PROCEEDINGS OF INTERNATIONAL SYMPOSIUM ON ANIMAL PRODUCTION AND CONSERVATION FOR SUSTAINABLE DEVELOPMENT

2018

-PART 1-
INTERNATIONAL SYMPOSIUM ON ANIMAL PRODUCTION AND CONSERVATION FOR SUSTAINABLE DEVELOPMENT 2018

ORGANIZER:
THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCE,
GIFU UNIVERSITY

-PART 2-
UGSAS-GU & BWEL JOINT POSTER SESSION ON AGRICULTURAL AND BASIN WATER ENVIRONMENTAL SCIENCES 2018

CO-ORGANIZER:
GIFU UNIVERSITY REARING PROGRAM
FOR BASIN WATER ENVIRONMENTAL LEADERS

DATE: OCTOBER 17-18, 2018
VENUE: 6TH FLOOR, UGSAS BLDG. GIFU UNIVERSITY, JAPAN

International Symposium
on Animal Production and Conservation for Sustainable Development 2018
Organized by The United Graduate School of Agricultural Science, Gifu University (UGSAS-GU)

PROGRAM
–PART 1-

DAY ONE: Wednesday, October 17

Time: 9:30-19:30
Venue: Main Seminar Room (6F in UGSAS Building, Gifu University)

Time Table

9:30-10:00 **Registration**

10:00-10:05 **Opening Remarks**
Prof. Masateru Senge (Dean of UGSAS-GU)

10:05-10:10 **Welcome Speech**
Dr. Fumiaki Suzuki (Executive Director and Vice President of Gifu University)

10:10-10:50 **Keynote Speech 01**
Dr. Yoko Tsukahara (International Goat Association, Langston University)

10:50-11:30 **Keynote Speech 02**
Assoc. Prof. Shigeru Ninomiya (Gifu University)

11:30-11:50 **Photo Shooting**

11:50-13:00 **Lunch Break**

Session 1 —Animal Production and Biotechnology—
Session Chair: ***Prof. Masato Yayota (Gifu University)***

13:00-13:25 **01.** ***Dr. Asif Mahmud (Globe Biotech Limited.)***

13:25-13:50 **02.** ***Dr. Sigit Prastowo (Sebelas Maret University)***

13:50-14:15 **03.** ***Dr. Shiori Miura (Niigata University)***

14:15-14:40 **04.** ***Assoc. Prof. Mohammad Nasir Uddin (Texas A&M University)***

14:40-15:20 **Keynote Speech 03**
Assoc. Prof. Atsushi Murai (Nagoya University)

15:20-15:30 **Coffee Break**

Session 2 —Avian Production and Reproduction—
Session Chair: ***Prof. Atsushi Iwasawa (Gifu University)***

15:30-15:55 **01.** ***Assoc.Prof. Ahmed Mohamed Hanafy Hassan (Suez Canal University)***

15:55-16:20 **02.** ***Dr. Hanny Cho Too***
(Livestock Breeding and Veterinary Department, Myanmar)

16:20-16:45 **03.** ***Prof. Anisur Rahman (University of Rajshah)***

17:10-19:10 **Dinner Meeting (At Gifu University Restaurant (2))**

DAY TWO: Thursday, October 18

Time: 9:30-17:40
Venue: Main Seminar Room (6F in UGSAS Building, Gifu University)

Time Table

9:30-10:00 **Registration**

10:00-10:40 **Keynote Speech 04**
Prof. Masato Yayota (Gifu University)

Session 3 —Animal Physiology and Production—
Session Chair: ***Assoc. Prof. Mohammad Nasir Uddin (Texas A&M University)***

10:40-11:05 **01.** ***Ms. Yuli Yanti (Gifu University)***

11:05-11:30 **02.** ***Dr. Adi Ratriyanto (Sebelas Maret University)***

11:30-11:55 **03.** ***Dr. Mei Matsuzaki (Shizuoka University)***

11:55-12:35 **Keynote Speech 05**
Assoc. Prof. Ikki Matsuda (Chubu University)

12:15-13:45 **Lunch Break**

Session 4 —Animal Conservation—
Session Chair: ***Prof. Shuichi Matsumura (Gifu University)***

13:45-14:10 **01.** ***Dr. Rizaldi (Andalas University)***

14:10-14:35 **02.** ***Prof. Irmanida Batubara (Bogor Agricultural University)***

14:35-15:00 **03.** ***Dr. Dewi Imelda Roesma (Andalas University)***

15:00-15:40 **Break & Preparation for Poster Presentation Session**

15:40-17:40 **-PART 2-** *Please refer to the next page for details.
UGSAS-GU & BWEL Joint Poster Session on Agricultural and Basin Water Environmental Sciences 2018

UGSAS-GU & BWEL Joint Poster Session on Agricultural and Basin Water Environmental Sciences 2018

Organized by The United Graduate School of Agricultural Science, Gifu University (UGSAS-GU)
Gifu University Rearing Program for Basin Water Environmental Leaders (BWEL)

PROGRAM
–PART 2-

DAY TWO: Thursday, October 18

Time: 15:40-17:40
Venue: Main Seminar Room (6F in UGSAS Building, Gifu University)

Time Table

15:40-16:45 **Poster Presentation**

17:20-17:35 **Best Presentation Award Ceremony**

17:30-17:40 **Closing Remarks**
Prof. Fusheng Li (Head of the Promotion Office of Gifu University Rearing Program for Basin Water Environmental Leaders)

Presenters

P01: ***Siyu Chen (UGSAS-GU; BWEL)***
P02: ***Masaya Toyoda (Graduate School of Engineering, Gifu University; BWEL)***
P03: ***Cahyo Wisnu Rubiyanto (UGSAS-GU; BWEL)***
P04: ***Wenjiao Li (Graduate School of Engineering, Gifu University; BWEL)***
P05: ***Wenzhuo Feng (UGSAS-GU; BWEL)***
P06: ***Ruoming Cao (UGSAS-GU; BWEL)***
P07: Huijuan Shao (UGSAS-GU; BWEL)
P08: Ali Rahmat (UGSAS-GU; BWEL)
P09: Shuailei Li (Graduate School of Natural Science and Technology, Gifu University; BWEL)
P10: Yuto Ito (Graduate School of Natural Science and Technology, Gifu University; BWEL)
P11: Fenglan Wang (UGSAS-GU; BWEL)
P12: Souzhu (UGSAS-GU; BWEL)
P13: Jobaida Akther (UGSAS-GU; University of Dhaka)
P14: Sharmin Sultana (UGSAS-GU)
P15: Tran Duy Quan (UGSAS-GU)
P16: Raj Kishan Agrahari (UGSAS-GU)
P17: Hend Altaib (UGSAS-GU)
P18: Panyapon Pumkaeo (UGSAS-GU)
P19: Muhammad Arifin (Graduate School of Natural Science and Technology, Gifu University)
P20: Yolani Syaputri (UGSAS-GU)
P21: Nayla Majeda Alfarafisa (UGSAS-GU)
P22: Akash Chandela (UGSAS-GU)
P23: Dina Istiqomah (UGSAS-GU; Gadjah Mada University)
P24: Kanthi Arum Widayati (Bogor Agricultural University)
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-PART 1-

INTERNATIONAL SYMPOSIUM ON ANIMAL PRODUCTION

AND CONSERVATION FOR SUSTAINABLE DEVELOPMENT

2018

KEYNOTE SPEECHES

ORGANIZER:
THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCE,
GIFU UNIVERSITY

Genetic improvement in goats for sustainable production

Yoko Tsukahara
International Goat Association
American Institute for Goat Research, Langston University

SUMMARY

Goats contribute food and economic security in the world. However, the production level is generally low, and internal parasitism has become a major constraint. Genetic improvement is one strategy to ease the problems and achieve sustainable production. Crossbreeding programs are commonly used to improve growth, dairy, and reproductive traits as well as resistance to internal parasitism especially in developing countries although the resulting composite breed is not always adapted to the area. A goat crossbreeding simulation model was developed to predict ant evaluate crossbreeding production scenarios. The International Goat Association that established in 1982 to promote goat research and development conducted an intensive study evaluating various goat projects in the world.

Introduction

Goats are well adapted to various environments and are produced all over the world. Goat production includes meat, milk, skin, fiber (cashmere and mohair), and manure that make significant contributions to the food and economic security of developing countries, especially in Asia and Africa. However, the supplies of goat products are often inadequate to meet the growing demand due to its low production levels as well as health problems.

Genetic improvement is one strategy to ease the problems and achieve sustainable production. Crossbreeding programs, which introduce high production levels of exotic gene to well-adapted indigenous breed in harsh environments, have been commonly used in developing regions. The traits of greatest importance in goat production include growth rate, milk production, reproductive performance, and resistance to internal parasitism. In general, exotic breeds are higher in milk yields and mature weights than the indigenous breeds, whereas indigenous breeds generally possess high reproductive performance and parasite resistance. Therefore, crossbreeding indigenous breeds with exotic breeds can be expected to improve their productivity with maintaining their high adaptability to the harsh environments.

The objectives of this paper are to provide an overview of research of crossbreeding programs focused on growth, dairy, and reproductive traits and resistance to internal parasitism in goats and to introduce activities of the International Goat Association.

Examples for crossbreeding systems in goats

Crossbreeding programs in goats typically employ exotic breed bucks of high performance, and small-sized indigenous does to produce F_1 breed, and then, backcross breeds (**BC**) by crossing exotic bucks with F_1 does (Hirooka et al., 1997). Figure 1 shows an example of crossbreeding programs involving indigenous Katjang breed with German Fawn breed in Malaysia. The objective of this program was to produce crossbreds combined high genetic potentials of growth rate and dairy performances of the German Fawn and the high fecundity, survivability, and adaptability to Malaysian environments of the Katjang. The goal of the program was to establish two composite breeds; the Jermasia which was a meat breed and composed 50% of each sire and dam breed genes and the Jermana which was a dairy breed composed 75% of the German Fawn gene (Mukherjee et al., 1985; Devendra, 2007).

Numerous crossbreeding programs of goats have been carried out in developing countries, but many of the programs were not successful or adapted to the areas. For instance, Jamnapari dairy goat from India has been used extensively in Southeast Asian countries to improve milk yield and body size of indigenous goats, however, the large mature body size of the resulted crosses can be a disadvantage during the feed shortage period in dry seasons. Ayalew et al. (2003) collected data on goat production from farmers to test the assumption that crossbreds would be more beneficial than indigenous goats under improved management and concluded better management of indigenous goats could improve household welfare without producing crossbreds. Tsukahara et al. (2011) developed a goat crossbreeding simulation model to predict and evaluate crossbreeding production scenarios. The model compared production efficiencies of crossbreeding systems in the tropic conditions and reported the greater production efficiency of composite breeds (F_1 and BC) than of indigenous in the intensive dairy production scenario and greater production efficiency and stocking rate of indigenous goats than of composite breeds under the integrated goat meat and oil palm production scenario.

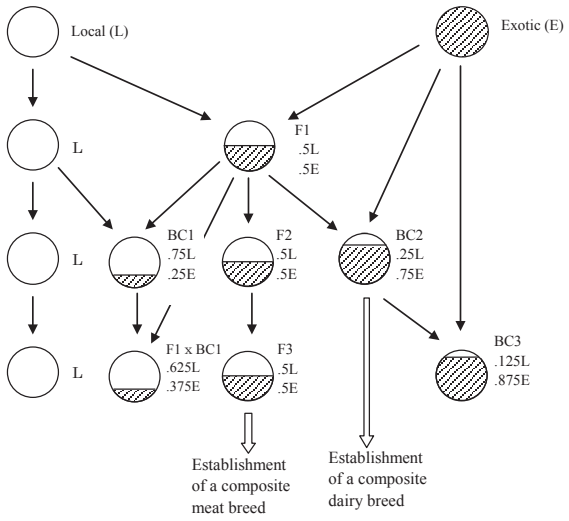

Fig. 1. A schematic diagram of crossbreeding systems between indigenous and exotic breeds. ⇨ shows *inter se* mating.

The estimation of crossbreeding parameters

Reliable crossbreeding parameter estimates are useful measures for designing a sound crossbreeding program. Crossbreeding parameters include additive genetic effects, dominant effect (heterosis effect), and the recombination loss or epistatic loss. Heterosis, which is a measure of non-additive genetic effects of both intra- and inter-allelic origin, is usually estimated as the amount by which the average performance of F_1 breed exceeds the average of the two parents (Jain, 1982). Thus, the amount of heterosis can be estimated from the difference between the averaged performances of F_1 breeds generated from the reciprocal crossing of sire and dam breeds and averaged performances of pure parental breeds. However, in goat crossbreeding programs in developing countries heterosis cannot be directly estimated from the method mentioned above, because reciprocal crosses have been rarely obtained (Hirooka et al., 1997; Tsukahara et al., 2008a). That is, the performance of exotic sire breed under the tropic conditions remains unknown in most cases because the germplasm of exotic breed is usually imported only male animals and/or through frozen semen. To solve the problem, Jain (1982) proposed an alternative procedure that estimates the effect of heterosis using the expectations of the mean performance of various grades of crossbreeding groups. Jakubec and Hyánek (1982) described another solution for estimation of heterosis using the magnitude of means of four types of crossbred populations (F_1, F_2, BC_1, BC_2) exceeding the mean value of the additive gene contribution of both the parental populations to the given generation mean. Kahi et al. (2000) pointed out that the merit of crossbred genotypes can be predicted by extrapolating the existing crossbreeding parameters. Hirooka et al. (1997) reported a method to estimate crossbreeding parameters on growth performance applying two mixed models; the Dickerson model (Dickerson, 1969, 1973) and the Kinghorn model (Kinghorn, 1980, 1983), utilizing data from a crossbreeding program involving the exotic German Fawn and the indigenous Katjang goats in Malaysia.

Growth traits

Growth traits including birth weight, weaning weight, and yearling weight are of importance for meat production and have been used to evaluate growth performance in goats. However, those traits could only observe fragmentary aspects of growth from birth to death (or culling) of goats. Instead, Tsukahara et al. (2008a) fit the longitudinal age-weight data from the crossbreeding program involving the exotic German Fawn breed and the indigenous Katjang breed to growth curve models and compared growth patterns of F_1, F_2 and BC_2 (75% German Fawn) does. This study showed the maturing rate of the backcross (BC_2) was higher than those of other genotypes, while the indigenous Katjang breed required more time to achieve the mature weight than other genotypes (Figure 2). Moreover, the significant influence of the genotype over mature weights and maturing rates were positively related in most cases and therefore, possible simultaneous improvement of the two growth traits by crossbreeding were suggested (Tsukahara et al., 2008a).

Dairy traits

Milk production is one of the most important products in goats. Goat breeding, for milk production in the United States and European countries, has been mainly conducted within-breed selection of pure breeds (Dubeuf and Boyazoglu, 2009). In contrast, crossbreeding to improve milk production of goats has been often implemented in developing countries under the tropics. Lee et al. (1975) reported that milk performances such as milk production and lactation periods of F_1, BC_2 (75% Saanen), and BC_3 (87.5% Saanen) were higher and longer than those of the Korean Native goats, whereas milk fat percentages of crossbreds were lower than that of the Native, further, there was no significant difference between purebred Saanen and BC_3 in milk production, lactation period, and milk fat percentage. Montaldo et al. (1995) compared dairy performance of high-grade ($\geq$ 15/16), and low-grade ($\leq$ 7/8 of any breed) crosses of Alpine, Granadina, Nubian, Saanen, and Toggenburg sires with local Mexican goats in northern Mexico, and reported that Alpine, Saanen and Toggenburg crosses had greater milk production and lactation length than Granadina and Nubian crosses or local does but no differences between high- and low-grade goats. Prasad and Sengar (2002) studied milk yield and composition of the dual purpose Barbari breed and its crosses with the Jamnapari, Beetal (milk breeds), and Black Bengal (meat breed) in India, and concluded that the breed effect was significant on milk yield and composition; Barbari sired Beetal crosses produced significantly more milk than the Barbari and the other crosses, and the milk fat percentage of Barbari sired Black Bengal crosses was significantly higher than the others.

Also, Ruvuna et al. (1995) investigated effects of crossbreeding on lactation curve parameters of Wood's

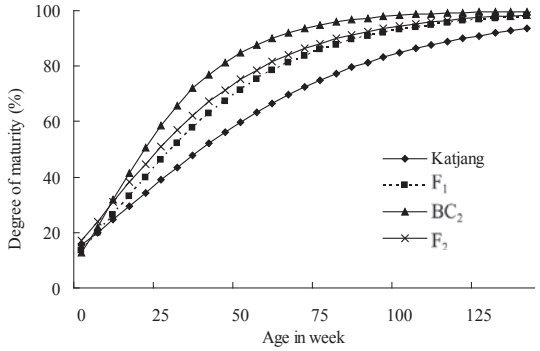

Fig. 2. Degree of maturity from time of birth to over 125 weeks of age by genotype in the Bertalanffy model.

equation ($Y_t = at^b e^{-ct}$), fitting data derived from a crossbreeding program conducted in Kenya involving two indigenous goat breeds (East African and Galla) and two exotic breeds (Toggenburg and Anglo Nubian). As a result, they reported that all lactation curve parameters (*a*, initial average milk yield: *b*, the rate of increase to peak production: *c*, the rate of decline after peak production) differed among the breed groups; the indigenous breeds ranked lowest for parameter *a* and highest for *c*, and the four-way cross was superior for peak yield, total lactation yield and the value of *b*.

However, most of the genetic improvement programs for dairy performance of indigenous goats have been failures and unsustainable (Devendra, 2007; Dubeuf and Boyazoglu, 2009). Serradilla (2001) reviewed reported experiments on the raising of high yielding breeds of goats under suboptimal conditions in different areas of the world, concluding some general reasons for impairing the success of these programs; 1) milk yield of F_1 does are intermediate between those of the parental breeds or, in some cases, closer to the exotic breed, nevertheless the effect of heterosis for milk yield has seldom been reported, 2) composition of milk, such as protein, fat, and total solids from F_1 does are either similar to those of local breeds or intermediate between those of the parental breeds, 3) backcrossed does generally do not show clear differences with F_1 for dairy traits, while does with a higher proportion of exotic genes (15/16) produced more milk than those with a lower proportion (7/8), 4) F_2 does show worst dairy performances than F_1 and backcrosses, and F_3 does are similar to those of local breeds.

Reproductive traits

Reproductive traits are a major determinant of productivity and economic viability (Mourad, 1994; Mellado et al., 2006). In particular, the reproductive rates of goats are undoubtedly the highest among the domestic ruminant species and the length of the breeding season shows distinct differences between breeds, hence, the reproductive performances of goats are considered more important than that of other domestic ruminant species. Despite the low heritability and viability of the reproductive performance, the genetic improvement of those traits through crossbreeding has still been expected.

Anous and Mourad (1993) characterized the Alpine milk breed, the Rove meat breed, and their first cross performance in France and reported that effect of the breed was not significant on fertility but highly significant on abortion and prolificacy of does. In addition, they showed that heterosis was positive and significant for prolificacy and fertility, while not for abortion rate. Montaldo et al. (1995) reported that litter size was greater in low-grade (≤ 7/8 of exotic breed) Saanen and Nubian crosses and the lower values were found in high-grade (≥15/16 of exotic breed) Toggenburg crosses, furthermore, litter weight means were significantly larger in high-grade Alpine and lower in high-grade Granadina. Tsukahara et al. (2008b) showed that crossbred goat groups showed higher performances in total litter weights and average litter weights than the indigenous breed, while litter size of crossbred goat groups was lower than that of the indigenous breed.

Though conception rates, service period, and litter size are generally treated as female maternal traits, influences of the sire of the fetus on these traits have been reported in cattle, sheep, swine, and mice (Kadry and Afifi, 1982/1983). Tsukahara et al. (2008b) estimated crossbreeding parameters to investigate the influence of parental genotypes and paternal heterosis on litter traits, regarding litter size, total litter weight, and average litter weight as traits of the litter using data from a crossbreeding experiment in Malaysia (Table 1). The results revealed that the genotype of sire had a significant and negative effect on litter size, and paternal heterosis had a significant and negative influence on total litter weight and average litter weight.

Table 1
Estimations of crossbreeding parameters for litter traits (Tsukahara et al. 2008b)

Parameters[1)]	Litter size	Total litter weight	Average litter weight
g^P	-0.212 **	0.23	0.475 **
g^M	0.158	1.712 **	0.984 **
h^P	-0.007	-0.21 *	-0.164 **

[1)] g^P, addirtive paternal breed effect; g^M, additive maternal breed effect; h^P, paternal heterosis effect
* $P < 0.05$, ** $P < 0.01$

Resistance to internal parasitism

Goats have peculiar features in adaptability such as anatomical, morphological, and physiological adaptation, and this unique character allows their wide distribution through the hot and cold climates (Devendra, 2007). This implies that high adaptability such as heat tolerance and resistance to diseases and parasites of indigenous goats in tropic regions are significantly related to their survivability. For example, Al-Busaidi et al. (2008) investigated significantly increased white blood cell counts and immunoglobulin levels of Dhofari goats, indigenous to Oman, during the summer, suggesting high resistibility of the breed under hot environment.

Internal parasitism has become a major constraint to small ruminant production all over the world due to widespread anthelmintic resistance. Ricordeau (1981) reported that the survival rate of progeny between

bucks selected for resistance to internal parasites (*Haemonchus contortus*) and untested does was much higher (71%) than that of controls (33%). Pralomkarn et al. (1997) reported that Thai native goat kids had greater resistance to *H. contorts* than crossbred (50% Thai native × 50% Anglo Nubian) kids although live weight gain was higher in crossbred kids than native kids. Fecal egg count is commonly used as an indicator of haemonchusis, and its heritability has been reported between 0.13 (75% Kiko × 25% Boer; Thomas et al., 2016) and 0.32 (crossbred cashmere-producing goats; Vagenas et al., 2002). Tsukahara et al. (2016) concluded that selection for resistance to *H. contortus* did not advisory affected growth performance of male goat kids in a central performance test in the south-central US.

Activities of the International Goat Association

The International Goat Association (IGA) promotes goat research and development for the benefit of humankind, to alleviate poverty, to promote prosperity and to improve the quality of life in the world. The main activities include 1) organizing an International Conference on Goats (ICG) every four years, 2) publishing Small Ruminant Research, the premier journal of goat and sheep research, and 3) promoting knowledge exchange to link research, production, marketing, and consumption of goat products. The IGA conducted an intensive study entitled "Scaling-Up Successful Practices on Sustainable Pro-Poor Small Ruminant Development" from 2011 to 2012 with support from the International Fund for Agricultural Development and reported success and failure of various goat projects in the world (IGA, 2014). The IGA is serving on the advisory council of the FAO and OIE coordinated program entitled "Global Strategy for the Control and Eradication of PPR (Peste des Petits Ruminants)" until the year 2030. IGA is holding the 13th International Conference in Eger, Hungary in September 2020.

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Animal welfare and behaviour

Shigeru Ninomiya
Faculty of Applied Biological Sciences, Gifu University, Japan

SUMMARY

When we consider animal welfare, Freedom to express normal behaviour, which is one of Five freedoms being recognized as a principle of animal welfare in the world is the most challenging issue. Applied Ethology has been working on the task but we can not understand animal behaviour completely. In this lecture I will talk about the relationship between animal welfare and behaviour. There are two theme, one is 'Is performing behaviours important for animals?' and the other is Environmental enrichment. I will also introduce our study regarding environmental enrichment for fattening beef cattle, which has been conducted for investigating their motivation to perform grooming behaviour at Minokamo Farm of Gifu university.

Introduction

Animal welfare in keeping animal has been a global issue and many countries have to deal with the issue. Five freedoms is well recognized as a principle of animal welfare and includes five aspects; Freedom from hunger and thirst, Freedom from discomfort, Freedom from pain, injury or disease, Freedom to express normal behaviour and Freedom from fear and distress (Farm Animal Welfare Council, 1992).

When we keep animals in artificial environments, Freedom to express normal behaviour is the most challenging issue. Applied Ethology has been working on the task, and the International Society for Applied Ethology holds scientific meeting every year. Although animal behavior is not perfectly explained by scientists, Applied Ethology and the society has revealed important knowledge related to behavioral restrictions in intensive husbandry systems (Mason & Burn 2011). In Japan, Japanese Society for Applied Animal Behaviour deals with this issue.

Is performing behaviours important for animals?

In artificial environments, animals are sometimes restricted to perform behaviours. Why does behavioural restriction decrease animal welfare? Animal behavioral needs will be generated by external stimuli such as stressors deriving from their environment or the method of animal care, or some internal factor in farm animals. This means that behavioral restriction would induce maladaptation to stressors or chronic stress (Ninomiya 2014).

Performing behaviour is also important for the other aspects of Five freedoms. For example, searching for resources will enable animal to get foods or water, and this meets Freedom from hunger and thirst. Moving to a more comfortable place will help animals to keep their homeostasis, and this meets Freedom from discomfort. Performing maintenance behavior (e.g. resting, grooming) will increase animal's physical health, and this meets Freedom from disease. Social behavior will improve affinity with peers, and this meet Freedom from fear and distress (Table 1).

Table 1 Five freedoms suggested by FAWC (1992) and animal behaviours to ensure them.

Five Freedoms	Animal behaviorr†
Freedom from Hunger and Thirst	Searching for resources
Freedom from Discomfort	Moving to a more comfortable place
Freedom from Pain, Injury or Disease	Maintenance behaviour
Freedom to Express Normal Behaviour	Normal Behaviour
Freedom from Fear and Distress	Social behaviour

† Environmental enrichment is conducted to stimulate behaviours.

Environmental enrichment

Environmental enrichment is one of animal management methods which enables animals to perform an adequate behavior for coping with stressors or satisfy their behaviour needs.

In zoo, environmental enrichment has been more popular in recent years. Environmental enrichment is also cited in modern intensive husbandry. In laying hens, an enrichment cage, which includes some devices for nesting, dust baths and perching are usually provided has been developed (Appleby & Hughes 1995). The devices enable laying hens to perform pre-egg-laying behavior, dust-bathing and perching. In a pig's housing system, bedded systems are accepted as environmental enrichment for pig behavioral needs for rooting (van de Weerd & Day 2009).

In cattle, devices for self-grooming were used (Pelley et al. 1995; Wilson et al. 2002). In fattening cattle farms, it is difficult to clean each cattle's body or increase frequency of muck managements because productive efficiencies per unit area and labor are sought for economic rationality. In the case where animals are dirty because of poor management of animal feces or urine, setting a device to enable animals to perform self-grooming behavior in housing will be a valid treatment for their welfare.

Our laboratory has been investigating the effect of grooming device in fattening beef cattle as environmental enrichment in terms of their motivation to perform grooming behaviour at Minokamo Farm of Gifu university. The device was combined by two brush. One brush measuring 50 cm in length and 10 cm in width was arranged together with a second brush in an inverted L-shaped pattern (Albert Kerbl, Buchbach, Germany). One brush was fixed horizontally about 130 cm from the floor and the other brush was attached to the wall of the pen.

Fig.1 Grooming device and fattening beef cattle.

Cattle's behaviour were recorded using CCD cameras and a digital video recorder and from the movie we observed their behaviour using a continuous behavioural recording. I will talk about the effect of the grooming device on performing grooming behaviour (self-grooming, social grooming) in fattening beef cattle in this lecture.

Conclusion

There have been many issues around animal welfare yet and Freedom to express normal behaviour is the most challenging issue. I have been studying animal welfare and behaviour, and the study introduced in this lecture is one part of my study. Animal behavior is not perfectly explained by scientists and therefore I hope that future study will clarify it.

Acknowledgement

I am grateful for conducting the study of environmental enrichment in fattening beef cattle by students of Laboratory of Animal welfare and behaviour, Gifu University, Japan.

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Maternal antibody transfer into eggs and new-born chicks in chicken and quail

Atsushi Murai
Graduate School of Bioagricultural Sciences, NAGOYA University

SUMMARY

In avian species, maternal immunoglobulin Y (IgY) is transferred into egg yolks of maturing oocytes to protect the new-born chicks against pathogenic attacks, but the molecular basis of IgY transfer is still unknown. To determine the structural requirements of IgY for selective transfer into avian egg yolks, recombinant wild-type IgY-Fc (WT) and its mutants were synthesized, and their uptakes into the egg yolks of chicken and quail were determined. The substitution of Y363 at the Cυ3 domain to alanine abolished the IgY-Fc uptake into egg yolks. Interestingly, substitution of G365 for alanine elevated IgY-Fc uptake. Characterization of several candidate IgY receptors responsible for maternal antibody transfer was performed. Our results emphasize the existence of a selective maternal antibody transport system recognizing the Cυ3 domain of IgY, which raises the possibility that an IgY with high transport ability might be engineered by genetic manipulation.

Introduction

In avian species, IgY, the counterpart to mammalian IgG, is transferred from the maternal blood circulation to the embryonic or new-born chick circulation. The process of avian maternal IgY transfer is divided into two steps: the first step is the transfer from the maternal blood to the egg yolks of developing oocytes, and the second step is the transfer from the egg yolks to the embryonic circulation through the yolk sac membrane in the developing eggs. The second step relies on an IgY-Fc receptor called FcRY (West et al., 2004). In the first step, however, the relevant receptor of the maternal IgY transfer is still unidentified

An understanding of the structural requirements of IgY and for selective transport into egg yolks is of importance for gaining insight into the molecular basis of maternal IgY transfer. In our previous study, the intact chicken IgY or its Fc fragment was incorporated into yolks of quail more effectively than Fab and $F(ab')_2$ fragments (Kitaguchi et al., 2008). The IgY heavy chain consists of four constant domains, and the Fc fragment mainly contains two constant domains on the C-terminal, the Cυ3 (C upsilon 3) domain and the Cυ4 domain (Fig. 1). Thus, the Cυ3 and Cυ4 domain are required for selective IgY transport into egg yolks.

To elucidate mechanism of maternal IgY transfer in birds, we investigated critical amino acid residues required for efficient IgY transport into the egg yolks by mutational analyses of selected residues located along the Fc domains of IgY. In addition, several candidate genes responsible for antibody transfer into egg yolks were characterized by gene expression analyses, immunohistochemistry and binding assay to IgY-Fc mutants.

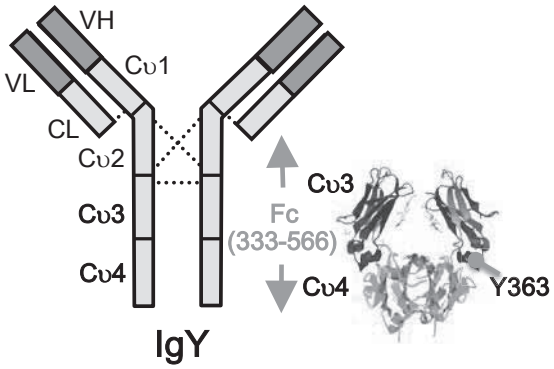

Fig. 1. The domain structure of chicken IgY (left) and conformation of IgY-Fc (right).

Material and Method

1. Measurement of intravenously-injected recombinant IgY-Fc concentrations in egg yolks

To synthesize chicken and quail IgY-Fc mutants, the cDNA encoding the IgY υ-heavy chain was isolated from a chicken and quail splenocyte cDNA library. The gene encoding the Fc regions was isolated by PCR from the template cDNA, and the PCR product was ligated into the mammalian expression vector, with a C-terminal His tag. The constructed expression vector was used to synthesize recombinant wild-type chicken and quail IgY-Fc (designated WT). The amino acid residues located on the Cυ3 domain of IgY-Fc were individually

substituted for alanine or other amino acid residues by site-directed mutagenesis (Fig. 1). The generated WT and mutant constructs were then transiently transfected into CHO-S cells, and the expressed WT and mutants were purified by His-affinity columns.

The purified proteins were labeled with digoxigenin. The regularly laying chicken and quail were injected intravenously with native and recombinant IgY-Fc or its mutants. Uptakes of the injected IgY-Fc into egg yolks were measured by ELISA.

2. Characterization of candidate IgY receptor genes for maternal antibody transfer

FcRY gene: To obtain a specific antibody against FcRY, rabbit was immunized with recombinant chicken FcRY. The obtained polyclonal antibody against FcRY was used for analysis of FcRY expression in maternal ovary and yolk-sac membrane of the developing embryos. Binding ability of IgY-Fc mutants to FcRY was measured by pull-down assay

Low-density lipoprotein receptor-related 2-like (LRP2L) gene: LRP2L gene was selected as a candidate gene by protein-protein interaction analysis between IgY and isolated ovarian sheets including oocyte plasma membrane. Body tissue expression pattern of LRP2L gene was characterized by reverse transcribed-PCR. Ovarian tissue localization and intensity of LRP2L expression was analyzed using polyclonal antibody.

Result and Discussions

1. Uptakes of IgY-Fc mutants in egg yolks

Among the 17 amino acid residues located on the Cυ3/Cυ4 interface of chicken IgY-Fc, the substitution of Y363 at the Cυ3 domain to alanine (Y363A) abolished the IgY-Fc uptake into egg yolks (Fig. 2). The comprehensive substitution of Y363 with other amino acids revealed that the only uptakes of the Y363F and Y363W mutants maintained the same degree of the WT, suggesting the residue at 363 needs to be allocated with aromatic amino acids to maintain the high transport ability.

Interestingly, when utilized quail IgY-Fc, the substitution of G365 with alanine (G365A) or with polar and nonpolar amino acid residues increased IgY-Fc uptake into egg yolks compared to the WT. These results raise the possibility that an IgY with high transport ability might be engineered by genetic manipulation.

2. Characterization of candidate IgY receptor gene

Western blotting and immunohistochemical analyses revealed that FcRY expresses in granulosa cell layer and theca layer of ovarian follicle in chicken, although its expression level was lower than that of yolk-sac membrane. Pull-down assay revealed that FcRY preferentially bound to WT compared with Y363A mutant, which showed a similar pattern of WT and Y363A transfer into egg yolks.

Another candidate gene, LRP2L, was highly expressed in ovary compared with other body tissues (liver, adipose tissue etc.). Microscopic analysis of ovarian follicles showed that LRP2L specifically expressed in oocyte plasma membrane within ovarian follicular tissues. These results suggest that LRP2L specializes in incorporation of egg yolk precursor in ovary. Binding analyses of both FcRY and LRP2L continues to be investigated.

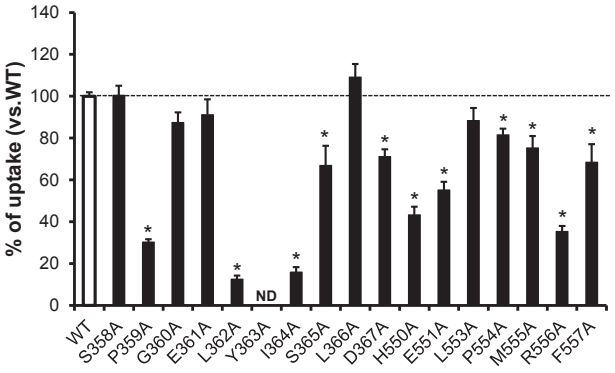

Fig. 2. IgY-Fc mutants with single alanine substitution and their uptakes into egg yolks of quail. *P<0.05 (vs. WT). n = 20 in WT and 4 or 5 in mutants.

Conclusion

Important amino acid residues (Y363 and G365) of IgY required for maternal IgY transfer into egg yolks were identified. Several candidate IgY receptor genes expressing in ovarian follicles were isolated and they are under investigation.

Acknowledgement

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Restoration and utilization of abandoned fields through goat grazing

Masato Yayota[1,2] and Kazuya Doi[3]
[1] Faculty of Applied Biological Sciences, Gifu University,
[2] Education and Research Center for Food Animal Health, Gifu University,
[3] The United Graduate School of Agricultural Science, Gifu University

SUMMARY

Many fields have been abandoned due to agricultural intensification over several decades in some developed countries. If fields are not managed properly, weedy and shrubby plants encroach the land, thereby degrading the fields and decreasing biodiversity. Goats do not have a strict preference to plant species while grazing and can survive in nutritionally harsh environments; therefore, they may be considered useful in weed management. The goal of our project was to manage abandoned fields using goats for restoring and conserving field environments. Our results showed that: 1) long-term (five years) goat grazing clearly changed the vegetational composition, increased the number of plant species, and improved plant diversity in an abandoned field, 2) The goats could maintain their nutritional status and body weight by grazing in the abandoned field for five years, and 3) cost of weed management through goat grazing was lesser or comparable to weeding by humans with a brush cutter. Thus, goat grazing is a feasible way for the sustainable management of abandoned fields.

Introduction

Agricultural intensification and a decrease in agricultural population have generated many abandoned fields in marginal areas over several decades in some developed countries. In Japan, the area of abandoned agricultural fields is more than 400,000 ha and more than half of these areas are now considered unsuitable for paddy and crop farming (MAFF 2018) due to encroachment and overgrowth of weedy and shrubby plants. Abandonment of fields may degrade the rural landscape (MacDonald et al. 2000) and increase the risk of wildfire (Osoro et al. 2007). Moreover, encroachment and overgrowth of weedy and shrubby plants reduce plant biodiversity. Thus, appropriate management measures should be implemented to restore and utilize these abandoned fields.

Goat grazing may be a feasible way for managing these abandoned fields (Fig. 1) because goats do not have a strict preference to plant species while grazing; they feed on grasses, forbs, and shrubs. They are capable of grazing and moving in hilly and steep areas that are mainly considered as marginal areas. Generally, abandoned fields are composed of semi-natural vegetation; however, this type of vegetation is often poorer in nutritional value than sown plant species. Moreover, vegetation and animal performance are largely influenced by stocking rate. Long-term grazing with different stocking rates induces qualitative and quantitative changes in field vegetation, leading to different animal performance and affecting sustainable grazing in the management of abandoned fields.

Fig. 1 Goat grazing as a method of weed management. Goats feed on a various plant species, create a patchy environment, and may recover abandoned fields in marginal agricultural areas.

In this brief review, we present the quantitative and qualitative dynamics of vegetation and goat performance in response to different stocking rates over a 5-year period. In addition, we discuss the economic potential of weed management through goat grazing by comparing this method with weeding by a brush cutter. Understanding these dynamics will facilitate the use of sustainable goat grazing systems to control abandoned agricultural lands and maintain biodiversity.

Vegetational dynamics with goat grazing

Long-term grazing with different stocking rates clearly affected botanical composition in an abandoned field. The dominant plant species at a high stocking rate (30 goats/ha) changed from bamboo (*Phyllostachys edulis*) to grass species over five years, while a dwarf-bamboo (*Pleioblastus argenteostriatus*) was continuously dominant in the paddock with a low stocking rate (14 goats/ha) throughout the five years. The number of plant species in the summer increased from 17-18 in the first year to more than 30 species in the fifth year, regardless

Fig. 2 Goats clearly alter the vegetational composition in abandoned fields. a. before the start of goat grazing in 2013, and b. after fiver years of goat grazing (2017).

of the stocking rate. Shannon diversity index also increased from approximately 2.0 in the first year to 2.5 in the fifth year at both high and low stocking rates (Doi et al. 2018a). These results suggested that goat grazing in an abandoned field for five years clearly changed the vegetational composition, increased the number of plant species, and improved plant diversity (Fig. 2).

Sustainable goat grazing

Goat grazing at various stocking rates (14-30 goats/ha), did not decrease forage biomass over time. Changes in the botanical composition affected the quality of the diet. At the high stocking rate, the diet quality, which was reflected by crude protein (CP) and neutral detergent fiber (NDFom), was relatively constant during the study period, whereas at the low stocking rate, diet quality decreased over the five years. This continuous decrease in the diet quality decreased forage intake. However, goats maintained their body weight over the five years at both stocking rates. Consequently, at the stocking rates studied, long-term goat grazing changed the quality of vegetation in an abandoned field; however, goats could maintain their nutritional status and body weight over five years (Doi et al. 2018b; 2018c). Thus, the use of grazing for the sustainable management of abandoned fields is feasible and does not have negative effects on the nutritional status of goats.

Economic potential of weeding by goats

Goat grazing is a feasible way to control field abandonment; however, its economic potential should be evaluated when we promote dissemination of this strategy. Economical sustainability is also crucial.

Compared with weeding by humans with a brush cutter, weeding by goat grazing largely reduced the working time for farmers or weeding workers. Moreover, weeding cost by goat grazing was comparable to or lesser than that by humans with a brush cutter, although the cost was varied according to grazing days and amortization period of grazing equipment such as pasture fence (Yayota et al. 2017). The results suggested that goat grazing is economical for managing field abandonment.

Future perspectives

Since the rapid economic growth after World War II, goat population in Japan has drastically decreased due to a reduction in demand for goat meat and milk; that is, the goat industry has remained stagnant over decades. Therefore, it is important that goats are given priority in the agricultural economy. Goats could be useful in restoring, managing, and conserving agricultural field environments. As shown here, this requires an ecological approach as well as an approach considering animal production science. In addition, its socio-economical value should not be forgotten, after all, goats are an important component of livestock.

Intensification of agricultural processes and livestock production is effective and inevitable for economic growth; however, it also negatively affects use of land and other resources, as well as the biodiversity and the rural landscape. Therefore, we should consider a more comprehensive solution for maintaining agricultural

environments.

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What we know and what we need to know about foregut-fermenting primates: a case of proboscis monkeys in Borneo

Ikki Matsuda

Chubu University Academy of Emerging Sciences; Wildlife Research Center, Kyoto University; Japan Monkey Centre; Institute for Tropical Biology and Conservation,

Universiti Malaysia Sabah

SUMMARY

Today, habitat destruction and poaching threaten nearly half of the free-ranging primate species with extinction. Hence, conservation programs have become integral aspects of zoological management, though studies in captivity have revealed that foregut fermenting primates such as colobines are historically difficult to maintain healthy in captivity, and that they have shorter lifespans than free-ranging individuals. Proboscis monkeys (*Nasalis larvatus*), endangered and endemic to Borneo, are the largest foregut fermenting colobines. They are notoriously difficult to maintain and breed in captivity. On the other hand, knowledge about the ecology of free-ranging proboscis monkeys is also incomplete because most studies have been conducted in mangrove and peat swamp forests from boats. However, by the 2000s, the attention raised for these unique monkeys had gradually attracted more researchers devoting their academic pursuits to the further understanding of the species, with studies still predominantly focused on their socio-ecology, but extending to genetic analyses, and other novel topics about their digestive capabilities and the sexual selection of enlarged noses. I summarized the findings from our proboscis monkey long-term project in Sabah, Borneo, Malaysia and discussed the conservation implications of our results

Background

Proboscis monkeys (*Nasalis larvatus*) have uniquely enlarged noses that are prominent adornments, especially for males (Koda et al. 2018), with enlarged and sacculated fore-stomach (Matsuda et al. 2015) (Fig. 1). However, for decades, knowledge about endangered proboscis monkey had been gleaned from limited information of their behavior from boat surveys along riverbanks where they rest in the early mornings and late evenings. This was due to the inhospitable swampy habitats they live in, which made them near impossible to track once they move into inland forests (Fig. 2). This resulted in patchy knowledge about the species' ecology.

Fig. 1 They are large, sexually dimorphic, and primarily arboreal, colobine primates. Adult males have the largest body size among colobine species (ca. 20 kg), whereas adult females are about half the weight of males.

We pioneered the first full-day continuous observations of proboscis monkeys by tracking them on foot in riverine forests in Sabah, Borneo, Malaysia. This long-term effort which began in 2005 led to the first systematic study of proboscis monkeys, revealing comprehensive information about their feeding and ranging behaviors; as well as novel findings about their incredible digestive strategy of rumination, the first documented among primate species (Matsuda et al. 2011).

Understanding the natural diet of species may provide useful information that can contribute to successful captive maintenance. A common problem experienced with captive foregut-fermenting primate (colobine) diets is that they are deficient in fiber and therefore highly digestible. This may contribute to gastrointestinal disorders often observed in zoos. Indeed, some colobines like proboscis monkeys are notoriously difficult to maintain and breed in captivity.

Here, I reviewed our *in-situ* and *ex-situ* proboscis monkey project to understand what we know and what we need to know about this endangered species for their long-term conservation.

Population status

Proboscis monkeys are classified as 'Endangered' according to the IUCN Red List of Threatened Species (IUCN, 2008), and listed under CITES Appendix I. Many proboscis monkey populations were formerly "naturally protected" by the fact that they live in swampy habitats, which are generally less threatened by human development compared to dry land forests due to their relative inaccessibility (Nowak 2012). However, in recent decades, modern technology made it increasingly easy to meliorate these habitats and reclaim swampland by filling it with compressed soil, so swamps quickly became a cheap source of land available for human exploitation, including industry and human settlements (Lhota et al. in press-a). Many populations are now found scattered in small patches of suitable habitats (Sha et al. 2008).

In our study conducted in the lower Kinabatangan region, Sabah, Malaysia, the relatively stable population was found within 10 years (2004-2014) because there were only minor losses of forest along the rivers where proboscis monkeys are generally found. These reduced losses were the result of increased protection measures for these habitats over that 10-year period in the Kinabatangan floodplain; however, certain areas within the proboscis monkey's range are still under threat and need to be protected to ensure the species' long-term survival (Matsuda et al. in press). Lastly, what we currently know about the overall population status of the species across its range (Borneo) is still based largely on "guesstimates" from some 2-3 decades ago. Therefore, further Borneo-wide population surveys will be helpful in clarifying the species' endangered status.

Fig. 2 Proboscis monkeys are endemic to the island of Borneo. They are restricted mainly to swampy habitats along waterways such as mangrove, peat swamp and riverine forests. Those habitats make continuous observations on foot very difficult.

Diet and digestive physiology

Proboscis monkeys are the largest colobines, with a diet consisting of various proportions of leaves (38%–73%), fruits (11%–50%), and flowers (3%–8%) (Bennett and Sebastian 1988; Boonratana 2013; Matsuda et al. 2009; Yeager 1989). Our study on their dietary choices considering a variety of nutritional and mechanical factors with diet digestibility found that the preferred leaves of proboscis monkeys not only contain more protein and less fibre but are also less tough and more digestible than the alternatives (Matsuda et al. 2017).

Proboscis monkeys are the only colobine species in which an apparent rumination of stomach contents has been observed under free-range conditions (Matsuda et al. 2011). However, displaying marker excretion patterns using fecal marker concentrations in four captive proboscis monkeys with a comparison of that in cattle, showed the separation of the solute and the different-sized particle markers, observed in cattle, did

not occur in proboscis monkeys; all markers excreted together at mean retention times of ca. 40 h (Matsuda et al. 2015). This indicates that the digestive physiology of proboscis monkeys is different from that of ruminants with typical characteristics. This may be the reason why the "suggestive rumination" is only a facultative strategy in this species.

Captive study

An approach to obtain information relevant for the improvement of diets in captive proboscis monkeys that are difficult to maintain healthy, is to compare the nutrient composition of feces from free-ranging and captive individuals. In theory, fecal material can be considered a proxy for diet intake integrated over a certain period of time. We found that feces from free-ranging monkeys contained more fiber and less metabolic fecal nitrogen than those from captive ones, indicating a less digestible diet in the wild. Our results suggest that free-ranging proboscis monkeys basically consume lower quality foods than captive individuals. Modifying the diets of captive proboscis monkeys to include more fiber, comparable to those of free-ranging ones, may contribute to their health and survival.

Outlook

I reviewed our *in-situ* and *ex-situ* proboscis monkey project which may shed light on the establishment of a constructive *in-situ* and *ex-situ* collaborative link to aid the management of their husbandry and possibly to provide necessary impetus for conservation and education initiatives, which will be beneficial for their long-term conservation. However, I also need to note that to ensure the long-term survival of proboscis monkeys, a multipronged approach would be needed according to the context and political situation within different proboscis monkey range countries, where mechanisms beyond scientific inquiry should be employed to augment actions that can tilt the balance towards the conservation of this unique species (Lhota et al. in press-b).

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-PART 1-

INTERNATIONAL SYMPOSIUM ON ANIMAL PRODUCTION

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Transgenic technology: A sustainable alternative in animal production

Asif Mahmud

Research and Development, Globe Biotech Limited, Dhaka, Bangladesh

SUMMARY

Molecular biology paved way by giving a new dimension to animal production. The emergence of transgenic technology has widened the scope of development in case of farm animals. It is one of the important tools to meet the future challenges for increased livestock production. The fast-growing human population increases the demand for basic needs of human being for their survival and well-being. Transgenic animals have roles as dairy cattle as well as in biomedical research such as gene therapy, disease resistance, drug testing, screening, toxicological screening, disease model production, production of pharmaceuticals, transgenic expression of immunoglobulins, xenotransplantation, new drug targets identification, drug development *etc*. Salmon is the first transgenic animal, which won US approval for food. The US FDA has approved a chicken that has been genetically engineered to produce a drug and goats that produce an anticoagulant. Clean meat can also be produced in the lab without raising a chicken or duck. Therefore, the genetically engineered animals and biotechnology will play a key role in the production of pharmaceutical proteins and bring about a complete refinement in animal production by increasing the quality and quantity of production, environment protection, maintenance of genetic diversity and overall improvement in animals' welfare.

Introduction

The fast-growing human population increases the demand for basic needs of man like food, machines etc. Being a citizen of one of the most densely populated countries in the world, we grew up with the thought that how the demand for basic needs can be assured. It is quite difficult to satisfy the need of food for its 170 million inhabitants without some miracle. Similar situation exists for other countries in the world as well as for developed countries at a different perspective.

Bangladesh is one of the most densely populated countries in the world. With a visionary leadership, relevant ministries nearly did that miracle. Bangladesh became a food surplus country and the 5th leading country in fresh water fish production. The meat production has also been constantly rising. As per Yearbook of Fisheries Statistics of Bangladesh 2016-17, consumptions of daily fish per person reached at 62.58 g. The big question is - are these achievements truly sustainable to satisfy the sustainable development goal (SDG). Relevant ministries have been focusing on intensive integrated farming system to increase the yield. Up to a certain extent it is logical but there is an obvious saturation point; as close as we will be moving towards that point the growth rate in yield will be nullified (Nag, 2018). Furthermore, agricultural land is in harsh competition with urban and industrial needs. Therefore, our main focus should be in obtaining high-yielding varieties of desired animals, which is truly a sustainable strategy. Several classical programs are in effect from relevant ministries to develop improved variety of animals to obtain higher yield by hybridization and natural selection method. However, the success of this method is limited and critically depends on availability of desired "super quality" among relevant species, which is very rare to find.

Over the ages of evolution and centuries of man-made selection processes those "super qualities" are almost used up. Therefore, it is highly unlikely to obtain a "super quality" animal for improving local varieties with ease. Another issue is that there are no controls of human over the natural hybridization and selection process of any animal. With the discovery of DNA double helix by Watson and Crick in 1953, the Human entered into a new era of knowledge - "The Genetic Edge". The art of genetic science started to become useful with the invention of polymerase chain reaction (PCR) technology that gave the power to pick a specific genetic element selectively from the pool of genetic materials. Over the periods, scientists have developed technologies to selectively transfer one gene of an animal to another, even from one species to another species, and found that

the transplanted gene is functioning in the new animal and executing its roles. This type of organism is called genetically modified organism (GMO); intra-species and inter-species gene transfers are called autogenic and transgenic GMOs, respectively. Generally, GMOs are organisms (i.e., microorganisms, plants or animals) in which the genetic material (DNA) has been transformed in such a manner that does not happen naturally.

Although transgenic livestock have had a high profile, the practical use of these animals has been restricted to medical applications like producing pharmaceutical proteins in milk, eggs etc. rather than livestock production that were originally envisioned. Use of lentivirus vectors (Lois *et al.*, 2002; Pfeifer *et al.,* 2002) and RNA interference (RNAi) (McManus *et al.,* 2002; Hannon *et al.,* 2002) to suppress the expression of specific genes offered the possibility of producing transgenic livestock more efficiently during 2002. CRISPR–Cas9 has revolutionized the generation of transgenic animals. This system has demonstrated an unprecedented efficiency, multiplexability, and ease of use, thereby reducing the time and cost required for genome editing and enabling the production of animals with more extensive genetic modifications (Shrock *et al.,* 2017). Other strategies for increasing the efficiency of CRISPR/Cas9 genome editing include inhibition of NHEJ (non-homologous end joining) or enhancement of HDR (homology directed repair) through chemical treatments (Maruyama *et al.,* 2015; Nakao *et al.,* 2016).

Methods

The CRISPR–Cas9 system has been used to generate a wide variety of transgenic animals via a number of common methods. These include: (i) germline editing via injection or electroporation of CRISPR–Cas9 reagents into early-stage zygotes or gonads, often used in model organisms, such as *Caenorhabtidis elegans*, *Danio rerio*, and *Drosophila melanogaster*, as well as in larger animals, (ii) primary cell editing followed by somatic cell nuclear transfer (SCNT), often used in larger animals, such as pigs and cows, and (iii) blastocyst injection with genetically modified ESCs followed by breeding to produce stable germline mutants, traditionally used in mice in the form of plasmids, mRNA preparations, as RNPs, or in viral vectors such as AAVs (Shrock *et al.,* 2017).

Small Model Organisms In the fruit fly *Drosophila,* CRISPR–Cas9 can be efficiently used to generate germline genetic modifications. CRISPR–Cas9 reagents can be injected into preblastoderm embryos, and cells from these embryos can be screened by PCR 24 h after injection. Pairs of gRNAs have been used successfully to generate defined deletions, and, in combination with single-strand oligo-deoxynucleotides, to induce Cas9-mediated homologous recombination (Yu *et al.,* 2013). This method has also been widely used with zebrafish. CRISPR–Cas9 reagents are injected into zygotes, or early-stage embryos to generate mosaic F0 animals. Mosaic adults are then crossed to generate homozygous mutants (Li *et al.,* 2016; Hwang *et al.,* 2013; Varshney *et al.,* 2015).

Rodents A major advance was reported by Jaenisch and coworkers (Wang *et al.,* 2013), in which they successfully used CRISPR–Cas9 in a one-step process to produce mice with mutations in multiple genes. This group demonstrated precise genome editing by co-injecting fertilized oocytes at the pronuclear stage with not just the Cas9 mRNA and sgRNA, but also single-stranded DNA oligos, enabling HDR based on the DNA oligos as templates. Blastocysts with these DNA oligo injections were implanted into the wombs of foster mothers. The time scale required for the generation of mice carrying multiple mutations using pronuclear injection was shown to be approximately one month, much shorter than the six-month to year-long time scale required by conventional methods. In addition to germline editing, ex vivo and in vivo gene editing has been demonstrated. Multiple systems have been developed to perform in vivo CRISPR–Cas9 editing in rodents. The necessary reagents are generally packaged in lentiviruses or AAV. Multiple tissues, such as lung (Sánchez-Rivera *et al.,* 2014), neurons (Swiech *et al.,* 2014), or liver (Cheng *et al.,* 2014) have been targeted. Delivery strategies using cationic nanoparticles have also been developed (Zuris *et al.,* 2014).

Larger Animals The production of larger transgenic mammals, including pigs (Li *et al.,* 2015) and cows (Gao *et al.,* 2017), has been accomplished via SCNT and/or pronuclear injection. SCNT involves genetically engineering primary cell lines in vitro, then fusing the nuclei of individual cells with enucleated porcine oocytes to produce genetically modified embryos (Verma *et al.,* 2015). At the blastocyst stage of development, these embryos are delivered to the uterus of a surrogate mother. Transgenic pigs may also be generated using zygote injection of the CRISPR–Cas9 reagents (Wang *et al.,* 2016).

Nonhuman Primates Although NHPs require larger facilities and longer time periods for experiments as compared with mice, they nonetheless play an important role in biomedical research (Chan, 2013) as they are phylogenetically closer to humans. The CRISPR–Cas9 system has been of great value for genome editing in NHPs. Indeed, highly precise genetic modifications in monkeys had not been feasible before the advent of CRISPR–Cas9, as the only technologies available for gene editing were viral vectors (Chan *et al.,* 2001; Niu *et al.,* 2010; Sasaki *et al.,* 2009). CRISPR–Cas9 has been used to precisely edit the genome of the Cynomolgus monkey. Using zygote injection, researchers introduced Cas9 mRNA and five gRNAs targeting the *Nr0b1*, *PPar-gamma*, and *Rag1* genes (Niu *et al.,* 2014). In the future, it is thought that genetically engineered NHPs may be very useful to study neurological disorders and human traits that cannot be easily recapitulated in common model organisms.

Applications

Biomedical Research Models for obesity and immunological, neurological, reproductive and haematological disorders, providing future hope for a variety of human therapeutic interventions through gene therapy. Transgenic animals can be used for disease resistance in humans and animals. Clements *et al.* reported that transgenic sheep have been developed that is resistant to Visna virus infection (Clements *et al.,* 1994). The transmission of bovine spongiform encephalopathy (Scrapie) is also prevented by the knock down of prion protein (Weissmann *et al.,* 2002). Transgenic mice have been developed that secretes recombinant antibodies in milk to neutralize the corona virus responsible for transmissible gastroenteritis (TGEV), an economically important disease in case of pigs (Castilla *et al.,* 1998). Transgenic dairy cows secreting lysostaphin into their milk have advanced resistance to mastitis due to protection provided by lysostaphin. It kills the bacteria *Staphylococcus aureus* in a dose dependent manner that protects the mammary gland against this major mastitis-causing pathogen (Donovan *et al.,* 2005).

Oncomouse was first transgenic animal to be patented. An activated human oncogene sequence is introduced into the animal at an early embryonic stage and it is found in its germ and somatic cells. The production of therapeutic proteins from transgenic animals usually involves their expression from mammary-gland specific promoters to drive secretion of the transgene into milk or an alternative is the use of kidney or bladder specific promoters that direct transgene expression to the urine, e.g., Prolactin for enhancement of immunity and Protein C for blood coagulation. Transgenic cattle harboring intact unrearranged human Ig heavy- and λ-light-chain loci were created. These 'transchromosomic' cattle were shown to produce human Ig. Primate-to-human organ transplantation ushered the age of xenotransplantation. It was recognized early on that for physiological, anatomical, ethical and supply reasons the pig was the best choice as a donor animal for vascularized organs. First published transgenic pig-to-primate xenograft used a novel transgenic delivery system for complement regulatory proteins in human (Byrne *et al.,* 1997). Transplant organs may soon come from transgenic animals. Currently, xenotransplantation is hindered by a pig protein that can cause donor rejection but research is underway to remove the pig protein and replace it with a human protein.

Transgenic Livestock Dairy cattle are likely candidates for transgenesis if the mammary gland is to be used as a bioreactor, as they produce about 10,000 liters of milk/year with 35 g protein/liter. The animals could be made to secrete nutraceuticals in milk that may have an impact over the growth of offspring. Physio-chemical properties of milk get altered while improving the milk composition and casein variants are the main target. Brophy *et al.* reported that cloned transgenic cattle have been developed that produce increased amounts of beta and kappa casein in milk that increase the value of milk in the production of milk-based products like cheese, yoghurt and also increase the shelf life of milk products (Brophy *et al.,* 2003). Transgenic animals could also be made to secrete antibodies in their milk that give resistance against several diseases like mastitis or to secrete antimicrobial peptides like lysozyme. Grosvenor *et al.* reported that the milk composition could also be changed by making the transgenic animals to secrete growth factors in milk consequently, affecting the growth and maturation of newborn offspring (Grosvenor *et al.,* 1993). Varieties of monoclonal and recombinant antibodies were produced in transgenic goats and cattle (Houdebine *et al.,* 1993; Grosse *et al.,* 2004). Kuroiwa *et al.* reported that transchromosomal animals could be used for the production of human therapeutic polyclonal antibodies (Kuroiwa *et al.,* 2002).

Transgenesis research with sheep, goat or pigs has

concentrated in the most part on utilizing their mammary glands as bioreactors for production of pharmaceutical proteins. Several conventional methods were used for the production of therapeutic proteins using bacteria, plants, yeast etc. but most of them were deficient in machinery for post translational modifications of eukaryotic genes. The transgenic livestock serve as potential bioreactors for the production of valuable proteins. Proteins like antithrombin III (AT III), tissue plasminogen activator (TPA) and antitrypsin have been derived from the mammary gland of transgenic sheep and goats. The human AT III (for the treatment of heparin resistant patients) is expected to be in market (Kues *et al.,* 2004). Glycosidase has been produced in the milk of transgenic rabbits, which is used in the treatment of Pompe diseases (Van *et al.,* 2001). Role of transgenic animals in carcass composition and growth enhancement are introduction of chicken ski gene has caused muscular hypertrophy in case of pigs and cattle (Bowen *et al.,* 1994). The acid meat gene or Rendement processing Napole gene has been involved in low yields of pork there by affecting the quality of meat in pig. Silencing the expression of this gene in pigs improve the quality of meat and modify the post mortem pH. There was a significant improvement in growth rate and feed conversion in transgenic pigs with human metallothionein promoter (Nottle *et al.,* 2001). Transgenic swine has been developed for production of functional hemoglobin having same oxygen binding capacity as that of normal human hemoglobin. It could be purified from porcine blood (D'Agnilalo *et al.,* 1998). The main potential application of transgenic animal is the production of recombinant and biologically active proteins in the mammary gland and this in turn could be used for the benefit of mankind. This is known as "Gene Pharming". Mammary gland is the favored site for production of these proteins since large quantities can be extracted and purified (Houdebine *et al.,* 1993). Farm animals like cattle and pigs could be used as an appropriate model for the study of human diseases like cystic fibrosis, cancer and neuro-degenerative diseases and their therapies (Li *et al., 2003*). Pigs could be used as an effective model for the study of growth hormone releasing hormone (GHRH) defects (Draghia *et al.,* 1999).

Limitations

Transgenic animals have large number of applications for the improvement of animal production and reproduction quality, improvement of productivity in animals, studies of various disease models and production of pharmaceuticals. However, there are many vital problems that need to be resolved for transgenic animal studies.

Dietary and Food Safety Concerns Food safety of bioengineered products is always a significant public topic. Some of the foreign gene and its promoter sequences from the virus may occur in the recipient animals in case of transgenesis. Homologous recombination or integration may cause the formation of new virus. Foreign gene inserted in the chromosome locus may also result in different genetic changes in different degrees, causing unintended effects. Transgenic animals may cause allergic reactions and there is increased risk of zoonotic diseases.

Environmental Impacts If transgenic animals are in the external environment and mating with wildlife then foreign gene may spread, which results in changing the species composition of the original genes, causing confusion in species resources. Decline in the genetic diversity may be there due to loss of wild allele. Once released into the environment, transgenic animals can disturb the ecological balance of species and genetic diversity of threatened species.

Ethics

As recent technology has accelerated the ability to create transgenic animals, ethical discussions have become increasingly relevant. Debates have been most intense regarding editing of the human genome, especially of the germline, but have also concerned editing of nonhuman animal genomes. For example, the proposal to use CRISPR–Cas9 gene drives to eradicate alleles or entire species from ecosystems has been the subject of ethical consideration. Use of animals in the transgenic research leads to greater suffering to the animals. But most people seem to accept some animal suffering to serve the basic interest and human welfare. Using animals for the production of therapeutic proteins, we reduce them to mere factories. Animals also are living beings and feel pleasure and pain just as we do. Animal sufferings are also caused by the expression of transgenes, inducing tumors or neurodegenerative diseases. Genetically altering the cells of an animal can lead to unknown side effects resulting from modifying genes. Even though humans may benefit from transgenic animals, the animal itself may not be benefited. Foreign genes might affect the host and bring threats to ecological balance and species diversity (Miao, 2013).

Conclusion

Transgenic animal production techniques have developed rapidly in the recent past and provided platforms for the preparation of transgenic animals. These techniques provide an entirely new alternative for the accurate modulation of genes for human use, particularly the production of drug, organ culture for human transplantation and their use in biomedical research will be significant. It is expected that with the development of simple and novel animal transgenic techniques will lead to more benefits and may help to alleviate or provide to cure certain diseases, which are presently incurable or expensive to treat. Production of transgenic animals and related products is still infancy in Bangladesh. Transgenic animal could be produced using different method, but no method seems to be perfect and has its own pros and cons. Despite these facts, transgenic animal production has a great prospect in animal pharming for human use.

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miRNAs and genes interaction as potential biomarker for farm animal fertility

Sigit Prastowo[1,*], Ahmed Amin[2], Nuzul Widyas[1] and Adi Rayriyanto[1]
[1] Department of Animal Science, Sebelas Maret University, Surakarta, Indonesia
[2] Department of Animal Production, Faculty of Agriculture, Cairo University, Egypt
[*]Corresponding author: prastowo@staff.uns.ac.id

SUMMARY
In current farmed animal practices, the success of a breeding program depends upon conventional animal reproduction. It is closely related to both male and female fertility which indicates the ability of an animal to produce offspring. Animals' fertility is often disturbed causing infertility cases which lead to genetic and economic loss especially when occur in superior animal population. MicroRNAs (miRNAs) are small noncoding RNA which work post transcriptionally to up or down regulate gene expression by binding into 3-UTR mRNA of specific target gene. One or many miRNAs can interact with many genes or with only one gene as the target. The aforementioned interaction was suggested as a fine tune process of gene expression which control specific function of animal metabolism in general, or specifically in animal fertility. There were many genes which have been reported to be responsible for specific function in animal reproduction process and these genes expressions were under the role of miRNAs. Hence, miRNAs and their interactions with genes which control male and/or female reproduction carry the potentials to be developed as fertility biomarkers. However, in every interaction occurrence between miRNA and gene related to its specific functions, further wet lab study is required as a validation procedure.

Keywords: miRNA, potential biomarker, farm animal fertility

Introduction
Micro RNAs (miRNAs) are non-coding RNAs that regulate gene expression post-transcriptionally [1]. It is belongs to a class of small (19–24 nt) regulatory RNA which able to control many cellular process by modulating the precise amount of proteins in the cell [2,3]. miRNAs have that roles through the recognition of sequences between miRNAs and their target genes [4,5]. Both cellular and extracellular miRNAs are found to be involved in regulating mammalian gene expression [6,7].
Because of its broader targeting abilities, miRNAs are to be found involved in most of biological processes and cellular pathways [8]. miRNAs are comprised of 1-5% of mammalian genome and according to bioinformatics analysis there are more than 60% mammalian genes could potentially target by a single miRNA [9]. Recently, 38.589 mature miRNAs were annotated in miRBase (http://www.mirbase.org/ released 22 at March 2018). In that database, there are 4719, 2145, 877, 4612, 260, and 1414 mature sequences for bovine, chicken, porcine, goat, sheep, and equine miRNAs respectively.
Similar to other biological process, most of animal reproduction event also controlled by many genes expressions. For example, miRNAs expression and regulation have been observed in bovine sperm [10], oocyte[11], ovary [3], zygote [12], and preimplantation embryo [13]. Previous study discover miRNAs expression in tissue specific and have temporal expression pattern in livestock [14]. However, to the best of our knowledge there were little studies performed to observe the role of miRNAs in animal reproduction by controlling specific gene expression. Those aforementioned background bring the authors, to show brief idea of using miRNAs as biomarker of farm animal fertility which control gene function in reproduction. This paper will cover information regarding miRNA biogenesis and its role in gene expression, the glance of miRNAs in animal reproduction both in male and female fertility, and an insight pipeline of miRNAs study to validate its interaction with specific gene expression.

A brief of miRNA biogenesis and its role in gene expression
miRNA biogenesis begins in the nucleus [15], initiated by miRNA gene transcription with the help of RNA polymerase II providing long precursor transcripts know as pri-miRNA. Mediated by Drosha and DGCR8A, pri-miRNA then proceeds into hairpin structure called pre-miRNA which has shorter sequence. The pre-miRNA then transported to cytoplasm facilitated by exporting 5 as transport enzyme. The pre-miRNA then edited by DICER resulting mature RNA, approximately 22 nt, and ready to target specific mRNA.
In its action, mature miRNA and Ago protein form a ribonucleoprotein complex, RISC, mediating post-transcriptional gene activity by bind to 3-UTR of mRNA target gene [15]. miRNA down regulate gene expression through mRNA translation inhibition or degradation. Several reports also reporting that miRNA can be function as post transcriptionally stimulation of gene expression [16–18]. Both down and/or up regulation of gene expression are the process of cellular fine tune or orchestrated protein level [19].

miRNA in male farm animal reproduction
Considering the role of male as sperm source, in this paper we only show information of miRNAs which have function or related to spermatogenesis and sperm quality parameters. Several studies, listed in Table 1, shows miRNAs related to spermatogenesis, sperm motility, and sperm abnormality. Sperm production and its quality are the most considerable trait for male fertility [20]. Any disturbance of sperm quality, would lead to low conception rate [21] or egg hatchability [22]. In this regards, losing fertility in male sometimes difficult to

diagnose and the major problems are coming from genetic defect [23].

Table 1. miRNAs in male animal reproduction

miRNA name	Pathway	Animal	Related reproduction process	Status	Source
miR-202	-	Chicken	Spermatogenesis	-	[24]
ssc-miR-10a-5p ssc-miR-125b ssc-let-7f ssc-miR-186	-	Porcine	Spermatogenesis	-	[25]
let-7a let-7d let-7e miR-22	-	Porcine	Sperm motility and abnormality	-	[26]
miR-17-5p miR-26a-5p miR-486-5p	PTEN	Bovine	High and low sperm motility	-	[14]
miR-122-5p miR-184	PI3K/AKT Signaling	Bovine	High and low sperm motility	-	[14]
miR-122-5p miR-184 miR-20a-5p	STAT3	Bovine	High and low sperm motility	-	[14]

miRNAs are play critical role in the regulation of target genes during spermatogenesis [27], for that they have close relationship with male infertility. In the previous study, miR-202 found to be upregulated in developing male chicken gonad, and also can be indicator for sexual dimorphic in chick embryo [24]. miRNA ssc-miR-10a-5p, ssc-miR-125b, ssc-let-7f, and ssc-miR-186 were reported highly expressed in porcine spermatogonia, pachytene spermatocytes, round spermatids, and spermatozoa respectively [25]. However, there were no information in both studies, to which gene those miRNAs targeted.

In sperm motility, it is reported that miRNA let-7d and let-7e were higher express in porcine sperm with low motility. Furthermore miRNA let-7a, let-7d, let-7e, and miR-22 were significantly increase in abnormal sperm when compared to the normal one [26]. Again, that study didn't show any gene as the miRNA target [26]. While in bovine, previous report show miR-17-5p, miR-26a-5p, miR-486-5p, miR-122-5p, miR-184, and miR-20a-5p have different expression in high and low sperm motility [14]. According to that study, these miRNAs are targeting PTEN and STAT3 pathway, and PI3K/AKT signaling.

miRNA in female reproduction

Several studies listed in Table 2, shows the function of miRNAs in female animal reproduction. These, various reproduction events are related to fertility in female animal. In this review, we only show studies which were related to ovaries, oocytes, and embryos.

Table 2. miRNAs in female animal reproduction

miRNA name	Gene(s)	Animal	Related reproduction process	Status	Source
miR-106a let-7	MYC WEE1A	Cattle	Oocytes	Predicted	[28]
miR-19b miR-130 miR-101 miR-19a	AMPKA1	Cattle	Granulosa cells	Predicted	[29]
miR-27b miR-372 miR-382	ACVR1 ID2	Equine	Follicle growth and maturation	Predicted	[30]
miR-34a	INHBB	Porcine	Granulosa cell apoptosis	Validated	[31]
miR-212	FIGLA	Cattle	Preimplantation embryo	Validated	[32]
miR-196a	NOBOX	Cattle	Preimplantation embryo	Validated	[33]

Oocytes known as female gamete which ovulated as the result of ovarian activity [34]. Any disturbance of ovarian activity lead to the female infertility as well as reducing reproductive efficiency. It would also increase the potential of economic loss in animal farming activity. Previous report shows miR-106a and let-7i were related to the oocytes development [28]. Both expressions were lower in oocytes, compared to cumulus and granulosa cells. It is well known that cumulus cells have an essential function in oocytes maturation [35], while after ovulation granulosa cells turn into luteal cells to produce progesterone for pregnancy support [36]. Other study reporting miR-19b, miR-130, miR-101, and miR-19a were predicted to be involve in bovine granulosa cell luteinization [29]. miR-27b, miR-371, and miR-382 were reported to be involve in preovulatory growth of equine follicle by altering ID2 levels [30], while miR-34a play role in granulosa cell apoptosis by targeting the INHBB gene in porcine ovary.

In the reproduction event related to embryo, we can show two studies of miR-212 and miR-196a were determining embryo preimplantation in cattle. Both miRNAs are expressed in oocytes and tends to increase in four and eight cell stage of embryos followed by a decline at morula and blastocyst stages. miR-122 demonstrated a temporary regulation during maternal to zygotic transition in bovine embryos by negatively regulate FIGLA mRNA [32]. Meanwhile miR-196a has been validated to be negative target of Newborn ovary homeobox gene (NOBOX) mRNA [33] which is important for early embryonic development.

miRNA validation study

In animal production area, growing evidences shows that miRNA regulate gene expression subsequent to animal production traits. For instance, there is a different of miRNAs expression between bulls with high and low fertility [37]. miRNA also involved in milk production [38], control postpartum period [39], chicken growth and carcass trait [40], pregnancy [41], and embryo viability [42]. Considering that promising functions, more study needs to be performed to understand the miRNA role and its application to animal production as biomarker or as a production enhancer agent [43].

So far, the bioinformatics prediction indicated that mammalian miRNAs able to regulates 30% of all protein-coding genes [44]. Most of miRNA control gene expression by binding to 3-UTR sequence of mRNA target gene at nucleotides number 2-8 which known as seeding region or binding site [45–47]. Since there are numbers of genes targeted by one miRNA or *vice versa*, the validation study of each miRNA targeted specific gene need to be performed. This work would inform the exact function of miRNAs or genes in the animal reproduction specifically.

As our experiences, miRNA validation study covering steps in dry and wet lab. Dry lab consists of in silico study for miRNA target prediction to specific gene, miRNA sequence similarity analysis, prediction of miRNA target in 3-UTR mRNA gene. Moreover, in wet lab covering plasmid construction by 3-UTR clone, followed with cell culture, and luciferase assay analysis which commonly performed using cell model.

In silico study to prediction miRNAs targeted to gene

According to the previous study [29], there were three steps as pipeline of in the miRNA prediction by in silico study method. First, miRNAs prediction tolls (databases) were employed to predict miRNAs targeted to specific

gene, namely MirTarget2 (www.mirdb.org), PicTar (http://pictar.mdc-berlin.de/), TargetScan (www.targetscan.org) and miRanda (http://microRNA.org). Another database also possible to be employed. Thereafter, common miRNAs that were found in all these databases were selected as candidate. Venn diagram would be great tool to select common miRNA as mentioned in the previous study [48].

miRNA sequence similarity analysis

In the market, the availability of miRNAs primer is commonly specific for human study, therefore for future miRNAs profiling in farm animal, a similar primer sequence to human will be use. For this, similarity sequence analysis become a reasonable thinking. The mature sequence of selected miRNAs candidate accessed from miRbase (www.mirbase.org), followed with sequence similarity analysis between human (hsa) vs animal miRNA, for example bovine (bta), avian (gga), porcine (ssc), goat (chi), sheep (oar), and equine (eca). These three letters in the bracket are identification for specific animal species. As an example, from previous study, Table 3 show the similarity analysis between human (hsa) vs bovine (bta) miRNAs.

Table 3. miRNA sequence similarity analysis[a]

miRNA	miRNA name	Mature sequence (5'-3')*	Similarity (%)
miR-323	hsa-miR-323a-3p	CACAUUACACGGUCGACCUCU	
	bta-miR-323	CACAUUACACGGUCGACCUCU	100
miR-23a	hsa-miR-23a-5p	GGGGUUCCUGGGGAUGGGAUUU	
	bta-miR-23a	AUCACAUUGCCAGGGAUUUCCA	-
miR-221	hsa-miR-221-5p	ACCUGGCAUACAAUGUAGAUUU	
	bta-miR-221	AGCUACAUUGUCUGCUGGGUUU	-

[a] Source: [29,48]; *) www.miRbase.com

Prediction of miRNA binding sites in 3-UTR gene

miRNAs which have 100% identical sequence between human vs specific animal, were predicted its 3-UTR mRNA binding sites (seeding region). At this point, several online software could be employed. A listed software provided online named tools 4miRs target prediction (https://tools4mirs.org/software/target_prediction/) will computationally calculate the potential target or miRNA in 3-UTR specific gene. In this point, FASTA sequence of 3-UTR gene and mature miRNA sequence were needed.

The listed software could give different results features due to different algorithm in Seed Region Match, Conservation, Free Energy, In-site Features and Accessibility Energy [49]. For this reason, selecting a potential candidate miRNA which is identified by all databases is a logic choice to avoid computational errors during identifying candidate of miRNAs.

3-UTR clone to construct plasmid

According to the pervious study [50], construct plasmids were generated by fusing 3-UTR gene fragment (seeding region) with luciferase gene in a pmirGLO vector (promega E1330). Other vectors supplied by other companies are also having possibility to use in this step. 3-UTR target gene which containing miRNAs binding site, cloned in the pmirGLO multiple cloning region. In addition, a mismatch sequence of miRNA binding site also cloned in pmirGLO vector.

Luciferase assay in cell model

Activity of the luciferase gene in the constructed plasmid can be measured using cell culture. In this regard cell types could be varied. The cells seeded at 2 x 10^5/µl in 24 wells plate and transfected with match and mismatch pmirGLO luciferase vector at confluency >70%. Along with that miRNA mimic and inhibitor also transfected using Lipofectamine 2000 as transfection agent. After 24 hour of culture, cell lysate then collected and store at -80°C until dual luciferase reporter assay performed [50]. Renilla activities is measured using a dual luciferase assay protocol on a Luminometer. The level of luciferase was compared to the un-transfected cells to ensure a proper transfection of the cells. The relative activity was calculated by normalization with the firefly activity.

Future perspective

A number of miRNAs expression have been identified related to both male and female reproduction events. Its expression, though not directly targeted to specific gene, already demonstrated as indicator of fertility in farm animal reproduction. There still long way to know each miRNA role to specific gene lead to the reproduction process. In this point, thousand interactions could be revealed between miRNA, gene, and fertility.

To know the exact function of selected miRNAs candidate, a series of profiling and validation study need to be performed. Moreover, along with the improvement of computational algorithm features in miRNA target prediction software, the more precise prediction for miRNA candidate selection would be resulted. So far, the computational method is still the golden attempt to start in revealing miRNA and gene interaction or in contrary.

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Generation of functional oocytes of common marmoset by xeno-transplantation of ovarian tissue

Shiori Miura[1], Yoshitaka Maeda[1], Jun Miyamoto[1], Ena Nakatsukasa[2], Nobuyoshi Fujisawa[1], Miki Miwa[3], Katsuki Nakamura[3], Kenji Sakimura[2] and Toshikuni Sasaoka[1,2]

1.Department of Comparative & Experimental Medicine, Center for Bioresource-based Researches/Bioresource Science Branch, Brain Research Institute, Niigata University

2. Department of Animal Model Development, Center for Bioresource-based Researches/Bioresource Science Branch, Brain Research Institute, Niigata University

3. Primate Research Institute, Kyoto University

SUMMARY

Common marmoset (Callithrix jacchus) is well known being a non-human primate experimental animal which is useful for translational research, for instance, researches of human brain function and various human disorders. However, it is extremely challenging to deal with numerous primates and breed them. Therefore, a few research facilities can maintain a relatively large number of common marmosets. Only limited laboratories can certainly create a genetically modified marmoset. Furthermore, considering that the reproduction engineering utilizing marmoset oocytes is not well established, it is essential to develop the way to get lots of oocytes. In order to the efficient utilization of the ovary, we have conducted xenotransplantation of the euthanatized marmoset ovaries to immunodeficient mice. Here we report that fully developed oocytes of common marmosets have been obtained from xenografted ovaries of common marmosets of immunodeficient mice. These results demonstrate that ovaries of euthanatized marmoset can be used as a source of generation of embryos. Now, we are studying at the effects of exogenous factors, i.e. reproductive hormone in order to collect oocytes more efficiently from the fresh and cryopreserved ovaries.

Introduction

The common marmoset (Callithrix jacchus) is a valuable laboratory animal, because of (1) similarity of metabolic, physiological, and anatomical characteristics to those of humans, (2) ease and safety in handling due to its small size and (3) high reproductive efficiency. In recent years, marmosets model is remarkable experimental model in fields such as brain research, infectious disease, reproduction, and aging1–3. The methods of superovulation and in vitro maturation of preimplantation embryos have not been established. There are many difficulties in terms of breeding and supplying of marmosets at a large scale.

Therefore, we focused on the ovaries of marmosets who were euthanized in other laboratories due to the end of the experiment or poor physical condition. If we can obtain matured oocytes, these will be effective source for supplying oocytes even in a small-scale laboratory. By reusing a tissue that is originally disposed as an invaluable genetic resource, it can contribute not only to reducing the number of marmosets used but also to reducing costs.

Ovarian transplantation is one of the clinical treatments to maintain an ovarian function. This technique allows for retention of hundreds of immature oocytes kept within the original ovarian tissue. In the ovary of the marmoset, there are a lot of immature oogonia than human 4. By effective utilization of immature oogonia existing in the ovary, we can obtain a sufficient number of fertilized eggs for animal production.

Here we report on the successful xenotransplantation of marmoset ovarian tissue to immunodeficient mice as shown by the development of mature antral follicles.

Material and Method

Ovaries were taken from euthanized adult marmosets and transported in Lifor solution at 4°C (Fig 1A). The

tissues were dissected into approximately 1 to 2 mm3 (Fig 1B) using a scalpel and scissors in Lifor solution and placed on ice until transplantation.

Ovarectomies of recipient mice were carried out prior to grafting to eliminate the influence of mouse ovaries (Fig 1C). Transplant surgery was performed on the left and right ovaries and kidneys as follows. The kidney was taken out of the body. The kidney capsule was incised with micro tweezers or injection needle, and a rounded glass tube was inserted to make a pocket. One to three ovary pieces were placed in pocket and the kidneys were returned to the abdominal cavity (Fig 1D).

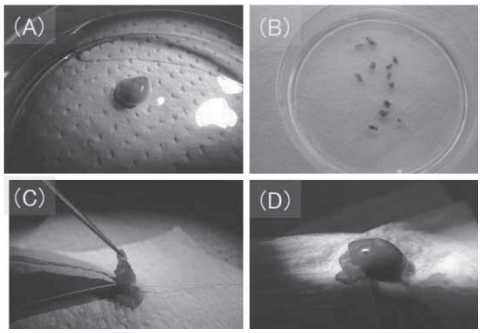

Fig.1 Ovary transplantation. The marmoset ovary (A) was dissected into approximately 1 to 2 mm3 (B). Ovarectomies were carried out prior to grafting (C), and ovary pieces were implanted under the kidney capsule (D).

From 18th day after marmoset ovarian transplantation, animals were administered with 7.5 units / 0.1 mL of recombinant human follicle stimulating hormone (rhFSH) every 24 hours subcutaneously. The graft tissues were stained with hematoxylin and eosin solution for histological evaluation of ovaries.

Result and Discussions

In this experiment, we aimed to obtain fertile oocytes by xenotransplantation of euthanized marmoset ovary to immunodeficient mice. The graft tissues survived under the kidney capsule for 31 days after transplantation (Fig 2).

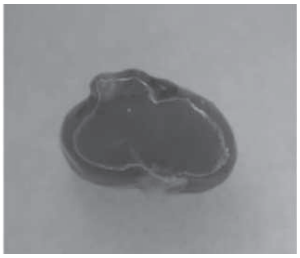

Fig 2. Engraftment of transplanted tissue.

Following xenotransplantation, it is confirmed the development of follicles at 600 μm or more in the recipient mouse with stimulation of rhFSH. Histological evaluation showed the existence of normal eggs in the follicle morphologically (Fig 3).

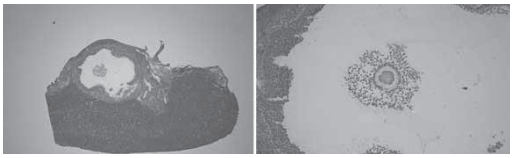

Fig 3. (A) Follicular development in grafts of marmoset ovarian tissue after xenotransplantation. Antral follicle in graft of tissue recovered 21days rhFSH treatment. Scale bar=1000μm, and oocyte (B) Scale bar=300μm.

Throughout the experiment period, it was revealed the presence of morphologically normal oocytes surrounded by cumulus cells. Depending on rhFSH stimulation, the number of oocyte tends to increase (table 1). In our experiments, it is difficult to collect samples with adjusted estrous cycle of recipient mice because we use ovaries took from the animals euthanized due to the completion of experiment or healthy condition. In marmoset, administration of FSH on day 9 or 11 improves the quality of eggs and fertilization rate of in vitro fertilization rate significantly5. More detailed examination is required as to whether this condition is similar to the case where the graft ovaries are transplanted into nude mice.

Table 1. Frequency of rhFSH stimulation and number of oocyte in the graft follicles.

Days after stimulation of rhFSH	Number of oocyte
0	1
7	2
14	3
21	1
30	4

Now, we are trying to establish experimental procedures regarding the hormone administration at pre/post transplantation, the storage method or freezing method of the ovary and grafting conditions to improve ovarian function after transplantation. We are aim to establish an economical and reliable systems enabling generalization of genetically modified marmoset model by ovarian xenotransplantation.

Conclusion

In this study, we succeed in the euthanasia marmoset ovary xenotransplantation to a nude mouse and it is histological confirms the survival of the grafts ovary morphologically.

Acknowledgement

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Marinobufagenin and preeclampsia in the squirrel monkey

Mohammad Nasir Uddin[1,2,3], Syeda H. Afroze[1,2,3], David CC Sprague[2,3] and Thomas J Kuehl[4]

[1] Texas A&M University College of Medicine, [2]Orion Institute for Translational Medicine, [3]Emergent Biotechnologies, [4]Baylor Scott & White Healthcare, Temple, Texas

SUMMARY

Our team seeks to understand the pathogenesis of preeclampsia (PreE), and develop strategies to lessen adverse outcomes. This study focuses on marinobufagenin (MBG) as a trigger of preE in a nonhuman primate, the squirrel monkey (Saimiri sciureus). We showed MBG in preE patients exceeds that in those with normal pregnancy. Others showed angiogenic imbalance in patients developing preE. In a rat model of preE, we found MBG increases prior to angiogenic imbalance and development of preE. MBG administration to normal pregnant rats induces angiogenic factor alterations, hypertension, proteinuria, and intrauterine growth retardation. We propose the novel hypothesis that MBG produces preE in a nonhuman primate and that this effect can be blocked or attenuated by RBG, and or a MBG antagonist. We proposed two hypothesizes: 1: We demonstrated that administration of MBG in early pregnancy leads to angiogenic factor imbalance and the onset of preE symptoms and signs in otherwise normal squirrel monkey pregnancies. The working hypothesis is that MBG elaboration early in the pregnancy induces hypertension, proteinuria, and angiogenic factor imbalance (increasing the angiogenic factor ratio) during and potentially following in vivo exposures. 2: We compared the ability of RBG and anti-MBG monoclonal antibody to attenuate the effects of MBG in vivo, using the squirrel monkey model. We demonstrated that MBG triggered preE features in pregnant squirrel monkeys including maternal effects and fetal growth retardation, which can be blocked by treatment with RBG, a structurally similar antagonist, or with anti-MBG antibody. These studies provide the first demonstration in a primate species of the potential for blockade of MBG-triggered pathways.

Introduction and Significance

Preeclampsia (PreE) is a disorder of pregnancy occurring in 3-10% of gestations (Pridjian and Puschett, 2002; WHO, 2000). The diagnosis of preE includes the de novo onset of hypertension and proteinuria mid-gestation or later, which may be accompanied by significant edema, intrauterine growth restriction (IUGR), and CNS complications. It is the second most frequent cause of maternal and fetal morbidity and mortality, accounting for about 60,000 maternal deaths annually worldwide. Marinobufagenin (MBG) is an endogenous mammalian cardiotonic bufodienolide with vasoconstrictive properties (Bagrov and Fedorova, 1998; Fedorova et al., 2001; Schoner, 2002). Increased circulating levels of MBG are found in volume expansion-mediated hypertension and in preE (Gonick et al., 1998; Lopatin et al., 1999; Vu et al., 2005). In an animal model of preE (Ianosi-Irimie et al., 2005), the urinary excretion of MBG is elevated prior to development of hypertension and proteinuria. Our overall goal is to understand the role of MBG in the pathogenesis of preE in a primate and to identify the therapeutic target. The objective of this proposal is to determine if MBG can cause features of preE and/or trigger the preE syndrome in a nonhuman primate. If so, we will determine if the effect of MBG is

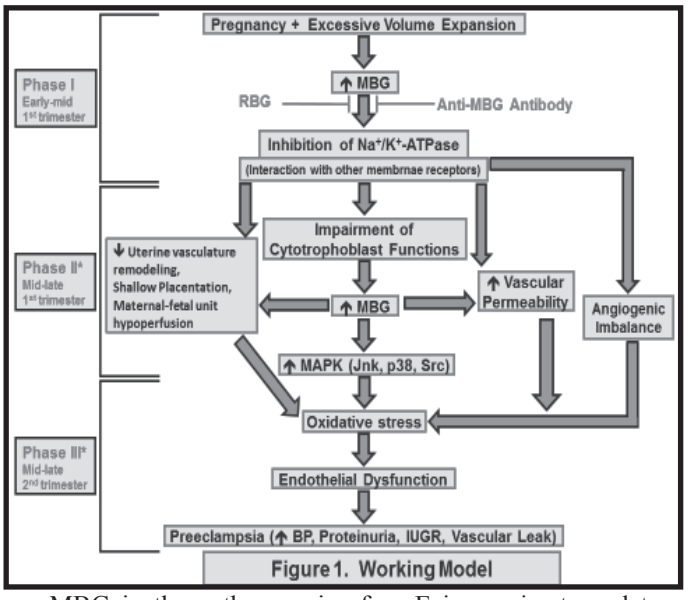


Figure 1. Working Model

prevented by concurrent treatment with resibufogenin (RBG), a structural antagonist of MBG, or with an anti-MBG monoclonal antibody. (Fig. 1) is that MBG targets vascular beds and causes vascular responses that induce the preE syndrome. This hypothesis is based on data in a rat model from our laboratory showing that MBG elaboration occurs early in preE, which then causes the preE syndrome with hypertension, oxidative stress, and angiogenic factor imbalance leading to endothelial dysfunction. The vascular response includes alterations in the circulating balance of angiogenic factors: both a decrease in the pro-angiogenic factors (VEGF, PlGF, TGF) and an increase in the anti-angiogenic factors (sFlt-1, sEng). Administration of MBG to rats beginning early in otherwise normal gestations induces hypertension, proteinuria, and fetal effects, which are characteristic of preE. We are well equipped to pursue this project because members of our team have developed a rat model of preE in which MBG is elevated prior to the onset of preE features. We have experience with telemetry monitoring of blood pressure and hypertension that can be applied to the squirrel monkey. We have extensive experience monitoring pregnancy and fetal growth in small, easily handled nonhuman primates, including the squirrel monkey. The rationale for the present study is based on our previous discovery that MBG administration causes a preE syndrome in rats. The potential significance of the current project is that successful completion will give us a non-human primate model of preE and identify therapeutic targets that might effectively prevent the MBG-induced preE syndrome in certain patient populations. A better understanding of these pathways in a primate has great potential for translation into clinical treatment and potentially prevention of preE.

Significant, reliable, and reproducible data have accumulated which favor a view that preE is a disorder of endothelial dysfunction (Roberts et al., 1989) involving oxidative stress. Methods for early diagnosis and treatment are unavailable, and prevention is currently not possible. Therefore, this illness represents an important example of a medical unmet need. It seems clear that preE is not a single disorder, but a syndrome with multiple pathophysiological triggers and mechanisms (Pridjian and Puschett, 2002). Recent data suggest that a significant segment of preE patients is responding to excessive secretion of marinobufagenin (MBG) (Puschett, 2006). Obstetricians have suspected that a vascular leak syndrome may develop in preE (Chesley, 1972; Gallery et al., 1979; Hays et al., 1985). A syndrome with many of the phenotypic characteristics of human preE results when pregnant rats are given weekly injections of desoxycorticosterone acetate (DOCA), and their tap water is replaced with normal saline (Ianosi-Irimie et al., 2005). This syndrome can be largely reproduced by the daily injection of MBG, beginning in early pregnancy (Ianosi-Irimie et al., 2005). We assessed vascular leak in this model and demonstrated greater leak in preeclamptic rats (PDS) than in normal pregnant (NP) animals (Uddin et al., 2009a). Angiogenic imbalance plays a role in the pathogenesis of preE in this rat model. However, the earliest event in the pathogenesis appears to be the secretion and elaboration of MBG (Agunannae et al., 2010). In these studies, MBG caused hyperpermeability of the endothelial cell layers by activating the MAPK and apoptotic signaling which was prevented by p38 inhibition (Uddin et al., 2009b). MBG also disrupted endothelial cell junctions, an effect prevented by p38 inhibitors (Uddin et al., 2009b). We found that the MAPK system is involved in the deleterious effects of MBG on cytotrophoblasts (CTBs), important for normal placental development (Uddin et al., 2008a; 2008b). When resibufogenin (RBG), an MBG antagonist, was given to rats that had been rendered hypertensive and proteinuric in the manner described above (Ianosi-Irimie et al., 2005), it reduced blood pressure to normal (Vu et al., 2006). The same was seen in normal pregnant animals given MBG to induce hypertension (Vu et al., 2005). When RBG was given early in pregnancy, it completely prevented all of the PE manifestations noted in the rat model, including IUGR and oxidative stress (Horvat at al., 2009, Uddin et al. 2010a; 2010b; 2011). These data suggest that RBG or anti-MBG monoclonal antibodies (Fedorova et al., 2008) may be important new therapeutic or preventative strategies for this syndrome. However, a primate model to test this concept is not available. Preliminary studies indicate that MBG induces features of the preE syndrome in pregnant rats including endothelial dysfunction, angiogenic imbalance, and intrauterine growth retardation (IUGR). The major objective of this proposal is to develop a primate model of preE and identify therapeutic targets that could effectively prevent the MBG-induced preE syndrome.

Our team seeks to understand the pathogenesis of preE, develop novel diagnostic biomarkers to predict disease

severity, and develop new strategies to lessen adverse outcomes. There are no compelling nonhuman primate models of preE, although preE features have been reported in nonhuman primates (Palmer et al., 1979; Hennessy et al., 1997; Podjarny et al., 2004), and nonhuman primates share some features of implantation that are important for understanding vascular dysfunction related to eclampsia and fetal growth restriction (Wulff et al., 2003; Carter, 2007). This application has a novel focus on MBG as a potential trigger of preE features in a nonhuman primate, the squirrel monkey. We showed MBG in preE patients exceeds that of those with normal pregnancy (Abi-Ghanem et al., 2011). We and others have demonstrated angiogenic imbalance in patients developing PE (Buhimschi et al., 2005; Molvarec et al., 2010; Abi-Ghanem et al., 2011). In a rat model of preE, we found MBG increases prior to angiogenic imbalance and that MBG acts on cultured cells to trigger hyperpermeability in human brain microvascular endothelial cell monolayers through disruption of tight junction proteins using MAPK pathways and to release an angiogenic factor, sFlt-1, into the culture medium (Uddin et al., 2009a). MBG administration to normal pregnant rats induces angiogenic factor alterations, hypertension, proteinuria, and intrauterine growth retardation (Ianosi-Irimie et al., 2005; Agunanne et al., 2009). We now propose the novel hypothesis that MBG induces features of preE and potentially triggers preE in a nonhuman primate and that these effects can be blocked or attenuated by RBG and or a MBG antagonist. We propose to instrument and monitor blood pressure in pregnancy, which is novel for this species, and to follow other preE features including fetal growth effects using minimally invasive techniques. If the results of these trials support the role of MBG as a cause of preE features and that one or more agents can block this effect without producing damaging fetal effects, it will open the door for studies of a number of innovative treatments in primates. Also, these studies may provide the first demonstration in a primate species of the potential for blockage of MBG-triggered pathways.

Methodology, Results and Discussion

We propose the novel hypothesis that MBG produces features of preE in a nonhuman primate and that this effect can be blocked or attenuated by RBG, an MBG antagonist, or by anti-MBG monoclonal antibodies. We demonstrate that administration of MBG in early pregnancy leads to angiogenic factor imbalance and the onset of preE symptoms and signs in otherwise normal squirrel monkey pregnancies. Administration of MBG in early or mid-gestation will lead to angiogenic factor imbalance and the onset of preE features in squirrel monkeys including hypertension, proteinuria, and fetal growth retardation. This hypothesis is supported by publications cited above (A. Significance) and by the observation that MBG is increased, and angiogenic imbalance is observed in preE patients. Urinary MBG and angiogenic factors were assayed in 30 preE and 40 normal pregnant patients. The MBG levels were higher in preE patients (Fig. 2; Abi-Ghanem et al., 2011).

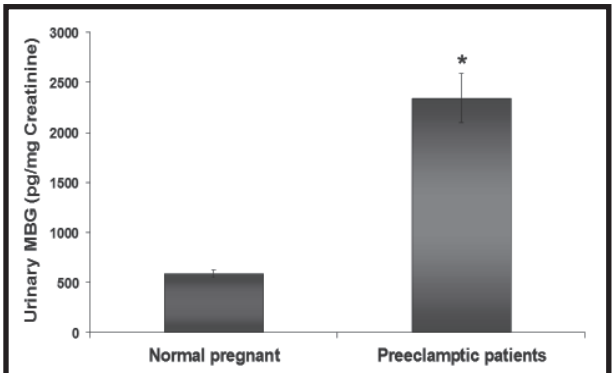


Figure 2. The mean Urinary levels (± SE) of MBG in normal pregnant (NP, n=40) and preeclampsia (preE, p=30) patients. The urinary levels of MBG are expressed in pg/mL ± SE. The mean urinary level of MBG in the preE group is significantly greater than that in the NP group (*p<0.05)

Angiogenic imbalance was observed in preE patients compared to normal pregnancies (Fig. 3). Our team is experienced working with primate and the urinary levels of MBG were measure in eight Owl monkeys at different gestational ages. The levels increase in early pregnancy (~52 days) then decrease minimum (~80 days), then increase toward term (~133 days) (Fig. 4).

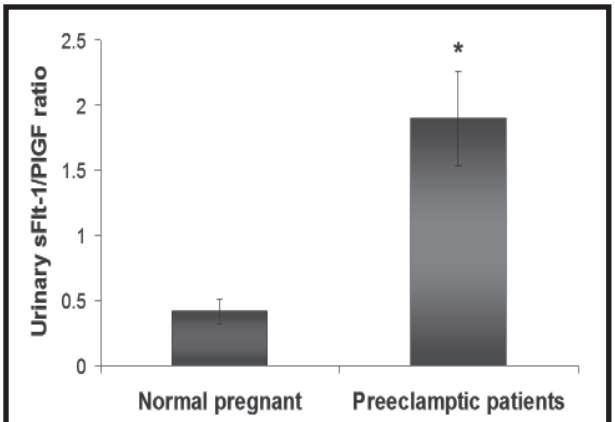


Figure 3. The mean Urinary ratio (± SE) of s-FLT-1/PlGF in normal pregnant (NP, n=40) and preeclampsia (preE, p=30) patients. The urinary ratio of s-FLT-1/PlGF is the indicator of angiogenic imbalance . The mean urinary ratio of s-FLT-1/PlGF in the preE group is significantly greater than that in the NP group (*p<0.05)

In this in vivo study, during one breeding season, 8 female squirrel monkeys will be identified in early pregnancy with gestational sacs, prior to appearance of the fetal pole with beating heart. They will be instrumented with a telemetry device to report blood pressure and heart rate while freely moving during a telemetry recording session in a cage with urine collection capabilities for 3 hours once or twice per week. We will assess the effect of MBG during two intervals in the approximately 20-week squirrel monkey gestation: one in the first trimester (with treatment in weeks 4-7) and one in the second trimester (with treatment in weeks 11-14).

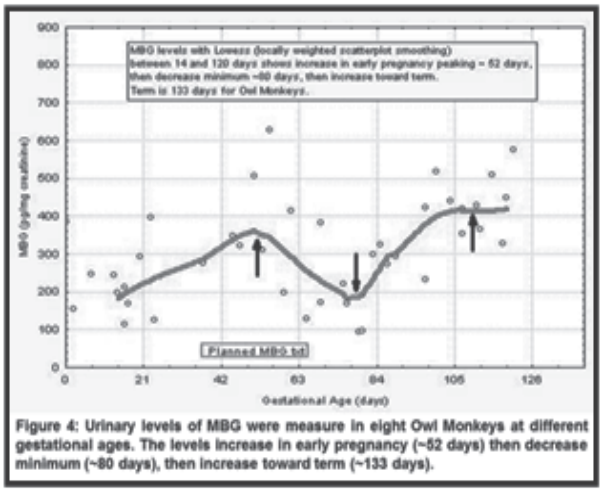


Figure 4: Urinary levels of MBG were measure in eight Owl Monkeys at different gestational ages. The levels increase in early pregnancy (~52 days) then decrease minimum (~80 days), then increase toward term (~133 days).

MBG is obtained from Cayman Chemical (Ann Arbor, MI), with a purity of >98%. Identity and purity will be confirmed by HPLC with MS analysis. Beginning 1 week after instrumentation, a blood sample will be drawn, and the animals (N=3 per group) will be treated with a minipump delivering 1) 7.65 µg/kg body weight / day of MBG in a DMSO/saline vehicle, or 2) vehicle alone for 28 days. For 1 week prior to and for four weeks of treatment, blood pressure and heart rate will be recorded using telemetry twice each week. Urine will be collected during the recording sessions (Table 1). Blood will be collected weekly during this study interval through the time of minipump removal and one week after pump removal. Fetal growth and development will be measured by ultrasound weekly. Following the removal of the minipump, observations will continue for the remainder of pregnancy with telemetry monitoring sessions and urine collections once in every two week and ultrasound assessment of fetal growth at two-week

Table 1: Plan for assessment of MBG as trigger for PE symptoms in pregnant squirrel monkeys

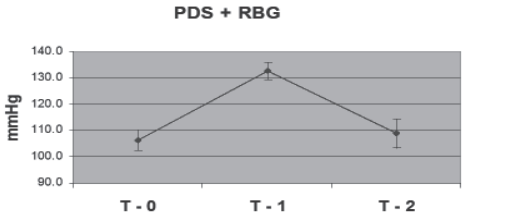
Gestation length		First trimester exposure group					Second trimester exposure group				
Days	Wks	US	Telemetry sessions	Urine	Blood	MBG or vehicle	US	Telemetry sessions	Urine	Blood	MBG or vehicle
0 to 7	1	X					X				
8 to 14	2	X					X				
15 to 21	3	X	2	X	Day 0		X	2	X		
22 to 28	4	X	2	X	Day 7	X	X	1	X		
29 to 35	5	X	2	X	Day 14	X	X	1	X		
36 to 42	6	X	2	X	Day 21	X	X	1	X		
43 to 49	7	X	2	X	Day 28	X	X	1	X		
50 to 56	8	X	2	X	Post-txt		X	1	X		
57 to 63	9		1	X				1	X		
64 to 70	10	X	1	X			X	2	X	Day 0	
71 to 77	11		1	X			X	2	X	Day 7	X
78 to 84	12	X	1	X			X	2	X	Day 14	X
85 to 91	13		1	X			X	2	X	Day 21	X
92 to 98	14	X	1	X			X	2	X	Day 28	X
99 to 105	15		1	X			X	1	X	Post-txt	
106 to 112	16	X	1	X			X	1	X		
113 to 119	17		1	X				1	X		
120 to 126	18	X	1	X			X	1	X		
127 to 133	19		1	X				1	X		
134 to 140	20	X	1	X			X	1	X		
141 to 147	21		1	X				1	X		
Term assessment of gestation length, infant size and condition, maternal weight											
Females weighed at 4, 8, 12, 16, and 20 weeks of gestation											

intervals.

Animals (N=3/group) treated during the second interval will be instrumented with telemetry at the same time as the first interval group, and their telemetry and urinary measures will serve as an additional control for manipulations. Similarly, the control group for the first interval can serve as an additional control for the treated animals in the second interval. During each interval, three animals will be treated with vehicle, and three will be treated with MBG. Plasma from blood samples will be assayed for MBG levels, angiogenic and anti-angiogenic factors (VEGF, PIGF, TGF-B, sFlt-1, and sEng). Urine will be assayed for protein levels, creatinine, MBG, and angiogenic factors.

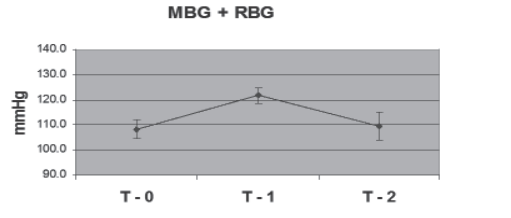
We anticipate that MBG will be increased in urine and blood during MBG treatment and that blood pressure will be increased. Proteinuria and fetal growth effects may not be altered by these short-term exposures. However, if MBG acts as a trigger, and provided 4 weeks of treatment is sufficient to create vascular injury, then we expect that effects will continue, leading to a worsening of preE features as pregnancy progresses. We anticipate that early treatment will be more effective than late treatment for this reason. Table 1 provides the schedule for animal manipulations in this experiment. The effect sizes for blood pressure, MBG levels, and angiogenic factors are expected to be large based on experience with pregnant rats, so that repeated measures of a small number of pregnancies will likely be sufficient to detect differences. Data will be expressed as means with SE, and results will be analyzed using analysis of variance with repeated measures. Nine pregnant squirrel monkeys will be used in this study during one breeding season. A pitfall of this study is that MBG at the dose extrapolated from previous rat experiments may not be sufficient to produce significant preE features in the squirrel monkey during or after the 4-week exposure or that MBG may not trigger a worsening condition. If this is the case, a power analysis using data from the initial experiment can be used to focus on a greater dose or longer exposure depending on how the blood levels of MBG compare to those measured in patients with preE. If effects are more prominent at one exposure time compared to another, this time will be used in a second experiment during a second breeding season with another group of nine pregnancies.

Compare the ability of RBG and anti-MBG monoclonal antibody to attenuate the effects of MBG in the squirrel monkey model. Our hypothesis is supported by materials cited above (A. Significance) and by the observation that RBG corrects the BP in PDS and MBG-infused hypertensive rats. When RBG was given early in pregnancy, it completely prevented all of manifestations of preeclampsia noted in our rat model, including IUGR and oxidative stress (Fig. 5: Horvat at al., 2009, Uddin et al. 2010a; 2010b; 2011). When given to pregnant rats rendered hypertensive by the administration of MBG, RBG resolves the hypertension (Fig. 6). Preliminary studies with a preE rat model: Female Sprague-Dawley



Ave BP	1063	132.5	108.8
St. Error	3.8	3.2	5.5
		p < 0.05	p < 0.05

Figure 5. RBG administration corrects the blood pressure (BP) in preeclamptic rats (PDS). T-0= initial; T-1= 7-10 days of pregnancy; T-2= final BP.



Ave BP	108.3	121.7	109.6
St. Error	4.2	4.2	3.2
		p < 0.01	p < 0.01

Figure 6. RBG resolves the BP in pregnant rats rendered hypertensive by the administration of MBG. T-0= initial; T-1= 7-10 days of pregnancy; T-2= final BP.

rats were mated with same-strain male rats, and pregnancy confirmed by the presence of vaginal plug. Pregnant rats were divided into 4 groups: group I = normal pregnant rats (NP); group II = pregnant rats that received deoxycorticosterone acetate (DOCA; 12.5 mg was given in a depot form (i.p.) in the beginning of the experiment, followed by a weekly injection of 6.5 mg), and whose drinking water had been replaced with 0.9% saline (PDS, 'pre-eclamptic' = pregnancy + DOCA + saline); group III = normal pregnant rats given daily injections of MBG (7.65 µg/ kg/animal weight/day) once pregnancy was established on day four of the experiment (NPM= normal pregnant + MBG). The group IV=PDS rats were injected with anti-MBG-P antibody, 2.2 mg/kg/d; on the 16th, 17th, or 18th day of pregnancy; PDS-MAB= PDS + anti-MBG antibody-P (3E9), n = 10.

Blood pressure was measured by the tail-cuff method (IITC Inc., LifeScience Instruments, Model 59). At day 19 of pregnancy, 24h urine collection was obtained. Animals were euthanized on day 19, and pups were counted, weighed, and examined. The group parameters are shown in Table 2. Vu et al. administered an intravenous dose of MBG antibody (antiserum MBG-P, Ab, 2.2 mg/kg) in PDS rats on the 16th, 17th, or 18th day of pregnancy (Vu et al., 2005; 2006).

If a preE syndrome is triggered, we will use the optimal interval for MBG treatment in a second series that divides MBG-treated pregnancies into three groups (N=3/group): 1) treated with MBG as in the first experiment, 2) given RBG at 30 µg/kg body weight / day in a set of daily injections beginning prior to implantation of the minipump delivering MBG, and 3) given anti-MBG antibody at 1 mg/kg body weight / day in daily injections as for RBG. With data from Specific Aim 1, it will be possible to perform a power analysis to better predict the needed sample size and to estimate the effects of these manipulations on pregnancy losses. If a progressing preE syndrome is not identified using short exposures, but short-term exposure is sufficient to induce preE features such as hypertension, the potential of RBG to block these effects can be measured. The treatment intervals and sampling schedule for this specific aim will be similar to that used in the first experiment (Table 1), and again the entire pregnancy will be evaluated to outcome variables.

Table 2. Blood pressure (BP), 24h urinary protein, pup number and percentage of abnormal pups in different groups of rats

Groups	BP (mmHg) Initial	BP (mmHg) Final	Protein (mg/24h)	Number of Pups	Percent of Malformed Pups
NP (n=10)	103±5	98±7	2.8±1.1	14.0±1.8	0
PDS (n=10)	101±8	141±9*	5.7±1.6*	10.2±1.5*	16*
NPM (n=10)	104±4	137±5*	6.4±1.8*	9.5±1.3*	18*
PDS-MAB (n=10)	102±8	100.8±8	3.1±0.9	13.6±2.1	0

The final (17-19 day of gestation) BP of PDS and NPM rats were higher compared to initial BP. However, there was no BP change in NP and PDS-MAB rats. The 24h urinary protein was significantly higher in PDS and NPM rats compared to NP and PDS-MAB. Number of pups decreased in PDS and NPM rats. The percentage of malformed pups were higher in PDS and NPM rats, however, there was no malformed pup in NP and PDS-MAB rats (*p<0.05 for all).

Use of telemetry to measure blood pressure and heart rate throughout pregnancy. In each pregnant female, a radio frequency device will be implanted for independent monitoring of blood pressure and heart rate. The devices from Data Sciences International (St. Paul, MN) as described by Watson, et. al. (2007) for the rat will be used in the squirrel monkey. Briefly, animals (about 800 g) are anesthetized with ketamine-xylazine. Then, a transmitter is implanted (s.c.) in the back. An attached pressure catheter is placed in the aortic lumen via the right carotid. Animals are allowed to recover from surgery for one week before treatments are initiated. Telemetric baseline blood pressure and heart rate are recorded during 3-hour sessions in a single cage twice each week. Measurement of the following six parameters occurs every 1 min. using the DataQuest System (Data Sciences): systolic blood pressure, diastolic blood pressure, mean blood pressure, heart rate, and activity level. These data are maintained in computer files as for each session for each animal (Fig. 7).

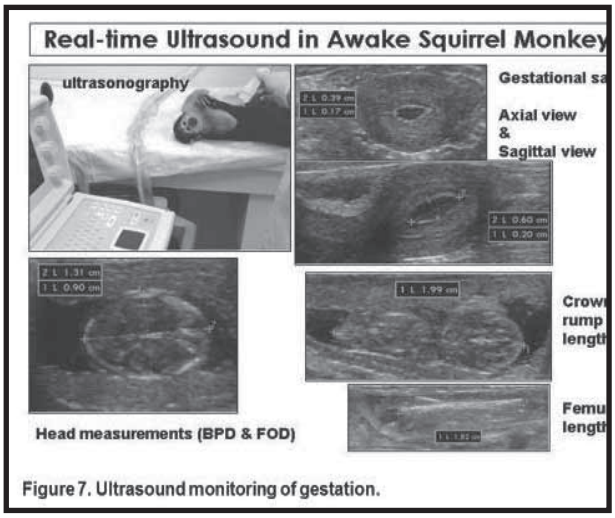


Figure 7. Ultrasound monitoring of gestation.

Measurement of MBG: Blood and urinary levels of MBG are measured by competitive ELISA according to the method most recently described by Abi-Ghanem et al. (2011). Urine samples are analyzed unprocessed, except for dilution with StartingBlockTM buffer. In contrast, serum samples are purified by solid phase extraction (SPE) on C18 Empore Disk Cartridges (Varian, Palo Alto, CA). The disk is first conditioned with 150 µL of acetonitrile, centrifuged at 280 x g for 1 min., then washed with water and centrifuged as above. The serum sample (300 µL) is then added to the disk with an equal volume of 10% acetonitrile in water, and centrifuged as above. Interfering substances are removed by sequential washes with Milli-Q water and 10% acetonitrile, and the sample is twice eluted with 150 µL of acetonitrile. Samples are vacuum-dried and reconstituted in 100 µL of StartingBlockTM buffer. Diluted urine samples and extracted serum samples are run in duplicate, and average MBG concentrations (pg/mL) are extrapolated from the standard curve using the StatLIA software.

Measurement of angiogenic factors: The angiogenic factors VEGF, PIGF, TGF-B, sFlt-1, and sEng are measured in the plasma and urine of the animals using commercially available kits (R&D Systems, Minneapolis, and Assay Designs, Ann Arbor, MI) at various time points of gestation (Agunanne et al., 2010).

Measurement of protein, creatinine, and other agents: Urine protein excretion is measured using the pyrogallol red method (Total Protein Kit, Micro Pyrogallol Red Method, Sigma). Each animal is housed separately in a metabolic cage. Creatinine is measured in blood and urine on a Nova 16 Analyzer (Waltham, MA), and the creatinine clearance (CCR) is calculated. Hematocrit is measured using a StatSpin MP Multipurpose Centrifuge (Norwood, MA) (Uddin et al, 2009a).

Selection of females, randomization, and baseline evaluations. Up to 9 pregnant adult females enrolled during each breeding season (December through April of each year). Females will be randomly assigned to treatment subgroups using a sealed envelope system. All will be weighed and evaluated with real-time ultrasound to verify normal anatomy and obtain baseline measurements of gestational sac for dating gestations.

Breeding, identification, and dating of pregnancies using real-time ultrasound. Squirrel monkey females are seasonal breeders with the breeding season for the colonies in central Texas running from December through April. Beginning in December each year, females enrolled in the trial will undergo ultrasound evaluation at weekly intervals until a pregnancy is identified (typically as a gestational sac, as seen in the figure). Then, pregnant females will be examined weekly during the administration of agents and each two weeks thereafter until term at about 20 weeks gestation. These exams are performed while females are briefly manually restrained. A typical exam takes 2-3 min., and images are documented. In the past three years, we have obtained these measures and developed growth curves for the squirrel monkey with induced or identified ovulation dates. These are shown in the growth curves from 45 pregnancies (Fig. 8).

Potential pregnancy interventions in the trial. The University of Texas MD Anderson Cancer Center animal care and use program has experience addressing the occasional need for surgical delivery, post-operative care, infant re-introduction, nursery rearing if needed, and management of spontaneous labor and delivery in a

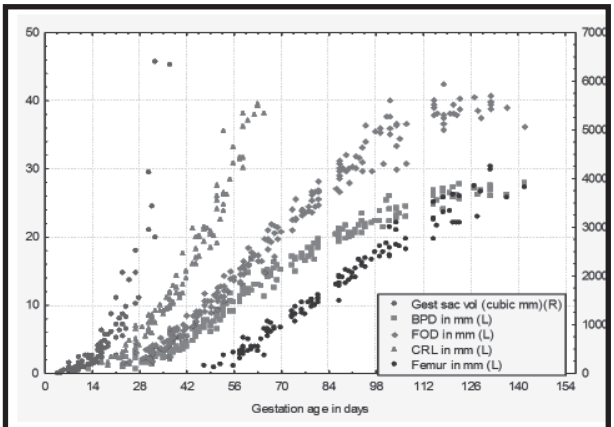
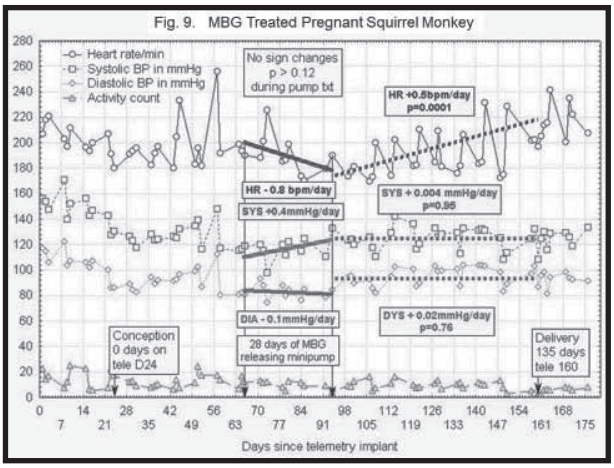


Figure 8. Ultrasound monitoring of 45 squirrel monkey pregnancies.

squirrel monkey breeding colony. The infants born in this study will be measured and evaluated. They will be made available for other research uses. C-section for clinical purposes is utilized in the squirrel monkey and subsequent pregnancy and delivery has been successful. As squirrel monkeys deliver large infants, malpresentation and maternal exhaustion can result in the need for clinical use of surgical delivery. If this occurs, animals will be cared for as appropriate by the animal facility veterinarians. As animals may develop more severe pregnancy complications including severe edema or even progression to seizures related to preE due to MBG treatment, clinical interventions including C-section to end pregnancy may be needed. Veterinarian oversight will be provided throughout the course of this trial to minimize threats to maternal well-being.

MBG treatment in pregnant squirrel monkey: We have measured the BP by telemetry device at different gestational ages of squirrel monkey. The data have shown in Fig. 9.



Squirrel Monkey Spontaneous Model of Preeclampsia: As shown in Fig. 10, the spontaneous model of squirrel monkey and its physical and biochemical features have been listed.

Ethical aspects and research material sharing for the proposed research. This research proposal uses pregnant squirrel monkey females and their fetuses with a minimum number of animals per group to obtain valid results. The acquisition, care, and use of animals will be in accordance with NIH Guidelines in an AAALAC-accredited institution following approval from the University of Texas MD Anderson Cancer Center IACUC. All applicable federal, state, and local laws and regulations will be followed. The most appropriate and humane form of euthanasia will be used, although this study is not planned as a terminal study. Wherever applicable, the research protocol will be reviewed and approved by the institutions' biohazard committees, although no Select Agents or recombinant genetic material is planned for use in these trials. Where possible, agents available in pharmaceutical grades for human clinical use will be utilized. Data, images, term infant monkeys, and fetal/placental tissues for monkey pregnancies, if produced, will be made available to the local research community.

Figure 10. Squirrel Monkey Spontaneous Model of Preeclampsia

- The face of preE in Squirrel monkeys.
- 2. We have 13 cases in 20 pregnancies.
- 3. sFlt-1/PlGF ratio of preE monkeys are higher than NP indicating angiogenic imbalance.
- 4. Proteinuria.
- 5. Limitation: No BP data.

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Impact of avian oviduct environment on preservation of mammalian spermatozoa

Ahmed M. Hanafy

Department of Animal Production, Faculty of Agriculture, Suez Canal University, 41522 Ismailia, Egypt-

College of Grassland Science, Gansu Agricultural University, Lanzhou, Gansu province, China

SUMMARY:

The unique features of avian reproduction include the secretion of the uterine fluid (UF) and the presence of specialized sperm storage tubules in the female oviduct. Numerous studies have demonstrated that the oviduct provides suitable environment for the resident sperm without the loss of their viability and fertility. It is reasonable to elucidate whether deposition of mammalian spermatozoa in the hen oviduct affects on extension the functional lifespan of sperm. Therefore, fresh rabbit semen were artificially inseminated into the oviduct of laying quail to examine the effect of its environment on migration and preservation of the sperm over an extended period. Following 0, 10, 30, 60, 90, and 120 min of artificial insemination, the oviduct of quail was dissected from the body cavity and cut into four parts. The individual segments of the oviduct were excised longitudinally, followed by washing with saline. The mucosal layer of each part was scraped using a scalpel. The viability of sperm in the washing buffer and mucosal layer was examined under a phase contrast microscope. Generally, the secretion of avian oviduct may prolong the survival rate and influence function of the mammalian sperm. This study has the potential for further development of reliable commercial extenders that contributes to livestock production and welfare at some time in the future.

Introduction

Artificial insemination (AI) is a powerful tool mostly utilized for livestock reproductive improvement. It is the most common technique in which collected semen can be diluted and mixed with an extender to generate hundreds of doses from a single ejaculate. Once the semen is diluted, it can be preserved for short periods (3-5 days) at 5 °C or for long periods of time (years) in gradually cooled to -195.6 °C using liquid nitrogen (Chupin and Thibier 1995). Recently, several extenders have been developed for liquid storage of semen, but none of them can store spermatozoa for more than 3 days without a decline in fertility (Vyt et al., 2004; El Azazzi and Hanafy 2015). Even though conventional extenders are also used to prevent cell membrane against damages during cryopreservation, addition of glycerol causes significant deteriorates in motility and viability of the sperm (Aires et al., 2003).

Avian, reptile, amphibian and some fish species are renowned to have a remarkable capability to store spermatozoa in female reproductive tract and sustain their fertility for long periods, up to months and years, dependent on species. Numerous studies have demonstrated that the oviduct of birds provide an environment appropriate for the resident sperm to maintain their survival and fertility potentials over prolonged periods. Particularly, their secretions of uterine fluids (UFs) are composed of energy source, mainly glucose, fatty acids and complex mixture of organic and inorganic salts (Ahammad et al., 2013; Huang et al., 2016). These UFs have been found to modulate motility, extend lifespan and maintain fertilizing capacity of the sperm throughout maintaining pH and providing a nutritive protective aqueous environment in the oviduct (Edward and Leach 1980; Holm et al., 2000; Ahammad et al., 2013).In addition, attachment intimate of sperm within an anatomical structure, sperm storage tubules (SSTs), in the female reproductive tract is the most important factor for attaining prolonged sperm storage. Inside SSTs, blind-ended reservoir, the lifespan of fertile sperm is considerably longer, up to 3-5 weeks in domestic fowl and 10-15 weeks in turkey, depending on the respective numbers of SSTs (Bakst et al., 2011). Most of the SSTs are predominant within the utero-vaginal junction (UVJ) mucosal folds that constitute a primary storage site (Bakst 1998). The secondary sperm storage location in the oviduct is situated within the distal infundibulum which is characterized by sub-epithelial tubular gland (Fujii and Tamura 1963). Although the exact approach by

which spermatozoa are preserved in the avian oviduct has not been completely clarified, abundant studies have been carried out to evaluate the environmental features of the oviduct and concluded their utility on resident sperms. Therefore, the aim of this study is to determine the role of quail oviduct environment on sperm survival of mammalian species. Three questions were postulated as follows: (1) whether rabbit sperm able to migrate in the oviduct of quail; (2) whether motility and viability of deposited sperm affects by environment of quail oviduct; and (3) whether the mammalian spermatozoa can bind within SST of the avian oviduct.

Material and Method

Animals

Mature female Japanese quails (*Coturnix japonica*), aged 20 weeks with proven fertility, were used in this study. At least one month before insemination, eighteen female quails, close in body weight (251.91±2.11g) and egg production rate (76.00±1.14%), were separated from male cages and maintained individually under photoperiod regimen of 16L: 8D. Experimental birds were fed laying ration (20% crude protein (CP) and 2900 kcal ME/kg) and supplied with water *ad libitum*.

One-year-old New Zealand White rabbit (*Oryctolagus cuniculus*) bucks (3.98 ± 0.1 kg body weight) were used for semen collection. Two mature bucks were housed in flat-deck cages with semi-opened system. Animals were fed a commercial standard diet that contains 18 % CP and 2700 kcal ME/kg. Drinking water was automatically offered freely.

Semen collection and evaluation

Fresh semen of rabbit bucks with normal sperm quality (Table 1) was collected by an artificial vagina. Immediately after collection, good quality ejaculates were mixed together and treated as one pooled semen sample. Sperm motility was assessed by visual estimation using a phase contrast microscope (Olympus, Japan). The sperm concentration was determined using a Bürker counting chamber and light microscopy at 400× magnification. For determination of progressive motility, a drop of 10 μl of semen was delivered onto a clean glass slide, covered with a cover slip, and estimated at 600× magnifications. Live/dead spermatozoa were examined on Eosin-Nigrosine-based stains smears using a phase contrast microscope. Prior to insemination, spermatozoa were kept at room temperature in darkness.

AI of female quail

For AI, 0.1 ml of undiluted fresh semen of rabbit was intra-vaginally introduced to the cloaca of female quail using a plastic dropper. At the time of AI, the female with the presence of an egg in the uterus, ascertained by palpation, was unused. Following 0, 10, 30, 60, 90, and 120 min of a single insemination, the oviduct of the female quail (n=3 per each time) was removed from the body cavity after slaughter and cut into four segments, infundibulum, magnum, isthmus and uterus. The individual parts of oviduct were excised longitudinally, followed by washing for two times with 10ml of the isotonic solution (0.9% NaCl) in Petri dishes. The washing solution was collected and centrifuged at 800× g for 5 min to precipitate and the final pellet was then resuspended in 1 ml of saline solution for spermatozoa observation. The mucosal layer of each segment was scraped using a scalpel. Viability of the sperm in washing buffer and in mucosal layer was examined under a phase contrast microscope at 600× magnification.

Results and Discussions

Sperm motility and viability are the main factor affecting sperm function for migration in the oviduct. As shown in Table (1), all obtained values of ejaculate volume, sperm cell concentration, progressive motility, and sperm viability of pooled ejaculates immediately before AI were in normal ranges of rabbit semen characteristics.

Table (1). Means ± SE of initial characteristics of semen samples collected from NZW rabbit bucks.

Parameters	Mean ± SE
Ejaculate volume (ml)	0.58 ± 0.10
Concentration (× 10^8/ml)	3.94 ± 0.21
Progressive motility (%)	78.18 ± 0.72
Sperm viability (%)	93.01 ± 0.59

SE= Standard error

In this study, the ability of mammalian spermatozoa to navigate and survive within avian oviduct has been observed. As shown in Table (2) and Fig (1), motile sperms were observed in the uterus and isthmus 10 min after AI. No sperm could be found in the uterine cavity 60 min after AI, demonstrating the importance of the oviduct cavity for assisting the transport of rabbit sperm into the infundibulum of quail. From this result, it could be proposed that the uterine fluids (UFs) did not harm the viable and motile sperm of rabbit (Ahammad et al., 2013). While immotile sperms were detected in uterus and isthmus after 30 min, it was still survived in magnum and infundibulum. After one hour of AI, motile sperm has only been observed in the infundibulum, but

immotile sperm was detected at the lower parts of the oviduct. Although immotile sperms were found in the magnum and infundibulum at 90 and 120 min of insemination, no motile or immotile sperms were observed in the other segments of the oviduct. These results showed that within a few minutes of semen deposition, rabbit sperm begin to migrate into the upper parts of the quail oviduct. Although spermatozoa oriented themselves in the oviduct, it is not yet known whether thermotactic, mechanotactic and/or chemotactic mechanisms guide sperm to transport throughout the oviduct. It has been reported that rabbit sperm tend to swim towards warmer temperatures in the reproductive tract of inseminated does (Bahat *et al*., 2003).

Table (3). Description of presence and viability of rabbit spermatozoa in mucosal layer collected from uterus and infundibulum of quail oviduct after AI.

Time(min)	Uterus	Infundibulum
0	N	N
10	N	N
30	M	N
60	M	M
90	M	M
120	M	M

M= motile sperm and N= no sperm was found.

It is worthy noted that SST not only act as a sperm storage site but may also play a role in selection of sperm that will be preserved. Based on the present findings, most of motile sperm which identified in the washing solution appear to be abnormal motile spermatozoa. This finding doesn't eliminate the possibility of sperm selection in the storage sites. Moreover, intact acrosome membrane was observed 1 h after AI in infundibulum part of the oviduct (Fig. 1). The percentage of spermatozoa that able to keep the intact acrosome can give an indication on the fertilizing capacity of sperm (Richardson et al., 1991). Prepare rabbit sperm to undergo the acrosome reaction relatively slowly is a significant factor that necessary for fertilizing success.

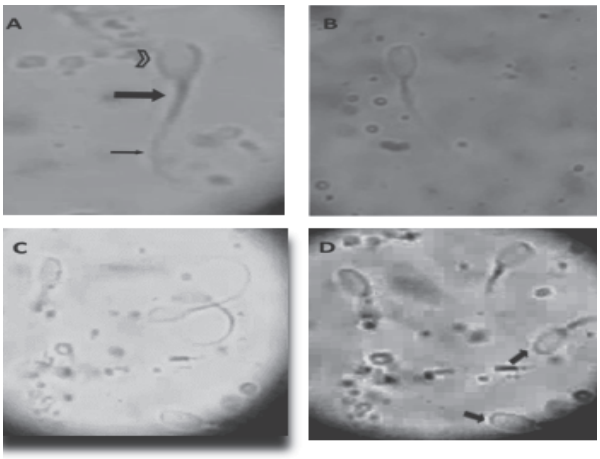

Fig 1. Scanning light microscope showing motile (A) and immotile (B) sperm of rabbit in washing buffer of oviduct segments after AI. After AI, uterus (C) and infundibulum (D) of female quails were washed at 10 and 60 min, respectively; and motile sperm was observed by a phase contrast light microscope at 600× magnification. While arrowhead, thick and thin arrows in A were showed the head, midpiece and flagellum of the sperm, respectively, small arrows in D were identified the acrosome area.

Table (2). Description of presence and viability of rabbit spermatozoa in washing buffer collected from segments of quail oviduct after IA.

Time	Uterus	Isthmus	Magnum	Infundibulum
0	N	N	N	N
10	M	M	N	N
30	IM	IM	M	M
60	N	IM	IM	M
90	N	N	IM	IM
120	N	N	IM	IM

M= motile sperm; IM= immotile sperm and N= no sperm was found.

To our knowledge, no information is published before on the viability and motility of mammalian spermatozoa entering the female reproductive tract of birds. Therefore, it might be interesting to evaluate whether rabbit sperm can bind within SST pockets of female quails and consequently prolonged flagellar activity and viability of spermatozoa. As shown in Table (3) and Fig (2), rabbit spermatozoa have the ability to interact and intimate associate within SST in the uterus and infundibulum. In contrast, no sperm storage site could be detected in the isthmus or magnum. The distribution of bind spermatozoa was higher in the infundibulum than the uterus segment 2 h after AI. Nevertheless, all heads of the sperm that were detected in the segments were extremely embedded in the SST, which allowed only the motile tails of these spermatozoa to be clearly visible. While mammalian sperm interacts with the surface of epithelial cells at the utero-tubal junction (Rijsselaere et al., 2004); Ito et al. (2011) speculated that resident sperm in the oviduct of quail "seems to be free from the epithelial cells of the SST" and most of them were sluggish. However, it is unclear whether the flagellar activity of buck sperm was related to the characters of sperm or to the technique of the study since the previous authors (Ito et al., 2011) generated an *in vitro* study in quail to determine the flagellar movement in the SST.

Because there are no eggs in the oviduct of used female quails, it is acceptable to presume that introduced sperm can reach the site of fertilization without an obstacle

from the downward egg. There is positive evidence that sperm quantity and motility are crucial for SST permeation (Froman et al., 2013). The number of sperm inseminated in this study may be more than that of native condition (Hanafy and Khalil 2015). Hence, the quantity, as well as quality, of spermatozoa plays a critical role in sperm entering and persistence in the SST. However, how sperm involved within SSTs and under which mechanisms still an unanswered question.

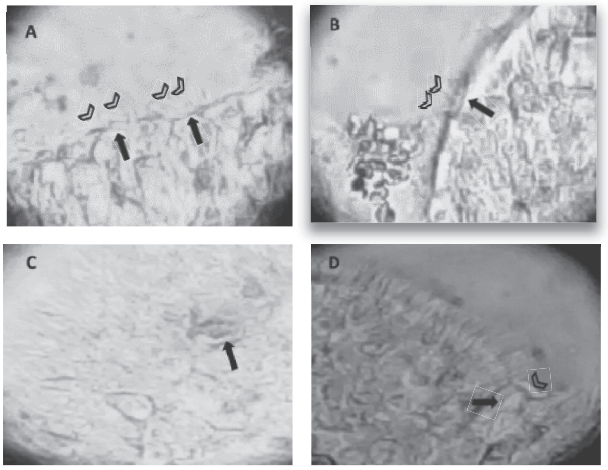

Fig 2. Scanning light microscope showing rabbit spermatozoa attached to SST in infundibulum (A and B) and uterus (C and D) of quail after 2 h of AI. Arrow indicates the SST and arrowhead shows the flagella of resident sperm in the SST. A representative photograph was detected by phase contrast light microscope at 600× magnification.

On the other hand, immune system of female quail is particularly active in the oviduct for protection against microbes and may also attack sperm as foreign bodies (Das et al., 2005b). Normally, oviductal cellular immune responses are suppressed in the SST of chicken to conserve storage sperm (Das et al., 2006b). While oviduct of quail might be considering sperm of rabbit as an unfamiliar body, the invasion may take time to build an effective level of immune response. Indeed, the motile sperm of rabbit may not harm by leukocytes of does that increase after coitus in the reproductive tract of the female (Taylor 1982). Altogether, even though this speculation at present is an imaginative idea; it is at least based on biological findings.

Conclusion

From this preliminary study three conclusions could be withdrawn: (i) 2 h after AI, spermatozoa of rabbit were mainly found in the SST of the uterus and infundibulum, (ii) the environment of oviduct influenced deposited sperm transport in the female genital tract of quail, and (iii) this result appeared to be open a wide discussion more than answering few questions for scientific communities.

Acknowledgement

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Possible roles of the yolk sac as a source of thyroid hormones during embryonic development of the chicken

Hanny Cho Too
Livestock Breeding and Veterinary Department, Myanmar

SUMMARY

In general, thyroid hormones (THs) are essential for the correct development of nearly every structure in the body from very early stages of development, yet the embryonic thyroid gland is not functional at these stages. To clarify the role of the egg yolk as a source of THs, I have analyzed the TH content in the yolk and the expression levels of the D1, D2, D3, TTR, and ALB gene in the same animal throughout the 21-day incubation period to gain a broad-range view of the roles of the yolk sac membrane in processing and supplying THs to the developing embryo. In addition, the expression of MCT8, MCT10, and OATP1C1 gene which are assumed to be responsible for transport of yolk THs in and out of the yolk sac membrane, was determined. To summarize, the expression of D3 and TTR in the earlier period, D2 and OATP1C1 in the later period, and D1, ALB, MCT8, and MCT10 were expressed in both periods of incubation.

Introduction

In all vertebrate species and invertebrate species so far, thyroid hormone (TH) is significant for regular functioning of almost all body's organs. The thyroid contains two hormones thyroxin (T4) and triiodothyronine (T3). T4 is the main hormone released from the thyroid glands and it is transformed into biologically active T3. Intracellular activation or inactivation of T4 and T3 is determined by three types of idothyronine deiodinases such as D1, D2, and D3 (Gereben *et al*., 2008). THs are transported throughout the body via the circulation. The major TH distributor in birds and other non-mammalian vertebrates are transthyretin (TTR) and serum albumin (ALB). TTR is one of the proteins in extracellular fluids, which binds the thyroid hormones T4 and T3. Visser *et al*., 2008 revealed that transport of T4 and T3 in and out of cells is controlled by several classes of transmembrane transporter, including members of the organic anion transporters family (OATP), L-type amino acid transporters (LATs) and monocarboxylate transporters (MCTs). Thyroid hormone transporters are necessary for the uptake of thyroid hormone into target tissues and they regulate intracellular T3 levels in a tissue-specific way. But the most important enzymes for TH metabolism are the iodothyronine deiodinases. The early embryo does not have a thyroid function and therefore it depends on the THs supplied by maternal hormone effect on the yolk of the chicken (Stijin *et al*., 2012). Some studies indicated that thyroid hormones are present in the egg yolk of chicken and quail but most of studies are focused on the late period of incubation. Thyroid hormone bound to yolk lipoproteins and enters embryo with non-specific uptake of yolk or by specific carrier (Mcnabb *et al*., 1997). In mammals, thyroid hormone reaches to the embryo by the potential effects of maternal thyroid hormones without crossing the placenta but in rats, some amounts of thyroid hormones do across the placenta (Fisher *et al*., 1977; Roti *et al*., 1983). Some studies indicated that thyroid hormones are present primarily in the eggs yolk of chicken and quail, 4-6 ng T4/g and 1.5-2.5 ng T3/g of yolk ((Mcnabb *et al*., 1997). Therefore, the objectives of my study are to observe the changes of iodothyronine deiodinase mRNA expression, carrier proteins and TH transporters

for transporting THs to the embryo before and after the onset of thyroid function. In addition, yolk TH content was examined by enzyme immuno assay.

Material and Method

Animals and Sampling

Fertilized eggs from layer strain (Hy-line) chicken were purchased from Japan Layer K.K., Gifu, Japan. The eggs were incubated at 37.8 ℃ and 70% relative humidity. The day of onset of incubation was labeled embryonic day 0 (E0). All the embryos (chicks) were piping with their beaks outside the eggshell at the time of E21 sampling. The yolk sac membrane was sampled daily from E4 embryos to 3-day-old chicks (C3) (n=7-9). The membrane was rinsed thoroughly with sterile phosphate-buffered saline (PBS) with several changes to remove remaining yolk, put on a piece of filter paper to absorb excess PBS, weighed on an electronic balance, and then immediately frozen in liquid nitrogen and stored at -85℃ until RNA extraction. The yolk contents were collected every other day (n=5), put into plastic tubes, weighed on an electronic balance, and stored at -30℃ for measuring the yolk TH content. All animal manipulations were approved by the Animal Research and Welfare Committee at Gifu University.

Quantification of mRNA expression

Total RNA was extracted from frozen yolk sac membrane using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNase-free DNase I (gDNA Remover, TOYOBO Co. Ltd., Osaka, Japan) was used to eliminate genomic DNA according to the manufacturer's instructions. Total RNA (1μg) was then reverse transcribed into first-strand cDNA using ReverTra Ace (TOYOBO) and a random primer (TOYOBO). The mRNA levels were determined by real-time PCR, performed in an Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA) with the two-step standard cycling program for the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent).

Measurement of yolk THs by enzyme immunoassay

THs were extracted from the frozen yolk using the methanol/chloroform extraction procedure of Wilson *et al*., 1997. T3 and T4 were measured using enzyme immunoassay kits (Immunospec Corporation, Canoga Park, CA, USA). According to the manufacturer's document, the cross-reactivity with related compounds other than T3 and T4 is less than 1.5%. The linearity of the yolk extract and the serum sample to the standard curve was confirmed by measuring serially samples with the assay buffer provided in the immunoassay kit. The inter-assay variations were 6.2-12.4% and 3.8-12.9%, respectively.

Results and Discussions

The present results indicated that the yolk weight decreased significantly and almost linearly during the incubation period (Fig. 1). As shown in Fig. 1, the total yolk TH content decreased in comparable tendency, and thus the yolk is likely to supply its components, including THs, to the developing embryo. The present study showed that the expression of TTR, whose protein has high affinity for THs, was very low in the yolk sac membrane from E13 onwards (Fig. 2B). A possibility is that ALB, whose gene expression increases between E1 and E18, contributes to TH distribution to the embryo in turn (Fig. 2B). These results seem to indicate that the yolk contains a sufficient amount of THs that can be supplied to the embryo even after the onset of thyroid function. It is assumed that TH inactivating D3, whose gene was expressed in almost the same period as TTR and ALB E14-E12, could regulate the amount of TTR and ALB-borne T3, thereby preventing unfavorable premature TH signaling in the earlier embryo. The expression of D2, the TH-activating deiodinase gene, was high between E14 and E21. This period corresponded to the increase of circulating T4, beginning at around E14 and reaching its peak on E20. The expression of OATP1C1, the gene of transmembrane transporter, was also high at around this period (Fig. 2C). It may be responsible for transporting plasma T4 into the yolk sac epithelial cells. The D1 gene expression was expressed almost throughout the 21-day incubation period (Fig. 2A). Thus, D1 in the yolk sac membrane could contribute to the recycling of iodine (by 5' –deiodination of rT3 to

T2) that cannot be supplied from outside the egg, rather than to the activation of T4 to T3.

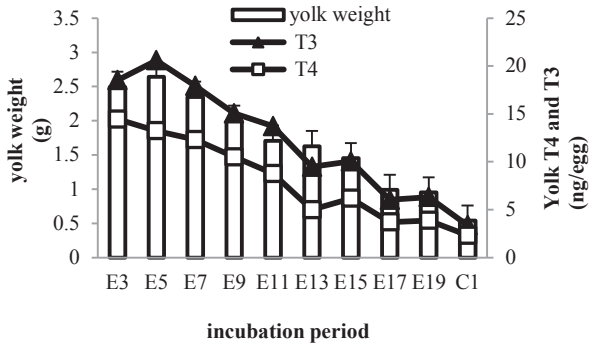

Fig.1. Yolk thyroid hormone content during embryonic development of the chicken by enzyme immune Assay. Each data presents mean ± SEM (n=5).

Conclusions

First, present results have confirmed previous observations that maternal THs in the yolk are available to the embryo (Mcnabb *et., al.* 1997). Second, this study has shown that yolk THs could be metabolized before their distribution to the embryo or action in the yolk sac membrane. A part of the maternal THs contained abundantly in the yolk is probably inactivated by D3, preventing unfavorable premature T3 signaling. The resulting rT3 could easily be deiodinated further by D1, which can be contributed to the recycling of iodine. The present study suggests that the yolk sac membrane not only transfers yolk THs to the embryo but also activates THs, thereby meeting the demands of the developing embryo and the yolk sac membrane.

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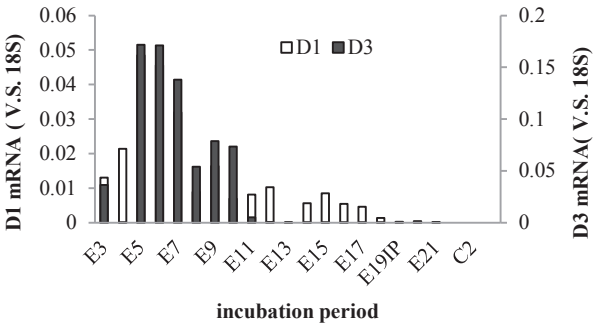

Fig .2 (A)

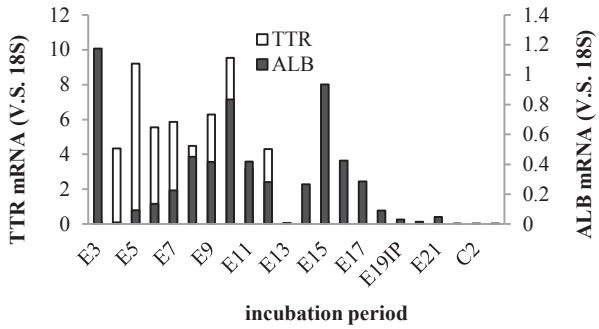

Fig. 2 (B)

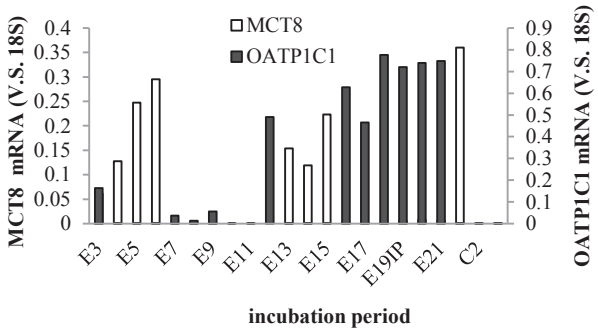

Fig. 2 (C)

Fig.2. D1, D3, TTR, ALB, MCT8 & OATP1C1 mRNA expression levels in the yolk sac membrane during embryonic development of the chicken by RT-PCR. (18S was used as an internal control) (IP=internal pipping) Each data presents mean ± SEM (n=7).

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Comparative studies of the morphology of oviduct of pre-laying, laying and regressing chickens from indigenous, sonali and RIR breeds (*Gallus domesticus* L.)

Md. Anisur Rahman*, Mst. Shamima Khanam and Mst. Sabiha Hossain
Department of Zoology, University of Rajshahi, Bangladesh. *Email: ar_zool.uk@fastermail.com

SUMMARY

The morphological measurements of length (cm) and weight (gm) of total oviduct from each reproductive stage (pre-laying, laying and regressing) of every breed (indigenous, sonali and RIR) were determined. The lengths (mean ± sd) of total oviduct for indigenous breed were 22.63±7.57cm (pre-laying), 50.76±2.17cm (laying), 44.93±9.37cm (regressing); for sonali were 9.53±1.05cm (pre-laying), 73.16±6.40cm (laying), 53.76±5.67cm (regressing) and for RIR were 13.53±0.73cm (pre-laying), 91.26±4.23cm (laying), and 83.93±2.48cm (regressing). The weights (mean ± sd) of total oviduct were 4.56±1.55gm (pre-laying), 32.30±1.94gm (laying), 19.83±7.65gm (regressing) for indigenous; 2.77±0.63gm (pre-laying), 51.53±7.52gm (laying), 27.90±13.64gm (regressing) for sonali and 3.70±1.90gm (pre-laying), 93.26±6.39gm (laying), and 59.30±2.44gm (regressing) for RIR breeds, respectively.

The mean values (F) of length of oviduct of each stage from different breeds were significantly different ($F_{3,16}$=31.49, pre-laying; $F_{3,16}$=12.29, laying; and $F_{3,16}$=11.06, regressing; (P<0.05)). Similarly, the mean values (F) of weight were significantly different ($F_{3,16}$=43.52, pre-laying; $F_{3,16}$=7.44, laying and $F_{3,16}$=13.50, regressing; (P<0.05). The reproductive fitness in chicken is assessed by the examination of oviductal parameters and this study is very much important in reproductive biology for the commercial egg and meat production through improved breeding plans essential to conserve the endangered birds from their extinction.

Introduction

Rapid growth of human population is a burning issue in developing countries like Bangladesh. The insufficient animal protein supply to increasing populations necessitates the increase of animal productivity which is on the other hand very much important for the conservation of endangered animals considering animal and human welfare. Poultry meat contributes 37 percent of total meat production and 22 to 27 percent of the total animal protein supply in the country (FAO, 2003). Annual per capita egg consumption was 32 eggs (Das et al., 2008), reached its highest level of 48 eggs in 2012 with a deficit of 53.85% (FAO, 2015) compared to the minimum requirement of 104 eggs/head/year and meat consumption was 2.92 kg/year to the minimum 7.67 kg/year (Begum, 2008). To reduce the deficiency, the gap between the continuous demand and short supply of animal proteins could be minimized through increasing chicken egg and meat production. The chicken egg and meat are used as an alternative among other sources of animal proteins because of their easy availability, low cost, high nutritional values and arbitrary choice.

The oviduct is a highly complex and dynamic organ with its coiled tubular structure in the reproductive system of avian species where ovulated ovum either being fertilized or not developed into egg. During the long journey through the entire oviduct, constituents of egg from the infundibulum, magnum, isthmus, uterus and vagina (Rahman, 2013) are sequentially secreted and deposited as layers around the ovulated ova (Rahman et al., 2007) is a necessary prerequisite for ′normal′ egg formation (Solomon, 2002). This egg production is of special interest to the commercial egg industry or producers (Chousalker and Roberts, 2008). Tremendous changes in the size of the chicken oviduct occurred during laying stage (Yu et a., 1972) indicating functional and healthy oviduct which is essential for both quantity and quality of egg (Saino et al., 2002). So, the morphological knowledge of reproductive tract is very much essential to the commercial producers and breeders for the production of egg and meat productive chicken. The disrupted activity or pathological changes can alter or deviate the function of the oviduct of a laying hen directly affects egg and eggshell quality (Chousalker & Roberts, 2008). Decline in egg and egg shell quality costs the egg industry millions of dollars every year (Abdel-Rahman et al., 2016) and ultimately decrease economic profitability (Chousalker and Roberts, 2008).

The avian oviductal tissues undergo dynamic morphological and functional changes during reproductive cycle. The most important processes involved in avian oviduct development and function seem to be extensive cell proliferation and differentiation of the luminal and glandular epithelium in Japanese quail (Rahman et al., 2007, 2009) and in chicken (Jeong et al., 2013; Hrabia et al., 2014). The morphometrical changes of oviduct at different developmental stages involved in the egg formation. The avian oviduct has been studied extensively in laying stage of different birds but a comparative analysis at different reproductive stages of different breeds is still in wanting. The length and weight of oviduct increased from pre-laying to laying and decreased from laying to regressing stage in chickens and thus this study provides the information for the proper understanding and identification of normal reproductive tract related to egg and meat production in chicken breeds. Therefore, the present work was designed to study the comparative morphology of oviduct of indigenous, sonali (crossbred; RIR ♂X Fayoumi ♀) and exotic RIR (Rhode Island Red) chicken breeds in their pre-laying, laying and regressing reproductive stages.

Material and Method

All procedures were approved by Animal Ethical Committee of Rajshahi University (390/320/IBSc). A total of twenty seven female chickens from three

chicken breeds (indigenous, n=9; crossbred sonali, n=9; and exotic RIR, n=9) were used for this study. Each breed was with three reproductive stages and they were pre-laying (n=3), laying (n=3) and regressing (n=3). These chickens were purchased from village markets (indigenous) and nearby farms (sonali and RIR) close to Rajshahi University. The chicken age ranged from 6-10 months for indigenous (pre-laying=6, laying=8, regressing=10); 3-19 months for sonali (pre-laying=3, laying=6, regressing=19) and 4-18 months for RIR (pre-laying=4, laying=10, regressing=18). They were allowed to acclimatize for two weeks with water and layer feed *ad libitum* and slaughtered by using halal method in the Genetics and Molecular Biology Laboratory. The length (cm) and weight (gm) measurements were taken by linear scale and electronic balance after the removal of whole oviducts from the experimental hens. Descriptive statistics and one-way analysis of variance (ANOVA) were subjected to evaluate morphological differences (mean±sd of length and weight) of each reproductive stage from three breeds and all statements of significance were assessed at $P < 0.05$.

Result and Discussions

The results for mean lengths (cm) of the oviducts in the three studied reproductive stages from each breed are presented in Table **1** and in Figure **1**. The mean lengths of oviduct varied in pre-laying stage ($F_{3,16}$=31.49, P<0.05) obtained from 6 months indigenous (22.63±7.5cm), 3 months sonali (9.53±1.05cm) and 4 months RIR (13.53±0.73cm) chickens. In laying stage the mean lengths were increased and achieved highest values comparing to pre-laying and regressing stages in three experimental breeds. Indigenous laying hens at 8 months showed mean length 50.76±2.17cm, sonali at 6 months 73.16±6.40cm and RIR at 10 months 91.26±4.23cm were statistically significant ($F_{3,16}$=12.29, P<0.05). After laying stage adult hens started to terminate egg production for physiological dysfunction and thus getting regressed. The mean values of oviduct length for regressing hens decreased from the laying stage and they showed significant difference ($F_{3,16}$=11.06, P<0.05) among indigenous (44.93±9.37cm) at 10 months, sonali (53.76±5.67cm) at 19 months and RIR (83.93±2.48cm) breed at 18 months.

The mean weights (gm) of the oviducts in the three reproductive stages from three breeds are presented in Table **1** and in Figure **2**. The total oviduct mean weights varied in pre-laying stage ($F_{3,16}$=43.52, P<0.05) in experimental different breeds (4.56±1.55gm for indigenous at 6 months, 2.77±0.63gm for sonali at 3 months and 3.70±1.90gm for RIR at 4 months). Due to accumulation of secretory substances in the cells for the egg production, the oviduct weights were rapidly increased in all three breeds and they showed significant variations among them ($F_{3,16}$=7.44, P<0.05). The laying hen oviduct mean weight for indigenous at 8 months was 32.30±1.94gm, for sonali at 6 months was 51.53±7.52gm, and for RIR at 10 months was 93.26±6.39gm. Due to regression, the hens showed decreased oviduct average weights which were comparatively less than the laying ones and they differed ($F_{3,16}$=13.50, P<0.05) from each other in three breeds (19.83±7.65gm for indigenous at 10 months, 27.90±13.64gm for sonali at 19 months and 59.30±2.44gm for RIR at 18 months).

Table 1 Length (cm) and weight (gm) of oviducts from pre-laying, laying and regressing reproductive stages of indigenous, sonali and RIR chicken breeds.

Morphological Parameters	Breeds	Reproductive Stages		
		Pre-laying (Mean±Sd)	Laying (Mean±Sd)	Regressing (Mean±Sd)
Length of oviduct (cm)	Indigenous	22.63±7.57	50.76±2.17	44.93±9.37
	Sonali	9.53±1.05	73.16±6.40	53.76±5.67
	RIR	13.53±0.73	91.26±4.23	83.93±2.48
F values (P<0.05)		F=31.49	F=12.29	F=11.06
Weight of oviduct (gm)	Indigenous	4.56±1.55	32.30±1.94	19.83±7.65
	Sonali	2.77±0.63	51.53±7.52	27.90±13.64
	RIR	3.70±1.90	93.26±6.39	59.30±2.44
F values (P<0.05)		F=43.52	F=7.44	F=13.50

There were differential growth patterns due to deposition of cellular components from the oviduct at various stages during a reproductive cycle (Yu et al., 1972) resulting morphological changes in length and weight of reproductive stages from different breeds were compared through statistical analyses in the present study (Table **1**). The total oviduct length and weight were lowest in pre-laying stages of all three chicken breeds and rapidly increased as a result of rapid development and growth of oviduct in laying stage and decreased in a similar manner during regression of the oviduct. The indigenous pre-laying chicken oviduct mean length of the present study at 6 months 22.63±7.57cm was higher than the mean length of 8.73 ± 0.27cm for 3 months and 9.93 ± 0.41cm for 5 months (Rahman et al., 1999) whereas it was lower than the 59.73 ± 0.85 cm at 5-6 months in local uttara fowl (Khan et al., 2017). The present mean weight 4.56±1.55gm for 6 months showed similarity with the report (6.34±1.54gm at 4 months Hyline layer) of Hrabia et al. (2014) though it was lower than the weight (31.73 ± 0.96gm) of local uttara fowl of 5-6 months (Khan et al., 2017). The sonali crossbred (Ambar et al., 1999) is a cross-breed RIFI (RIR male X Fayoumi female) introduced in Bangladesh for well adaptation to the environment (FAO, 2015). The productivity of sonali was more profitable compared to RIR and Fayoumi (Rahman et al., 1997), improved only in egg production in reciprocal cross FIRI (Rahman et al., 2004) whereas Khawaja et al. (2013) reported that FIRI chickens were better performer in all traits than RIFI. To our knowledge the oviductal length measurement was lacking in pre-laying stage of sonali breed and in the present study we found its mean length 9.53±1.05cm at 3 months age. The mean weight 2.77±0.63gm of this study was compatible with the oviduct weight (5.05gm) of the pre-laying stage (4 months) of the commercial hybrid Dekalb strain (Yu et al., 1972). RIR birds at 5 months had oviduct with 22.7 ± 0.727cm length and 18.5 ± 0.485 gm weight (Banerjee et al., 2006) and for same age pre-laying showed 32.21cm length and 22gm weight (Singh, 2001). ISA brown chicken oviduct length increased depending on age (3months, 9.97±0.27cm; 5 months, 11.33±0.32cm) (Rahman et al., 1999). The present findings bearing the mean length at 4 months

13.53±0.73cm and mean weight 3.70±1.90gm of RIR chicken at pre-laying stage were not in agreement with the findings of different workers.

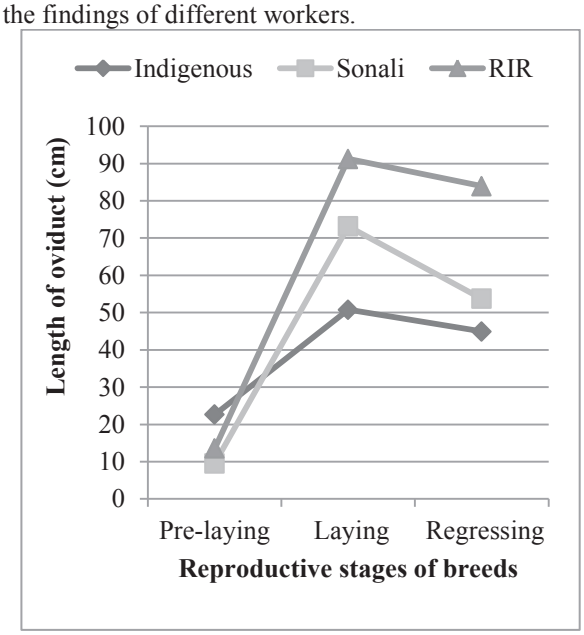

Fig. 1 Comparison of total oviduct length (cm) from pre-laying, laying and regressing stages of indigenous, sonali and RIR chicken breeds.

There are many reports on oviductal morphological measurements (length and weight) of adult laying hens of indigenous, exotic RIR and a few on cross bred sonali chickens. Indigenous adult laying hens (*Gallus gallus dometicus*) between 12-18 months had mean length 71.85±5.45cm and mean weight 56.15±9.67gm (Mohammadpour et al., 2012), 8-11 months 46.18±9.49cm and 24.45±1.81gm (Mishra et al., 2014) and only mean length at 9 months was 69.02 ± 0.93cm (Rahman et al., 1999), at 8 months 45.0cm length and 23.60gm weight in native guinea fowl (Abdul-Rahman et al., 2018). The entire oviduct was weighed 19.77±1.48gm for normal feathered, 19.8±3.50gm for Naked Neck and 25.33±5.52gm for Frizzled Feathered in three Nigerian indigenous adult laying chickens (Mahmud et al., 2017) and indigenous adult turkey hens (*Meleagris gallopavo*) at 11-14 months had mean length 87.57±37cm and weight 59.66±1.52gm (Mirhish and Nsaif, 2013). From the current study it was evident that the mean length and weight from indigenous chicken 50.76±2.17cm 32.30±1.94gm at 8 months was compatible with the reports except Mohammadpour et al. (2012) for more age, Mahmud et al. (2017) for different genotypes and Mirhish and Nsaif (2013) for different species.

The crossbred Cairo L-2 strain matured sexually at 5 months and oviduct weight was 43.1±1.27 gm (Nassar et al., 2017). F_2 generation from a cross between White Leghorn (WL) and Dongxiang Blue-shelled (DX) layer chickens displayed the oviduct length 48.18±6.70cm and weight 41.46±9.69gm (Shen et al., 2017). The commercial hybrid Dekalb strain oviduct weight at laying (14months) 51gm (Yu et al., 1972). All these findings of cross bred layers were not comparable with the present results with mean length and weight values 73.16±6.40cm and 51.53±7.52gm for 6 months. The RIR layer showed the mean length at 5-6 months was 68.50±0.39cm (Naragude et al., 1999), laying stage after 5 months the length was 67.74cm and weight 77.2 gm (Singh, 2001). The total length of oviduct at 10 months was 68 cm (Rahman, 2013), at 13 months was 63.26 ±2.51cm and weight was 41.64±7.08 gm (Shyam, 2007), ISA brown chicken oviduct mean length was 73.45±0.96cm at 9 months (Rahman et al., 1999), laying turkey had mean length 72.8±5.3cm (Parto et al., 2011). Very recently, Preedaa et al. (2018) reported the mean length of WH Layer at 11 months was 71.85±0.56 cm and weight was 52.50±1.24gm. The oviduct during laying stage increased rapidly with highest values (91.26±4.23cm and 93.26±6.39gm at 10 months) and thus the present findings of RIR at laying stage were superior to others reports.

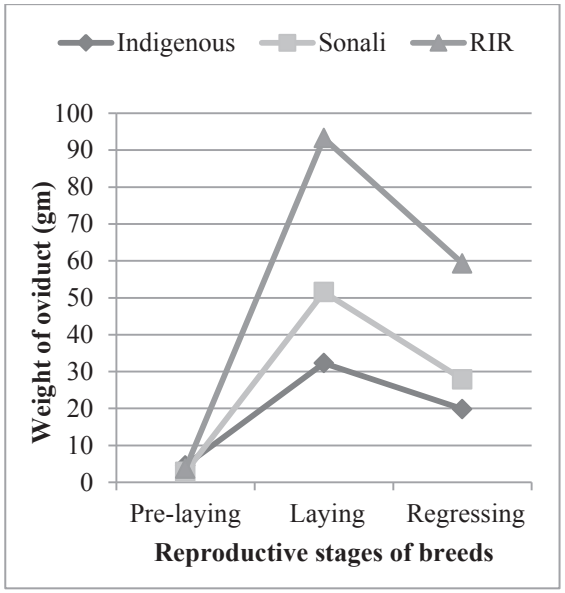

Fig. 2 Comparison of total oviduct weight (gm) from pre-laying, laying and regressing stages of indigenous, sonali and RIR chicken breeds.

The commercial hybrid Dekalb strain oviduct weight at regressing stage 4.6 gm (19 months) (Yu et al., 1972), 14-16 months showed 38.20±4.20gm total oviduct weight with a regressing rate (Yu et al., 1973). The reduction of oviduct occurred during induced molting in WH hens (Sudaresan et al., 2006) and in laying Hyline brown females (64.98 ± 1.91 g to 41.05 ± 2.76 g at 13 months) (Leśniak-Walentyn and Hrabia, 2016). The total weight of oviduct varied with stage of ovarian development ($F_{5,54}$=31.9, P<0.001) *i.e.* oviduct weight regressed from its laying stage of its follicular absence in a passerine bird (Williams and Ames, 2004). Khokhlov (2008) reported that dynamic increase occurred in infundibulum of Lohman Brown hens in laying stage and decreased in regressing stage. The present results of regressing stages (indigenous

44.93±9.37cm, 19.83±7.65gm; sonali 53.76±5.67cm, 27.90±13.64gm; RIR 83.93±2.48cm, 59.30±2.44gm) from three breeds were comparable with the abovementioned findings.

The breeds of present study are used by egg producers and breeders for dual purposes *i.e.* egg and meat production and of them the sonali and RIR are reared commercially where the eggs of sonali are used only in hatcheries for meat production. In pre-laying stage, immature chickens do not produce egg and their oviduct development with increased length and weight is associated with differentiation and specialization of cells from the particular segments of oviduct for the production of egg-specific proteins in laying stage. The molting or regressing process leads to dynamic changes in morphology, physiology and function of the reproductive tract of laying hens (Jeong et al., 2013) and thus egg laying got terminated. The present results explored the fact that the increased morphometric values of oviduct have been found in laying stages in all breeds comparing to that of pre-laying and regressing reproductive stages. The oviducts from RIR hens had largest length and heaviest weight of oviduct during laying and then regressed in regressing stages and the indigenous had highest values only in pre-laying stage (Table **1**, Figure **1** & **2**). Considering all, the present findings made clear comparison among three reproductive stages in chicken breeds on the basis of oviduct morphometry and also provided a hierarchical order as RIR>sonali>indigenous. The changes of morphological parameters of oviduct related to the egg production efficiency in the studied breeds and we are eagerly waiting for the investigation to be conducted in the next.

Conclusion

The mean lengths and weights of the entire oviduct of indigenous, sonali and RIR hen breeds at pre-laying, laying and regressing stages were significantly different ($P<0.05$). All the three breeds had similar gross and morphometric patterns of oviduct increase from the pre-laying to laying and decrease from laying to regressing stages. So, this study in addition to its contribution to the knowledge of comparative oviductal morphology at different reproductive stages up to different breeds, has also established a baseline data for further comparative gross and morphometric reproductive studies in another breeds. A better understanding of the morphological aspects of oviduct will provide the basis for improvements in the efficiency of chicken egg production and it will help to develop improved technologies for the propagation of endangered chicken breeds as well as other species of poultry.

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Effect of total mixed ration ensiled with fermented juice of epiphytic lactic acid bacteria on fermentation quality and energy expenditure

Yuli Yanti[1,2] and Masato Yayota[3,4]

[1]The United Graduate School of Agricultural Science, Gifu University, Japan

[2]Department of Animal Science, Faculty of Agriculture, Universitas Sebelas Maret, Jalan Ir Sutami No. 36A Kentingan Surakarta, Jawa Tengah, Indonesia

[3]Faculty of Applied Biological Sciences, Gifu University, Japan

[4]Education and Research Center for Food Animal Health, Gifu University (GeFAH), Japan

SUMMARY

This aim of this study was to determine the effect of total mixed ration (TMR) ensiled with fermented juice of epiphytic lactic acid bacteria (FJLB) on fermentation quality and energy balance in ewes. The TMR were prepared from rice straw, corn stover, brewer grain, tofu waste, steam flaked maize and mineral-vitamin. The treatments were: CON (no silage additive added), FJLB, COM (commercial additive) and MIX (FJLB+COM). TMR was fermented for minimum 2 months. Four Suffolk ewes with the rumen canullas (7.5 ± 2.2 years old; 51.0 ± 19.5 kg) were assigned into 4 × 4 Latin square design. The TMR was offered at the amount of 2% of BW on dry matter (DM) basis All the parameters observed in fermentation quality were not affected by silage additives ($P>0.05$), except for acetic acid concentration ($P<0.01$). The COM and MIX treatment has higher acetic acid concentration than CON treatment ($P<0.01$). The chemical composition of TMR silage was not affected by the silage additives ($P>0.05$). However, the DM content in FJLB treatment tended to be higher than that in COM treatment ($P=0.06$). The silage additives did not show clear effect ($P>0.05$) on the gross energy (GE) intake, digestible energy (DE), energy digestibility, methane (CH4) energy and metabolizable energy (ME). The TMR silage prepared from agriculture by-products with FJLB has good fermentation quality and no negative effect on methane emission and energy utilization..

Keywords: energy utilization, fermentation quality, FJLB, methane emission, TMR silage

Introduction

Agricultural by-product has been known as feed for livestock in developing countries where the crop production, such as rice paddy and maize, are abundant in amount. However, the utilization of these crop by-products is challenged by its low nutrient content; mainly structural carbohydrates that will lead to low in digestibility then finally result in low animal production. Further, in the process of fermentation in the rumen, 2-12% of ingested gross energy is converted to methane, which leads to reducing the efficiency of feedstuff utilization. Feed that contains many structural carbohydrates produces more methane emission in the rumen than feed contained low non-structural carbohydrates (Archimède et al. 2011).

To supply the balanced nutrient to livestock, agricultural by-product could be combined with other feed source, such as food by-product, into total mixed ration (TMR). Food by product usually in wet type therefore easy to deteriorate. Ensilaging the TMR is one of technology to preserve the nutrient content of TMR. An additive could be added to silage to improve the fermentation quality. Fermented juice of epiphytic lactic acid bacteria (FJLB) is a potential silage additive that has been proved to increase the fermentation quality of forage silage.

In our previous study, the TMR silage with FJLB contained similar amount of fiber to non-additive treatment. However, when FJLB treatment combined with commercial silage additive, the fiber content of TMR silage was improved (Yanti and Yayota, 2017). It may suggest that TMR silages with FJLB might mitigates the methane emission and improve energy utilization of agricultural by-products.

Therefore, we hypothesized that FJLB treatment on TMR silage that prepared from agriculture by-product

will lead to reduce methane emission and more efficient energy utilization in ruminants. The objective of this study was to determine the effect of TMR ensiled with FJLB on fermentation quality and energy balance in ewes.

Materials and Methods

The proportion of material in TMR was 25, 23, 19.5, 19, 12, 1.5% for rice straw, corn stover, brewer grain, tofu waste, steam flaked maize and mineral-vitamin, respectively. TMR was formulated to meet or exceed the nutrient requirement of sheep according to NRC (2007).
All the materials were mixed manually and added silage additive according to the following treatments. The treatments were: CON (no silage additive added), FJLB, COM (commercial additive: "Si-Master AC"®, Snow Brand Seed Co.,Ltd., Sapporo, Japan), and MIX (FJLB+COM). The FJLB were prepared from Italian ryegrass modified by Burenook et al. (2016). FJLB was sprayed 1% of fresh matter. The commercial additive diluted and sprayed according to the factory recommendation. TMR were packed into polyethylene bag doubled with flexible container bag (capacity 100 kg). Silages were made in four replications for each treatment.

Four Suffolk ewes with the rumen canullas have been used (7.5 ± 2.2 years old; 51.0 ± 19.5 kg), then they were assigned into 4 × 4 Latin square design. The ewes were housed in a metabolic cage individually. The TMR was offered at the amount of 2% of BW on dry matter basis. Adaptation was 8 days followed by collection period for 6 days in each period. Fecal and urine were collected and handled.

The chemical composition of TMR silage and feces was analyzed by the AOAC methods (AOAC, 2007). The volatile fatty acid (VFA) in TMR silage was assayed using gas chromatography. Fleig point was calculated as described by Denek and Can (2006).

The gross energy (GE) concentration of the samples was determined with a bomb calorimeter (CA-4PJ, Shimadzu Corp., Kyoto, Japan). Digestible energy (DE) was computed from difference GE intake and fecal energy. Metabolizable energy (ME) was computed from DE minus the urinary energy (UE) and energy losses from methane. Methane production was estimated according to Blaxter and Clapperton (1965).

Results

All the parameters observed in fermentation quality were not affected by silage additives ($P>0.05$), except for acetic acid concentration (Table 1). The COM and MIX treatment has higher acetic acid concentration than CON treatment ($P<0.01$). The chemical composition of TMR silage was not affected by the silage additives ($P>0.05$). However, the DM content in FJLB treatment tended to be higher than that in COM treatment ($P=0.06$). The silage additives did not show clear effect ($P>0.05$) on the GE intake, DE, energy digestibility, CH4 energy and ME (Table 2).

Discussion

The Fleig point in all silages was over 97.99, indicating that all TMR silages had very good quality. Wet type of food by-products including tofu waste and brewer grain contained lactic acid bacteria (LAB: 9 × 107 and 6.4 × 107 cfu/g, respectively. Tanaka et al. 2001). Thus, it would be sufficient to produce lactic acid and lowering pH to achieve good fermentation quality because well preserved silage will be obtained when LAB level reaches 105 cfu/g (Cao et al. 2016).

Acetic acid content in COM and MIX treatment were higher than that in CON treatment. It can be explained that LAB in COM (Lactobacillus paracasei) was heterofermentative type that can convert lactic acid into acetic acid. The heterofermentative LAB may also present in FJLB, because acetic acid in FJLB treatment tended to be higher than CON treatment and becoming greater when combined with commercial additive (MIX). Other parameters in silage fermentation quality did not differ among the treatments. Again, this was because non-additive treatment also contained sufficient LAB for getting favorable fermentation.

GE intake was similar in ewes fed TMR silage with different silage additive. This result might be caused by the similar palatability of all TMR silage due to similar silage quality. The DE was not different among the treatments. This is because similar chemical composition in all TMR silages led to have a similar rumen degradation and nutrient absorption in the intestinal tract. Predicted energy losses as methane in this study was 0.08 of GE, and this value agreed with the value of predicted methane in Adesogan et al. (1998) that often quoted for feeds at maintenance. This result implied that TMR silage prepared from agriculture by-products does not have any adverse effect on methane emission.

In conclusion, TMR silage prepared from agriculture by-products with FJLB has good fermentation quality and no negative effect on methane emission and energy utilization.

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Table 1. Fermentation quality and chemical composition of TMR ensilaged with fermented juice of epiphytic lactic acid bacteria

Item	Treatment				*P*-value
	CON	FJLB	COM	MIX	
Fermentation quality					
pH	4.58 ± 0.13	4.41 ± 0.13	4.25 ± 0.10	4.33 ± 0.10	0.303
Lactic acid (g/kg DM)	31.51 ± 3.40	37.43 ± 3.78	37.80 ± 4.36	40.15 ± 2	0.379
VFA (g/kg DM)					
Acetic acid	13.22 ± 2.88^{b}	16.72 ± 0.78^{ab}	21.46 ± 1.59^{a}	24.22 ± 1.21^{a}	0.005
Propionic acid	0.89 ± 0.34	0.92 ± 0.27	0.96 ± 0.31	1.08 ± 0.36	0.976
Butyric acid	0.46 ± 0.09	0.27 ± 0.10	0.42 ± 0.15	0.1 ± 0.06	0.125
Fleig point	97.99 ± 5.55	106.23 ± 4.86	109.35 ± 3.03	108.52 ± 3.94	0.301
Chemical composition					
DM	38.0 ± 0.33	38.8 ± 0.44	37.1 ± 0.45	38.4 ± 0.35	0.055
CP (%DM)	12.5 ± 0.16	12.9 ± 0.28	13.2 ± 0.20	13.1 ± 0.31	0.227
aNDFom (%DM)	48.9 ± 1.08	47.6 ± 1.41	47.7 ± 0.74	46.9 ± 0.34	0.581

CON: control (no additive added), FJLB: fermented juice of epiphytic lactic acid bacteria additive, COM: Commercial silage additive, MIX: FJLB+COM, VFA: volatile fatty acid, CP: crude protein, aNDFom: α-amylase neutral detergent fiber exclusive ash, DM: dry matter. The values with different superscript within the same row are significantly different at 5% level. Values are mean ± standard error.

Table 2. Effect of different silage additives in TMR silage prepared from agriculture by-product on energy utilization in ewes

Parameters	CON	FJLB	COM	MIX	*P*-value
DMI (g/kg $BW^{0.75}$/day)	48.3 ± 3.25	45.9 ± 3.37	39.5 ± 5.19	44.2 ± 1.9	0.404
GEI (MJ/kg $BW^{0.75}$/day)	0.9 ± 0.06	0.9 ± 0.06	0.8 ± 0.07	0.8 ± 0.04	0.415
DE (MJ/ kg $BW^{0.75}$/day)	0.6 ± 0.03	0.6 ± 0.03	0.5 ± 0.04	0.6 ± 0.05	0.663
CH_4 energy (MJ/100 MJ GE)	8.5 ± 0.09	8.4 ± 0.11	8.4 ± 0.17	8.4 ± 0.19	0.898
ME (MJ/$BW^{0.75}$/day)	0.5 ± 0.03	0.5 ± 0.02	0.4 ± 0.03	0.5 ± 0.05	0.490

CON: control (no additive added), FJLB: fermented juice of epiphytic lactic acid bacteria additive, COM: Commercial silage additive, MIX: FJLB+COM, DMI: dry matter intake; GEI: gross energy intake; DE: digestible energy; ME: metabolizable energy.

Values are mean ± standard error.

Potential use of inulin as a prebiotic to maintain gastrointestinal health in monogastric animals

Adi Ratriyanto*, Eka Handayanta, Nuzul Widyas and Sigit Prastowo

Department of Animal Science, Faculty of Agriculture, Sebelas Maret University, Surakarta Indonesia

*Corresponding E-mail: ratriyanto@staff.uns.ac.id

SUMMARY

Due to the ban of antibiotics in animal production, new concepts have been developed to secure animal health and performance. Prebiotics are naturally occurring compound considered to beneficially affect the composition and activity of the microflora in the gastrointestinal tract (GIT), thus, may exert positive influences on immune response of the animals. Inulin has attracted considerable attention as a non-digestible oligosaccharide with prebiotic properties and offered as potential substitutes for in-feed antibiotics. It is a polydisperse carbohydrate consisting mainly of β-(2,1) fructosyl-fructan links and shows a degree of polymerization ranging from 20–60 fructose units. Inulin is not hydrolytically digested by mammalian enzymes in the upper GIT, but is selectively fermented in the lower tract and thus may support the growth of beneficial bacterial populations, such as *Bifidobacteria* and *Lactobacilli.* Microbial fermentation results in the formation of short-chain fatty acids, mainly acetic, propionic, and butyric, as well as lactic acid, resulting in a lower pH and prevention of enteric colonization of potentially harmful bacteria. Furthermore, there is evidence that inulin addition to the diet improved crude ash, crude fiber and neutral detergent fiber digestibility, which would be concomitant with the maintenance of a beneficial intestinal environment.

Introduction

The use of antibiotics as feed additive was banned firstly in Sweden in 1986, followed by some other countries and the European Union in 2006 [1]. In Indonesia, the use of antibiotic growth promoters has been banned in 2017. During the first years after restriction of in-feeds antibiotics in Sweden, there were increase in the use antibiotics as therapeutic [1]. Therefore, there is growing interest on natural substances as substitute of in-feeds antibiotics to secure the animal health and performance, including prebiotics [2].

Prebiotics are oligosaccharides or polysaccharides that are not hydrolytically digested by mammalian enzymes in the upper gastrointestinal tract (GIT), but are selectively fermented in the lower tract and thus may support the growth of beneficial bacterial populations, such as Bifidobacteria and Lactobacilli [3]. Certain dietary non-digestible oligosaccharides (NDO), such as inulin and oligofructose are considered as prebiotics [4]. Inulin and other NDO are considered as potential substitutes for in-feed antibiotics due to their negative impact on the growth of harmful bacteria [3]. Inulin is a polydisperse carbohydrate consisting mainly of β-(2,1) fructosyl-fructan links and shows a degree of polymerization (DP) ranging from 20–60 fructose units [5]. It is originated, for example, chicory roots (*Cichorium intybus*) and Jerusalem artichoke tubers (*Heliantus tuberosus*) [2]. This review focuses on the effect of inulin on performance traits, intestinal microbes and nutrient digestibility in monogastric animals.

Effects of inulin on animal performance

The prebiotic function of inulin has been confirmed in studies with humans and animals [6,7]. Studies in humans suggest that inulin is indigestible in the upper part of GIT [6], which was confirmed by studies in pigs [8]. In their observation, Yasuda et al. [8], found the lack of inulin degradation in the upper GIT of pigs. By contrast, results of other studies, however, revealed that about 57% of inulin was already digested in the stomach and small intestine of pigs [9]. The higher pre-cecal digestibility of inulin in pigs compared to humans could be due to higher numbers of bacteria in the small intestine of pigs [9].

Generally, inulin can be utilized by Bifidobacteria, Lactobacilli, Bacteroides, and Enterococci but cannot be utilized by E. coli [2,10]. It is fermented by anaerobic bacteria to be utilized as energy source, thereby

providing carbon skeletons for their own growth [10]. The mechanisms through which beneficial bacteria may exert their inhibitory effects on potentially harmful bacteria are partly related to the production of short-chain fatty acids (SCFA) and lactic acid during fermentation, resulting in a lower pH and prevention of enteric colonization of potentially harmful bacteria [4]. In addition, inulin is supposed to improve the absorption of certain minerals in pigs [11] and poultry [12], and to stimulate the synthesis of B-vitamins [4].

Beneficial effects of inulin in maintaining intestinal health is associated with improvement in animal performance. Studies on the effects of inulin on animal performance are presented in Table 1. Inclusion of inulin in the diet improved performance of broiler chickens [13,14], laying hens [15,16], pigs [17] and rabbits [18]. This improvement is associated with modification in substances which involved in protein or energy metabolism such as carnitine, creatine and albumin [18,19]. Moreover, Sevane et al. [20] observed that inclusion of inulin in the diet improved number of genes, processes and pathways with putative involvement in growth, performance and immune status of chickens. The lactic acid-producing bacteria are considered to be immunomodulatory which directly or indirectly affect the GIT and systemic defense functions. Dietary inulin that increase the density of these bacteria may enhance defense mechanisms of the host and increase resistance to various health challenges [21].

Table 1. Effects of inulin on performance, immune systems and blood metabolites

Animal	Inulin Effects	Reference
Broiler	↑ performance and carcass traits	Praveen et al. [13]
Broiler	↑ performance	Kareem et al. [14]
Broiler	↑ feed intake ↑ intestinal immune function (IgA concentration in cecum and mucin mRNA expression in jejunum)	Huang et al. [29]
Broiler	↑ growth performance ↓ blood lipids (triglyceride and VLDL)	Taraz et al. [30]
Broiler	↑ serum sodium levels, creatine kinase, creatinine	Ates et al. [19]
Broiler	↑ number of genes, processes and pathways with putative involvement in growth and performance ↑ immune immune status	Sevane et al. [20]
Broiler	↑ weight gain, feed efficiency	Izadi et al. [31]
Broiler	No effects on performance No effects on ND antibody titer	Elrayeh et al. [32]
Broiler	↑ weight gain	Nabizadeh [33]
Broiler	↑ weight gain	Rebolé et al. [7]
Broiler	↑ growth performance	Awad et al. [34]
Quails	No effects on performance and carcass traits	Majewska et al. [35]
Laying hens	↑ egg production and quality ↓ blood triglyceride and cholesterol	Sang-Oh et al. [15]
Laying hens	↑ egg production and quality	Chen et al. [16]
Laying hens	↑ eggshell weight and strength ↑ ash, calcium, and phosphorus in tibia	Chen et al. [12]
Rabbits	↓ mortality rate ↑ performance ↑ blood hemoglobin, albumin	Attia et al. [18]
Pigs	↑ weight gain and feed efficiency ↑ immunoglobulin A and G	Grela et al. [17]
Pigs	↑ weight gain ↑ plasma trace elements (Zn, Fe, Cu) and hemoglobin	Samolińska and Grela [11]

↑= increased, ↓= decreased

Effects of inulin on intestinal bacterial populations and microbial metabolites

Studies pertaining to effects of dietary supplementation with inulin on intestinal bacterial populations (Table 2) indicated inconsistent results in pigs [22,23], and poultry as well [7,24]. Loh et al. [22] and Eberhard et al. [25] did not find a stimulation of Bifidobacteria and Lactobacilli in the duodenum, jejunum, cecum and colon of pigs due to dietary inulin supplementation. By contrast, Lynch et al. [23] found an increase in the cecal Bifidobacteria populations in pigs, accompanied with a decreased E. coli population following inulin supplementation in the diet. Similarly, inulin supplementation increased the number of Bifidobacteria and Lactobacilli in broilers [7], laying hens [15] and turkeys [26], but inhibited E. coli and Salmonella growth in the small intestine and ceca of broilers [26–28].

Based on the findings on inulin degradation and its effects on microbial populations, some authors concluded that the prebiotic effects of inulin in pigs are less pronounced compared to those observed in humans, poultry and rodents [9,25,36]. However, it should be noted that the discrepancy between studies on inulin degradation and effects on microbial populations may be attributed, at least in part, to differences in the methodology and origin of the test substrates [8,36], the age of the animal and type of legumes and cereals in the diet [25]. As was pointed out by Henry and Saini [37], legumes and cereals, such as soybean meal, lupines wheat, barley and, commonly used in feed formulation contain substantial amounts of NDO, which may have masked and diluted the effect of dietary inulin

supplementation. The prebiotic efficiency of NDO depends also on the DP of the fructans. NDO showing a longer DP are more resistant to fermentation, thus leading to fermentation more distally in the GIT [38]. Moreover, dietary NDO may not selectively increase Bifidobacteria when the initial level of the indigenous population is already relatively high prior to dietary inclusion [22].

Table 2. Effects of inulin on microbial populations and metabolites in the gastrointestinal tract

Animal	Inulin Effects	Reference
Broiler	↓ Salmonella counts	Huges et al. [28]
Broiler	↑ faecal lactic acid bacteria ↓ Enterobacteriaceae counts ↑ faecal acetic acid, propionic acid, SCFA	Kareem et al. [14]
Broiler	↑ Bifidobacteria counts ↓ E. coli counts	Nabizadeh [33]
Broiler	↑ Bifidobacteria and lactobacilli counts ↑ butyric and d-lactic acids	Rebolé et al. [7]
Broiler	↑ acetic acid No effects on intestinal bacterial communities	Rehman et al. [24]
Broiler	↑ Bifidobacteria, Lactobacillus counts ↓ E. coli counts	Xu et al. [27]
Local chickens	↓ intestine pH ↑ Lactobacilli ↓ E. coli	Krismiyanto et al. [39]
Laying hens	↑ Bifidobacterium and Lactobacillus population ↓ Escheria coli and Salmonella	Sang-Oh et al. [15]
Turkey	↑ Bifidobacteria, Lactobacillus counts ↓ E. coli counts	Juskiewicz et al. [26]
Turkey	↑ faecal microbial activity ↑ short-chain fatty acids	Stanczuk et al. [40]
Pigs	↑ Bifidobacteria	Metzler et al. [41]
Pigs	No effect on Bifidobacteria and Lactobacilli ↑ butyric acid ↓ acetic acid and total SCFA	Loh et al. [22]
Pigs	No effect on Bifidobacteria and Lactobacilli	Eberhard et al. [25]
Pigs	↑ Bifidobacteria ↓ E. coli	Lynch et al. [23]

↑= increased, ↓= decreased, SCFA= short-chain fatty acids

Effects of inulin on nutrient digestibility

Studies in poultry showed that inulin increased crude protein and fat digestibility, amino acids and fatty acids digestibilities [42] associated with modification in intestinal morphology [31,33] as presented in Table 3. Different to poultry, studies in piglets revealed only minor effects of inulin on nutrient digestibility [9,36]. The addition of 2% inulin to a diet of growing pigs improved ileal and total tract crude ash [9,36] and ileal crude fiber digestibilities [9], indicating an enhanced fermentation of fiber in the small intestine of pigs. Furthermore, supplementation of inulin in the diet did not affect total tract dry matter, organic matter, crude protein, neutral detergent fiber and energy digestibilities in pigs [43].

The improvement in nutrient digestibility due to NDO supplementation would be concomitant with the maintenance of a beneficial intestinal environment [2,9]. Furthermore, the lack of an effect of inulin supplementation in several studies may have been due to a masking or dilution effect of native NDO from cereals and legumes used in these diets [44]. It is possible, that minimal effect in nutrient digestibility is linked to a lower intestinal transit time, a common attribute of fermentable fiber [3]. In addition, it has been well established that the extent of microbial fermentation and nutrient digestibility is partly dependent on the source and level of NDO supplementation and transit time of digesta in the GIT [44].

Table 3. Effects of inulin on nutrient digestibility and intestinal morphology

Animal	Inulin Effects	Reference
Broiler	↑ jejunum histomorphometry	Izadi et al. [31]
Broiler	↑ intestine and cecum length	Elrayeh et al. [32]
Broiler	↑ intestinal morphology	Nabizadeh [33]
Broiler	↑ crude protein and fat digestibility, amino acids and fatty acids	Alzueta et al. [42]
Broiler	↑ intestinal morphology and nutrient absorption	Awad et al. [34]
Laying hens	↑ small and large intestine length	Chen et al. [16]
Rabbits	↑ crude protein digestibility	Attia et al. [18]
Pigs	↑ crude ash digestibility	Branner et al. [36]
Pigs	↑ crude ash and fiber digestibility	Böhmer et al. [9]
Pigs	No effects on nutrient digestibility	Pierce et al. [43]

↑= increased, ↓= decreased

Conclusion

Inulin is potential to be applied in the diet of monogastric animals and has been shown to improve animal performance including poultry, rabbits and pigs due to its mode of action in modification of intestinal microbes. It cannot be enzymatically digested in the upper part of gastrointestinal tract, but selectively fermented by beneficial bacteria such as Bifidobacteria and Lactobacilli. Increasing number of these bacteria stimulate the production of short-chain fatty acids and

lactic acid, lead to reduction in harmful bacteria and improvement in intestinal health. In addition, supplementation of inulin in the diet increase the digestibility of specific nutrients. Although there are inconsistent effects on animal, inulin offers as an interesting alternative feed additive in animal production.

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Mechanisms of sperm-storage in the avian oviduct

○Mei Matsuzaki and Tomohiro Sasanami
Faculty of Agriculture, Shizuoka University

SUMMARY

Although successful fertilization depends on timely encounters between sperm and egg, the decoupling of mating and fertilization often confers reproductive advantages to internally fertilizing animals. In several vertebrate groups, which employ internal fertilization, post-copulatory sperm viability is prolonged by storage in specialized organs within the female reproductive tract. In birds, ejaculated sperm can be stored in a quiescent state in motility within oviductal sperm storage tubules (SSTs), thereby retaining fertilizability for up to 15 weeks at body temperature (41 °C); however, the mechanism by which motile sperm become quiescent within SSTs is currently unknown. Here, we show that low oxygen and high lactic acid concentrations are established in quail SSTs, thus resulting sperm motility inactivation in the SSTs. These findings suggested a novel physiological role of lactic acid for the sperm quiescence in the SSTs and opened up a new opportunity for technological improvement in prolonging sperm longevity at ambient or body temperature.

Introduction

In internal fertilizers, successful fertilization depends on the timely arrival of both sperm and egg at the site of fertilization. To achieve this, females of some of these species are capable of storing spermatozoa in their reproductive tracts until their eggs are ready to be fertilized. This phenomenon is common in many non-mammalian animals, including insects, fish, amphibians, reptiles, and birds.

In avian species, sperm storage tubules (SSTs), simple tubular invaginations located between the vagina and uterus, serve as sperm storage sites. Once ejaculated, spermatozoa migrate to and stay in the lumen of SSTs, where they can remain their fertilizability for long periods of time (up to 15 weeks) at a normal body temperature of 41 °C. Previous studies have explored the physiological roles of SSTs in sperm quiescence (Sasanami et al., 2013); however, the underlying molecular mechanism of this process has remained a mystery. In this study, we hypothesized that SSTs contain a substance(s) that renders sperm motility inactive. Based on this hypothesis, we aimed to identify the component(s) responsible for sperm quiescence within SSTs.

Material and Method

Excised quail UVJ tissues were minced in extraction buffer and extracted for 3 h on ice. The UVJ extracts were separated by gel-filtration chromatography, high-performance liquid chromatography (HPLC), and preparative thin-layer chromatography (PLC). The fractions were tested for their activity of sperm motility inactivation and the molecular structure of purified bioactive component was determined by NMR analysis.

We examined the synthetic pathway of bioactive component by incubating the isolated SST with or without an inhibitor of glycolysis. We also addressed the mechanism of how this component inhibits sperm motility by measuring sperm dynein ATPase activity and axoneme sliding in the presence of the active component.

Results and Discussions

We first identified sperm motility inactivating factor from the UVJ extracts. The UVJ extracts were separated and tested inhibitory effects on sperm motility. As the result, lactic acid was identified as a sperm motility inactivation factor from the bioactive fraction. The lactic acid concentration in the SST cells was higher than that of UVJ epithelial cells (Fig.1; Matsuzaki et al., 2015). In addition, the lactic acid production in the SSTs occurred in the presence of glucose and significantly inhibited by the addition of 2-deoxyglucose, an inhibitor of glycolytic enzyme (Fig.2; Matsuzaki et al., 2015). Therefore, it was

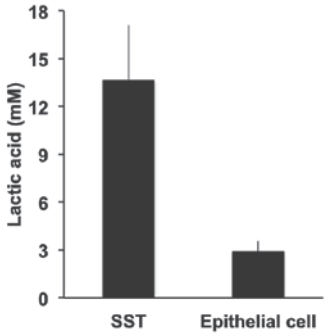

Fig.1 Measurement of lactic acid in the SST cells.

suggested that SST cells are able to produce lactic acid via glycolysis. Subsequently, we performed histochemical analysis and it was revealed that the mitochondrial activity of SST epithelial cells was suppressed due to the limited oxygen supply around the SST cells. In addition, the expression of monocarboxylate transporter 4 (MCT4), which is responsible for lactic acid transport was evident in the SSTs by RT-PCR analysis. Based on these observations, it is suggested that lactic acid is synthesized in the SST epithelial cells via glycolysis and might be transported to the lumen through the function of MCT4.

Second, we investigated the mechanism of sperm motility inhibition by lactic acid. Consequently, it was observed that sperm motility was inhibited not only by lactic acid, but also by other organic or nonorganic acids. According to the measurement of extra- and intracellular pH of sperm (pH_e and pH_i) in the presence of various organic acids, pH_i was decreased in response to pH_e decrease by the addition of any acids (Fig.3; Matsuzaki et al., 2015). Furthermore, pH_e and sperm motility had a strong positive correlation, suggesting that inactivation of the sperm motility is caused by decreasing luminal pH_i due to the presence of high

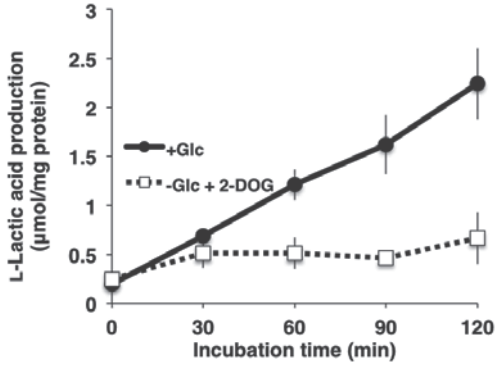

Fig.2 Lactic acid production in isolated SSTs.

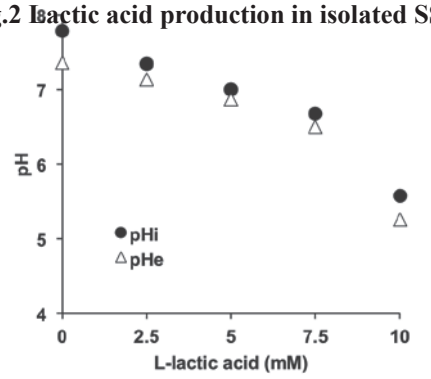

Fig.3 Relation between extra- or intracellular pH and lactic acid concentration.

concentration lactic acid. On the other hand, sperm dynein ATPase activity as well as axoneme sliding of de-membrane sperm decreased in a pH-dependent manner. Hence, it follows that the quiescence of sperm flagellar beating is related with decreasing dynein ATPase activity induced by intracellular acidification. In fact, pH_i of the resident sperm in the SSTs were decreased approximately 6.3.

Conclusion

In this study, following conclusions were obtained.

(1) Lactic acid was identified as sperm inactivation factor derived from the UVJ extracts.

(2) SST epithelial cells produced lactic acid from glucose via the glycolysis under hypoxic conditions, and probably released into SST lumen via MCT4.

(3) Sperm motility suppression during the storage in SSTs was caused by decreasing dynein ATPase activity resulted in intracellular acidification of sperm in response to low pH of SST lumen.

These findings suggested that lactic acid produced in the SST cells reduces sperm motility during storage. However, we were unable to produce fertilized eggs by artificial insemination using in vitro stored sperm, indicating that sperm preservation requires unknown factor(s) other than lactic acid. Further studies are needed to uncover the mechanism of sperm storage in avian species.

Acknowledgement

This work was supported by a Grant-in-Aid for Scientific Research (B) (General) (24380153 to T.S.), a Grant-in-Aid for Scientific Research in Innovative Areas (24112710 to T.S.), a Grant-in-Aid for Challenging Exploratory Research (25660211 to T.S.), Japanese Association for Marine Biology (JAMBIO) (No. 24–64 and No. 25–57 to T.S.), Toukai Foundation for Technology (No. 7 to T.S.) and Japan Advanced Plant Science Network.

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Threats and conservation of Sumatran primates

Rizaldi

Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Indonesia

SUMMARY

Sumatra is a rich biodiversity island laid on the tropical region of Indonesian territory. It is home to about 17 species of non-human primates including great apes (orangutans), gibbons, macaque monkeys, leaf monkeys, and slow loris. All those primate species live in various habitat types range from natural forest, degraded forest to human habituated environments. Development of agricultural industries and other human facilities across the island have given threats to all species at different intensity levels. The threat intensities depend on several factors; forest-dependent species, landscape contour, diet composition and living in close contact with the human. Habitat loss and fragmentation are the most serious threat to those primates although other threats are also alarming. Even though primates are very important biodiversity richness in the island, I found fewer efforts have been made for protection and lack studies have been conducted to support conservation. Study on geographical distribution and population status of each species are urgently needed in order to suggest authorities to make appropriate conservation plans and to take immediate actions.

Here, I am presenting distribution surveys of primate populations conducted in the central region of Sumatra. This surveys aimed to identify ongoing threats to each species in this region and to understand their current distribution due to habitat alteration. This survey found tiny scattered populations of leaf monkeys, a moderate population of macaques and very limited distribution of gibbons. Large oil palm plantation area could not be tolerated by any primate species but scattered forest remnant and rubber gum forest remains in the plantation have compensated dietary requirements of leaf monkeys and macaques. I am also presenting a comparative study of agile gibbon- a forest-dependent species. This study compared between living in fragmented and non-fragmented forest habitat in order to understand how forest fragmentation threats the gibbons. I analyzed demographic parameters, ranging behavior and food resources of the gibbons. I have observed population increased of agile gibbons (*Hylobates agilis agilis*) living in fragmented forest habitat although they have very limited home ranges compared to non-fragmented habitat. We will discuss minimum habitat requirement for a sustainable population of the purely arboreal primates. These studies suggest an immediate evaluation of the conservation status of Sumatran primates especially each species and sub-species of leaf monkeys. Recommendation for in-situ conservation, partnerships and applicable management conservation would be discussed.

Ex situ conservation of Javan pangolin (*Manis javanica*) in Bogor Agricultural University, Indonesia

Chairun Nisa[1,2], Irmanida Batubara[2,3], Burhanuddin Masyud[2,4] and Entang Iskandar[2,5]

[1]Faculty of Veterinary Medicine, [2]Tropical Biopharmaca Research Center, [3]Department of Chemistry Faculty of Mathematics and Natural Sciences, [4]Faculty of Forestry, [5]Primate Research Center, Bogor Agricultural University, Indonesia

SUMMARY

Javan pangolin is an unique mammals that belong to protected species according the Indonesian regulation, and listed as critically endangered species in IUCN red list, and APPENDIX I of CITES. However, exploitation of the animals still occurring massively that so conservation of this animal is strongly needed. The captive breeding is the best alternative solution and legally justified for sustainable using. Bogor Agricultural University has concerned on conservation of the Javan pangolin since 2011 and has engaged maintenance in captivity started on 2014 till now. Firstly the study on ecological aspect, such as population and habitat characteristic was done, and continued with development study of pangolin captive breeding. The study started with adaptation of behavior and daily activity of pangolins in captive breeding cage, along with study of feed formulation as a substitute for natural feed, and continued with study of breeding and reproduction technology and management. The results showed that Javan pangolins had a good adaptability and were able to survive in captivity as an artificial habitat. A key factor in the life of the Javan pangolins in captivity was determined by the successful provision of artificial feed formulas as an alternative to natural feed substitution. Javan pangolins shown positive in pairing and social interaction as an early indicator of breeding and reproductive processes in captivity. Implementation of Javan pangolin breeding and reproduction technology in captivity is still an important challenge in the captive process.

Introduction

Javan pangolin (*Manis javanica*) is one of Indonesia's unique biodiversity assets. It is known as pangolin or scaly anteater because of its scaly and ant-eating body (Figure 1). In the classification of pangolins belongs to the order Pholidota which only has one family Manidae, one genus *Manis* and eight species. *M. javanica* is one of four species distributed in Asia, while four other species in Africa. Currently the pangolins belong to the protected animals under Law no. 5 of 1990 and PP No.7 of 1999, as well as a list of red list or critically endangered species by IUCN, although CITES still places it in the Appendix II list.

At present, the exploitation of pangolins has reached an alarming level, so that the population in nature is under serious threat that can lead to the extinction of these animals. Pangolin is widely exploited because some people, especially the Chinese people, believe in treatment and healing using pangolin body parts. Pangolin scales have long been used in TCM (traditional Chinese medicine) and are believed to be a cure for certain diseases, such as rheumatism, skin, fever, even tumors or cancer; whereas the meat is believed to increase vitality (Bräutigam *et al.* 1994). This has led to massive exploitation of pangolins.

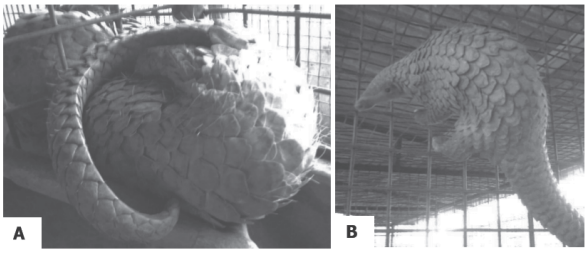

Fig.1. Javan Pangolin with scaly body A. roll up and B doing activity in the cage

In the past ten years, anteaters are thought to be the most smuggled animals from Indonesia, with the ultimate goal being mainly China. While conservation efforts both in situ and ex situ are still far from expectations. One of the

ex situ conservation efforts that has been carried out is breeding, but the success rate is still low. This is mainly due to the constraints of specific natural feeds, which are ants and termites (expensive in prices and in-continuous supply), and still lacking data on reproductive biology information. So, the aims of the study is to conserve Javan pangolin by captive breeding as the best alternative solution and legally justified for sustainable using.

Material and Method

Bogor Agricultural University started the Javan pangolin conservation on 2011 till now. The research scheme is shown in Fig 2. The first stage is study on ecological aspect of Javan Pangolin: population and habitat characteristic (2011-2012). The location for this study is in a number of sites that become part of the area of spread, namely: Java Island, especially West Java, Kalimantan (Central Kalimantan and South Kalimantan) and Sumatra especially North Sumatra.

The second stage is development study of pangolin captive breeding (2013-now). On this stage three stage of research are conducted, namely

a. The study of behavioral adaptation and daily activity of pangolins in captive breeding cage.
b. The study of feed food as a substitute for natural food
c. The study of breeding and reproduction technology and management

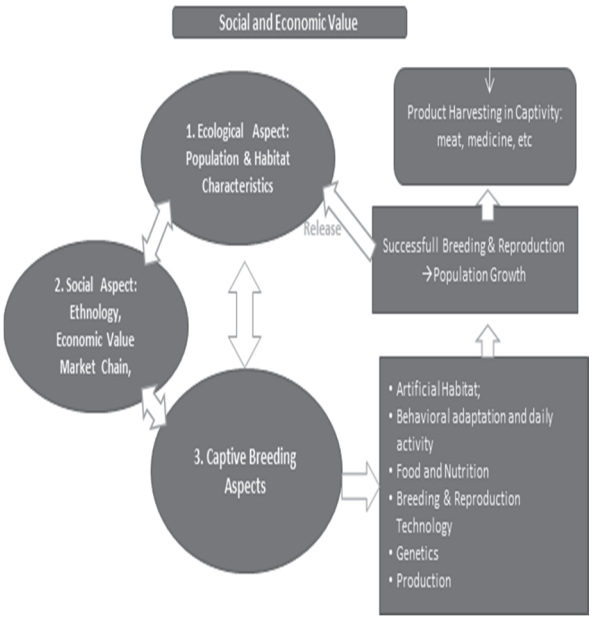

Fig. 2 Research Scheme

Result and Discussions

Study on ecological aspect of Javan Pangolin: population and habitat characteristic (2011-2012).

The research started on 2011 with the population and habitat characteristic in West Java, Central and South Kalimantan, and North Sumatera. The objective of this study is to get an overview of population conditions and habitat characteristics as a basis or reference for constructing and designing of artificial habitat in captivity.

At present the condition of pangolin populations in nature is under serious threat. From the results of ecological studies and pangolin populations in several regions of the three major islands in Indonesia, namely: West Java, North Sumatra, South Kalimantan, and Central Kalimantan, shows that the pangolin population in these locations is already very low at only 3-80 pangolins, even in some rare locations (population < 3 tails) (Masy'ud et al., 2012). Pangolin make nest hole in the ground or in the tree trunk. The habitat of Pangolin and their nest in the nature is shown in Figure 3.

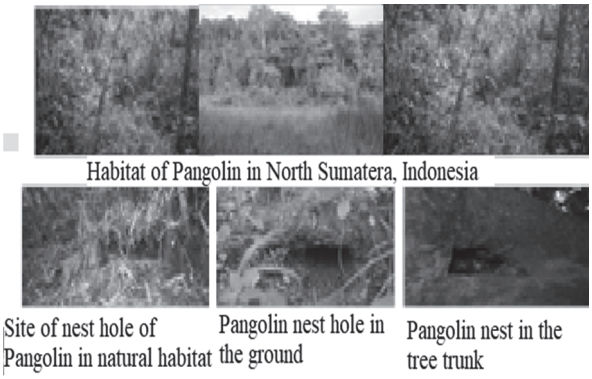

Fig. 3 Pangolin habitat and nest

The illegal hunting of pangolin directly from nature which is carried out continuously, uncontrolled illegal trade and utilization without being followed by breeding activity will result in the extinction of these animals. In addition, the pangolin population decline is also thought due to the conversion of functional areas of pangolin habitat into fields or plantations, resulting in habitat destruction and reduced availability of pangolin feed in nature. Pangolins eat ants (ordo Hymenoptera), termites (ordo Isoptera) and red earth ants (*Myrmicaria sp.*) as the most preferential or preferable feed in their natural habitat.

This animal is a solitary and nocturnal animal, so it only

has afternoon to night activities (Novriyanti 2011). So far, some zoos and conservation centers, such as Singapore Zoo, Taipei Zoo Taiwan, and Carnivore and Pangolin Conservation Project (CPCP) Vietnam, have been successful in keeping pangolin to produce puppies from marriage in captive, adapting feeding replacements from natural feed which is relatively expensive and difficult to maintain continuity of availability.

Development Study of Pangolin Captive Breeding (2013-now)

Captive breeding is one of the *ex situ* conservation efforts, although the level of success is still low. So far, the information about the successful maintenance of pangolins in captivity by providing substitute feed formulas has only been reported by several zoos abroad, such as the Singapore Zoo and Taipei Zoo.

TropBRC has 4 pangolins in the captive breeding; 2 is female and 2 is male (Figure 4). Each pangolin was maintaining individually, that in each cage facilitated with connecting door. Generally, pangolin was shown well adaptive in artificial habitat (cage). The pangolin in captive breeding was doing rest in the wooden box or in the logs as the enrichment of the cage, eating, moving in the ground of cage, and doing social interaction between male and female Pangolin (Figure 5).

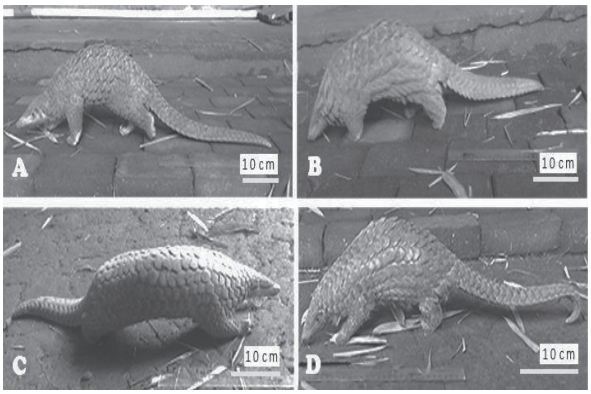

Fig. 4 Pangolin in captivity: A. female-1, B. male-1, C. female-2, D. male-2

Since the natural food for Pangolin is difficult to find, the supply is not continuous, and the price is also expensive, we performed the study on artificial feed formulation for substitution the natural feed. This study is started on 2015. The formula was adopted from Singapore Zoo, but modified using local feed. The objective of the step is to create or find the optimum artificial feed formula to replace or decrease the use of natural food for pangolins in captivity, calculate feed consume and positive effect to growth (FCR, feed conversion rate), body weight and survivality of pangolin in captivity. Artificial feed formula is made by utilizing chicken egg and meat, meal worm, cricket powder and other supplement, combine with ant egg (local: kroto). The study conducted this year to adapt pangolins with an artificial feed formula showed good results. Feed formulations that were suitable and sufficient for the metabolic needs of the pangolin body. Our research team has successfully adapted pangolins that were kept in captivity with artificial feed formula substitutes (Nisa' *et al*. 2015).

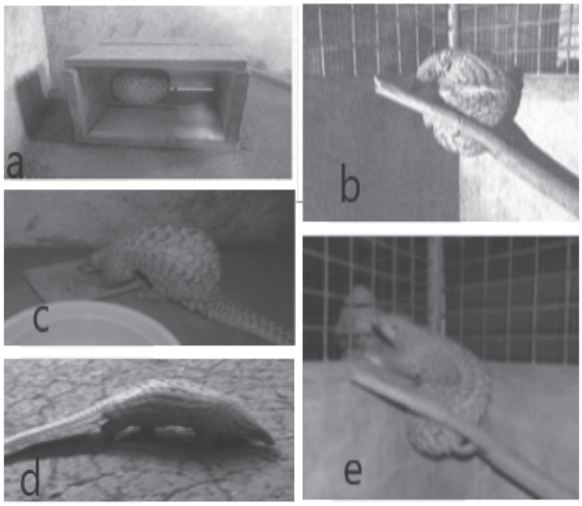

Fig. 5 Pangolin in captivity: a. rest in wooden block, b. rest in logs, c. feeding activity, d. walking in the ground, e. social interaction between female and male

Morphological information of female reproductive organs of pangolins (*M. javanica*) (Rachmawati, 2011) and males (Akmal, 2014) have been reported. These things are expected to become a database for the success of pangolin breeding in Indonesia. So, for the next study will conduct is related to the reproductive behavior of pangolin in captivity, supported by analyses of hormone profile and steroid metabolite of female reproductive hormone of pangolins. In an effort to provide greater space for a pair of pangolins to be mated, the connecting door between the two cages is opened. For hormone analyses will be carried out non-invasively, using fecal and urine samples as well as invasively using blood samples. The results of this study are expected as an initial effort to breed pangolins in captivity to support ex situ conservation.

Information of female reproductive biology such as the characteristics of the ovary and estrous cycle as well as monitoring methods are very important because the

success of animal breeding depends on female fertility (Agil, 2007). One method of monitoring the estrous cycle is through the profile of the reproductive hormone, especially estrogen and progesterone. Pangolin is an animal that easily stressed, so the monitoring method should be avoid the animal stress, such noninvasive method. The profile of hormone can be determined using noninvasive method from fecal and urine sample, as well invasive method using blood sample.

Reproductive hormones are steroid hormones which after metabolic processes, especially in the liver, their metabolites will be excreted through feces and urine. Excretion of reproductive metabolites varies in shape and amount in each species. Generally reproductive hormone metabolites are excreted through feces and urine in the form of oestron, oestradiol, pregnanolon or pregnanediol. In some species hormone metabolites are excreted mostly through feces, while some other species through urine. Although hormonal profile analyses of several animal species has been studied, careful use of this noninvasive method carried out in new species must really be the main concern. Characterization of types of reproductive hormone metabolites and hormone assay validation that will be used must be done carefully when measuring hormone levels of new species, so that the results are accurate and can be used to examine reproductive problems of the species (Agil, 2007).

Conclusion

Javan Pangolins have a good adaptability and are able to survive in captivity as an artificial habitat. One of the key factors in the life of the Javan pangolins in captivity is determined by the successful provision of artificial feed formulas as an alternative to natural food substitution. Javan Pangolins shown positive pairing and social interaction as an early indicator of breeding and reproductive processes in captivity. The implementation of javan pangolin breeding and reproduction technology in captivity is still an important challenge in the captive process.

Acknowledgement

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Molecular Analysis of *Tor* spp. (Cyprinidae) in Sumatra

Dewi Imelda Roesma, Djong Hon Tjong, Warnety Munir, Dyta Rabbani Aidil
Biologi Department, Faculty of Mathematics and Natural Sciences, Andalas University

SUMMARY

The molecular study among *Tor* spp. in Sumatra (*T. douronensis, T. tambra, T. tambroides* and *T. soro*) has been conducted using partial sequence of Cytochrome b (Cyt b) and Cytochrome Oxidase I (COI) genes. DNA amplification was done using PCR machine. Analysis was done on 53 sequences of Cyt b (588 bp) and 61 sequences of COI gene (572 bp). Sequence divergences among *Tor* spp. in Sumatra were less than 7% and 3.5% for Cytb and COI respectively. While sequence divergences between *Tor* spp. in Sumatra and *Tor spp.* in other region in Asia were less than 7.3% and 4.5% for Cytb and COI respectively. The low of sequence divergences of *Tor* spp. do not support the separated them into four different species.

Introduction

Tor (Cyprinidae) is the genus of freshwater fish which commonly known as Mahseer or Semah group, widespread in the Trans Himalaya region (Thailand, Nepal, India and Myanmar) and Southeast Asia (Thailand, Cambodia, Vietnam, South China, Malay Peninsula and Indonesia) (Kottelat et al., 1993, Rainboth, 1996, Kottelat, 2013). *Tor* lives in a clean and fast flowing water river habitat inland and also found in cold water in high altitudes area and warm water such as India (*T. putitota* and *T. tor*) (Roberts, 1999; Bhatt et al., 2004). *Tor* species became one of the fishes with high economic value and became the favorite in the fishing game or ornamental fish (Ng, 2004).

Currently, *Tor* comprised of 10 species (Kottelat et al., 2013) which previously consisted of 20 species (Kiat, 2001). There are still many conflicting in the classified of *Tor* species, raising doubts on the clarity of *Tor* taxonomy. *Tor* is distinguished from other Cyprinidae groups based on the presence of the lobe (median lobe) at the bottom of the mouth (Zhou and Chu, 1996). In Indonesia, (Haryono and Tjakrawidjaya, 2006) reported from the Zoology Museum Bogoriensis there are four species namely *T. soro*, *T. douronensis*, *T. tambra,* and *T. tambroides*. According to Kottelat et al. (1993), the four species are distinguished only based on the presence and size of the median lobe, there are no other characteristics that clearly distinguish among the species.

The studies of *Tor* in Indonesia is limited to morphological studies (Haryono and Tjakrawidjaya, 2006), gonad development (Haryono, 2006), genetic structure of *T. tambroides* in South Sumatra and genetic variation of *T. tambroides* in Batang Tarusan river (Wibowo et al., 2013; Wibowo and Kaban, 2015), and the genetic variation of *T. douronensis* in West Sumatra (Roesma et al., 2017). Although *Tor* in Indonesia, especially in Sumatra, became one of the market commodities, the cultivation of *Tor* has not been done in well. The overfishing, habitat degradation or other threats could have a future impact on *Tor* population stability in nature, while, the study of *Tor*, especially in their genetic diversity, has not been finished. The breeder reported that the cultivation business was not satisfactory because of lack of support for genetic data. De Silva et al., (2004) concluded that the genetic information is necessary for the succes of the cultivate efforts.

Currently, molecular studies have been conducted in *Tor* groups using gene markers on mitochondrial DNA (cytochrome b and cytochrome oxidase I) because they have conserved regions, evolutionary rate is faster and are able to categorize at species level (Esa et al., 2006; Nadiatul et al., 2011; Wibowo and Kaban, 2015). Based on the previuos studies, an intensive study of *Tor* spp. in Sumatra using molecular analysis (cytochrome b and cytochrome oxidase I gene) is necessary to establish a clear taxonomy, to know the closeness of the relationship to the success strategy of the crosses in cultivation and appropriate conservation efforts considering the geographical and environmental conditions.

Material and Method

Samples collection

A number of 28 individuals of *Tor* spp. were collected from 13 populations (25 individuals) in West Sumatra, 2

populations (3 individuals) in Jambi for Cyt b and 15 populations (19 individuals) in West Sumatera, (2 individuals) in Jambi and 4 populations (7 individuals) in North Sumatera for COI analysis. A piece of the tissue samples stored into Eppendorf tubes (1.5 ml) which containing 96% ethanol PA. Individual samples were stored within 10% formalin for a while and replaced with 70% ethanol for long term storage

DNA Isolation, PCR and DNA Sequencing

DNA isolation was performed on the protocol Kit INVITROGEN PureLink™ Genomic DNA Mini Kit. The result of DNA isolation was used for amplification using PCR method with thermal cycler (SensoQuest). Primary used were *forward* 5' CGA TTC TTY GCN TTC CAY TTC YT 3' and 5' CCT CCR ATC TTC CGA TTA CAA GAC 3' (reverse) for Cyt b and *forward* (Fish F1: 5 'TCAACCAACCACAAAGACATTGGCAC3') and *reverse* (Fish R1:5'TAGACTTCTGGGTGGCCA AAGAATCA3') for COI gene. The PCR product were checked in 2% agarose at a voltage of 100 V, power 20 W for 50 min. Good quality PCR product were used as samples for sequencing in MacroGen USA DNA Sequencing Laboratory.

Data Analysis

A total of 61 sequences of COI gene were analyzed consisting of 28 *Tor* spp. sequences in Sumatra and 33 sequences taken from Genbank NCBI included out group species (Table not shown). DNA sequences (forward and reverse) were assembled and edited using the DNA STAR program (Burland, 2000).DNA sequence of *Tor* spp. in Sumatra compared with the sequences contained in NCBI, to see the similarity of all sequences in *http://blast.ncbi.nlm. nih.gov/Blast.cgi*. All sequences were aligned using the Clustal X program (Thompson, 1997). Sequences that have been align were edited using BIOEDIT program (Hall, 1999). The number of polymorphism sequences of the nucleotide sequences was analyzed using the DNA Sequence Polymorphism 5.10 (Rozas, 2003). Sequence divergences of all sequences was analyzed using the Kimura-2 parameter model (K2P) in the MEGA 6.0 program (Tamura, 2013). The phylogenetic tree was reconstructed based on four methods; Neighbors Joining (NJ), Maximum Evolution (ME), Maximum Likelihood (ML) and Maximum Parsimony (MP) using MEGA 6.0 (Tamura, 2013).

Result and Discussions

Molecular Analysis:

Cyt b Gene

A 588 bp from 740 bp which was successfully amplified were analyzed for 53 individual Cyt b gene sequences where 25 of them were obtained from the Genbank, NCBI. The average of total nucleotide composition was A: 29.60%, C: 30.20%, T: 27.70% and G: 12.50%. There were 241 bp (40.99%) variable site and 347 bp (59.01%) as a conserved site of a total of 588 bp without insertion or deletion. In the total of 241 variable sites, 165 bp (82.57%) as parsimony site and 42 bp (17.43%) as singleton site. In a total, there were 327 mutations among all sequences with the transition is more common than transversion (Ts : Tv) with bias value R: 3.998**.** Particularly to *Tor* spp. in Sumatra, 60 substitutions were observed consisting of 50 transitions and ten transversions without insertion and deletion.

Construction of a phylogenetic tree using four methods (ML/NJ/ME/MP) in order to know the relationship between *Tor spp.* which represented by NJ tree was shown in **Fig. 1**. NJ tree show similar topologies for ML, ME and MP with a confidence level bootstraping 1000 times using the MEGA Version 6.0 program. Based on the analysis of the phylogenetic tree using four methods, three main clusters were obtained, supported by highly bootstrap value 87/88/84/88 for ME/NJ/ML/MP, respectively. Phylogenetic analysis showed the monophyletic relationship between *Tor* spp. in Asia with *Cobitis elongatoides* and *Cobitis taenia* as outgroup.

The first cluster consists of four species of *Tor* in Sumatra (first subcluster and second subcluster) and three species of *Tor* from China (third subcluster). The second cluster consists three species of *Tor* in Sumatra and *T. tambroides* from Genbank (first subcluster), and *T. putitora*, *T. khudree*, and *T. tor* India (second subcluster). The third cluster consists of *T. qiaojiensis* and *T. hemispinus* and the genus which are said as a synonyms of *Tor*, included *Neolissocheilus, Labeobarbus,* and *Barbus*. The sequence divergences were calculated using Kimura 2 parameter method in MEGA Version 6.0 program. Overall, *Tor* spp. in Asia has a sequence divergences 0.0%-7.9% and *Tor* spp. in Sumatra with sequence divergences 0.0%-6.9%.

According to Kartavtsev (2011), in the vertebrate, the value of distance between sequence of Cyt b gene of the species in the same genus is 10.69 ± 1.34. Based on that value, all the species in the first cluster and first subcluster in the second cluster should be consist of only one same species.

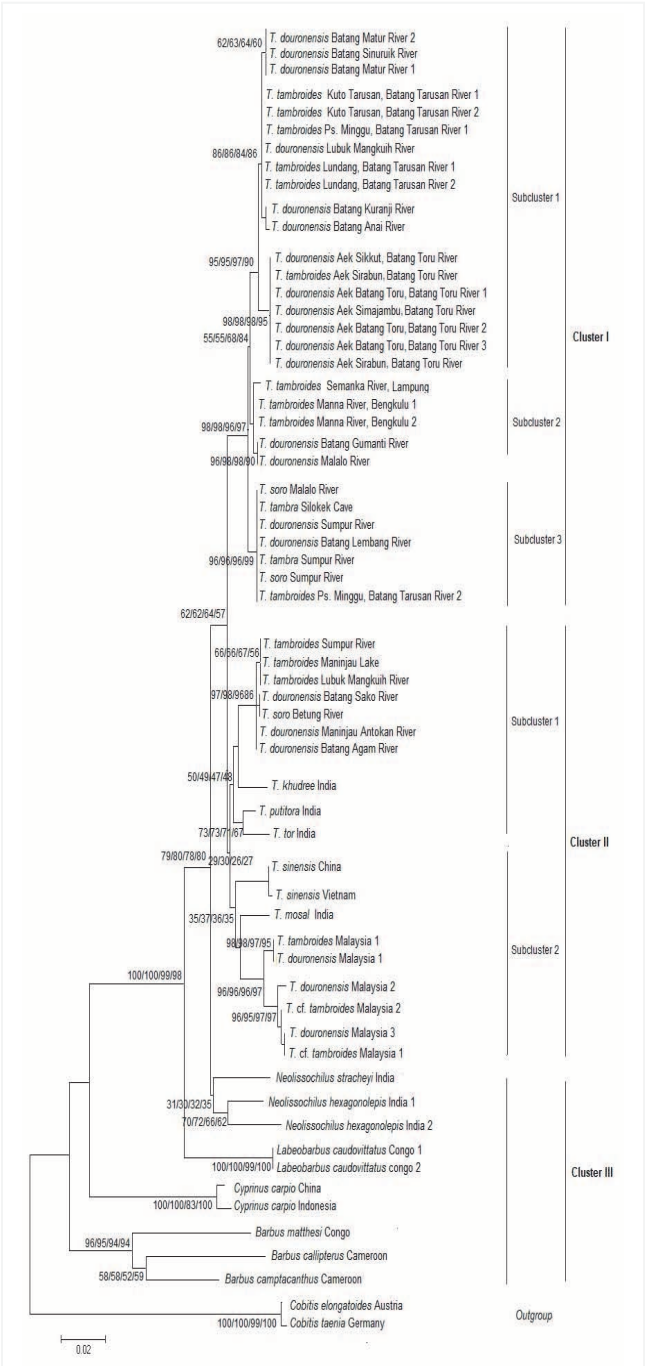

Fig. 1 The Phylogenetic Tree of *Tor* spp. based on Cyt b gene using bootstrap 1000 (ME/NJ/ML/MP)

COI Gene

Part of the COI gene was analyzed from 61 individuals (33 of them from the Genbank, NCBI) using forward (FISH F1) and reverse (FISH R1) primers. As such as 572 bp of COI gene sequences were analyzed from the total length 720 bp which was successfully amplified.

The average of total nucleotide composition was A: 26.40%, C: 27.60%, T: 29.60% and G: 16.40%. The quantity of A + T (56.00%) is higher than G + C (44.00%) which is characteristic for the genome of the vertebrate mitochondrial as well as reported in other Cyprinidae groups (Esa et al., 2012). There were 178 bp (31.12%) with the variable site and 394 bp (68.88%) with the conserved site of a total of 572 bp without insertion or deletion. In the total of 178 variable sites, 165 bp (92.17%) as parsimony site and 13 bp (7.83%) as singleton site. In a total, there were 247 mutations among all sequences with the transition is more common than transversion (Ts : Tv) with bias value R: 5.584. Particularly to *Tor* spp. in Sumatra, 31 substitutions were observed with 28 transitions and three transversions without insertion and deletion.

Construction of phylogenetic tree using four methods (ML/NJ/ME/MP (**Fig. 2**.). The NJ tree show similar topologies to ML, ME and MP with a confidence level bootstraping 1000 times using the MEGA Version 6.0 program. Based on analysis of the phylogenetic tree using four methods, three main clusters were obtained, supported by highly bootstrap value 98/98/96/97 for ME/NJ/ML/MP, respectively. Phylogenetic analysis showed monophyletic relationship between *Tor* spp. in Asia and the out-group (*Cobitis elongatoides* and *Cobitis taenia*).

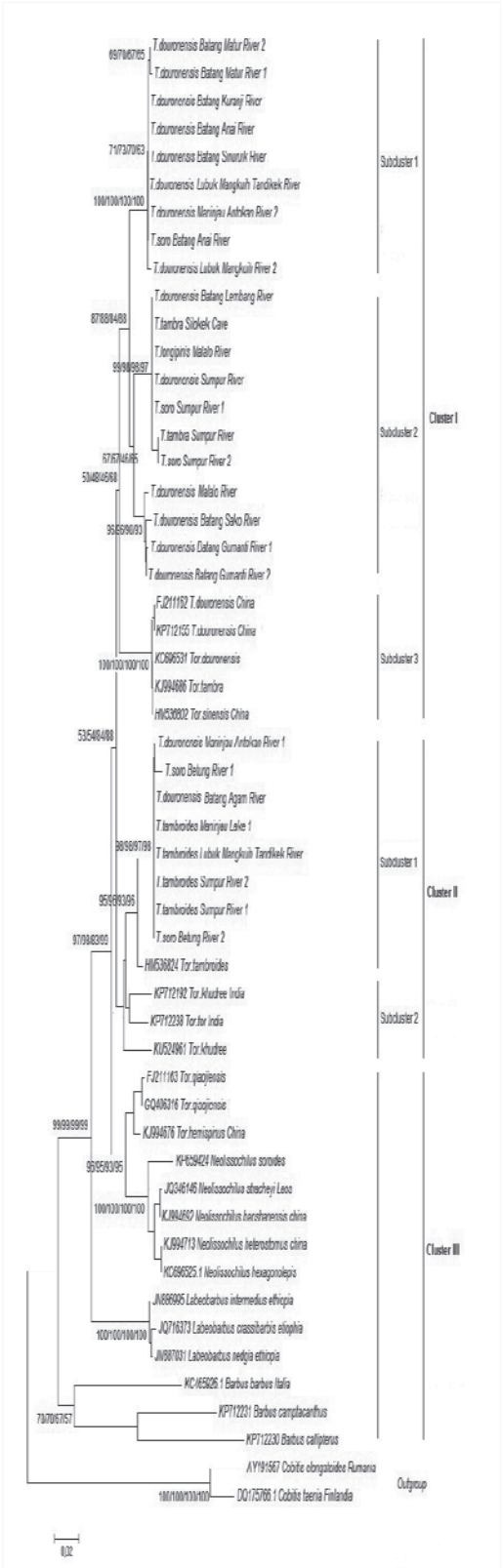

Fig. 2 The Phylogenetic Tree of *Tor* spp. based on COI gene using bootstrap 1000 (ME/NJ/ML/MP)

The first Cluster consists of four species of *Tor* in Sumatra (first, second, and third subcluster with sequence divergences 0.7%-1.4% between them). The second cluster consists of three species of *Tor* in Sumatra, *T.* putitora, *T.* khudree and *T. tor* India (first subcluster) and other *Tor* species from Asia (second subcluster). While the third cluster consists of the genus which are said as a synonyms of *Tor*, included *Neolissocheilus, Labeobarbus, Barbus* and *Cyprinus*. The sequence divergences is calculated using Kimura 2 parameter method in MEGA Version 6.0 program. Overall, *Tor* spp. in Asia has a sequence divergences 2.5%-3.5% and *Tor* spp. in Sumatra with sequence divergences 0.0%-1.4%. Again, according to Kartavtsev (2011), in the vertebrate, the value of distance between sequences of CO1 gene of the species in the same genus is 9.66 $\pm$ 0.72. Based on that value, all the species in the first cluster and first subcluster in the second cluster should be consist of only one same species.

The result of the present study using Cyt b and COI gene showed the overlapping placement of all the species of *Tor* in Sumatra. Nguyen et al. (2008) reported that the unresolved relationship between *Tor* spp. especially on *T. douronensis* with other species which formed from three distinct lineages. Diversity study of *T. tambroides* and *T. douronensis* in Malaysia (Nguyen et al., 2006), showed that poorly resolved on *T. tambroides* and *T. douronensis* taxonomy status with share the some haplotype that allegedly due to gen flow, morphological identification mistake or hybridization between species.

As previously described, these four species of *Tor* were hard to be distinguished morphologically, the grouping into different species generally based on presence and the size of the median lobe (Kottelat et al., 1993). Controversial taxonomy status of *Tor* group had occur since long time ago (Rainboth, 1996; Robert and Kottelat, 1993). The result of present study do not support the separation of four species (*T. douronensis, T. tambroides, T. tambra* and *T. soro*). The result of present study is expected to be a reference for the researcher to conduct the future studies for example in the breeding management of *Tor* species and the determination of conservation status.

Conclusion

The molecular analysis of present study using Cyt b and COI gene do not support the separation of four species (*T. douronensis, T. tambroides, T. tambra* and *T. soro*).

Acknowledgement

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-PART 2-

UGSAS-GU & BWEL JOINT POSTER SESSION ON AGRICULTURAL AND BASIN WATER ENVIRONMENTAL SCIENCES

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Stemflow generation and dissolved organic matter properties in elated to tree size and rainfall characteristic in a subtropical evergreen broadleaved forest of central Japan

Siyu Chen [1], Ruoming Cao [1], Shinpei Yoshitake [2] and Toshiyuki Ohtsuka [3],

1. **The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan**
2. **Takayama Experimental Field Station, River Basin Research Center, Gifu University, 919-47 Takayama, Gifu 506-0815, Japan**
3. **River Basin Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan**

INTRODUCTION

Stemflow variability has been linked to meteorological and biological factors. The meteorological factors include rainfall amount, intensity and duration; wind speed and direction during rainfall events (Levia et al., 2011; Pyker et al., 2011; Van Stan et al., 2011). Linear relationship between stemflow volume and rainfall amount has been reported by many researchers (Price and Carlyle-Moses, 2003; Yang et al., 2008; Van Stan and Levia, 2010). However, the effects of rainfall intensity on stemflow volume remain unclear. Some studies showed that stemflow volume decreased with the rainfall intensity and indicating that heavy rainfall may overload stemflow pathways through the canopy (Crockford and Richardson, 2000; Carlyle-Moses and Price, 2006; Staelens et al., 2008), while some studies found that stemflow increased with rainfall intensity (Van Stan et al., 2014, Zhang et al., 2015). However, the reverse results may due to the great difference of the maximum rainfall intensity in these studies, for example, the maximum rainfall intensity was up to 72mm/h in the research of Crockford and Richardson (2000), while the maximum rainfall intensity was only 4.1mm/h in the study of Van Stan et al., (2014). The biological factors include canopy morphology, diameter at breast height (DBH), basal area, bark morphology and branch angle, etc (Van Stan et al., 2011; Van Stan et al., 2014; Zhang et al., 2015). Previous studies have shown that the bark morphology plays a crucial role in stemflow volume and is a key constraint at smaller rainfall magnitudes, bark water storage capacities differed significantly among different species (Levia and Herwitz, 2005; Carlyle-Moses and Price, 2006; Van Stan and Levia 2010). And forest canopies had a strong impact on the redistribution of rainfall and ecohydrological processes occurring in forested landscapes (Owen et al., 2006). Stemflow volume has been investigated to bary greatly as a function of tree species. For example, coniferous trees were more inclined to generate stemflow than broadleaved trees (Huber and Iroume, 2001); stemflow production of deciduous trees varies over time as a result of foliation and defoliation (Staelens et al., 2008)

Therefore, the present study investigated stemflow production of different tree sizes in more than 100 individual rainfall events during 2 years (2016-2017) in a natural *Castanopsis cuspidata* evergreen forest with different tree sizes (evergreen broadleaved forest) of central Japan, emphasized on the stemflow of individual tree at individual rainfall event scale. Accordingly, the novelty and main objective of this study is to analyze the tree size associates with rainfall characteristics effects on stemflow generation basing on the large data base at individual rainfall event scale. We hypothesized that: (1) rainfall amount and intensity have different influences on stemflow volume, stemflow initiation threshold,stemflow percentage and funneling ratio (FR); (2) tree size is an important factor to control the stemflow generation efficiency (stemflow volume, stemflow initiation threshold, stemflow percentage and FR) at individual tree scale, bigger trees would be more effective at generating stemflow; (3) DOM properties of stemflow may be related to tree size.

MATERIALS AND METHODS

The study site is located on Mt. Kinka, central Japan. A 0.7 ha study plot (70 m × 100 m) was established on the lower slopes of Mt. Kinka (ca. 60 m a.s.l., 35° 26′ N, 136° 47′ E) in 1989. The study area has a subtropical monsoon climate and the study forest is an evergreen broadleaved forest, which has a basal area of 46.1 m2 ha-1, the dominant tree species of this forest is Castanopsis cuspidata, which accounted for 87.86% of basal area and is also the dominant canopy tree species in the study plot (Chen et. al, 2017). The annual mean temperature is 16.1 °C, and the mean temperature in the coldest month, January, and the hottest month, August, are 4.4 °C, and 28.0 °C, respectively. The study area has abundant precipitation, with an annual average precipitation of 1926 mm during 2016-2017. The climatic data was collected at a weather station near the study plot.

To measure stemflow hydrologic flux, 12 individual trees of dominant species (C. cuspidata) were selected for observation, including 4 DBH classes (20-30 cm, 30-40 cm, 40-50 cm, >50 cm) with 3 individual trees in each DBH class. Stemflow volumes were collected from June 2016 to December 2017 and represented 111 rainfall events. Stemflow samples of 12 trees were collected from the sample reservoir tank twice a month for chemical analysis.

RESULTS AND DISCUSSION.

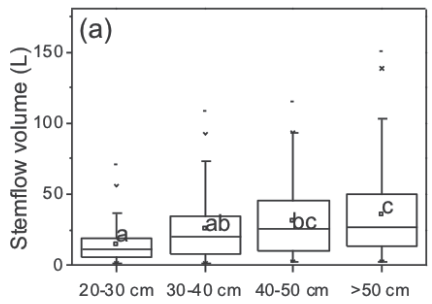
Results indicated that individual stemflow volume of *C. cuspidata* had an exponential relationship with incident rainfall depth, stemflow volume increased with increasing rainfall depth and followed a positive exponential function ($p < 0.001$), which was more significant in smaller (20 cm < DBH < 30 cm) trees. In general, individual stemflow volume was increasing when rainfall intensity was less than 15 mm/h, however, when rainfall intensity was greater than 15 mm/h, stemflow tended to decrease with increasing rainfall intensity.

Moreover, stemflow volume increased with increasing DBH class, DBH > 50 cm class produced the highest amount of stemflow (average = 14.87 L), more than twice of 20-30 cm DBH's (average = 35.65 L) (Fig. 1a). Further analysis revealed that stemflow volume of different DBH class demonstrated great differences under rainfall events with different rainfall intensity. Significant differences were found among different DBH class during the rainfall events with rainfall intensity was less than 15 mm/h, which were the larger trees produced more stemflow than the smaller trees, the

average stemflow volume of DBH class > 50cm (35.8 ± 3.1 L) was nearly 3 times as the 20-30 cm'(13.2 ± 1.2 L). Whereas the average stemflow volume was 32.9 ± 9.8, 42.0 ± 9.9, 36.2 ± 9.1 and 38.6 ± 10.0 for 20-30 cm, 30-40 cm, 40-50 cm and DBH >50 cm, respectively during the rainfall events with the rainfall intensity was more than 15 mm/h, the differences among different DBH class were not significant.

Rainfall depth and intensity had a greater impact on stemflow percentage of lager trees (DBH > 40 cm) than that of small trees (DBH < 40 cm), and stemflow percentage of DBH > 40 cm trees decline sharply with increasing rainfall up to 50mm (Fig.1b). Moreover, a negative exponential relationship was observed between stemflow percentage of incident rainfall and rainfall intensity, Stemflow percentage decreased with increasing rainfall intensity, and the stemflow percentage decreased rapidly when rainfall intensity less than 15 mm/h, then the stemflow percentage tended to be stabilized no more than 5%. Comparing the stemflow percentage in different DBH class, we found significant differences between different DBH class, stemflow percentage of 20 – 30 cm DBH class was least (average = 3.8 ± 0.15%), while the DBH > 50 cm DBH was largest (average = 10.6 ± 0.64%), stemflow percentages of 30 – 40 cm and 40 – 50 cm DBH class was 6.4 ± 0.27 % and 9.0 ± 0.51%, respectively. Moreover, the variabilities of stemflow percentages were different under different rainfall intensity, the difference was significant among different DBH class when rainfall intensity was less than 15mm/h, whereas the rainfall intensity was more than 15mm/h, the stemflow percentage appeared to be unaffected tree sizes.

Similar to stemflow percentage, negative exponential relationships were found between FR and incident rainfall depth / rainfall intensity across all DBH class, and the correlations were more clear when tree size more than 40cm. In contrast to stemflow percentage, the FR of *C. cuspidata* was highest in 20 – 30 cm DBH class with an average of 17.9, and lowest in DBH > 50 cm class with an average of 9.0. For 30 – 40 cm and 40 – 50 cm DBH class, FR averaged 15.8 and 11.6, respectively (Fig. 1c). The variabilities of FR among tree sizes also different during different rainfall intensity, while the rainfall intensity was less than 15mm/h, significant difference was not found among the smaller trees (DBH < 40 cm), but found in the bigger trees (DBH < 40 cm). However, while rainfall intensity was more than 15mm/h, significant difference was found in smaller trees (DBH< 40cm), and no significant difference in bigger trees (DBH > 40cm).



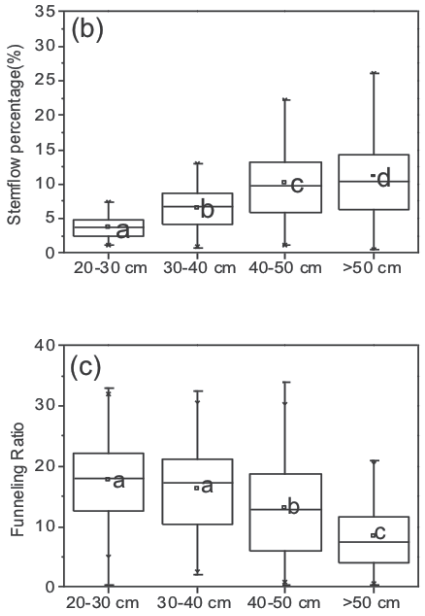


Fig. 1: Box plots depict (a)stemflow volume, (b)Stemflow percentage, (c) Funneling Ratio(FR) of different DBH class for *C. cuspidata* (p<0.05).

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Future changes of intensity and structure for landfalling typhoons in Japan by pseudo-global warming experiments

Masaya Toyoda, Jun Yoshino, and Tomonao Kobayashi
Environmental and Renewable Energy Systems Division, Graduate School of Engineering, Gifu University

INTRODUCTION

Japan is a typhoon-prone country where typhoons constantly approach with an average of 11.5 in a year and make landfall with an average of 2.9 typhoons in a year. The IPCC Fifth Assessment Report (IPCC, 2013) suggests future changes in the characteristics of tropical cyclones may occur, while there is a significant amount of uncertainties in their predictions. In order to estimate future change of coastal disasters such as storm surge and high wave, it is important to project the intensity and structure of future-climate typhoons. Although there are several researches on the future changes for intensity and track of future typhoons based on the statistical approaches (Tsuboki et al., 2014; Toyoda et al., 2017), there are few reports investigating intensity and size of future typhoons based on the dynamic approaches.

In this study, for a total of 52 typhoon which made landfall in Japan islands from 2000 to 2017, ensemble pseudo-global warming experiments (CMIP5 RCP8.5 scenario: 2080 - 2099) are conducted using a high-resolution typhoon model. Based on the experimental database, we investigate the future change of the maximum wind speed (MW) and the radius of maximum wind speed (RMW) at landfall time in Japan.

COMPUTATIONAL METHOD

(1) Target typhoons

As target typhoon in this study, a total of 52 typhoons from 2000 to 2017 which made landfall in the Japan islands are selected. Fig. 1 shows the tracks of all 52 typhoons provided by the Japan Meteorological Agency (JMA). According to the JMA record, 53 typhoons made landfall in Japan during 2000 to 2017. Only one case, that is Typhoon CHATAAN (2002), is excluded from the target typhoons because it is difficult to calculate the whole life of the typhoon due to the deficiencies of input initial and boundary conditions.

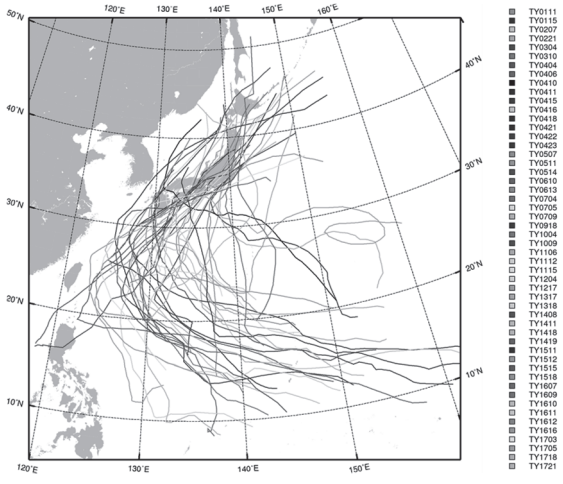

Fig.1 The track of 52 landing typhoons in 2000 ~ 2017 (JMA Best Track) covered in this research

(2) High-resolution typhoon model (HTM)

The high-resolution typhoon model (hereafter HTM) is used to simulate the intensity and size changes of overall life of typhoons. HTM is based on the mesoscale meteorological model MM5 (Dudhia, 1993). The automatic movable nesting technique and the several kinds of physical parameterizations (e.g. the ocean mixed layer, dissipative heating, and sea-spray processes) are introduced into MM5 to express realistic typhoon intensity and structure accurately (Yoshino et al., 2013). The triply nested computational domains used in this study have a horizontal grid spacing of 27-km (D1), 9-km (D2), and 3-km (D3), respectively. The movable nesting technique is applied to both D2 and D3.

(3) Present climate and future climate experiments

In this study, first of all, present climate experiments are carried out using NCEP FNL data as initial, boundary and assimilation conditions. We conduct future-climate experiments by applying the method of pseudo-global warming experiment (Sato et al., 2007) to estimate future changes of intensity and structure of typhoons in comparison with present climate observations. The input conditions for the pseudo-global warming experiment are created by adding monthly average global warming difference (GWDs) to NCEP FNL. The GWDs are the difference of monthly mean fields between the future climate present climate. Temperature, Sea surface temperature, geopotential height, east-west wind speed, north-south wind speed and relative humidity are used as the 3D monthly mean fields derived from the general circulation model GCMs. In this study, the monthly mean fields in the future climate are averaged during 2080 - 2099 by a total of 8 GCMs of the CMIP5 RCP8.5 scenario (high-level reference scenario: supposed not to do policy mitigation policy) In addition, the monthly mean fields in the present climate are averaged during 2000-2019 by 15 GCMs of the CMIP5 historical scenario (2000-2005) and 8 GCMs of the CMIP5 RCP8.5 scenario (2006-2019).

RESULTS AND DISCCUSION

(1) Present climate experiments

First, we discuss the results of the MW and RMW of 52 typhoons simulated by the present climate experiments. The MW and RMW simulated by HTM are smoothed in 6 hours. Figure 2 compares the observed and simulated MW of 52 typhoons at the landfall time. Figure 3 also compares the observed and simulated RMW of 52 landfalling typhoons. In terms of MW, the bias error is +1.27 m/s, the root means square error is +5.35m/s, and the correlation coefficient is 0.75. In terms of RMW, the bias error is +10.0 km, the root means square error is +29.6 km, and the correlation coefficient is 0.73. Therefore, it can be said that the results of the simulated global warming experiments in the next section is highly reliable.

(2) Pseudo-global warming experiments

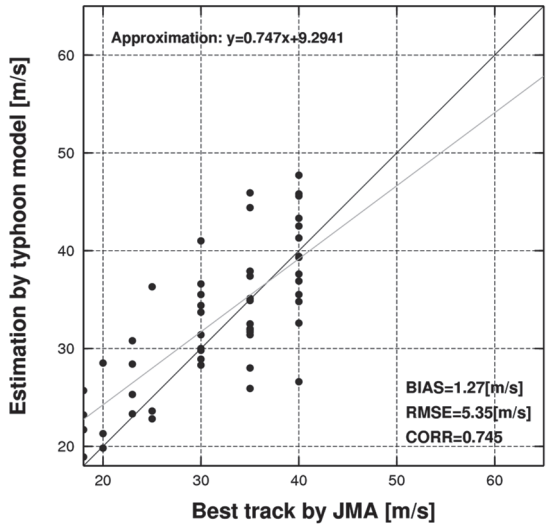

Fig.2 A scatter diagram and statistical scores of the MW between estimation by CNTRL and observation by JMA.

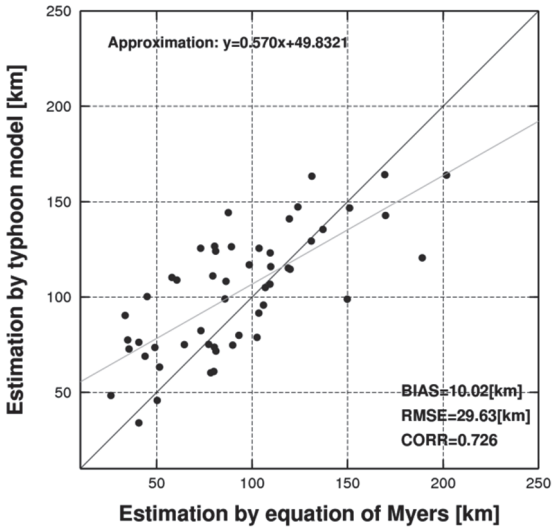

Fig.3 A scatter diagram and statistical scores of the MW between estimation by CNTRL and observation by JMA.

Next, we compare with pseudo-global warming experiments present climate experiments and discuss future changes in MW and RMW at typhoon landfall time. (Table.1). The average MW and the average RMW at landfall time are 32.9 m/s and 102.5 km in the present climate and are 38.4 m / s and 90.2 km in the future climate, respectively. The future changes are + 5.48 m/s and -12.3 km, and there are significant differences in one-tailed test at a significance level of 5%. 41 typhoons show that the MW are increased, and the greatest change between the future climate and present climate is +22.5 m/s by Typhoon USAGI (2007). In addition, 14 landfalling typhoons corresponding to about 27% of the total typhoons are categorized as the strength of either "very strong" or "furious" under the future climate. On the other hands, 32 typhoons indicate that the RMW is decreased, and the largest future change is - Km by Typhoon PABUK (2011). Therefore, future landfalling typhoons in Japan tend to increase the MW and to decrease the RMW. It is expected that the future trends of typhoon intensity and structure provided by this study will be useful to establish and redevelop the policy of typhoon-related disaster mitigation and prevention.

Table.1 Summarize of results of pseudo-global warming experiments (Bold number mean significant results).

	Present climate	Future climate	Future change
Maximum wind speed (m/s)	32.9	38.4	**+5.48**
Radius of maximum wind speed (km)	102.5	90.2	**-12.3**

CONCLUSION

In this study, we conducted present climate experiments and pseudo-global warming experiments for 52 landfalling typhoons in Japan from 2000 to 2017. It became clear that under the future climate, the maximum wind speed of typhoon is increased, on the other hands, the radius of maximum wind speed tends to be decreased by the future global warming. The changes in the characteristics of landfalling typhoons in Japan are considerable in the future. It is expected that future trends of typhoon intensity and structure obtained in this research can be utilized for disaster prevention and disaster reduction measures in the future.

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Historical of agriculture transition and livelihood change in northern Laos after road construction: Case study in Sone District, Houaphan Province

Cahyo Wisnu Rubiyanto[1] and Isao Hirota[2]
1. **The United Graduate School of Agricultural Science, Gifu University, Japan**
2. **Faculty of Applied Biological Sciences, Gifu University, Japan**

INTRODUCTION

Rural area has characterized as low population size, low educational level, low socioeconomic status, high rates of chronic disease and mortality, and low access to health care services (Sharkey and Horel 2008). In Laos, 80% of population live in countryside (UNDP 2004), where they have low standards of infrastructure, especially roads. Road construction is one of important factors altering rural livelihoods. Improving road access is considered to be an effective way of improving livelihoods and reducing rural poverty (Rigg 2006; Warr 2006).

Local people in Laos have heavily depended on natural resources and now the livelihood system is about to change. The impacts of road construction bring various kinds of changes of local society and nature. The expansion of agricultural field and following destruction of habitats of various natural resources might be observed at local level on one hand, and it can bring opportunity to access more cash income source and contribute decrease poverty on the other hand. To know the dynamics of local livelihood and natural resource from various aspects is indispensable to present sustainable system and relationship between society and nature.

This study compares two villages with different histories and conditions of road; (1) Houay Su village (middle access) and (2) Bong village (bad access), in Sone district, Houaphan province, northern Laos after preliminary survey of 14 villages in the district and examine how road construction influence the dynamics of natural resources and rural livelihood. This study aims to clarify the historical rural livelihood change by focusing on commercialization of agricultural products and non-timber forest products (NTFPs), economic improvement at village and household levels, historical transition of agricultural system such as expansion of agricultural land and following deforestation of surrounding environment between two villages..

MATERIALS AND METHODS

The livelihood change is described based on statistic data and by interviewing key informants of each household and person in charge district related to local livelihood. After the collection of both quantitative and qualitative data were collecting through focus group discussion and individual interviews. In addition to participatory observation relating to research objectives, seventy households of two villages were chosen and interviewed with semi-structured questionnaire. In this study, wealth ranking methods were applied for analyzing collected data and information.

RESULTS

Livelihood changes in both villages are shown in Fig. 1 and Fig. 2 Fig. 1 shows that the villagers in Bong village have cultivated rice in upland field. Number of household collecting NTFPs have increased and red mushroom has increased drastically since 2015. Number of cultivating maize had increased until 2014 then decreased in 2015. Recently, number of cultivating new cash crops such as cassava have increased.

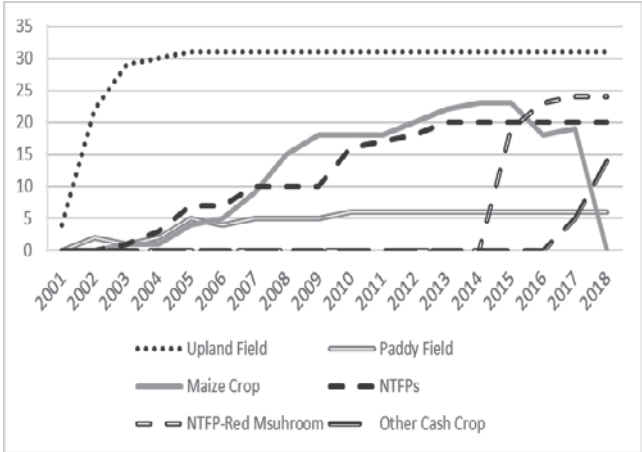

Fig. 1: Change of number of households from 2001 to 2018 in each livelihood activity in Bong village.

Fig. 2 shows that the villagers in Houay Su village mostly cultivate upland rice and collect NTFPs. Paddy field had expanded steadily along with the improvement of access and it provided opportunity and flexibility to local people to expand cash crops to get more income. Number of household cultivating maize gradually increased until 2016, but decreased drastically in 2017 and was followed by increase in new cash crops such as coffee, cassava, cardamom and *Amorphophallus* sp. Almost all villagers cultivate coffee since 2017 supported by Lao Z49 organization from the United States. They chose Houay Su village to cultivate coffee as an alternative of opium poppy cultivation.

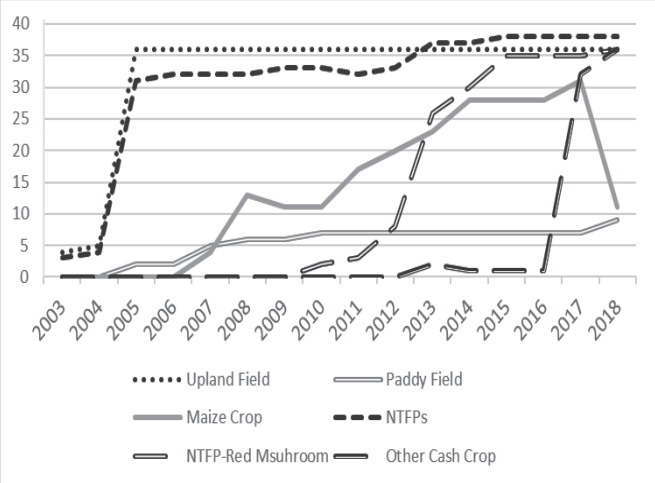

Fig. 2: Change of number of households from 2001 to 2018 in each livelihood activity in Houay Su village.

DISCUSSION

This study clarified that road construction has given impacts directly to agricultural activities and livelihood changes. The village committee in Bong village allocated 2 a per person per household to all households, so, the household who have a lot of number of family, they will get more paddy area to provide their food security. Even though the villagers had changes their livelihood in the last decade, they still hold out to cultivate rice in upland field and collecting NTFPs especially red mushroom as their livelihood strategies. On the other hand, cultivating rice in upland field and collecting NTFPs are the main livelihood in Houay Su village, and in the recent years, agricultural land has expanded to include new cash crops. The people in Bong village with bad accessibility did not have alternative cash crops because of less opportunity with traders and development projects. The people in Houay Su village with good accessibility have many alternative cash crops for sustainable livelihood. However, cultivating cash crops related to expansion of agricultural land and it might be influences the environmental consequences of expansion, as driver of deforestation; replace forests, degraded forests, or grasslands (Gibbs et al. 2010).

ACKNOWLEDGMENTS

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Antibiotics resistance genes in vermicomposting of fruit and vegetable waste added with excess activated sludge

Wenjiao Li [1], Guangyu Cui [1], Yasushi Ishiguro [2] and Fusheng Li [2]
1. **Graduate School of Engineering, Gifu University, Japan**
2. **River Basin Research Center, Gifu University, Japan**

INTRODUCTION

Fruit and vegetable waste is generated during production, processing and consumption. Conventional methods used for treatment and/or recycling of fruit and vegetable waste include incineration, landfill, anaerobic digestion, feeding and composting. Compared to these methods, vermicomposting has the advantages of effective stabilization of the organic waste through the combined involvement earthworms and bacteria in decomposition of the easily decaying organic constituents and of higher reuse value for its final product as fertilizers or soil conditioner.

On the other hand, activated sludge is the main byproduct of sewage treatment process and is also an organic waste, which is mainly consisted of microorganisms and can be treated by vermicomposting. The rich content of proteins in activated sludge may promote the growth of earthworms, thus improving the stabilization efficiency of fruit and vegetable waste and may improve the value of the final products as fertilizers due to the high content of nitrogen and phosphorus. Our previous results on vermicomposting of fruit and vegetable waste indicated that adding excess activated sludge could play a positive role in the growth of earthworms, decomposition efficiency of fruit and vegetable waste and the fertilizer value of the final products.

However, numerous studies have identified that activated sludge is a significant reservoir of antibiotics resistance bacteria (ARB) and antibiotics resistance genes (ARGs) due to overused antibiotics and their metabolites discharged into the sewage system. Some researchers mentioned that ARGs might proliferate in sludge due to the preferential survival and selection pressure of ARB during wastewater treatment. Adding activated sludge into the vermicomposting system treating fruit and vegetable waste can bring ARGs into the system. As a result, depending on the fate and behavior of ARGs in the vermicomposting process, the safety of the final product as fertilizers may differ greatly. Nevertheless, information regarding the fate and behavior of ARGs during vermicomposting of fruit and vegetable waste added with excess activated sludge is not available.

Accordingly, the main objective of this study was to investigate the occurrence and behavior of ARGs during vermicomposting of five types of fruit and vegetable waste added with excess activated sludge.

MATERIALS AND METHODS

1. Vermicomposting experiment

The earthworm species *Eisenia foetida* was used. Fruit and vegetable waste included banana peels, cabbage, lettuce, carrot and potato. Excess activated sludge was collected from a slaughterhouse wastewater treatment plant and was used after dewatered. A mixture of soil with final vermicompost from our previous study was used as the bed material to avoid the threat to earthworms' survival from leachate generated during vermicomposting. Eleven plastic containers were used as the reactors for vermicomposting. Each reactor included two compartments, namely the treatment compartment and the bed compartment. The image diagram of the reactors is shown as **Fig.1**. The reactors were divided into a control group and a test group. The bed compartment was filled with 100 g (wet basis) of bed material, and then 10 earthworms (0.35 - 0.50 g/worm) were invited. For the treatment compartment of the control reactors, 100 g (wet basis) of banana peels, cabbage, lettuce, carrot, potato and sludge were added respectively. For the treatment compartment of the test reactors, 100 g (wet basis) of the mixture of each type of fruit and vegetable waste with sludge were added respectively at the mixing ratio of 3:2 (fruit and vegetable waste : sludge). All reactors were kept in dark and were run under a constant temperature (25 ℃).

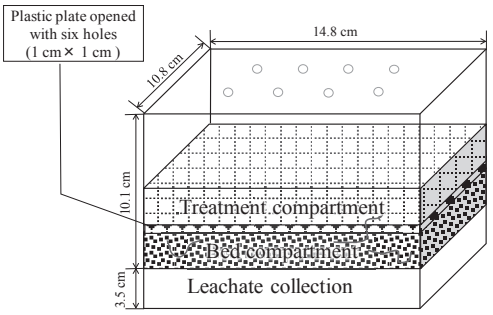

Fig.1 Image diagram of each vermicomposting reactors.

2. DNA extraction and quantitative PCR (qPCR)

Frozen dried samples were used for extracting and purifying total genomic DNA with the DNA extraction kit (MOBIO, USA) based on manufacturer's manual.

Typical ARGs targeted in this study included *tet G*, *tet M*, *tet X*, *sul 1* and *qnr S*. Class 1 integrase (*intl 1*) was also targeted. The choice of these ARGs for study is because tetracycline resistance genes, sulfonamides resistance genes and quinolone resistance genes have been widely detected from wastewater treatment plants due to the overuse of the relevant antibiotics for animal production and in human medical care. The abundance of the ARGs, *intl 1* and bacterial 16S rDNA gene was determined by the quantitative PCR instrument (TP800, Takara, Japan). The standard DNA of *tet G*, *tet M*, *tet X*, *sul 1*, *qnr S* and *intl 1* were constructed by the TA-cloning method (Takara, Japan). Each gene was quantified in triplicate for each sample.

RESULTS AND DISCUSSION

1. Abundance of ARGs and intl 1

Compared to the fruit and vegetable samples before adding the sludge, the samples after mixing the sludge showed apparently higher values for the abundance of ARGs and *intl 1* before the start of vermicomposting. As displayed in **Fig. 2**, *tet G* in the treatment compartment increased from 3.82E+06, 5.27E+05, 4.39E+05, 1.00E+06, and 8.81E+05 copies/g-dry to 9.47E+07, 4.92E+07, 8.67E+07, 1.01E+08, and 8.98E+07 respectively in the treatment for banana peels, cabbage, lettuce, carrot and potato after adding the sludge. The observed increases can be easily explained as the result brought about by rich *tet G* in the sludge (1.44E+08 copies/g-

dry). Similarly, significant increases were also observed for other genes except *qnr S* in the treatment for lettuce, as could be seen from **Table 1**. For *qnr S* in the treatment for lettuce, since the abundance of *qnr S* in lettuce (4.62E+05 copies/g-dry) was closer to that in the sludge (5.27E+05 copies/g-dry), a significant increase after adding the sludge was not revealed.

After vermicomposting, for the treatment of fruit and vegetables added with the sludge, the results in **Fig. 2** showed that *tet G* in the treatment compartment decreased. For the treatment without adding the sludge, *tet G* showed a general trend of increases for all fruit and vegetables. The values of the abundance of *tet G* in the treatment compartment of the treatment with the addition of the sludge were markedly higher than the treatment without the addition of the sludge. For *tet M*, *sul 1*, *qnr S*, and *intl 1*, similar results with *tet G* were found in the treatment compartment after vermicomposting. For *tet X*, however, the abundance in the treatment compartment before vermicomposting fell in the range of 2.62E+04 – 2.38E+06 copies/g-dry for all reactors, and increased to 6.94E+04 – 3.27E+07 copies/g-dry after vermicomposting.

In this study, the material in the bed compartment after vermicomposting was considered as the final product. No significant changes of *tet G* were found after vermicomposting in all bed compartments except for the treatment for potato, sludge, and the cabbage added with sludge, as could be seen from **Fig.2**. The changing extent of other ARGs and *intl 1* in the final product with the addition of sludge was shown in **Table 1**. Apart from the changes revealed for *intl 1*, *tet G*, and *sul 1* in the treatment for cabbage after adding sludge, and the changes revealed for *qnr S* in the treatment for banana peels after adding sludge, no significant changes were observed in the final product of other treatments. Meanwhile, even if abundant ARGs were introduced into the vermicomposting system by adding sludge, the ARGs were not remained or transferred into the final product during the vermicomposting process. Considering both the treatment compartment and bed compartment, the reduction of ARGs was detected during the vermicomposting. Huang et al. (2018) reported a similar result on the changes of *tet C*, *tet G*, *tet M*, *tet O*, *tet W* and *tet X* during vermicomposting treatment of sludge, with the reduction rate of the genes being 83.1%, 39.6%, 99%, 80.2%, 94.1% and 60.9%, respectively. The reduction is probably due to the reason that earthworms regulated the bacterial community and their environment in vermicomposting system.

2. Abundance of 16S rDNA gene

The abundance of 16S rDNA reduced significantly in all treatment compartments after vermicomposting except the treatment for banana peels, cabbage and its mixture with sludge. The largest decrease occurred in the treatment for carrot (from 3.27E+10 copies/g-dry to 8.52E+09 copies/g-dry), followed by the treatment for sludge (from 2.44E+10 copies/g-dry to 5.70E+09 copies/g-dry). A previous study reported decreases in the abundance of 16S rDNA for all treatments of excess activated sludge with three different earthworm densities. The decreases are probably due to the rapid depletion of nutrients by the intensified digestion of earthworms (Cui et al. 2018). In contrast, different results were observed in the bed compartment of the treatments for banana peels, potato, the mixture of banana peels and sludge, the mixture of lettuce and sludge. The most significant increase occurred in the bed compartment of the treatment for potato (from 2.76E+10 copies/g-dry to 3.72E+10 copies/g-dry). Huang et al. (2018) revealed an increase of 16S rDNA during vermicomposting due to the effect of earthworms in enriching the agricultural probiotics in the final vermicomposting product. It is worth to notice that a significant decrease (from 2.76E+10 copies/g-dry to 1.75E+10 copies/g-dry) in the bed compartment was detected for the treatment for cabbage. The significant difference of the ARGs in the bed compartment for the treatment of cabbage with and without the addition of sludge shown in **Table 1** can probably be explained by the changes of 16S rDNA.

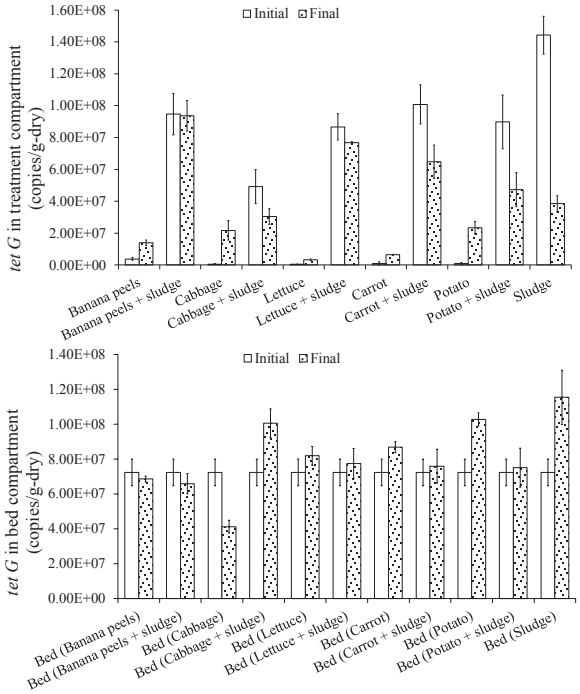

Fig. 2 Changes of *tet G* in the treatment compartment and bed compartment before and after vermicomposting. Data are represented as mean and standard deviation (n = 3).

Table 1 The changing extent of ARGs and *intl 1* after adding sludge in the treatment compartment before vermicomposting and the bed compartment after vermicomposting. Values show the percentage of changes in the treatment compartment, and the values in the parentheses show the percentage of changes in the bed compartment.

	intl 1 (%)	*tet G* *(%)*	*tet M* (%)	*tet X* (%)	*sul 1* (%)	*qnr S* (%)
Banana peels	816.9 (19.6)	2376.9 (-3.9)	2398.4 (0.9)	1779.6 (22.5)	2108.0 (51.9)	1195.2 (684.8)
Cabbage	5028.7 (104.9)	9247.3 (143.9)	3089.1 (44.0)	2915.1 (63.5)	34136 (335.4)	100.7 (-55.7)
Lettuce	11543 (-45.0)	19647 (-5.4)	33684 (35.0)	4382.8 (-68.7)	71718 (-41.9)	-1.2 (186.6)
Carrot	181.5 (-20.4)	9964.3 (-12.6)	6077.3 (-35.1)	5327.1 (-56.3)	4691.8 (-25.5)	671.3 (-7.3)
Potato	66016 (-17.0)	10095 (-26.9)	20682 (19.3)	4745.3 (-28.4)	74554 (-26.0)	4014.3 (22.4)

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Mating-type diversity in *Phytophthora colocasiae* causing leaf-blight of taro in Japan

Wenzhuo Feng[1], Kayoko Otsubo[2], Ayaka Hieno[2], Haruhisa Suga[3] and Koji Kageyama[2]
[1] The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[2] River Basin Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[3] Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott) is one of the most popular staple crops for its edible corms and leaves in Japan. Recently in Japan, especially in Ehime, Miyazaki and Kagoshima prefectures where are the major production areas for taro, the disease of taro leaf blight has occurred since 2014 and is expanding year by year. The causal agent was isolated from diseased leaves and identified as *Phytophthora colocasiae* Raciborski. The pathogen can asexually produce zoosporangia, and zoospores released from them can spread rapidly via water during the frequent heavy rain and typhoon period. It has been reported that in naturally infested soils, the soil survival of *P. colocasiae* sporangia is less than 21 days, lasts for 3 months in taro leaves, and lasts for more than 107 days in soil at –1,500 J/kg matrix potentials. Therefore, in addition to zoosporangia, the mating-type of *P. colocasiae* isolates that is related to long-lived oospore formation is very important for determining a primary inoculum.

Phytophthora colocasiae has been reported in many locations, such as Southeast Asia and the Pacific. The species was previously described as only existing heterothallic isolates, depending on the presence of opposite mating types (A1 and A2) to form oospores, until Lin and Ko (2008) found seven homothallic A1A2-type isolates in Taiwan. However, oospores seemed difficult to form in most of the areas because of non-coexistence between A1 and A2 type there (Narula and Mehrotra 1980; Ann et al 1986; Tyson and Fullerton 2007; Mellow et al. 2018). Up to now, there is no report about the mating type of *P. colocasiae* in Japan. Therefore, in this study, our objectives were to investigate the mating-type diversity, and analysis their distribution in taro fields in Japan.

MATERIALS AND METHODS

Collection and maintenance of isolates

A total of 150 isolates were isolated and obtained from the diseased taro leaves and petioles of 9 fields in Kagoshima, 30 fields in Miyazaki and 15 fields in Ehime prefecture (Table 1). All isolates were maintained on corn meal agar or potato-dextrose agar media at 20°C in the dark.

Mating type testing

All isolates were inoculated on 5 ml V8 medium (1 ml V8 juice, 12.5 mg CaCO3, 0.1 g agar and 4 ml distilled water) at 25°C in darkness, respectively. Two heterothallic isolates obtained from taro fields (EPC201522 and EPC2017Ko1) were selected as standard type strains and strictly verified that the former isolate is A1 mating type and the latter is A2 mating type.

Totally, three V8-medium plates were prepared for each isolates, one for incubating alone, and the other two for pairing it with A1 strain of EPC201522sh1 and A2 strain of EPC2017K01sh, respectively. The oospores in plates for pairing were examined after incubation for 6 days and 10 days. The absence of oospores at the interface between colonies indicated the same mating type, while the presence of oospores indicated the opposite mating type. The plates for incubating alone were examined twice after more than a month to determine whether the isolate is heterothallic or homothallic based on the formation of oospores.

RESULTS AND DISCUSSION

Mating-type diversity

In all, there were five kinds of mating types confirmed from the 150 isolates: two kinds of heterothallic isolates of A1 and A2 types, and three kinds of homothallic isolates that generated oospores in the plates of incubation alone, containing A1 type which generated oospores with pairing of A2 strain, A2 type which generated oospores with A1 strain, and A1A2 type which generated oospores with both of A1 and A2 strains. This complex mating-type diversity in *P. colocasiae* has not been reported all over the world.

Mating-type distribution in taro fields

Table 1. The mating-type distribution in three prefectures

Location		Number of fields	Number of isoltates	Heterothallic		Homothallic[a]		
				A1	A2	A1	A2	A1A2
Kagoshima	1	1	5	0	2	0	2	1
	2	2	3	0	2	0	0	1
	3	2	14	0	5	0	9	0
	4	1	6	1	2	0	2	1
	5	3	31	0	11	0	20	0
	Total	9	59	1	22	0	33	3
Miyazaki	1	2	3	0	3	0	0	0
	2	1	1	0	1	0	0	0
	3	6	9	0	5	0	4	0
	4	14	24	1	12	0	10	1
	5	1	1	0	1	0	0	0
	6	2	3	0	1	0	2	0
	7	1	1	0	1	0	0	0
	8	1	1	0	1	0	0	0
	9	2	6	0	3	0	3	0
	Total	30	49	1	28	0	19	1
Ehime	1	14	41	6	24	0	9	2
	2	1	1	0	1	0	0	0
	Total	15	42	6	25	0	9	2

[a]Homothallic isolates that generated oospores in the plates of incubation alone, containing A1 type which generated oospores with pairing of standard A1 type, A2 type which generated oospores with standard A2 type, and A1A2 type which generated oospores with both of standard A1 and A2 types.

Totally, we investigated the mating-type of 150 isolates from 54 fields in Japan (Table 1). One, 22 and 36 out of 59 isolates in Kagoshima, 1, 28 and 20 isolates out of 49 isolates in Miyazaki, and 6, 25 and 11 out of 42 isolates in Ehime prefecture were identified as A1 type, A2 type and homothallic, respectively (Table 1). These results showed that in Japan the most isolates were A2 type, only a few isolates were A1 type and more than 44% isolates were homothallic. On the other hand, 23 of 32 fields, where two or more isolates were collected, were found multiply infested

Table 2. The mating types in fields where two or more isolates were collected from

Mating types	Number of fields
A1 only	0
A2 only	8
Homothallic only	1
A1and A2	2
A1 and homothallic	0
A2 and homothallic	16
A1, A2 and homothallic	5
Total	32

with A1 and A2, A2 and homothallic, or A1, A2 and homothallic (Table 2). Hence, this complex and similar mating-type distribution in the three prefectures indicated that the possibility of the cross-infestation of *P. colocasiae* in Japan would be very high.

Mating types in one leaf

Two or three isolates were collected from the different disease spots in each of 13 leaves. Based on the analysis of mating-type, as shown in Table 3, a total of eight leaves were infected with multiple mating types, including one leaf infected with A1 and homothallic and seven infected with A2 and homothallic. This results indicated the disease spots in one leaf could be caused by different source of *P. colocasiae.*

Table 3. The mating types of *P. colocasiae* in each leaves

Location	No.(leaf)		Number of isolates	Heterothallic		Homothallic		
				A1	A2	A1	A2	A1A2
Kagoshima	1	1	3	0	2	0	1	0
		2	2	1	0	0	1	0
	2	1	3	0	2	0	1	0
		2	3	0	3	0	0	0
		3	3	0	1	0	2	0
	3	1	3	0	1	0	2	0
		2	3	0	0	0	3	0
		3	2	0	1	0	2	0
	4	1	2	0	1	0	1	0
		2	3	0	0	0	3	0
	5	1	3	0	0	0	3	0
		2	2	0	0	0	2	0
		3	3	0	1	0	2	0

Segregation of mating type in one isolate

We selected two heterothallic isolates of each A1 and A2 types and six homothallic isolates for single-hypha separation (Table 4). Six single-hyphae cultures segregated from the two A1 isolates and 15 single-hyphae cultures from the two A2 isolates were confirmed as the same type with their own original isolates. Among the 19 single-hyphae cultures from five homothallic A2 type isolates, four kinds of types, including heterothallic A1, A2 and homothallic A2, A1A2 types were found. The other one homothallic A1A2 isolate segregated into 12 single-hyphae cultures. Three, one, seven out of the 12 cultures were confirmed as heterothallic A1, A2 and homothallic A2 types, respectively. In addition, the remaining one culture was confirmed as homothallic A1 type that was firstly found (Table 4). These results suggest that the homothallic isolates will include both of heterothallic and homothallic features, and that heterothallic isolates will have a stable property.

Table 4. The mating-type analysis in single-hyphae cultures of different type isolates

Isolate		Type	Number of single-hyphae cultures	Heterothallic		Homothallic		
				A1	A2	A1	A2	A1A2
Heterothallic	EPC201509	A1	2	2	0	0	0	0
	EPC201522	A1	4	4	0	0	0	0
	KS17Ai1-2	A2	10	0	10	0	0	0
	EPC2017Ko1	A2	5	0	5	0	0	0
Homothallic	EPC201527	A2	4	0	1	0	2	1
	EPC201534	A2	6	0	0	0	6	0
	KS16TaOKI3	A2	3	1	2	0	0	0
	KS16TaOKI4	A2	3	0	0	0	2	1
	KS16TaOki5	A2	3	0	1	0	2	0
	KS16TaYo2	A1A2	12	3	1	1	7	0

In this study, a complex mating-type diversity in *P. colocasiae*, including heterothallic isolates of A1 and A2 types and homothallic isolates of A1, A2 and A1A2 types was presented in Japan. This situation might be one of the reasons that the disease are expanding. The oospores with longevity and durability generated easily can survive in severe environment safely such as winter. The sexual reproduction would introduce a longer-lived form of isolate and increase the chances of generating new and potentially more threatening *P. colocasiae* genotypes.

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Estimation on nitrogen deposition into Takayama forest over three years

Ruoming Cao[1], Siyu Chen[1], Shinpei Yoshitake[2] and Toshiyuki Ohtsuka[2]
1. **The United Graduate School of Agricultural Science, Gifu University**
2. **River Basin Research Center, Gifu University**

INTRODUCTION

Nitrogen (N) will input into forest ecosystems from atmosphere by precipitation which is called N deposition in forms of dissolved organic nitrogen (DON) and dissolved inorganic nitrogen (DIN). Nowadays, more and more N input into ecosystems mostly due to human activities, such as agricultural and industrial activities. There are various influences of N deposition on forests. Increasing N deposition could have a rise in biomass production by 0.31 Pg C yr^{-1} (Thomas et al. 2010) and canopy uptake or leaching occurred in response to N deposition (Schwarz et al. 2014; Izquieta-Rojano et al. 2016). On the other hand, the environmental consequences are also serious faced with increased N deposition, for example, nitrate leaching to groundwater (Gundersen et al. 2006). Studies on N deposition in Europe and China have evolved well, especially focusing on dissolved inorganic nitrogen (DIN) deposition which is known to have anthropogenic source. And dissolved organic nitrogen (DON) gradually is attracting attention as significant part of atmospheric N deposition (Cornell 2011). There are few studies on N deposition in Japanese forests.

In Takayama forest site which is an Asia flux network forest, compartment models of carbon pools and fluxes have indicated (Ohtsuka et al. 2007; Chen et al. 2017). It has become clear that where and how the forest stores carbon. But there is no monitoring the N fluxes by N deposition. It is not yet clear that temporal dynamics and effect of N deposition in the forest site. This study aims to estimate DON and DIN deposition input into Takayama forest during three years and to identify level and dynamics of N deposition during growing season and snow season, and response of canopy to N input into forest.

MATERIALS AND METHODS

Study site–The study site is a cool-temperate deciduous broad-leaved forest (Takayama forest) in central Japan (36°08'N, 137°25'E, 1420 m a.s.l.). Dwarf bamboo densely cover the forest floor. The climate is cool-temperate with growing season (May–Nov) and snow season (Dec–April).

Experimental setup—During growing season (May–Nov), we set up the samplers of bulk precipitation (3 replicates) near 1 ha plot, samplers of throughfall above bamboo (TF_a) (9 replicates) and throughfall below bamboo (TF_b) (9 replicates), and samplers of stemflow (SF) (9 replicates) within 1 ha plot. The volumes of water samples were measured by cylinder (5L) or rain gauge and water subsamples were collected by 100 ml bottles at once per month from 2016 to 2018. During snow season, we collected snow samples (3 points) at each 10 cm distance from snow surface to soil surface and transformed melted sample to 100ml bottles.

Chemical analysis—After being filtering by 0.45μm membrane filter, we measured the concentrations of total dissolved nitrogen (TDN), NH_4^+ and NO_3^-+NO_2^- (DIN) by a QuAAtro 2-HR nutrient analyzer. DON was calculated by difference between TDN and DIN.

Calculation– N fluxes were calculated based on volume and concentrations. In order to evaluate the interactions of canopy to N deposition, we used net TF. Net TF was performed in terms of flux data: Net $TF_a = TF_a + SF - BP$, Net $TF_b = TF_b - TF_a$. In this study, stemflow of dwarf bamboo was neglected.

RESULTS

N deposition via bulk precipitation–About 11.5 kg N ha^{-1} yr^{-1} of dissolved N was deposited into the study site by BP with 32 % snowfall flux. The percentage of DON to TDN amounts to 72 % (Table 1).

Temporal dynamics of N deposition–Average monthly concentrations of DON in BP followed a clear seasonal pattern in 2015 and 2016, with the highest concentration on May 2015 or June 2016 and tended to decrease over subsequent time. However, there was no clear seasonal pattern in 2017 (Fig.1). The dynamics of DIN concentrations revealed decreasing trends from spring to winter in 2015 and 2017. But there was no clear seasonal pattern in 2016 (Fig.1). For average DON flux, higher flux was deposited during summer, except 2017 when N deposition in autumn also higher (Fig. 1).

Canopy exchange–There was positive relationship between rainfall and DON fluxes in net TF_a but negative relationship between water fluxes in TF_b and DON Fluxes in net TF_b ($P < 0.05$) (Table 2). It suggested that DON was leached by tree canopy however uptake by bamboo canopy. For DIN flux, there was significantly positive correlation between rainfall and DIN fluxes in TF_a ($P < 0.05$), but no significant correlation existed between water fluxes in TF_b and DIN fluxes in net TF_b (Table 2). It turned out that DIN flux was leached by tree canopy, whereas dry deposition significantly influenced DIN flux in TF_b.

DISCUSSION

N deposition–DIN flux was within the range of Japanese and European studies (Cape et al.2012; Mitchell et al. 1997), whereas lower than that in China (Jia et al. 2014), where is faced with serious environmental problems. DON flux coincide with that in Guangzhou city in China but at higher level compared to Europe (Li et al. 2012; Cape et al. 2012). DON contribution in Takayama forest was greater than most studies (2 % ~ 28 % in European forests, Cape et al. 2012; 7 % ~ 67 % in Chinses forests, Zhang et al. 2012), but was in accordance with that in the remote Tibetan area of China (Zhang et al. 2008). A major contribution of DON to TDN in the study site showed that importance of DON deposition in the forest site. Seasonal pattern of DON concentration maybe due to nitrogen fertilizer use in spring, which was also reported by Izquieta-Rojano et al. (2016) who argued that DON deposition was mainly dominated by agricultural activities. And snow contribution cannot be ignored based on the characteristic of snow season existed.

Canopy exchange–The leaching of DON by tree canopy is general. However, DON uptake is less reported (Izquieta-Rojano et al. 2016). DON was uptake by understory bamboo canopy implied that DON flux was an important source for

nutrient supply in the study site.

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Table 1. Average nitrogen concentration and average annual flux in bulk precipitation during three years (May 2015–April 2018). Values in parentheses are standard deviation (n = 3). Different letters are significantly different between concentrations or fluxes of dissolved nitrogen in snowfall and rainfall (P < 0.05). DIN = Dissolved inorganic nitrogen, DON = Dissolved organic nitrogen, TDN = Total dissolved nitrogen.

	Rainfall	Snowfall	Total (kg N ha^{-1} yr^{-1})	Snowfall /Total
Concentration (mg L^{-1})				
DON	0.39 (0.03) a	0.32 (0.16) a	–	–
DIN	0.13 (0.04) a	0.23 (0.09) a	–	–
TDN	0.53 (0.04) a	0.53 (0.19) a	–	–
Precipitation (mm)	1543 (17.7)	670 (125)	–	–
Flux (kg N ha^{-1} period^{-1})				
DON	5.96 (0.52) a	2.28 (1.45) b	8.24 (1.01)	0.27 (0.14
DIN	1.66 (0.04) a	1.56 (0.79) a	3.22 (0.76)	0.46 (0.1
TDN	7.61 (0.55) a	3.84 (1.96) a	11.5 (1.42)	0.32 (0.1
DON/TDN	0.78 (0.01)	0.58 (0.11)	0.72 (0.05)	–

Table 2. Spearman correlation between rainfall amount (mm) and net throughfall above bamboo (Net TF_a) (kg N ha^{-1} episode^{-1}) or between water depth of TF_a (mm) and net throughfall below bamboo (Net TF_b) (kg N ha^{-1} episode^{-1}) (n = 23). DIN = Dissolved inorganic nitrogen, DON = Dissolved organic nitrogen. * means significant at $P < 0.05$.

	NH_4–N	NO_3–N	DIN	DON
Net TF_a	0.14	0.12	0.42*	0.52*
Net TF_b	–0.03	0.08	0.20	–0.46*

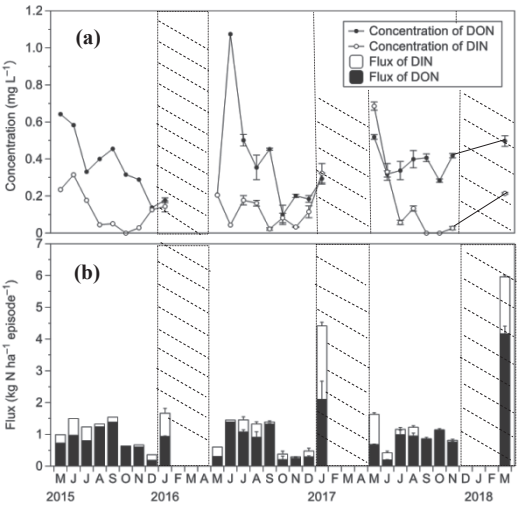

Fig. 1. Mean monthly concentration (mg L^{-1}) (a) and mean flux (kg N ha^{-1} episode^{-1}) (b) in bulk precipitation from May 2015 to April 2018. Shaded areas show snow season. Fluxes on January 2016, January 2017 and March 2018 stand for fluxes during snow period between two adjacent years. Error bars show the standard error (n=3). DON = Dissolved organic nitrogen, DIN = Dissolved inorganic nitrogen.

Immobilization of cesium in contaminated forest soil using various additives: evaluation based on the inhibition effect on its transfer to grass

Huijuan Shao[1] and Yongfen Wei[1, 2]
1. The United Graduate School of Agricultural Science, Gifu University, Japan
2. River Basin Research Center, Gifu University, Japan

INTRODUCTION

Soil contamination of radionuclides released from nuclear accidents and nuclear tests has been a serious environmental problem in the world (Ashraf et al. 2014). Due to the Fukushima Daiichi Nuclear Power Plant accident on March 11, 2011, a large amount of radionuclides was released into the northeastern part of Japan. Cesium-137 (^{137}Cs) is of a special concern due to its long half-life (about 30 years), high solubility in water and strong affinity to soil. Due to its biogeochemical similarities with potassium (Zhu and Smolders, 2000), many studies have reported that vegetation takes in deposited ^{137}Cs from soil through their roots, and translocate it quickly to the leaves.

A majority of the land heavily contaminated with ^{137}Cs in northeastern areas of Japan is covered by forests (Hashimoto et al. 2012). Extensive decontamination measures including topsoil removal, landfilling and soil washing have been conducted in Fukushima prefecture. These measures are highly effective at reducing the degree of contamination, but undoubtedly they will cause environmental destruction and are too expensive to be implemented in the large scale of contaminated forest. Therefore, to develop an effective, eco-friendly and low-cost technology for treatment of ^{137}Cs-contaminated forest soil is strongly desired.

In-situ immobilization, which aims to inhibit the transfer of ^{137}Cs from soil to vegetation and water bodies by increasing competitive ion concentration or fixation capability by application of certain additives is a promising technology (Lee et al. 2009). To generate information that can be used for immobilization technology, the evaluation of inhibition effect of three low-cost adsorbent additives on Cs transfer from soil to vegetation was performed in this study. Meanwhile, the distribution of Cs on different parts of vegetation was also examined.

MATERIALS AND METHODS

Soil and additive samples—Soil was collected from the surface layer (0–15 cm depth) after removing plant and litter layers in the evergreen coniferous forest located along Ijira Lake in Gifu Prefecture, Japan. Three additives including coconut shell biochar (BC), incinerated sewage sludge ash (ISSA, before phosphorus recovery), and natural zeolite (Zeolite) were obtained from TAKII Seed Corporation, North Wastewater Treatment Plant of Gifu City, and SINKOU SUNRISE Corporation, respectively. Soil and additives were all air-dried at room temperature and then sieved with a 2 mm of mesh. The basic physicochemical properties of soil and additives are summarized in **Table 1**. As shown, soil had the lowest values of pH, cation exchange capacity (CEC) and specific surface area (SSA); while biochar had the highest values of pH, organic matter content, CEC and SSA.

Experimental design—Stable ^{133}Cs was used in this study since its chemical behavior for vegetation was reported to be similar with that of ^{137}Cs (Burger et al., 2018). CsCl solution was uniformly mixed with soil and the Cs contamination level was set to be 100 mg/kg dry weight. 480 g of soil was placed in each plastic pot and the soil was then well mixed with BC, ISSA or Zeolite at a ratio 1:10 (additive: soil) by hand until homogeneity was achieved. In addition, no additive applied soil was used as control for comparison. Each treatment had three replicates.

Pot experiment and analysis—Napier grass (*Pennisetum purpureum*) was selected and cultivated inside a glass greenhouse in Gifu University. During the whole cultivation period from 2017/11/02 to 2018/06/02, the maximum and minimum temperatures in the greenhouse were 40 °C and –3 °C, respectively, and no additional fertilizer was applied. After harvest, the grasses were washed carefully, separated into three parts (leaf blade, leaf sheath and root), then freeze-dried, shattered by a crusher and digested with nitric acid in an autoclave. The Cs concentration of the digested samples was measured using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700X). The distribution of Cs in the dried grass sample was analyzed using scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM/EDX, Hitachi SU3500). EDX analysis were performed at the acceleration voltage of 20 kV.

RESULTS AND DISCUSSION

Cesium concentration accumulated in different parts of the grass—As shown in **Fig. 1**, as compared with control (without additive), all three additives obviously declined Cs concentration in the grass. And especially biochar (BC) showed the lowest value; the Cs concentration in the grass was reduced by 93.5 - 97.1%. The increase of potassium concentration after additive addition is considered to be the most likely reason for this reduction due to the increased fixation capability of potting soil after additive addition increased (data not shown). Among different parts of Napier grass, leaf blade possessed the highest Cs concentration. This may indicate that for Napier grass the leaf blade was the main organ to accumulate Cs (Kang et al. 2012).

Table 1 Physicochemical properties of the soil and additives used in this study

Property	Soil	Biochar	Incinerated sewage sludge ash	Zeolite
pH (H_2O)	4.88 ± 0.13	9.25 ± 0.21	5.27 ± 0.15	6.23 ± 0.11
Organic matter (%)	15.85 ± 0.87	81.31 ± 2.14	0.27 ± 0.07	0.38 ± 0.07
Cation exchange capacity (cmol/kg)	24.77 ± 2.35	47.83 ± 2.36	30.15 ± 1.18	87.58 ± 2.35
Specific surface area (m^2/g)	21.63 ± 1.25	157.14 ± 3.29	34.93 ± 1.86	43.39 ± 2.51

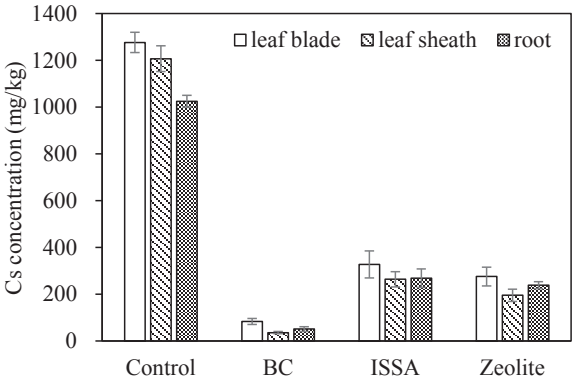

Fig. 1 Cesium concentration in different parts of grass under different additive additions (BC: biochar, ISSA: incinerated sewage sludge ash)

Distribution of cesium in different parts of the grass— According to **Fig. 2** and **Fig. 3** obtained from SEM/EDX analysis, it is obvious that Cs was unevenly distributed in different parts of the grass, and its concentration inside the root was higher (0.15%) than that on the outside surface (0.08%). Xylem (plant vascular tissue) inside the root is considered to have mainly contributed to the result since it conveys water and dissolved minerals from the roots to the rest of the grass.

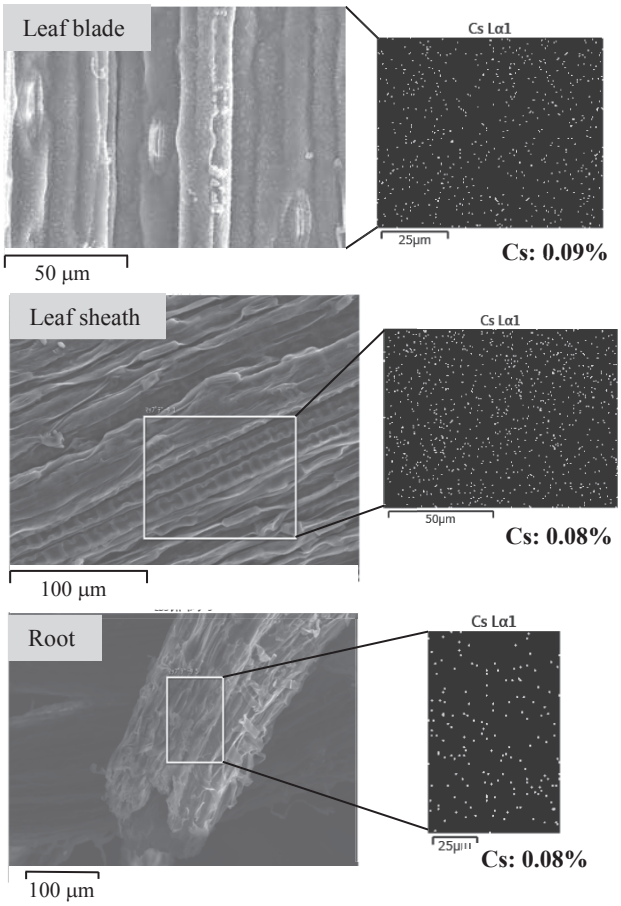

Fig. 2 Cesium distribution on the outside surface of different parts of the grass (Cs: 100 mg/kg; control)

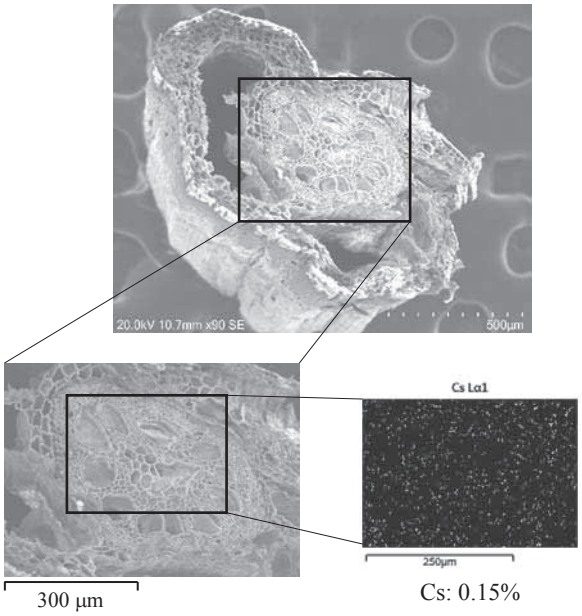

Fig. 3 Cesium distribution inside the grass root (cross section; Cs: 100 mg/kg; control: without additive)

In addition, in biochar addition case, the Cs concentrations on all parts of the grass (0.02% in average) were lower but the potassium (K) concentrations were higher by comparing with that of control (data not shown). It indicated that the increased K concentration via biochar addition was one likely reason to inhibit Cs uptake by grass root due to the competition between Cs and K.

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Hydrological characteristics under different forest types in central Japan

Ali Rahmat[1], Keigo Noda[2], Kengo Ito[2] and Masateru Senge[2]
1. **The United Graduate School of Agricultural Science, Gifu University, Japan**
2. **Faculty of Applied Biological Sciences, Gifu University, Japan**

INTRODUCTION

In Japan after World War II many natural forest was change to coniferous plantation forest for timber production. The plantations were dominantly formed by converting native broadleaf forest to coniferous forest (Hirata et al., 2006). Converting vegetation type have big impact on hydrological cycle in watershed scale (Bosch and Hewlett, 1982, Chen et al., 2009). In Japan, forest covers most mountainous region located upstream of agriculture and urban areas. From viewpoint of water resource management, the forested areas are considered to be water sources (Sawano et al., 2005; Kumagai et al., 2014) and are intimately linked to downstream ecosystems (Gomi et al., 2009). Converting broadleaf forest to coniferous forest may influence the hydrological condition, where this condition must be considered by water resource management to make policy in future.

The objectives of this study is to compare the hydrological characteristic between deciduous broadleaf and evergreen coniferous forest. The results of this study will provide useful information for water resource management in such areas.

MATERIALS AND METHODS

The Kuraiyama experimental forest is located in Gero City, Gifu prefecture, Japan, between longitude 137°11′-137°14′ E and latitude 35°58′-36°01′ N. Elevation is between 820-1451m). The investigation carried out in No.10 evergreen coniferous and No.12 deciduous broadleaf. There is a right triangle weir has been built at each stream of basin and data logger has been placed at the bottom of stream. The precipitation data collected using tipping bucket rain gauge, has been placed in No.12 deciduous broadleaf basin.

The No.10 basin forest is an evergreen coniferous forest of Chamaecyparis obtuse (dominant tree species). This basin located in the south of experimental forest, elevation between 926-1278 m and area is 0.6 km2. 74% of the basin is covered by 40-50 years old artificial coniferous forest, 18% are broadleaf forest and 8% are a natural coniferous forest.

The No.12 basin forest is a deciduous broadleaf forest of Quercus sp (dominant tree species), the area is at the south of No.10 basin area. Elevation between 909-1278 m, area is 0.73 km2. 77% of the No.12 basin is covered by deciduous broadleaf forest, 14% is covered by 50-70 years old artificial coniferous forest, and 9% is covered by natural coniferous forest. The ground is also covered with a high density of bush, sasa bamboo grass, and litter layer.

RESULTS

The flow duration curve (FDC) is a plot is a plot shows the percentage of tie that flow in a stream is likely to equal or exceed some specific value of interest. FDC is one way to look water discharge characters. Based on **Fig.1**, FDC showed, more than 95% daily discharge in No.12 deciduous broadleaf is higher than in No.10 evergreen coniferous. However, in **Fig.1** clearly seen around 2% the discharge in No.10 evergreen coniferous is higher than No.12 deciduous broadleaf.

In **Fig. 2** showed, the peak discharge, direct runoff, runoff coefficient in No.12 deciduous broadleaf forest is lower than in No.10 evergreen coniferous forest, but relationship between precipitation and direct runoff in the No.10 evergreen coniferous is closer compared in No.12 deciduous broadleaf.

In **Fig. 3** showed, in the different amount of precipitation event, the results are consistent in all event, the peak discharge in No.10 evergreen coniferous is higher than in No.12 deciduous broadleaf. However, the discharge in No.10 evergreen coniferous faster decline after reach the peak compared to discharge in No.12 deciduous broadleaf. In **Fig. 4**, clearly seen snow depth in No.12 deciduous broadleaf is deeper than in No.10 evergreen coniferous.

DISCUSSION

The result showed that the annual runoff from the coniferous evergreen forest was smaller than that from the broadleaf deciduous forest (**Fig. 1**). One of the reasons of this difference in the annual runoff can be the phenological difference between broadleaf deciduous and coniferous evergreen forests. While coniferous evergreen forests always keep leaves, broadleaf deciduous forests lose almost all leaves in autumn. Due to no leaves in broadleaf deciduous forests during winter season, the more snow can reach the ground. In contrast, coniferous evergreen forests intercept a lot of snow by canopy, and thus, the less amount of snow reaches the ground (**Fig. 4**). The highest interception for the broadleaf deciduous forest was 29% in UK (Herbst et al., 2008), while that for the coniferous evergreen forest was 45% in Sweden (Alavi et al., 2001). Leaf shape and configuration affect LAI and water storage (Jonckheere et al., 2004, Keim et al., 2006). Some leaves only store water as a thin coating whilst others also store in capillary spaces between leaves. For these reasons, flat leaves (deciduous species) store water less than trees with needle leaves type (coniferous species) (Keim et al., 2006). Water storage by mature deciduous with and without leaves is 0.2-2 mm and 0.03-0.8 mm respectively (Leyton et al., 1967), and 0.1-4.3 mm for mature coniferous tree (Link et al., 2004). Thus, if we can assume that annual interception of both forest types is comparable, annual ET of coniferous evergreen forest is larger than that of broadleaf deciduous forest. Then, under the same climatic conditions, we can conclude that annual runoff from coniferous evergreen forests is smaller than that of broadleaf deciduous forests. For short-term runoff (based on rainfall event) characteristics between different forest types, found that the discharge in a coniferous evergreen forest was always higher than that in a broadleaf deciduous forest regardless of the magnitudes of storm events: a small autumn rainfall, a middle typhoon storm, and a large typhoon storm.

Cypress plantations usually exhibit overstocked stands having sparse or no understory vegetation cover due to the low light conditions (Onda et al., 2010). The similar phenomenon was observed in this research about little or no understory vegetation cover in coniferous forest. On the other hands, shows a high dense understory vegetation cover in a broadleaf deciduous forest. Consequently, poor litter layers or no understory vegetation which were strongly affected by

coniferous evergreen forest induced high peak discharge and high surface runoff compared with the broadleaf deciduous site. Sakai et al. (2009) also indicated that a litter layer had become very sparse and soil surface was exposed at both cypress and cedar plots, while the deciduous plot was covered by a thick litter layer and understory vegetation. The existence of the understory vegetation cover can delay the speed of surface runoff and reduce peak discharge in broadleaf deciduous forests. Sakai et al. (2009) and Hirano et al. (2009) suggested that surface runoff at the cypress and the cedar plot was a root flow (shallow flow through the root layer because of no litter layer) and that the surface runoff at the broadleaf deciduous plot was a litter flow due to its thick litter layer.

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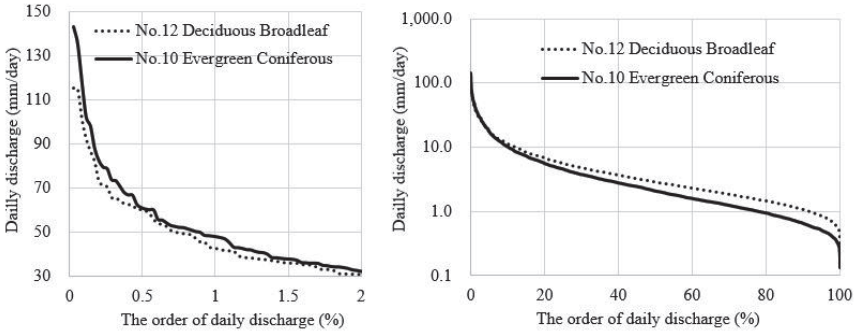

Fig.1 Flow duration curve from 2008-2017

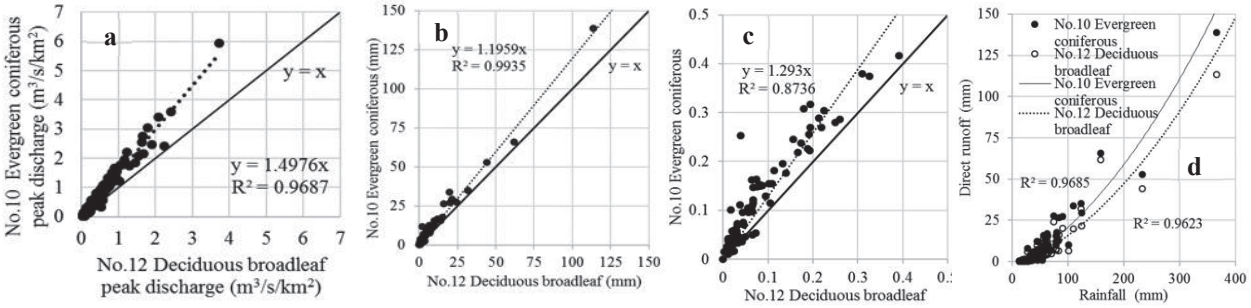

Fig.2 Peak discharge (a), direct runoff (b), runoff coefficient (c), relationship rainfall with direct runoff (d) in No.12 deciduous broadleaf and No.10 evergreen coniferous

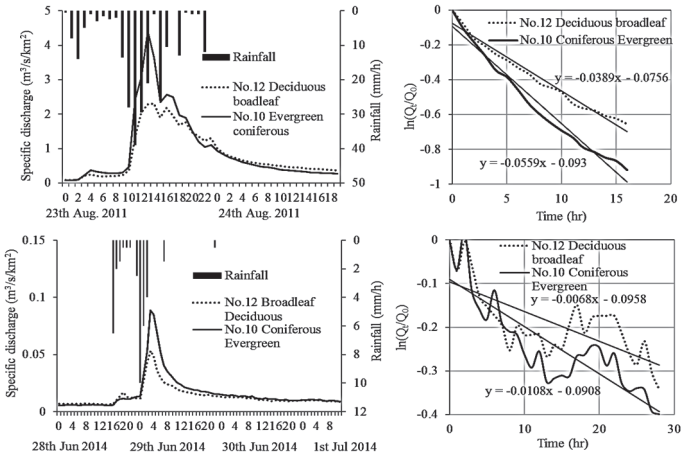

Fig.3 Hydrograph No.12 deciduous broadleaf and No.10 evergreen coniferous (up: 100 mm/event, down: 35 mm/event)

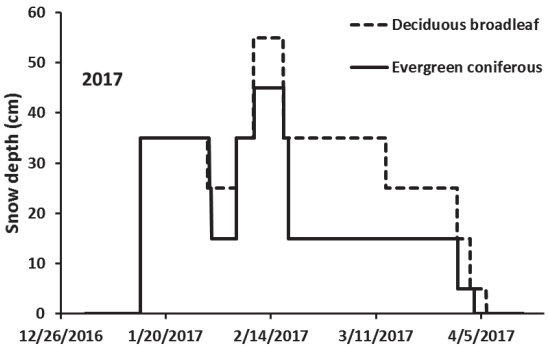

Fig.4 Snow depth in No.12 deciduous broadleaf and No.10 evergreen coniferous

Changes of pathogenic bacterial indicators during vermicomposting treatment of sewage sludge

Shuailei Li[1], Guangyu Cui[2] and Fusheng Li[3]
1. **Graduate School of Natural Science and Technology, Gifu University, Japan**
2. **Graduate School of Engineering, Gifu University, Japan**
3. **River Basin Research Center, Gifu University, Japan**

INTRODUCTION

Sewage sludge is not only rich in nutrients, but also contains a large number of pathogenic microorganisms. Improper treatment and disposal will carry pathogenic pathogens into the environment, causing disease transmission, thus seriously threatening public health and ecological security. Vermicomposting is an environmentally friendly and sustainable urban sludge treatment technology. In particular, it is an effective management tool for organic solid waste for developing countries owing to its low operational cost and high application value for generation of vermicompost that can be used as fertilizers or soil modifiers.

Despite numerous studies (Monroy et al., 2009; Henault-Ethier et al., 2016) conducted on pathogenic bacteria survival in vermicompost, a better understanding of the mechanisms involved is still necessary. One of the major obstacles to the widespread acceptance of vermicomposting as a general organic solid waste management alternative is the insufficient removal potential of pathogenic microorganisms during vermicomposting (Edwards et al., 2010) Therefore, to study the behavior and elimination mechanism of pathogenic bacteria in the process of sludge treatment can not only provide risk assessment for the treatment of sewage sludge by earthworms, but also improve the efficiency of sludge sanitation and safety utilization. The goal of the present study is to evaluate the changes of total coliforms, fecal coliforms and *E. coli* during the treatment of sewage sludge under different density of earthworms and its possible mechanism.

MATERIALS AND METHODS

Return activated sludge—Returned activated sludge was collected from the North Wastewater Treatment Plant of Gifu-city, Japan. It was used as the test sludge after sedimentation and centrifugation.

Earthworm—Earthworm used in this study is *Eisenia foetida,* and the average weight is 0.30g. The earthworms had been fed with sewage sludge for six months in the laboratory.

Experimental set up— The experiment was designed for three groups. Each reactor was loaded with 500 g of sludge, and 20, 40 and 60 earthworms were introduced into the reactor respectively (referred here after as E20, E40 and E60). In addition, a control group E0, referred to the reactor added with 500g sludge but without earthworms, was also designed. The reactor was placed in a constant temperature chamber at 25℃ and sampled once a week for 31 days.

Physicochemical parameter— Water content, organic matter (OM), dehydrogenase activity (DHA) of the wet samples collected during composting were determined, together with pH, electrical conductivity (EC), dissolved organic carbon (DOC) and C/N ratio using the samples after drying.

Microbial analysis—Total coliform and *E. coli* were analyzed by plate count method. In brief, 2g of centrifugal sludge was added to an 18 mL tube containing physiological saline solution and oscillation was performed for 30 minutes at 200 rpm for distribution of bacteria uniformly in the tube. 1mL of the solution was taken and injected into another tube containing 9 ml saline solution for dilution. This process was repeated in the same way till 10^2, 10^3,10^4 folds' dilutions were achieved. 1mL of the sample was poured to a Petri dishes containing Chromocult® Coliform Ager (Merk KGAA, Darmstad, Germany) and incubated for 24h at 37℃. All dark-blue to violet colonies were counted as *E. coli*, and all salmon to red colonies were counted as total coliform. Fecal coliforms were analyzed by M-FC method.

Statistical analysis— Statistical analysis was done using SPSS 19.0. One-way analysis of variance (ANOVA) was used to test the significance of observed differences over time. Principal component analysis (PCA) was conducted based on CANOCO 5.0.

RESULTS AND DISCUSSION

Pathogenic bacterial indicators—The **Fig. 1**, **Fig. 2**, and **Fig. 3** showed the influence of earthworms on total coliforms, fecal coliforms and *E. coli* respectively.

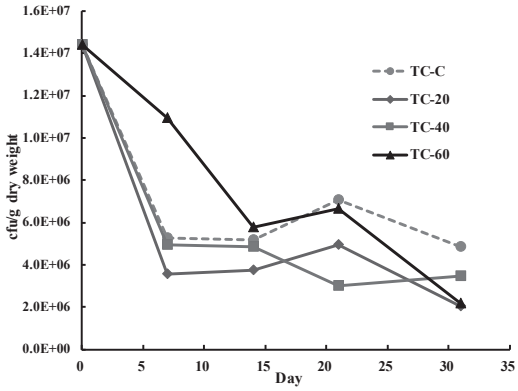

Fig. 1 Changes of total coliform during vermicomposting

As shown in **Fig. 1**, the number of total coliforms in compost products decreases with the change of composting time. A week later, it showed a slow downward trend. Compared with the control group, under the condition of high earthworm density, the total number of coliforms was higher than that of the low density group. The removal rate of total coliforms can reach 86%.

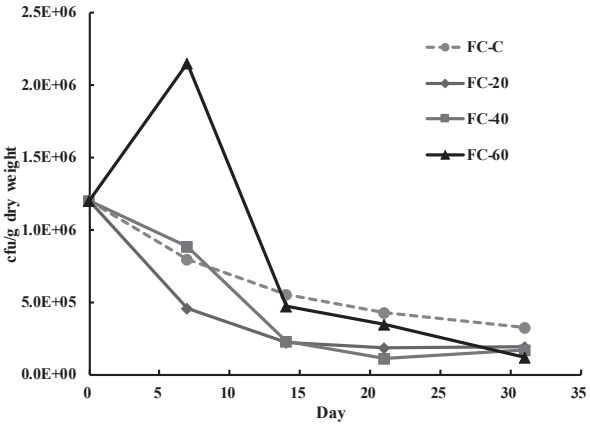

Fig. 2 Changes of fecal coliforms during vermicomposting

The number of fecal coliforms in E0, E20 and E40 groups decreased gradually during composting. The number of fecal coliforms in the group of E60 increased abruptly on the seventh day, then decreased gradually.

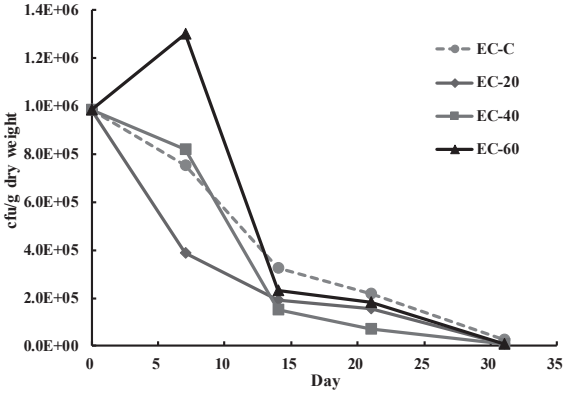

Fig. 3 Changes of *E. coli* during vermicomposting

As **Fig. 3** shows the number of *E. coli* decreased gradually during the process. Compared with the changing trend of fecal coliforms shown in **Fig. 2**, the changing trend of the number concentration of *E. coli* was consistent with fecal coliform.

Chemical properties—As shown in the **Fig. 4** and **Fig. 5**, variation of OM (Organic Matter) and DOC (Dissolved Organic Carbon) reflected the changes of nutrients during the vermicomposting. It also indicated that the vermicompost tended to stabilization.

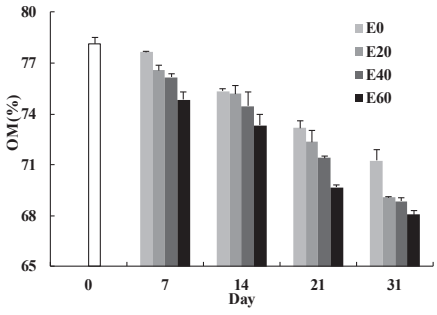

Fig. 4 Variation of organic matter during vermicomposting

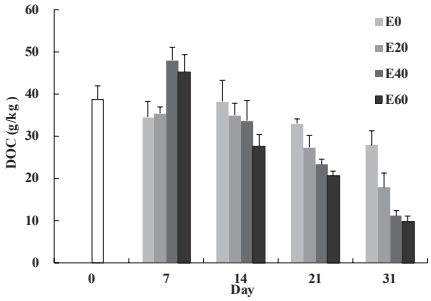

Fig. 5 Variations of DOC during the vermicomposting

Compared with the initial sludge (78.14%), the organic matter in the final compost product (68.86%) decreased by about 10%. The higher the density of earthworm, the faster the organic matter will decline. During the treatment, DOC decreased gradually. The higher the density of earthworm, the more the DOC concentration will drop.

Correlation analysis— Correlation analysis were conducted by SPSS 19.0 and CANOCO 5.0. The **Fig. 6** showed the results of redundancy analysis (RDA). The abundance of the pathogenic bacterial indicators had a strong positive linear correlation with OM, DHA and DOC that could be probably linked to such nutrients as labile C and labile sugars.

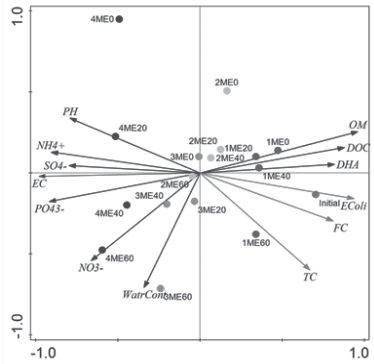

Fig. 6 Redundancy analysis

In the experiment, we found that the density of earthworm doesn't play a noticeable role in the reduction of pathogenic bacteria. The abundance of the pathogenic bacterial indicators had a strong correlation with OM and DOC that could be probably linked to nutrients in the substrate. Research to clarify the relation between pathogenic bacteria and nutrient should be carried out.

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Estimation experiment of complex terrain atmospheric structure coefficient C_n^2 by weather forecast model

Yuto Ito[1], Tomonao Kobayashi[2] and Jun Yoshino[2]
1. **Graduate School of Natural Science and Technology, Gifu University, Japan**
2. **Faculty of Engineering, Gifu University, Japan**

INTRODUCTION

Recently, the propagation of laser is becoming more important for transmission system realization of mass data. A barometer of the degree of a disturbance of atmosphere that influences on the propagation of laser is C_n^2. However the appropriate model for estimation has not been drawn up. Then to make the appropriate model for estimation of C_n^2 when you set the certain weather conditions and environmental conditions is a problem for transmission system realization.

In this research, I'll estimate C_n^2 express a disturbance of atmosphere by using weather forecast model (WRF). And I'll give a possibility of the numerical model to compare the model and observation.

MATERIALS AND METHODS

(1) Analytical method and term

Joint observation was conducted by some companies on a disturbance of atmosphere of the laser propagation at JAXA Taiki aerospace experiment station in Taiki town, Hokkaido in December 2016. This disturbance of atmosphere was considered from optical and meteorology perspectives. This joint observation was also carried out at Shonan campus of Tokai University in Hiratsuka city, Kanagawa prefecture, in September 2017 and March 2018. In this research, I'll estimate C_n^2 by weather forecast model for the joint observation in December 2016 and March 2018, and its result is compared with the observation result. In two joint observation points, altitude and land use are markedly different. So I'll compare and consider about the influence of this terrain on C_n^2 estimation by numerical model.

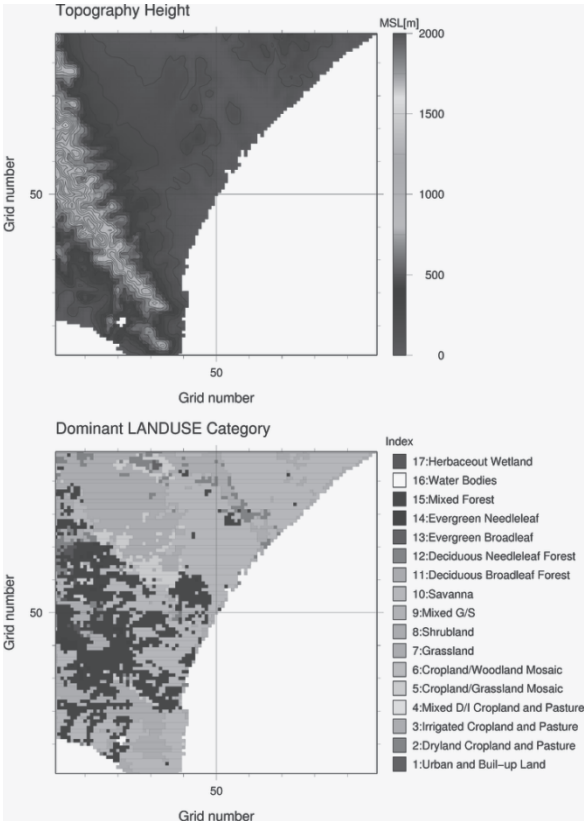

Fig.1 altitude and land use distribution of Taiki town, Hokkaido（JAXA Taiki aerospace experiment station）

(2) The influence of terrain condition on C_n^2 estimation

Figure1 and 2 shows that altitude and land use distribution of Taiki town, and Hiratsuka city used in numerical calculation model by WRF Domain2 to discuss influence of terrain conditions. In figure1,2 target point is (50,50). Grid size is 1km.Figure3.4 shows that correlation between result of C_n^2 estimation and result of C_n^2 observation. As shown in Fig.1, there is a sea in the southeast direction, but the target area is flat and land use is almost uniform. So as shown in Fig.3 that reproducibility of C_n^2 estimation is high. But as shown in Fig.2 that there are somewhat ups and downs, especially land

Fig.2 altitude and land use distribution of Hiratsuka city, Kanagawa prefecture（Shonan campus of Tokai university）

use is complicated around Tokai university Shonan campus. In a smaller scale, there are many lecture buildings and high trees in campus and you can easily imagine that an air current is small and complicated that can not be reproduced by WRF. So as shown in Fig.4 shows that reproducibility of C_n^2 estimation by WRF is low. According to these results, accuracy of C_n^2 estimation is influenced by topography and land use. In an atmospheric turbulence field, Monin-Obukhov similarity can usually be applied as a turbulent structure. The influence of the ground surface has a significant influence on the atmosphere and disturbance within the atmosphere boundary layer close to the ground surface targeted in this study. C_T^2 and C_n^2 are calculated by sensible heat flux and friction speed estimated using WRF by similarity of Monin-Obukhov in the ground boundary layer.

RESULTS AND DISCUSSION

(1) Result of analysis

From Fig.3, some data of C_n^2 estimation by WRF is underestimated compared to the C_n^2 observation. We know that this underestimated period is the nighttime zone. WRF estimates that the sensible heat transport is small and atmosphere is stable. However there are actual temperature fluctuations. In other periods, the C_n^2 estimation by WRF is highly reproducible for the C_n^2 observation.In this figure, C_n^2 estimation by WRF is smaller compared to C_n^2 observation sunset to sunrise. In this figure, C_n^2 estimation in daytime. On the other hand, estimation is bigger than observation in nighttime. Moreover, the plot is spread widely as a whole, the correlation between C_n^2 estimation and C_n^2 observation is low, and the estimation accuracy of WRF seems to be low. However, if you distinguish daytime and nighttime, correlations can be seen.

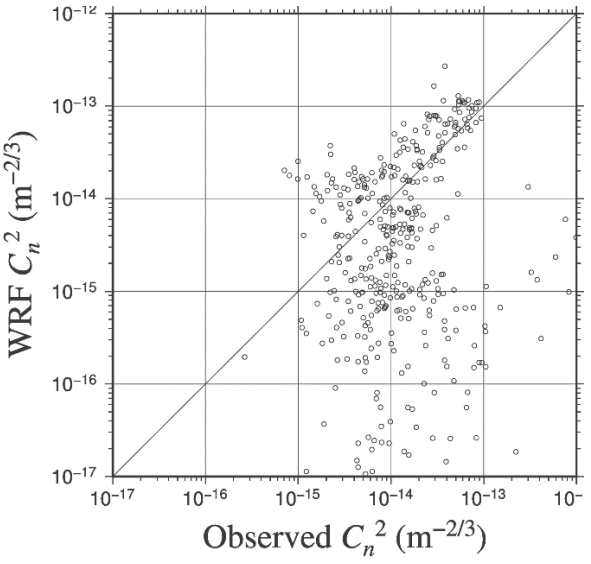

Fig.3 Correlation between result of C_n^2 estimation and result of C_n^2 observation（JAXA Taiki aerospace experiment station）

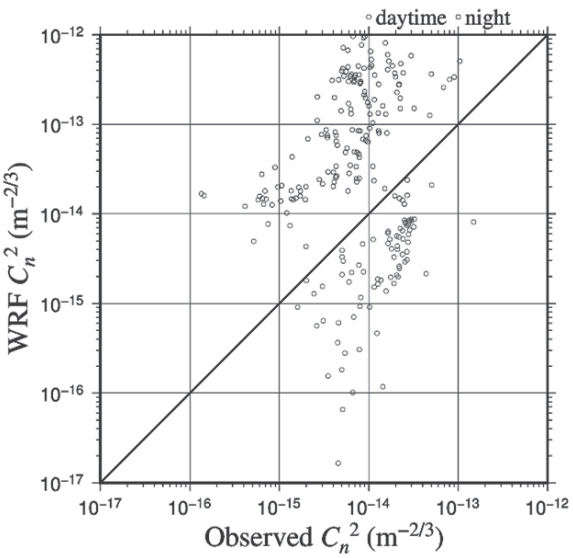

Fig.4 Correlation between result of C_n^2 estimation and result of C_n^2 observation（Shonan campus of Tokai university）

CONCLUSION

In this research, we reproduced the state of the atmosphere for joint observation of Taiki town in December 2016 and Hiratsuka city in March 2018 by WRF. So we estimated C_n^2. Furthermore we compared its result with observation. The reproducibility of estimation is influenced by altitude and land use.

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Estimation of small hydropower generation using river discharge water in headwork

Fenglan Wang[1], Masateru Senge [2] and Keigo Noda [2]
1. The United Graduate School of Agricultural Science, Gifu University[1]
2. Faculty of Applied Biological Sciences, Gifu University, Japan[2]

INTRODUCTION

In recent years, receiving the influence of action for reduction of greenhouse gas (CO_2) and accident at Tokyo Electric Power Company's Fukushima No. 1 nuclear power plant， people began to focus on renewable energy such as solar, wind power, hydropower, geothermal, biomass and wave power. We have great renewable energy recourse to make generation. It is purely domestic energy that we do not need to import the energy sources from other countries.

It has been worked eagerly for promotion of introduction of renewable energy use. Thus some strategies or policies were taken by government, like Feed-in-tariff law, taking 2002 as the beginning year to develop the renewable energy and etc. Tariff price of small hydropower (SHP) generation, 35.7 yen for 1 kWh when the output is below 200kW, 30.45 yen when the output is from 200kW~1000kW for 1 kWh and 25.2 yen when the output is from 1000kW~30000kW for 1kWh (Agency for Natural Resources and Energy, 2002), also encourage the diffusion of SHP generation.

There is no clear definition of small hydropower in Japan. However, according to the output, it is classified as large hydropower, middle hydropower, small hydropower, mini hydropower and micro hydropower. In this research, we refer facilities with output less than 1000kW to small hydropower. SHP generation is a kind of distributed energy and facility utilization rate is higher in the renewable energies.

In Japan, it has great potential SHP generation, and there exists many unused falling heights to be utilized for making SHP generation. For SHP generation using agricultural water supply facilities, utilization of adjustment reservoir and distribution water to make generation has been planning and carrying out rapidly in recent years.

Headwork is one of agricultural irrigation facilities developed since ancient times to intake the agricultural water from the rivers.

In this research, we focus on the discharge water from the upstream part to the downstream part of headwork to evaluate the generation potential with using its' falling highs and this discharge water. We would like to evaluate the possibility of generation utilizing the discharge water of headwork by analysis of annual generation and monthly generation

MATERIALS AND METHODS

There are total 8 sites in Gifu prefecture and Aichi prefecture are estimated totally. They are in ONYU headwork, FURIKUSA headwork, KANSAGAWA headwork, MUROMATSUBARA headwork, HOSOGAWA headwork, OKAJIMA headwork, INUYAMA headwork and MEIJI YOUSUI headwork.

We plan to use the sixteen years' data of flow rate (2002~2017) to estimate the generation capacity. Among them, for MEIJI YOUSUI headwork, 21 years' data (1997~2017) is used and for INUYAMA headwork, 10 years' data (2008~2017) is used.

The generation output(kW) was calculated with using the form: $\mathbf{P = g \times Q \times H \times \eta}$,

(p: generation output[kW], g: gravitational acceleration (9.8) [m/s²], Q: flow rate[m³/s]), H: drop, η: general efficiency [0.72＝0.825(water wheel efficiency) × 0.875(generator efficiency)].

For the calculation process, firstly, the excess probability (is shown as percentage, to make the daily flow rate of headwork in descending order, then to divide that rank by the total number of days to change them to percentage). Secondly, the utilization rate was calculated when the flow rate corresponding to excess probability 10% to 90% was taken as the maximum water usage. Thirdly, the relations between maximum output and utilization rate is estimated. Fourthly, the output is obtained when the utilization rate is 60% and it is used as maximum output in the calculation. Last, the actual annual and monthly generation capacity are calculated with using maximum output when the utilization rate is 60%. The usage rate of small-hydropower power generation equipment is estimated to be at least 60% as a standard for commercialization according to Energy · Environment Conference Cost etc. Verification Committee in Japan. Some conceptions are related in this paper. Here, I will introduce these conceptions. Annual generation capacity: annual generation amount when using the maximum water usage (maximum water usage: the maximum value of the flow rate that a facility can use). Actual generation capacity: annual generation amount when using actual water usage (actual water usage: actual flow rate with maximum water usage as upper limit). Utilization rate (%): division of actual annual generation capacity and annual generation capacity.

RESULTS

For the results of this thesis, I will introduce the results of one site-MEIJI YOUSUI headwork as an example.

Figure1 shows the excess probability. Figures 2 shows the relation between maximum output and utilization rate, and we can get the output when the utilization rate is 60% from this figure. Then, we set this output as maximum output and obtained the annual and monthly generation capacity (Figure 3 and Figure 4). For MEIJI YOUSUI headwork, the maximum output is 947 and we got the annual generation capacity from 3196~6609MWH (average annual generation is 4971MWh) and average monthly generation is 414 MWh, the biggest generation is 577MWh in October and 303MWh in June. It obtained the relatively stable generation through the year.

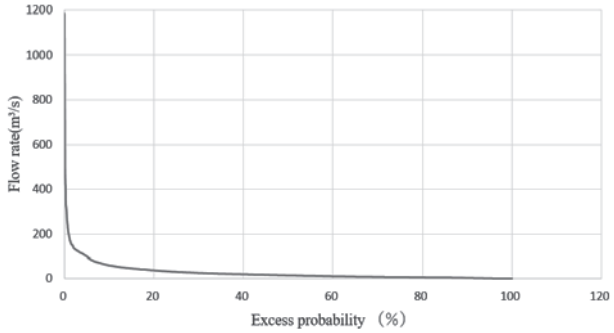

Fig.1 Relation between flow rate and excess probability

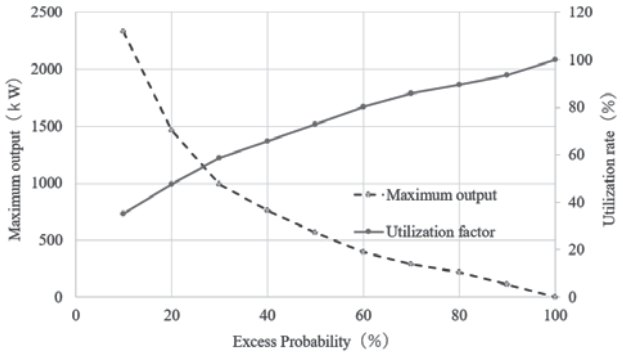

Fig.2 Relation between utilization rate and maximum generation output.

Until now, we finished the calculation of potential generation for seven sites. And it is made a conclusion in a table

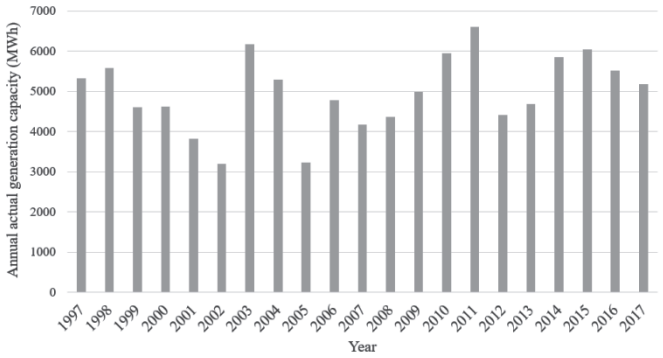

Fig.3 Annual actual generation capacity when maximum generation output is 947kW.

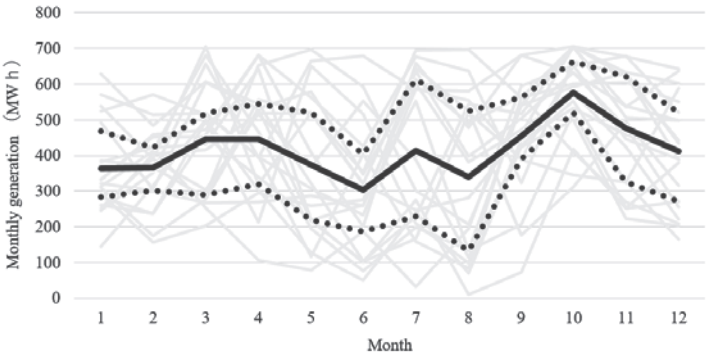

Fig.4 Monthly generation capacity when maximum generation output is 947kW.
*(Solid line is average value; Dotted lines are value of 25% and 75%; Gray line is annual generation for each year).

Table1 annual and monthly generation capacity for seven research sites.

Generation capacity / Site names		Inuyama	Meiji Yousui
Maximum output	kW	2002	947
Annual generation ave.	MWh/Y	10524	4971
Monthly generatio ave.	MWh/M	942	414
Monthly generatio max.	MWh/M	1315(Mar.)	577(Oct.)
Monthly generatio min.	MWh/M	396(Jun.)	303(Jun.)

DISCUSSION

Until now, we have finished the calculation of potential generation for seven sights. From the present results, From the present results, we got the relatively big potential generation and quite stable monthly generation capacity. The annual average actual generation we got totally is 18976MWh for 5 research sites. It can provide for 2447 person's annual electricity (In Japan, annual electricity consumption of per person is 7753kWh.

To complete this thesis, it needs to clarify some water rights problems related in using the discharge water in headwork. And also, the basin condition (such as: the resource of water in headwork and where to be flown into) each headwork needs to be evaluated clearly.

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A study on Kotsu-yousui area regional water requirement for paddy field by estimating the repeated use of water field

Suozhu[1], Masateru Senge[2] and Keigo Noda[2]
1. **The United Graduate School of Agricultural Science, Gifu University**
2. **Faculty of Applied Biological Sciences, Gifu University, Japan**

INTRODUCTION

In recent years, the agricultural land area in Japan tends to be decreasing due to the increase of general-purpose fields, the reduction of repetitive use due to separation of irrigation and drainage canal, the aging of agricultural workers, urbanization and etc. Based on this background, we choose the Kotsu-yousui as the research area, at first, we will reorganize the irrigation and drainage system in accordance with the current gross area and irrigation and drainage system, then we estimate the quantity of planned water intake based on the CB method using a new irrigation and drainage system, aiming to grasp the present water demand.

MATERIALS AND METHODS

The First of all, we will give an explanation of geographical condition and water condition of Kotsu-Yousui area which is the investigation site of this research. The field survey and analysis were conducted to reorganize the irrigation and drainage canal. According to the field survey, we grasped the profit paddy field area in the target field survey area and confirmed the irrigation and drainage canal in the paddy field, and made new blocks.

In order to grasp the amount of required water, we conducted a survey to measure the flow rate of rice paddy canal. We selected 28 observation points of important river and irrigation canal in three water type IWAKURA Yousui, Aisekawa Yousui, Shinkostu Yousui in Kotsu-yousui Area, We measured the actual flow rate for five times in 2015 according to the regional water management.

The CB method is a method created by improving both of the water requirement in depth and water balance method. While it is considering the reduction and repetitive use within the wide area of paddy filed, when it can be assumed as the flowing water in irrigation and drainage canal is near to the most steady state, that is, we planned to estimate the maximum required water amount in the period of continuous dry weather in the training period.

In order to investigate the maximum requirement water amount, area of paddy field in 2016 was calculated based on the map and the field survey, and the transition (changes) of paddy field area and building area were compared in 1978, 1990 and 2016.

According to the survey and research results, the decrease of agricultural land and the increase of building land were observed in Kotsu-yousui area, and the change of land use accompanying with urbanization became clear. In particular, in Kita Nagoya city, paddy field area decreased remarkably. As a result, it was found that when the maximum required amount is estimated by using the CB method, the actual water intake is excessive.

RESULTS

In Kotsu-yosui district, we plan to use the CB method which is a calculation method of the irrigation requirement in consideration of the repetition use, and aims to grasp the actual situation of the water supply management irrigation requirement in the district that could not evaluate impact statement of the possibility of water quantity in the district. And, we aim to estimate the actual condition of distribution of management water.

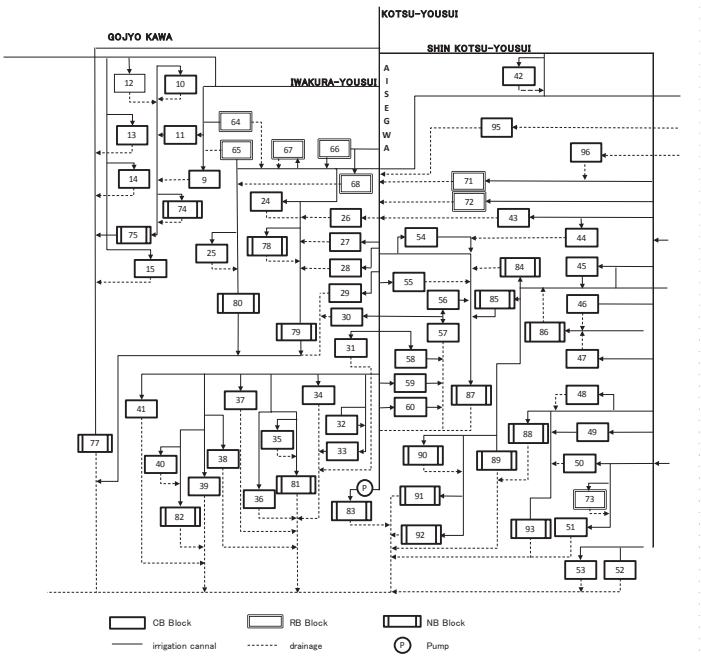

Fig. 1 Blocks of Kotsu -yousui area (1978).

DISCUSSION

In this study, we measured the amount of water intake in Kotsu-yousui area and proposed a decision result. I want to continue this study in order to make more accurate judgment. In the Kotsu-yousui area there are many rivers, the main water system joins together with the river and we will estimate the quantity of the final drainage water and evaluate the influence of the final drainage water of rice paddy on the repetitive use structure of agricultural water in the wide paddy field of Kotsu-yousui, in combination with the quantity water intake.

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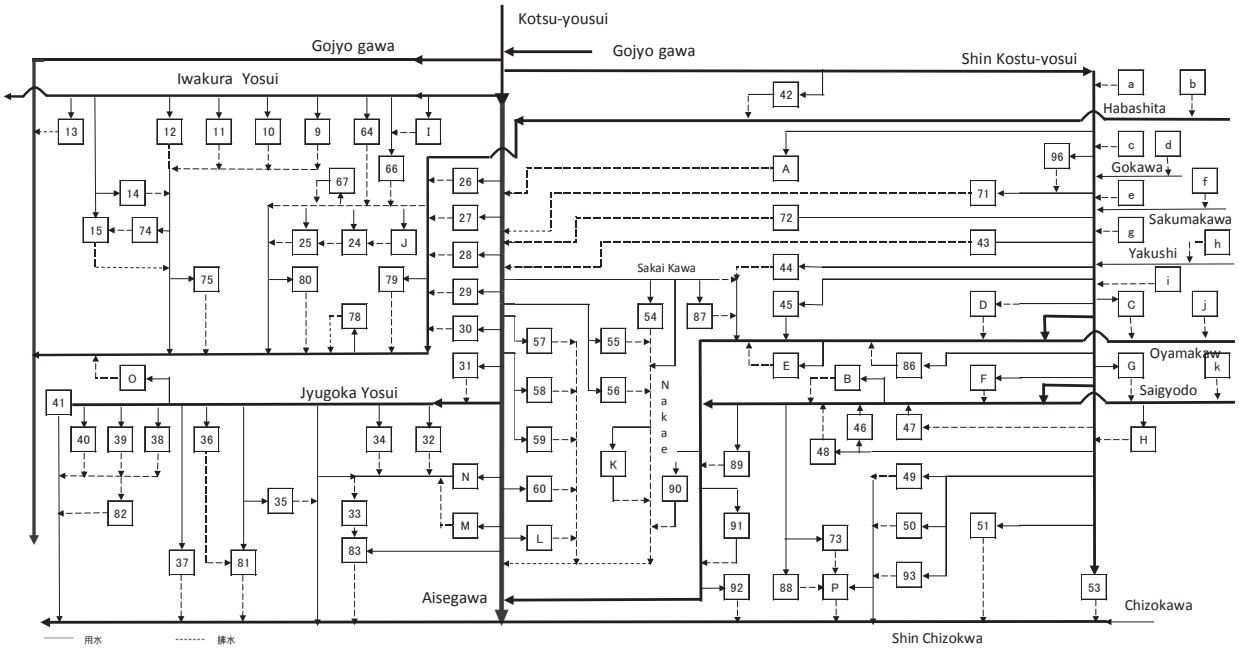

Fig2. Blocks of Kotsu-yousui area (2018).

Recombinant ovine angiotensinogen as a promising substrate for measuring renin concentration

Jobaida Akther[1,2], A. H. M. Nurun Nabi[2], Satoshi Ohno[3], Takashi Yokogawa[3,4], Tsutomu Nakagawa[5], Fumiaki Suzuki[5] and Akio Ebihara[4,5]
1. The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
2. Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka 1000, Bangladesh
3. Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
4. Center for Highly Advanced Integration of Nano and Life Sciences, Gifu University (G-CHAIN), 1-1 Yanagido, Gifu 501-1193, Japan
5. Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

INTRODUCTION

Renin is the key enzyme of the renin–angiotensin–aldosterone system (RAAS) which plays an important role in circulatory hemodynamics through its effects on vascular tone, salt and water homeostasis [1,2]. This enzyme cleaves its substrate, angiotensinogen (ANG), to produce the decapeptide angiotensin I (Ang-I), which is subsequently converted into angiotensin II inducing a vasoconstriction as well as aldosterone release [1]. Plasma renin activity (PRA) measurement got much importance as a substitute of RAAS activity evaluation [3–5]. PRA was also found to be associated with increased risk of myocardial infarction, cardiovascular and all- cause mortality in hypertensive patients [6–9]. Now-a-days direct renin assays that measure renin as mass concentration being faster, methodologically simpler and results, being independent of ANG concentration are progressively replacing traditional PRA assay [10]. Several comparative studies showed that the reproducibility of plasma renin concentration (PRC) is higher than that of PRA among laboratories [10]. PRC is defined in two approaches [11]. One is plasma renin concentration measured by immunoassay (ir-PRC), which is expressed as mass concentration. The other is plasma renin concentration measured by activity assay (ac-PRC). ac-PRC is determined by measuring an enzymatic activity of renin with an excess amount of renin substrate to avoid the influence of variation in plasma ANG concentration [11].

Ovine ANG (oANG) has been shown as a better substrate to measure ac-PRC [11]. Assayed with human renin, oANG shows 4.4× lower Michaelis constant (K_m) and 2.1× higher turnover number (k_{cat}) than human ANG, thereby leading to about 9× higher catalytic efficiency (k_{cat}/K_m) [12,13]. When oANG is utilized in ac-PRC measurement, a less amount of ANG is needed to reach a saturating concentration of substrate owing to the lower K_m.

We have recently reported a method of producing recombinant oANG at a milligram level using *E. coli* expression system [14]. Recombinant oANG produced by this method retains the similar activity and stability to that of expressed in mammalian cells. Since a large amount of oANG is available now, we wish to establish a better clinical assay for renin concentration measurement using our cost-effective recombinant oANG.

MATERIALS AND METHODS

Production of oANG using E. coli cells- The production of recombinant oANG in E. coli was performed as described previously [14].

Physical properties of oANG- Fractions were pooled from different stages of recombinant oANG preparation and subjected to SDS-PAGE using SuperSep Ace 12.5% gel, 13 wells (Wako, Tokyo, Japan). DSF experiments were performed as described previously. To estimate the melting temperature (Tm), the Boltzmann equation was used to fit the fluorescence data using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA) and the calculation templates. The T_m values were expressed as the mean ± standard deviation.

Kinetic analysis- Various concentration (0.08 – 1.5 μM) of recombinant oANG were incubated with recombinant human renin standard (27 pM) at 37°C for 30 min in 1× ES. The rate of Ang-I generation was determined by an enzyme-linked immunosorbent assay (ELISA) [15]. The K_m and maximum velocity (V_{max}) were estimated by Hanes-Woolf plot using GraphPad Prism 7.0 software. The kcat of renin-ANG reaction was calculated using the following formula:

$k_{cat} = V_{max}$ / renin concentration

Establishment of calibration curve of renin concentration using recombinant oANG- Ang-I specific ELISA [15] was used to measure the amount of Ang-I produced from various concentrations of renin. Microplate Manager 6 Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to calculate amount of Ang-I produced from the absorbance. Enzymatic activity of renin (i.e., renin activity) is defined as follows:

Renin activity = [pg amount of Ang-I / (0.1mL × 2 h ×1000)] ng Ang-I mL^{-1} h^{-1}

Renin activity measurements were repeated six times (n = 6) to establish a calibration curve of renin concentration.

RESULTS

Production of recombinant oANG- Recombinant oANG was expressed in *E.coli* cells and purified to homogeneity using two steps of column chromatography (Fig. 1). The final preparation contains oANG protein alone (lane 5, Fig. 1). About 300 μL of 291 μM recombinant oANG was obtained from 0.5 L of *E.coli* cell culture, with a yield of about 9 mg per liter of culture.

To examine the protein quality, the absorbance of recombinant oANG (1:20 diluted) was measured with a scan range of 240-340 nm. The absorbance at 340 nm was almost zero, indicating that there was no aggregation in the protein preparation. Maximum absorbance was 0.562 at 279 nm and minimum absorbance was 0.015 at 340 nm.

Protein stability analysis of recombinant oANG- DSF was used to examine the stability of recombinant oANG. The DSF results showed that oANG undergoes a two-state transition during the course of thermal denaturation. The T_m of recombinant oANG was 52.96 ± 0.22°C.

Establishing calibration curve of renin concentration using recombinant oANG- Various concentrations of renin in pM range were prepared using recombinant human renin standard, and the renin activities were measured at 0.8 μM of oANG (5 times higher than K_m value). A linear calibration curve was obtained by plotting the renin activity versus renin concentration (0.2 – 1.5 pM), with a slope of 2.10, a y-intercept of 0.02, and R^2 of 0.99 (Fig. 2). This result demonstrates a strong linear relationship between renin activity and renin concentration. An intraassay CV of < 20% was found in the six repeating experiments.

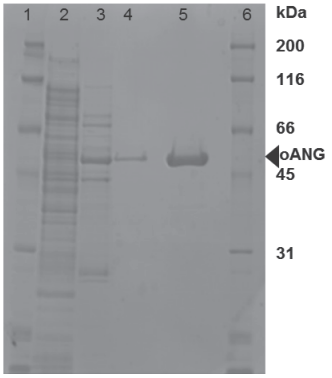

Fig. 1. SDS-PAGE analysis of recombinant oANG. The stages of oANG production were separated by SDS-PAGE and stained with CBB. Lane-1,6: molecular weight marker; Lane-2: cell supernatant after sonication; Lane-3: after affinity column chromatography; Lane-4: after ion-exchange column chromatography; Lane-5: concentrated oANG after purification (5 μg).

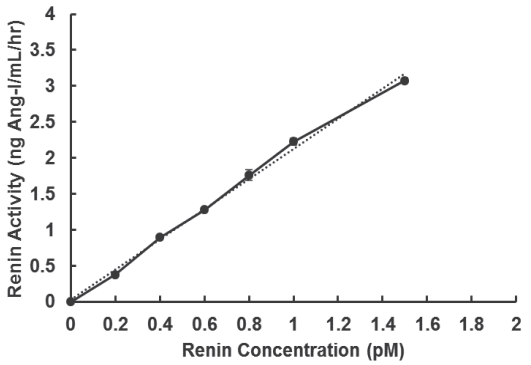

Fig. 2. Renin concentrations vs. renin activity plot. Linear regression analysis produced a slope of 2.10, a y-intercept of 0.02 and R2 of 0.99 (n = 6) suggesting linear relationship between renin concentration and renin activity. Error bars are showing standard error of mean values ranging from 0.02 - 0.07.

DISCUSSION

Renin concentration in plasma has significant prognostic value in patients with cardiovascular and renal diseases. The source of renin substrate, ANG, to be used in the assay method for renin evaluation is limited. This limitation can be solved using recombinant oANG expressed in E. coli cells, based on our results. With this recombinant oANG, picomolar amount of renin can be measured from known renin activity, which should be utilized in the clinical assay for measuring PRC.

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Frequency of *FUM21* mutation causing fumonisin non-producibility in *Fusarium fujikuroi* strains

Sharmin Sultana[1], Miha Kitajima[2], Hironori Kobayashi[2], Ryuou Yamaguchi[2], Masafumi Shimizu[2], Koji Kageyama[3] and Haruhisa Suga[4]

1. **The United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan**
2. **Fac. of Applied Biological Sciences, Gifu University, Gifu**
3. **River Basin Research Center, Gifu University, Gifu, Japan**
4. **Life Science Research Center, Gifu University, Gifu 501-1193, Japan**

INTRODUCTION

Fumonisin is a kind of carcinogenic mycotoxin produced by 15 species of *Fusarium fujikuroi* species complex (Rheeder et al. 2002). Fumonisin causes intestinal cancer and neural tube defect in human and several disease in animals (Marasas et al. 2004). *F. proliferatum* and *F. fujikuroi* are considered as potential fumonisin producers on rice (Cruz et al. 2013). The frequency and production level of fumonisin of *F. fujikuroi* are partly attributed to different assay method and different growth media used in individual investigation (Cruz et al. 2013, Bolton et al. 2016). On the basis of fumonisin production we classified *Fusarium fujikuroi* into two group such as F-group, produces fumonisin in significant level and G-group does not produce fumonisin in detectable level. Fumonisin production is dependent on a biosynthetic gene (*FUM*) cluster that composed of 16 genes (Proctor et al. 2003). The FUM cluster was detected not only from F-group strains but also from G-group strains. FUM21, a Zn(II)-2Cys6 DNA binding transcription factor, positively regulates *FUM* gene expression and is required for fumonisin biosynthesis (Brown et al. 2007). Without a functional FUM21 lacked transcripts from two biosynthetic genes *FUM1* and *FUM8* and failed to produce FB_1 (Brown et al. 2007). It has been revealed that low level of expression of *FUM21* is the cause of low fumonisin production in a *F. fujikuroi* strain (Rosler et al. 2016). Information of the genes affecting fumonsin producibility can be a target for chemical development to inhibit this mycotoxin production. The information is also important for accurate assessment of fumonisin production risk of *F. fujikuroi*.

In previous study, genetic mapping using the crossing progenies between a fumonisin producing strain Gfc0825009 (G9) and a non-producing strain Gfc0801001 (G1) indicated that fumonisin non-production in G1 attributes to the FUM cluster (北嶋 2012). PCR results indicated that G1 retain entire FUM cluster. Some important genes tested were expressed in G9 but only *FUM21* and *FUM1* expression were detected in G1 in RT-PCR result. Though *FUM21* expression was detected in G1, lack of remaining three *FUM* gene expressions suggested dysfunction of *FUM21* in G1. We identified the causative mutation in *FUM21* and its distribution in *F. fujikuroi* strains.

MATERIALS AND METHODS

- Ninety five *Fusarium fujikuroi* strains, fumonisin production of that had been investigated in previous report were used (Suga et al. 2014).
- Genomic DNA was extracted from 3–4-day old mycelium (2–3 cm diameter) cultured on potato dextrose broth (PDB) by using potassium ethyl xanthogenate solution, as previously described (Suga et al. 2014).
- Confirmed transcription of some *FUM* gene of G1 and G9 by RT-PCR after culturing by fumonisin induction medium. RT-PCR products were subjected to 2% electrophoresis gel.
- *FUM21* from G9 was cloned into a transformation plasmid vector. Point mutation was also created in *FUM21* of the plasmid.
- Fumonisin production recovery of transformants was determined by ELISA (detection limit is 0.22 ppm) with culturing in corn medium for 10 days in 25º C. Fumonisins in three of each transformant series including previous studies (Kobayashi 2013) were quantified by LC-MS/MS analyses.
- PCR-RFLP (Restricted Fragment Length Polymorphism) was developed to determine g.2551 G> T (p.G 678 *) of *FUM21* of *F. fujikuroi* using dCAPS primer.

RESULTS and DISCUSSION

RT-PCR of *FUM* genes

DNA with expected size of all genes tested were detected in G9 by RT-PCR but it was detected only for *HistoneH3*, *FUM21* and *FUM1* in G1 (Fig. 1A and 1B). Lack of transcription of multiple *FUM* genes suggested dysfunction of the transcription factor FUM21 in G1. Positive conversion of *FUM6*, *FUM8* and *FUM10* expression by integration of *FUM21* was confirmed in a transformant FfT21FUMKOD (#2) (Figure 1C).

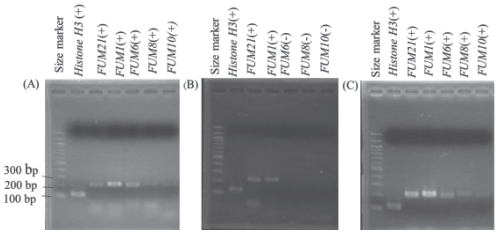

Fig. 1: RT-PCR results of (A) G9, (B) G1 and (C) FfT21FUMKOD-2. (+) indicated the expected size of RT-PCR product was detected, (-) not detected.

***FUM* gene complementation by transformation**

Fumonisin production recovery was failed by complementation of only *FUM21* but it was succeeded by *FUM6/ FUM7/ FUM8/ FUM10/ FUM11/ FUM2/ FUM13* (小林 2013, FfDTFUM21_6_13 in Fig. 2). In order to identify which of g.888G>C (p.D261H) or g.2551G>T (p.G678*) (Fig. 3) is the causative mutation of fumonisin non-production in G1, complementation of *FUM21* with either of these point mutation was conducted in G1.The plasmid that carry a point mutation at 888th (pDT21G888C-1) and 2551th (pDT21G2551T-2) nucleotide in *FUM21* of G9 was created and transformed into FfT67831011213-30 (a transformant of G1 carrying *FUM6/ FUM7/ FUM8/ FUM10/ FUM11/ FUM2/ FUM13* regions of G9) (Fig. 2). None of the transformants recovered fumonisin production in case of pDT21G2551T-2 (FfDTFUM21G2551T_6_13 in Fig. 2) while five

transformants recovered fumonisin production in case of pDT21G888C-1 (FfDTFUM21G88C_6_13 Fig. 2). These results suggested that g.2551G>T (p.G678*) rather than g888G>C (p.D261H) is one of the cause of fumonisin non-production in G1 (Fig. 3).

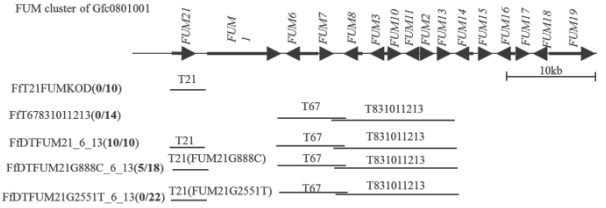

Fig. 2: Result of *FUM* gene complementation in G1 by transformation (the number of fumonisin producing transformants/ the number of investigated transformants). Here, "T" is DNA fragment from fumonisin producing strain G9.

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MTEPIVFVEDNPAGVDSGHKPRFRRRRGACESCKRRKVRCNGSNPCNQCQKSSIDCQYKASSWKTNDGYTERGPSESPLP 80
MTEPIVFVEDNPAGVDSGHKPRFRRRRGACESCKRRKVRCNGSNPCNQCQKSSIDCQYKASSWKTNDGYTERGPSESPLP 80

IARGSLTPPQTVTLPSPIGAVTYTGSVEESSASGIDGSDPHSLSMRLSNTGSTINTDSTDISQWQDWLINSDVPFGDSMQ 160
IARGSLTPPQTVTLPSPIGAVTYTGSVEESSASGIDGSDPHSLSMRLSNTGSTINTDSTDISQWQDWLINSDVPFGDSMQ 160

LTGTSDDFLTDWLDPQLVNPPNTAAITQTLSGENLSRPSGLLLGTADPLPSNSDSDLNGTRNELIASFFHKLRSQRPFIL 240
LTGTSDDFLTDWLDPQLVNPPNTAAITQTLSGENLSRPSGLLLGTADPLPSNSDSDLNGTRNELIASFFHKLRSQRPFIL 240

CDGSSETILNNNTMGHREFHDTKFISRCLDTCYADPEGIRVFLERKSVDSVADEVAKGASAVDRETSVLFHSVMAIGCHG 320
CDGNSETILNNNTMGHREFHHTKFISRCLDTCYADPEGIRVFLERKSVDSVADEVAKGASAVDRETSVLFHSVMAIGCHG 320
          g.888G>C(p.D261H)
LSLEQGHHTIGKQKYSVSWIFKEALYMRQNLRDKPTLRGLQALLTMAYFSGRVGDDSTSSLLADAAVCAQTLELHSASAI 400
LSLEQGHHTIGKQKYSVSWIFKEALYMRQNLRDKPTLRGLQALLTMAYFSGRVGDDSTSSLLADAAVCAQTLELHSASAI 400

EKQYNSSSEQQVVAKRALWFLNSLEKPRCLAEGLLPLIHDDLIDYDPPSSASHSPDEVDWFAINARFATICYSIIRERPRG 480
EKQYNSSSEQQVVAKRALWFLNSLEKPRCLAEGLLPLIHDDLIDYDPPSSASHSPDEVDWFAINARFATICYSIIRERPRG 480

KLGRSSPRRGQGQASQQSASSTISRIESLLDEWRGDLPFASESNATESNEFAALTCSERRHRIKCLNKYWSAVIATHSGQ 560
KLGRSSPRRGQGQASQQSASSTISRIESLLDEWRGDLPFASESNATESNEFAALTCSERRHRIKCLNKYWSAVIATHSGQ 560

ARVVVVDGGEGVGSSKERCVEAAQEILKNSHYITSTDILYDISLYYYITVATRVIMTAVIREAFAGDLAEDRVRKNTKIP 640
ARVVVVDGGEGVGSSKERCVEAAQEILKNSHYITSTDILYDISLYYYITVATRVIMTAVIREAFAGDLAEDRVRKNTKIP 640

RKETRYGSLTSYMGIAIGLFSRLSLDIDVPVDEVTELGKLGRQIMKCQ                                  688
RKETRYGSLTSYMGIAIGLFSRLSLDIDVPVDEVTEL                                             677
                                  g.2551G>T(p.G678*)
```

Fig. 3: Comparison of the amino acid sequences of FUM21 of fumonisin producing strain (upper) G9 and fumonisin non-producing G1 (lower).

Frequency of *FUM21* mutation in *F. fujikuroi* strains

We developed PCR-RFLP that can determine g.2551G>T in FUM21 (Fig. 4).

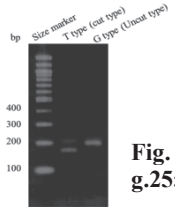

Fig. 4: PCR-RFLP for determination of g.2551G>T in FUM21.

All F-group strains tested showed uncut type (i.e. G type) whereas 35 (81%) out of 44 G-group strains showed cut type (T type) (Table 1).

Table 1. Frequency of *FUM21* mutation in *F. fujikuroi* strains.

Group	Strain	FUM21g2551+D3:D3:D40	Group	Strain	FUM21g2551+D3:D3:D40
G-group	Gfc0625010	G,T,A,A,T	G-group	Gfc0825002	G,T,A,A,T
G-group	Gfc0801001h	G,T,A,A,T	G-group	Gfc0825003	G,T,A,A,T
G-group	Gfc0801003	(G,T,A,A,T)	G-group	Gfc0825004	G,T,A,A,T
G-group	Gfc0801002	G,T,A,A,T	G-group	Gfc0825005	G,T,A,A,T
G-group	SMN86-2h	G,T,A,A,T	G-group	Gfc0825006	G,T,A,A,T
G-group	Gfc8707249	G,T,A,A,T	G-group	Gfc8707123	G,T,A,A,T
G-group	Gfc8707642	G,T,A,A,T	G-group	Gfc8707182	G,T,A,A,T
G-group	Gfc1004002	G,T,A,A,T	G-group	Gfc1004001	G,T,A,A,T
G-group	MIE92-4	G,T,A,A,T	G-group	GL25	G,T,A,A,T
G-group	Gfc9424707	G,T,A,A,T	G-group	GL27	G,T,A,A,T
G-group	Gfc0625002	G,T,A,A,T	G-group	GL28	G,T,A,A,T
G-group	Gfc0625004	G,T,A,A,T	G-group	Gfc0925005h	G,T,A,A,T
G-group	Gfc0625005h	G,T,A,A,T	G-group	Gfc8707117	G,T,A,A,T
G-group	Gfc0625006	G,T,A,A,T	G-group	Gfc0925011	G,T,A,A,T
G-group	Gfc0625007	G,T,A,A,T	G-group	Gfc0925012	G,T,A,A,T
G-group	MYG92-10	G,T,A,A,T	G-group	Gfc0625001h	G,T,A,A,T
G-group	Gfc0925010	G,T,A,A,T	G-group	Gfc0901009h	G,T,A,A,T
G-group	Gfc0901002	G,T,A,A,T	G-group	Gfc1034001h	G,T,A,A,T
G-group	Gfc0901005	G,T,A,A,T	G-group	APF06083h	G,T,A,A,T
G-group	Gfc9424702h	G,T,A,A,T	G-group	GL24h	G,T,A,A,T
G-group	Gfc9424703	G,T,A,A,T	G-group	MAFF235949h	G,T,A,A,T
G-group	Gfc0825001h	G,T,A,A,T	G-group	Gfc0625008	G,T,A,A,T

Table 1 (continue)

F-group	Gfc0009063h	T,C,G,C,C	F-group	Gfc1041003	T,C,G,C,C
F-group	Gfc0825009h	(T,C,G,C,C)	F-group	Gfc1041010	T,C,G,C,C
F-group	41-116	T,C,G,C,C	F-group	Gfc1041011	T,C,G,C,C
F-group	41-108	T,C,G,C,C	F-group	Gfc1025029	T,C,G,C,C
F-group	Gfc1004003h	T,C,G,C,T	F-group	Gfc1025037	T,C,G,C,C
F-group	Gfc0009110	T,C,G,C,C	F-group	Gfc1006007	T,C,G,C,C
F-group	Gfc0009105h	T,C,G,C,C	F-group	Mo309	T,C,G,C,C
F-group	Gfc0825011h	T,C,G,C,C	F-group	41-79h	T,C,G,C,C
F-group	41-84	T,C,G,C,C	F-group	Gfc1025091	T,C,G,C,T
F-group	Mo141	T,C,G,C,T	F-group	Gfc0921041	T,C,G,C,C
F-group	IBR89-1	T,C,G,C,C	F-group	Gfc0921034	T,C,G,C,C
F-group	Gfc1034002	T,C,G,C,C	F-group	Gfc1019001	T,C,G,C,C
F-group	Gfc0921039h	T,C,G,C,C	F-group	Gfc1019003h	T,C,G,C,C
F-group	Gfc1043035	T,C,G,C,C	F-group	Gfc1006002	T,C,G,C,C
F-group	Gfc0009117	T,C,G,C,C	F-group	Mo136	T,C,G,C,C
F-group	Gfc0825007h	T,C,G,C,C	F-group	Gfc1019004	T,C,G,C,C
F-group	Gfc0921002	T,C,G,C,C	F-group	Gfc1006001	T,C,G,C,C
F-group	Gfc0921009	T,C,G,C,C	F-group	Mo80	T,C,G,C,C
F-group	Mo78	T,C,G,C,C	F-group	Gfc0921040	T,C,G,C,C
F-group	Gfc1043037	T,C,G,C,C	F-group	Gfc0921014	T,C,G,C,C
F-group	Gfc1043045	T,C,G,C,C	F-group	Gfc1016022	T,C,G,C,C
F-group	Gfc1043032h	T,C,G,C,C	F-group	Gfc1016024	T,C,G,C,C
F-group	Gfc1043046	T,C,G,C,C			

According to our previous study multiple causative mutations are present in G1 and g.2551G>T (p.G678*) is one of them. In future we further identify additional mutations and the variation of mutations in G-group strains.

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Suitable culvert configuration for prevention the risk of dam failure due to hydraulic fracturing adjacent to culverts in embankment dams

Duy Quan Tran[1], Shinichi Nishimura[2], Masateru Semge[2] and Tatsuro Nishiyama[2]
1. **The United Graduate School of Agricultural Science, Gifu University, Japan.**
2. **Faculty of Applied Biological Sciences, Gifu university, Japan.**

INTRODUCTION

Hydraulic fracturing is generally considered as one of the most probable causes leading to the concentrated leakage and incidents of many fill dams soon after the completion of the dam's construction (Sherard et el. 1972). For predicting the occurrence of hydraulic fracturing at any point in a fill dam, the normal stress of the point is compared with the water pressure at that point. If the water pressure is higher than the normal stress, it is thought that hydraulic fracturing has occurred. Previous studies revealed that the risk of hydraulic fracturing increases in cases where the arching action is present (Ngambi et al. 1997). Due to this arching action, the stress in fill dams can be reduced to a level lower than the water pressure.

It is generally identified that there are high risks of dam incidences resulted from arching action and hydraulic fracturing adjacent to the culverts of the fill dams where elastic moduli between the culvert materials and the fill soils are significantly different (Sherard et el. 1972, Ngambi et al. 1997). Hence, selection a suitable culvert configuration for reducing the risk of hydraulic fracturing is truly necessary. Culverts utilized in practice generally have a pipe shape, a box shape or a horseshoe shape. Past researches have revealed, however, that using culverts with these shapes brings about the potential risk for inducing arching action and hydraulic fracturing (Ngambi et al. 1997, Tran et al. 2018). The purpose of this study is to find the relationship between culvert configurations and arching action as well as the risk of hydraulic fracturing adjacent to culverts by using finite element analyses. The results of the simulations suggest some suitable culvert shapes to prevent hydraulic fracturing around the culverts of agricultural dams with low to medium heights.

MATERIALS AND METHODS

Materials—To investigate the effects of the different culvert shapes on the risk of hydraulic fracturing in fill dams, some background information on a dam failure was utilized here as a case study. The case study dam is an agricultural dam called KE 2/20 REC dam in Central Vietnam. The dam was put into operation in July 2008. However, in June 2009 – just under one year after it was put into operation - the dam broke at the location of the pipe culvert. Previous study has suggested that the cause of the dam failure is associated with the hydraulic fracturing and arching action occurring adjacent to the pipe culvert. In this study, therefore, the physicomechanical properties of the fill soil material is taken from the previous investigation and are summarized in Table 1 (Tran et al. 2018).

Figure 1 shows the culvert shapes considered in this study. The dimensions of these culverts, including the height of 1.1 m and the width of 1.2 m, are kept to be similar to those of the pipe culverts in the case study dam. In Fig. 1a, a box-shaped culvert is displayed. In the case of Fig. 1b, the box-shaped culvert (Fig. 1a) is changed by inclining the chamfers (*IG*=1.0) in variations of 0.1 m in the vertical direction and 0.1 m in the horizontal direction. In Fig. 1c, the culvert shape is modified by a combination of inclining chamfers (*IG*=1.0) and slanted walls with a gradient (*G*).

Table 1: Material properties

Total density (ρ):	2.018 Mg/m^3
Dry density (ρ_d):	1.673 Mg/m^3
Coefficient of permeability (k):	6.247×10^{-5} cm/s
Elastic modulus (E):	16800 kPa
Poisson's ratio (v):	0.3

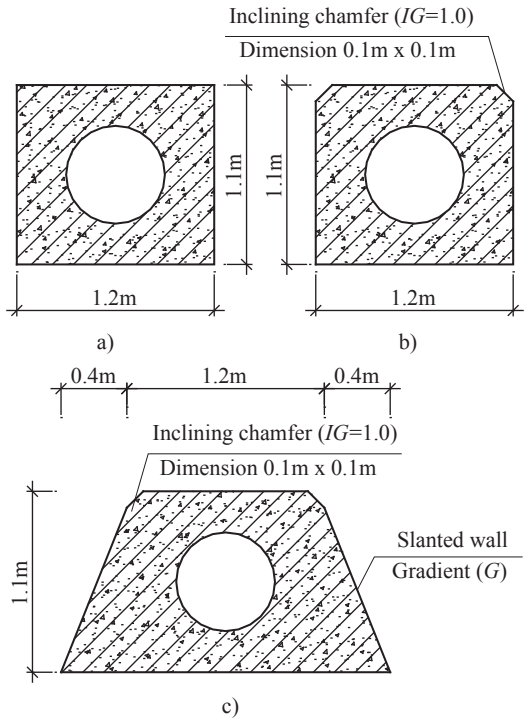
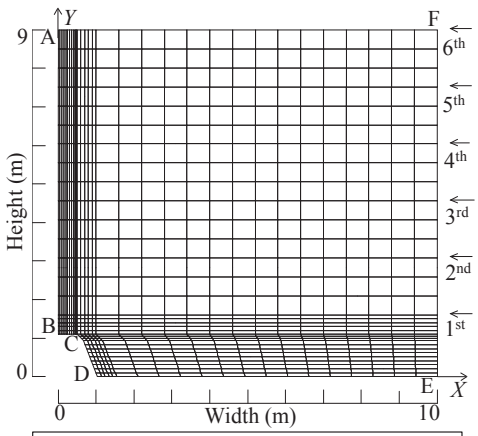


Fig.1: Cross section of culvert

Numerical analysis—The finite element method (FEM) is often utilized in studies of the stress-strain distribution in dams as well as in investigations of hydraulic fracturing in fill dams. Based on finite element analyses, the stress distribution around a culvert can be determined, after which the possibility of hydraulic fracturing can also be predicted by comparing the stress with the water pressure at corresponding locations. In this research, therefore, build-up analyses with 6 layers of fill soil using FEM was simulated to evaluate the likelihood of hydraulic fracturing around culverts with different configurations. Figure 2 shows the finite element mesh for a representative case of the culvert shape (Fig. 1c) containing the inclining chamfers (*IG*=1.0) and the slanted walls with a gradient of *G*=0.4. This model consists of 1024 elements and 3201 nodal points. All elements are eight-node quadratic quadrilateral elements and elements adjacent to culvert have smaller dimension than the others in order to improve accuracy and detail of stress distribution around the culvert.



Boundary conditions:
+ AB and EF: X-direction fixed, Y-direction free.
+ BCD: X- and Y- directions fixed.
+ DE: X-direction free and Y-direction fixed.

Fig.2: Cross section of culvert

RESULTS AND DISCUSSION

Potential for hydraulic fracturing adjacent to box-shaped culverts—The distribution of normal stress minus water pressure (σ_n-W) versus the distance along the culvert periphery (L) for the box-shaped culverts (in Figs. 1a and 1b) are shown in Fig. 3. In this figure, the distance (L) is calculated from the origin at the midpoint of the culvert top (similar to B in Fig. 2). As seen in Fig. 3, in both cases of the box-shaped culverts, the normal stress on the sides of the culverts are significantly reduced to be really lower than the water pressure due to the effect of the arching action. The maximum value of (σ_n-W) at the sides of the culverts is about -6 kN/m^2. Therefore, it can be concluded that there are actual risks of hydraulic fracturing adjacent to the box-shaped culvert as well as the box-shaped culvert with the inclining chamfers.

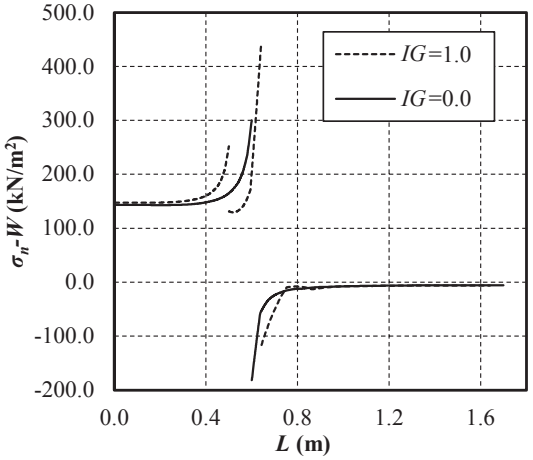


Fig.3: Distribution of normal stress (σ_n) minus water pressure (W) around box-shaped culvert (Fig. 1a – IG=0.0) and box-shaped culvert with inclining chamfers (Fig. 1b – IG=1.0)

Effect of the gradient of slanted walls on the risk of hydraulic fracturing—Figure 4 shows the distribution of (σ_n-W) around the culvert shape in Fig. 1c, including the inclining chamfers (IG=1.0), and the slanted walls with gradients varying from 0.1 to 0.5. It can be seen that the minimum value for (σ_n-W) increases when the gradient of the slanted walls is changed from 0.1 to 0.5. It seems to suggest that the effect of arching action might be lowered in cases of higher gradients of the slanted walls. The results in Fig. 4 also reveal that when the gradients are equal to or higher than 0.4, the risk of hydraulic fracturing along the outlet conduits can be controlled.

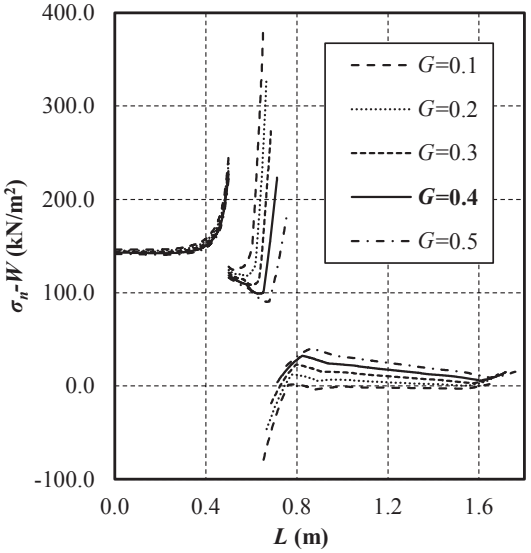


Fig.4: Distribution of normal stress minus water pressure (σ_n-W) around culverts with inclining chamfers (IG=1.0) and slanted walls (Fig. 1c)

CONCLUSION

Based on the results discussed in the previous section, the following conclusions are drawn:

(1) There is a potential for hydraulic fracturing close to box-shaped culverts and box-shaped culverts with inclining chamfers because the normal stress on the sides of the culverts is reduced by arching action to point of being lower than the water pressure.

(2) Culverts with slanted walls can reduce the risk of hydraulic fracturing compared with culverts with vertical walls. In the Japanese standard, the slanted walls with gradient's values from 0.1 to 0.3 are suggested for applying to construction. Based on the simulated results in previous sections, however, we recommend that the gradient of 0.4 or higher values should be applied to practice to control the potential for hydraulic fracturing around the culverts.

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Expression GWAS-An approach for understanding the *ALS3* Signal Transduction Mechanism

Raj Kishan Agrahari[1*], Ayan Sadhukhan[2*], Yuki Nakano[1], Satoshi Iuchi[3], Masatomo Kobayashi[3], Sanjib Kumar Panda[4], Yuriko Kobayashi[1, 2] and Hiroyuki Koyama[1, 2]

1. **The United Graduate School of Agricultural Science, Gifu University**
2. **Faculty of Applied Biological Sciences, Gifu University, Gifu 501- 1193, Japan**
3. **Expermental Plant Division, RIKEN Bio Resource Centre, Tsukuba, Ibaraki 305-0074, Japan**
4. **Department of Life Science, Assam University, Silchar 788011, India**

***These authors contributed equally to this work**

INTRODUCTION

Aluminum (Al) toxicity in acid soils (pH < 5.5) is a severe global problem that affects greater than 30% of the world's arable land, with this being a major limiting factor for crop productivity in these areas (von Uexkull and Mutert, 1995). Physiologically Al toxicity includes severe root growth inhibition coupled with reduction in shoot biomass and crop yield (Kochian, 1995). At the cellular level, Al toxicity results from deleterious effects of the Al^{3+} ion, which is the toxic species of Al that predominates under acidic conditions. Al^{3+} targets are both external, including the cell wall (Tabuchi and Matsumoto, 2001), and intracellular including the cytoskeleton (Blancaflor et al., 1998; Grabski and Schindler, 1995), ion transporters (Huang et al., 1992), constituents of signaling systems (Haug et al., 1994; Jones and Kochian, 1995), and various other cellular components and enzymes (Jones and Kochian, 1997), many of which are negatively charged or coordinate positively charged cofactors (Kochian, 1995).

Previously, a molecular genetics approach was employed using *Arabidopsis thaliana* as a model system in order to identify factors required for Al resistance and/or tolerance in plants (Larsen et al., 1996, 1997). From this approach a unique als-3 mutant was found to comprise with distinct characteristics (Larsenet al., 1996). Al-dependent phenotypes of als3 include complete arrest growth of the primary root, lateral roots, and the shoot. *ALUMINIUM-SENSITIVE 3* (*ALS3*) encodes a half type ATPase is involved in the Al-translocation process, while the gene is inducible in the shoots under Al stress, this gene is involved in the redistribution of toxic Al away from sensitive tissues thereby conferring Al tolerance. But the detailed regulatory mechanisms leading to the induction of *ALS3* under Al stress remain unclear. Here we present our work regarding the new signaling molecules upstream to *ALS3*.

MATERIALS AND METHODS

Plant Materials

Seeds of natural *A.thaliana* accessions and KO lines were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA), the Nottingham Arabidopsis Stock Center (Nottingham, UK), and the RIKEN BioResource Center (Tsukuba, Japan), We treated 100 *A.thaliana* accessions with 25 μM Al for the GWAS. Progeny lines were obtained by controlled self-pollination method. The homozygocity of KO lines was confirmed by PCR using SALK recommended methods.

Plant Growth Condition

Germinated *A.thaliana* seedlings were grown on mesh and transferred to to modified 2% MGRL (pH was 5.6) culture solution (Fujiwara et al., 1992). The solution was changed every 2 days, and grown under controlled environmental conditions for 10 days (Sawaki et al., 2016).

Gene Expression Analysis

10 days old seedlings were then transferred to the treatment solution containing 25 μM Al. after a 24-h stress treatment, the shoots were collected and freeze in liquid nitrogen, and crushed using tissue homogenizer. Total RNA was extracted from the shoot and used for cDNA. *ALS3* expression level was determined by real-time qPCR.

Genome-wide Association Study

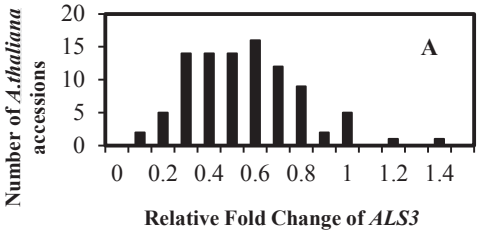
The GWAS calculations were completed using the compressed mixed linear model (Yu et al., 2006; Zhang et al., 2010) of the TASSEL program as previously described (Kobayashi et al., 2016). The genome-wide SNPs used in this study were obtained from a public database. Missing data or a minor allele frequency below 10% was removed. Genes nearest to the most significantly associated SNPs were annotated according the TAIR 10 database.

ALS3 expression analysis in KO line

Total RNA was extracted from a 24-h stress (25 μM Al) treated shoot sample, and used for cDNA. *ALS3* expression level was determined by real-time qPCR.

RESULTS & DISCUSSION

From a GWAS on *ALS3* expression we found 22 SNPs ($P<10^3$) to be strongly associated to *ALS3* expression. Role of the genes, linked to the most significant SNPs, in regulating *ALS3* was validated by reverse genetics.



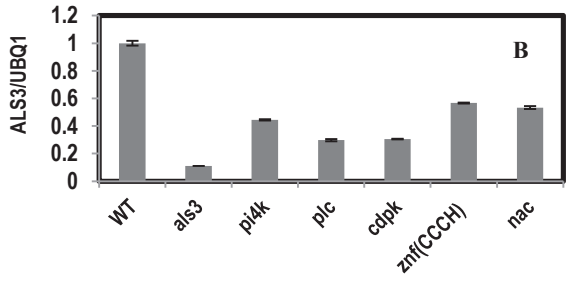


Fig 1. (A)Histogram of *ALS3* expression in *A. thaliana* accessions. (B) Expression of *ALS3* in T-DNA insertion mutants of GWAS detected genes.

As *ALS3* encodes a putative ABC transporter homologous to a predicted bacterial metal resistance protein, responsible for Al tolerance, though it's signaling is unclear. We did GWAS to identify a signaling cascade regulating *ALS3* expression under Al stress. From the GWAS we successfully identified *ALS3* signaling cascade that provide genetic evidence of the involvement of phospholipids signaling under Al stress.

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A Survey of GABA production from different species of *Bifidobacterium*

Hend Al taib [1], Takumi Morioka [2], Mayuko Abe [3], Izumi Nomura [1] and Tohru Suzuki[1,2]

1. **The United Graduate School of Agricultural Science**
2. **Graduate School of Natural Science and Technology**
3. **Faculty of Applied Biological Sciences**

INTRODUCTION

Bifidobacterium is one of the most important probiotics which normally inhabits the gut of all mammals including animal and human. It exerts its function mainly through existence as a part of the gut microbiota. Gut microbiota represents 10 times as many cells as that in our body and 150 times as that of our genomes. Therefore, it is often called the forgotten organ.

Recent years have witnessed the rise in the gut microbiota brain axis as a major topic of interest in biology (Grenham et al 2011). Gut-brain axis refers to the interplay between gut and brain which occurs through many pathways. One connection pathway occurs through neurotransmitters which transfer from gut to brain and vice virsa by specific signals. These signals pass between the enteric nervous system of the gut and the central nervous system (CNS) of the brain. Recently microbes have proved to produce several neurochemical transmitters which drive the name microbial endocrinology (Lyte et al 2011). Those materials have the same structure as that of mammals. Such producing microbes are also named *psychobiotics* (Dinan et al 2013).

Bifidobacterium has proved to produce several neurotransmitters. One of the most important neurotransmitters, recently shown to be produced by bifidobacteria, is Gamma-aminobutyric acid (GABA). GABA is the major inhibitory neurotransmitter in the CNS. It also has an immunomodulatory function through the existence of its receptors in most of the immune cells (Jin et al 2013). Basically, GABA is biosynthesized by decarboxylation of glutamate by the action of glutamate decarboxylase encoded by *gad*B and transferred to outside of the cell through glutamate/Gamma amino butyrate antiporter encoded by *gad*C(R Dhakal et al 2012). As it has an important function on both neuro-immunomodulation level and little investigations are available about its productivity from *Bifidobacterium*, a detailed study for its production from *bifidobacterium* genes is needed. Here, A survey of GABA production ability and producing gene prevalence within the genus *Bifidobacterium* has been performed.

MATERIALS AND METHODS

1. *Bacterial strains and culture condition.*

More than 25 different bifidobacteria species have been selected from the National Bio Recourse Project (NBRP), strains have been selected according to the homology of *gad* genes. MRS was supplemented with 1% monosodium glutamate. Monosodium glutamate acts as a precursor of GABA in the media.

2. *Selection a suitable prebiotic enhancer by using flora model to mimic in vivo environment.*

To maintain high GABA production on a long run in the gut, it was needed to select a good prebiotic enhancer. Flora model (Takagi et al. 2016) has been selected to mimic inside environment for a selection of best prebiotic enhancer. Different resource of oligosaccharides has been added to flora model jars. The final GABA production has been estimated with HPLC

3. *HPLC Screening of GABA production*.

HPLC condition has been managed as follow; Reverse phase chromatography with cadenza column CD-C18 (250x4.6mm i.d.,3µm), Column oven at 35°C, flow rate 0.8ml/min. A gradient system elute has been used (water-acetonitrile, 2-propanol, TFA) The NBD-F pre-column derivatization has been used for GABA production estimation according to the method by (C Aoyama et al. 2003). Each sample has been prepared in triplicate. Biological error has also been considered by the triplicate culture of each sample.

4. *In silico analysis of Gad gene prevalence and homology in bifidobacterium producer strains*.

Using CLC working bench ver.8, the multigene alignment has been performed for gad genes. NCBI has been used to obtain the available genome sequences. Whole genome sequence analysis has been performed using Illumina Miseq analyzer of GIFU University for one high producer and another nonproducer strain of bifidobacteria.

RESULTS and DISCUSSION

1. *B. adolescentis shows the highest GABA production.*

GABA standard curve has been estimated with equation y=443.34x, R^2=0.998. The values were normalized considering the internal standard. B. *adolescentis* laboratory strain no.4-2 has shown to be the highest in GABA production up to about 3 g/L (after two overnight incubation). The whole genome sequence of this strain has been done and *gad*B and *gad*C have been found in the genome. Another strain B. *adolescentis* JCM 1275 has shown zero production of GABA and the whole genome data also shows the absence of *gad*B encoding Glutamate decarboxylase and *gad*C encoding glutamate/GABA antiporter genes which means those two genes are essential for GABA production in genus *Bifidobacterium*. This data confirms the result obtained by the Russian research group (R.A. Yunes et al 2016)

2. *Manno Oligo Saccharides (MOS) have shown to enhance GABA productivity in flora model.*

The MOS has enhanced GABA production compared to Faracto Oligo Saccharides (FOS)and Dextrins (Dex) table1. This could be referring to the ability of bifidobacteria to digest Oligosaccharides into short chain fatty acids.

The added oligosaccharide	Normalized GABA peak µg/L
Control (no additives)	99.2
Flora model+0.5%MOS	145
Flora model+0.5%Dex	82.1
Flora model+0.5%FOS	146

Table.1. the estimated GABA production in flora model under addition of various resources of oligosaccharides, the process estimated for fecal samples from bifidobacteria carrier volunteers. It confirms that MOS and FOS are two good prebiotic enhancer for bacterial growth in flora model which mimic the gut environment.

3. *A high homology gene set for GABA production exists within the genus Bifidobacterium.*
   The multigene alignment shows high homology in the sequences of *gad*B genes which confirm that this gene is essential for GABA production in the genus *Bifidobacterium.* The pyridoxal phosphate binding motif PLP binding motif has also confirmed to be homologues in this gene. PLP is an important cofactor for glutamate decarboxylase activity. Once it is found means this gene should have some activity. The multigene alignment data is shown in fig.1.

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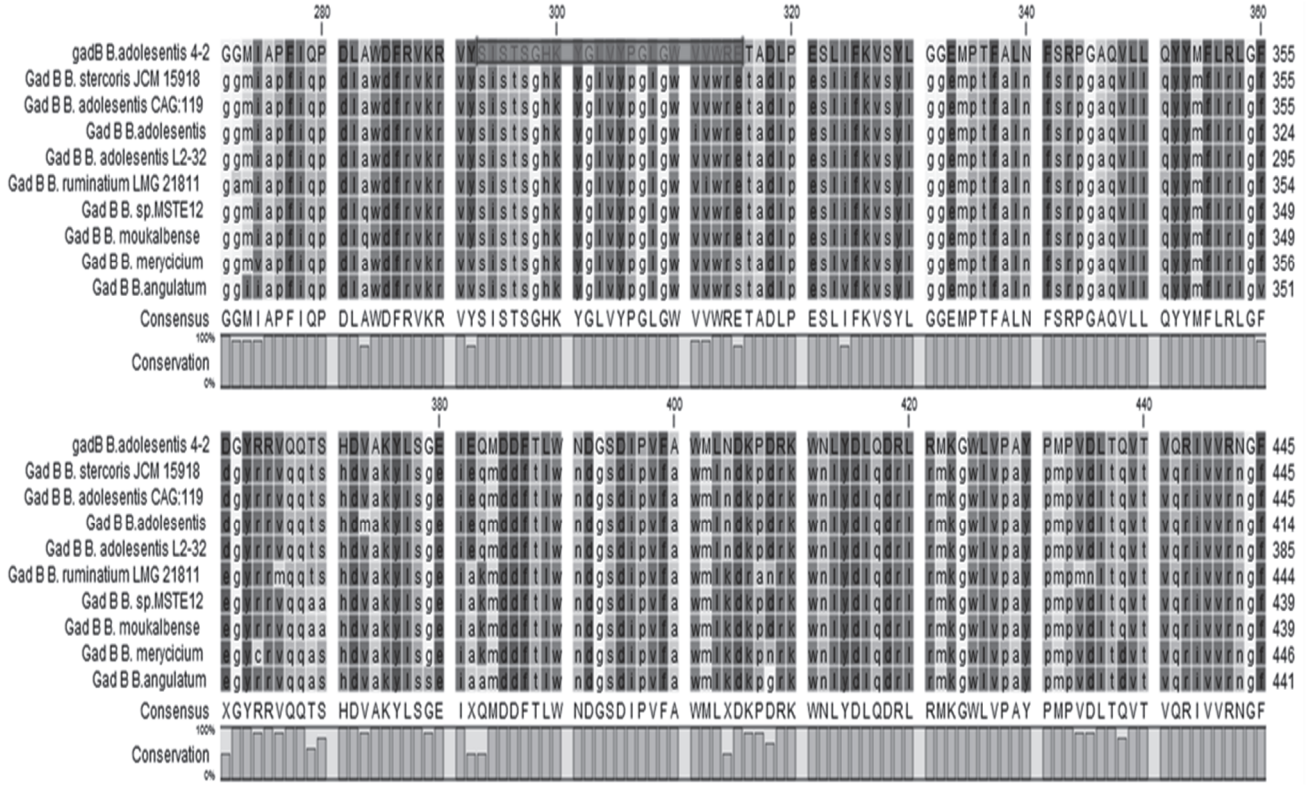


Fig.1 Multifragment alignment for *gad*B gene in 10 strains of bifidobacteria. It shows a high consensus sequence of *gad*B gene and also a high similarity in PLP binding motif marked by the gray rectangular in the upper part of the figure. The software used was CLC working bench ver.8.

Identification of bacteria from bioaerosol at AIST, Tsukuba, Japan

Panyapon Pumkaeo, Wenhao Lu, Youki Endou, Tomohiro Mizuno, Junko Takahashi and Hitoshi Iwahashi
The United Graduate School of Agricultural Science, Gifu University
Faculty of Applied Biological Sciences, Gifu University

INTRODUCTION

Bioaerosols are atmosphere particles, mists or dust of μm range, associated with metabolically active or inactive viable particles. They contain living organism's included microorganisms such as viruses, bacteria, and fungi also plant material as well as pollen. For study about microbial diversity in human and environment, Next Generation Sequencing (NGS) become a famous. NGS is a novel method of DNA sequencing that quickly and efficiently read the underlying sequence of an organism by means of massively parallel sequencing. The aim of this study is identifying bacteria which contained in environmental samples by using NGS.

MATERIALS AND METHODS

This study monitored the environmental sample (Bioaerosols) from November 2013 to January 2014 for 50 days using air samples were collected at AIST, Tsukuba, Japan. Samples were bio-analyzed using a next-generation sequencing method. In this study, we used two NGS platform. The sample was detected bacteria. The sample was divided into three subgroup and two subgroups total 6 samples according to the size of its bioaerosols, large subgroup contains bioaerosols whose diameter is bigger than 3.3μm, and small subgroup contains those smaller than 3.3μm

RESULTS

There are three groups of bacteria samples. Each collected in different time. Hits by days shows found that quantities of bacteria DNA appear equally in the bioaerosol samples. Results of taxonomy of bacteria, the 16s rDNA sequences were divided into 505 phylotype. (data not show) The bacteria composition was included in 41 class. (Figure.1) The main class were *Actinobacteria*, *Alphaproteobacteria*, *Bacili* and *Clostridia* that found in the all sample.181 Family were classified. (Figure.2) The high abundance of bacteria was *Clostridiace*, *Lactobacilliacae* and *Methylococaceae*. We can see that results of bacteria identification have no distinctions neither in different period nor between smaller and larger subgroups that means bacteria are not suitable for being regional-specific and seasonal-specific organisms.

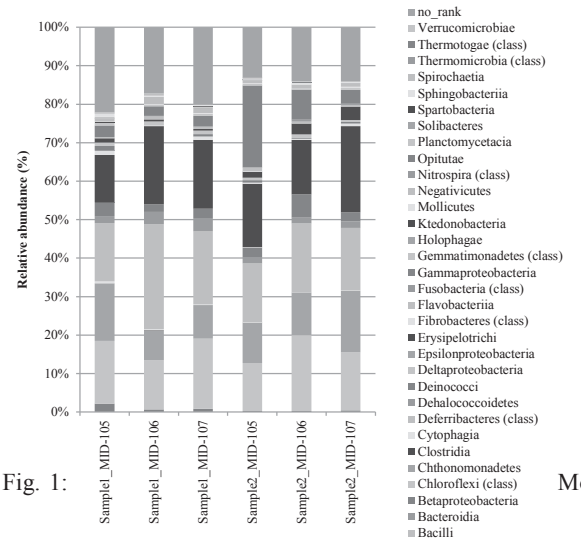
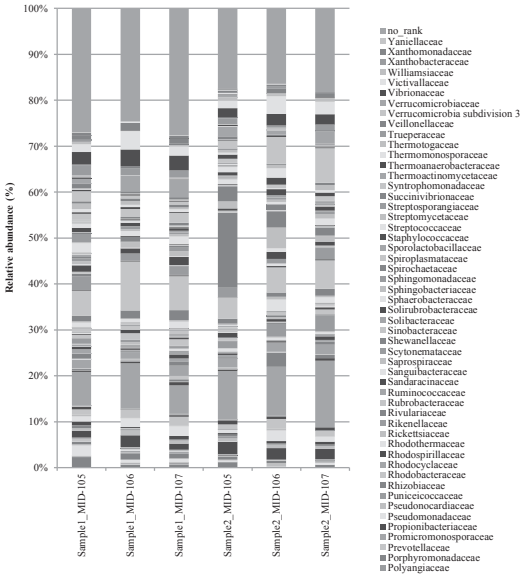


Fig. 1: Most abundant bacterial groups identified using Next Generation Sequencing at the class level.

Fig. 2: Most abundant bacterial groups identified using Next Generation Sequencing at the family level.



DISCUSSION

For bacteria, the hits of read by days was showed that quantities of bacteria DNA appear equally except group 2 in which smaller subgroup contains only half amounts of DNA comparing to larger subgroup (data not show) Although several factors may interfere the sampling and DNA extraction. (Núñez, et al., 2017) found that depletion in the airflow rate in sample during collection caused of different of DNA concentration in the replicates. In this study, we tend to believe that it is mis-operation during DNA extraction that caused deviation. In the dust samples collected at AIST, *Actinobacteria, Alphaproteobacteria, Bacili* and *Clostridia* that found in the all samples. (Figure1) Common in the aerosol *Bacilli* were identified. (Srivastava, Singh, & Jain, 2012) Some of *Actinobacteria* were detected in the dust event samples that collected at the high altitudes over the Noto Peninsula, Japan. (Maki, et al., 2017) In this study the information of bacteria in the aerosols and in the future, biological trace information will be study such as eukaryote, plant and animal in the different areas.

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The role of combination of color and scent for attracting pollinators in generalist flowers

Muhammad Arifin[1] and Tomoko Okamoto[2]
1. **Graduate School of Natural Science and Technology, Gifu University, Japan**
2. **Faculty of Applied Biological Sciences, Gifu University, Japan**

INTRODUCTION

Pollination, defined as a transfer of pollen from anther to stigma, is important event of plant life cycle. Plant uses flower as advertisement for attracting pollinating animals (Fishman and Hadany 2015). Generally, pollinators use visual signals, including flower size, color and shape, and olfactory signals, referred as floral scent, to navigate for visiting flowers, while plants can attract effective pollinators and avoid inappropriate flower visitors using visual and olfactory signals (Chen et al. 2010; Okamoto et al. 2015).

The pollination mode is classified into generalized and specialized pollinating according to the range of pollinating animals. Generalized pollination defined as use of several to many species of the available pollinators, in other hand, specialized pollination use of one or few species of available pollinators (Ashworth et al. 2004).

Floral color and scent have been focused by many pollination ecologist as important traits for pollination success (Fenster et al. 2004; Majestic et al. 2007). Some previous studies revealed that white colored flowers blooming in daytime can attract a wide variety of pollinators (McCall et al. 2013; Reverte et al. 2016). Several field studies revealed that color morphs exhibited significant differences in floral scent profiles (Majestic et al. 2007, Majestic et al. 2010). The phenotypic integration between scent and colour was directly linked to plant–pollinator interactions (Kantsa et al. 2017). It is thought that some scent compounds are biosynthesized in same pathway or regulated by same factors with pigments. However, relationships between the combination of floral color-scent and type of flower visitors in unclear.

Here we report the progress of field observation to reveal the pollinator assemblage and flower color of various flowering plants.

MATERIALS AND METHODS

Study plants and field sites

We focused on the flowering plants with generalized pollination system. Up to now, we observed 25 species of both native and invasive flowers. The observation was conducted on spring to autumn 2018 during the flowering season of each species. Field site of this study were at several locations in Gifu Prefecture, Japan.

Collection and analysis of flower visitor

Insect visitors were observed and collected throughout the period of flowering season of each species. Because weather conditions influence the foraging activities of insect, we observed and collecting flower visitors only in the daytime on sunny day (0900 h to 1500 h). The observation periods of each flower vary from 30 minutes to 120 minutes because some flowers showed very low frequency of insect visitation. There is no nocturnal observation in this study.

At the field site, flower visitors were collected using hand net after confirming insects landed on the flowers. Identification of the specimens was carried out in laboratory. In order to distinguish the pollinator and flower visitor, we confirmed the presence of pollen grains on the insect body. Quantification of pollen loads placement on insect body was also recorded.

Floral color measurement

In this study, the terms that we used to describe colors were based on human visual spectrum (~380 nm to 780 nm). DIC color guide tools were used to measure the petal color of each species.

Floral scent collection and analysis (plan for next season)

We will collect floral scent of each species by head-space sampling in situ. Floral scent collection will be performed in sunny day during the peak of insect visitation (1000 h and 1400 h).

Collected samples will be analyzed using gas chromatography mass spectrometry (GC-MS) to reveal the floral scent profiles.

RESULTS

A wide array of insect visitor in 25 plant species were recorded. The visitors vary in total number and types between species (Table 1). Hymenopteran insect, wasps and bees, were the most common insect in generalist flowers. Moreover, the numerous flower visitors tend to prefer white colored flower both in native and invasive flowers. In contrast, invasive flower species with purple color such as *Baptisia australis* and *Verbena rigida* were visited by very few of flower visitors. Because we are continuing field observation, the statistical analysis will be conducted after finishing to collect data.

DISCUSSION

The data of flower visitors, revealed that there was a clear difference in total numbers and type of insect visitors on each flower color. The white colored flower tend to attract wider types of insect visitors with high number than another color. This result supports the recent studies (McCall et al. 2013) according to feeding activities of insects, indicating numerous insects prefer white floral color over others.

Nevertheless, there was no rule such certain color attract specific type of pollinators. Influence of other floral traits, or the possibility of trait combinations, seem to have great effect for attracting the flower visitor. Our temporary result, only provided from color trait, thus, further studies in floral scent should be conducted.

Our data also suggest a unique interaction case in invasive flower species. Invasive flower such as *Baptisia australis* and *Verbena rigida* visited by only few insect. In contrast, another invasive species such as *Mentha suaveolens* attracted a huge number of insect visitors including native insects. This might impact directly and indirectly to the change of community-level interactions in the ecosystem. To survive and become successful invader, the invasive flower might construct novel interaction with resident native iwhich already present in their new habitat. The pollination in invasive flowers is carried out by the native pollinator belonging to alike functional group as

the pollinating plants in their native range (Stout and Tiedeken 2016). However, the mechanism of attractiveness for invasive species to native pollinators deserve further special study.

ACKNOWLEDGMENTS

We are grateful to Nobumitsu Kawakubo for introducing the study sites and providing DIC color guide tools.

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Table 1. Insect observed visiting flowers at the study sites

Flower species	Origin	Flower Colour	Observation period (Min)	No. of insects visitor	Types of insect visitors
Rhaphiolepis indica var. umbellata	Native	White	60	30	Wasp, bee, beetle, fly
Ligustrum obtusifolium	Native	White	60	31	Wasp, bee, beetle, hoverfly, butterfly, bug
Photinia glabra	Native	White	60	17	Beetle, hoverfly
Baptisia australis	Invasive	Deep purple blue	75	4	Wasp, bee
Deutzia crenata f. plena	Native	Red purple and white	60	34	Wasp, beetle, butterfly, ant, thrips
Lespedeza spp.	Invasive	Vivid red purple	60	13	Wasp, bee, ant
Castanea crenata	Native	Light greenish yellow	65	25	Wasp, bee, beetle, fly, thrips
Ampelopsis glandulosa var. heterophylla	Native	Dull yellow green	70	46	Wasp, bee, beetle, fly, ant, thrips
Ammi majus	Invasive	White	60	11	Wasp, bee, beetle, fly, hoverfly
Oenothera speciosa	Invasive	Pale red purple	60	12	Wasp, ant, thrips, homoptera
Astragalus sinicus	Invasive	Vivid reddish purple	90	24	Wasp, bee, beetle, fly, hoverfly, butterfly, ant, thrips
Verbena rigida	Invasive	Deep purple	120	3	Hoverfly, butterfly
Leucanthemum vulgare	Invasive	White	60	18	Wasp, beetle, ant, thrips, bug
Erigeron philadelphicus	Invasive	Reddish white	90	40	Wasp, bee, beetle, fly, hoverfly, butterfly, bug
Circum japonicum	Native	Dull reddish purple	120	19	Bee, fly, butterfly
Oxalis articulata	Invasive	Vivid red purple	60	18	Wasp, bee, beetle, ant
Euonymus japonicus	Native	Very pale yellow green	60	75	Wasp, beetle, fly, hoverfly, butterfly, ant
Stenactis annuus	Invasive	Light grayish purple	30	40	Wasp, fly, hoverfly, butterfly, bug
Mentha suaveolens	Invasive	White	60	96	Wasp, beetle, butterfly, thrips, bug, mosquito
Salvia lutescens	Native	Dull purple blue	90	47	Wasp, bee, fly, hoverfly, butterfly, moth, bug
Taraxacum officinale	Invasive	Vivid greenish yellow	60	75	Wasp, bee, fly, ant, thrips
Cayratia japonica	Native	Yellow green	90	36	Wasp, bee, beetle, ant, thrips
Phytolacca americana	Invasive	Pale yellow green	70	20	Wasp, beetle, ant, thrips
Liriope platyphylla	Native	Pale bluish purple	60	69	Wasp, bug, ant, thrips
Enkianthus perulatus	Native	White	60	21	Wasp, bee, beetle, fly

Isolation and characterization oF *Lactobacillus spp.* from virgin coconut oil, West Sumatera

Yolani Syaputri[1,3], Sumaryati Syukur[2], Endang Purwati[3], Hitoshi Iwahashi[1]
1. The United Graduate School of Agricultural Science, Gifu University, Yanagido 1-1, Gifu City, 501-1193, Japan
2. Laboratory of Biotechnology, Departement of Chemistry, Faculty of Math and Natural Sciences, University of Andalas, Padang 25163, Indonesia
3. Laboratory of Biotechnology, Departement of Animal Nutrition, Faculty of Animal Husbandary, University of Andalas, Padang 25163, Indonesia

INTRODUCTION

Virgin Coconut oil is an essential oil in West Sumatra and reported to against bacteria, fungi, viruses, and protozoa (Kamdem, Guerzoni, Baranyi, & Pin, 2008). Virgin Coconut Oil is extracted from the kernel of matured coconut and has been widely utilized in food, medicine, and industry (H Ibrahim, et al., 2017). The common process to make Virgin Coconut Oil is a fermentation process without heating and adding harmful chemicals compound. This process makes Virgin Coconut Oil has a good quality (Suryani, et al., 2014). *Lactobacillus plantarum*, *Lactobacullus thermobacter*, *Corineaebacterium bovis*, *Corineaebacterium xerocis* and *Micrococcus luteus* were reported in coconut milk (Suryani, et al., 2014). One of the functions of Lactic Acid Bacteria is bio-preservative. in this study, was conducted to determine the species of Lactic Acid Bacteria in Virgin Coconut Oil as bio-preservative.

MATERIALS AND METHODS

Sample Collection—An advanced commercial Virgin Coconut Oil sample which made in west Sumatra was collected randomly in Padang city.

Isolation and Characterization of Lactic Acid Bacteria from Virgin Coconut Oil (VCO)—Serial dilution technique until 10^{-8} was used for isolation Lactic Acid Bacteria in MRS broth (Becton, Dickinson and Company - USA), plate on MRS agar at 37˚C for 24 hours in anaerobic condition. Lactic Acid Bacteria (LAB) was grown on MRS broth and MRS agar and incubated at 37°C for 24 hours (Syukur, Fachrur, Jamsari, & Endang, 2014). One colony was selected randomly based on the shape, size, and color of the colony. Isolate was stored at -80°in MRS Broth containing 20% glycerol (v/v).

Molecular Identification of Isolates

DNA Isolation—Bacteria were grown in MRS Broth at 37˚C for 18 hours. The cells were harvested, and to extract bacterial genome were used Extrap Soil DNA Kit Plus Ver.2. (Nintetsu Sumikin Kankyo Kabushiki Gaisya, Japan) according to the manufacturer's protocol. Electrophoresis was performed on 1% agarose gel in TAE 1X buffer and photographed under UV light.

16S rRNA Sequencing—The 16S rRNA gene fragment of ~1.5 kb was amplified by using a pair of universal primers 27 F: (5'- GAGTTTGATCCTGGCTAG-3') and 1525 R: (5'-AGAAAGGAGGTGATCCAGCC-3'). Polymerase chain reactions (PCR) were carried out in a Fast reaction Tube (Applied Biosystems, USA) in a total volume of 25 µl containing 12.5 µl 2 × Green Master Mix PCR (Promega, USA), 1.25 µL of each primers 27F and 1492R (concentration 0.05 pmol/µL), 9 µL nuclease free deionized water and 1 µL template DNA, running under the following temperature program: initial denaturation of DNA for 5 min at 95 °C, 25 cycles of 1 min at 94 °C, 1 min at 56°C, and 1.5 min at 72 °C; and final extension for 7 min at 72 °C. 5 µl aliquots of the PCR products were analyzed by electrophoresis using 1% (w/v) agarose gel in Tris Acetic acid EDTA (TAE 1X) buffer at 100 V for 30 min. The gel was then placed in Electronic U.V. Transilluminator to detect the presence a band of 1500 bp. The size of the DNA fragments was estimated using a FastGene 100 bp DNA Ladder (Nippon Genetics, Germany). Fast Gene™, Gel/PCR Extraction kit (Nippon Genetics, Germany) was used for purification before sending the extracted DNA for sequencing, according to the manufacturer's instructions. An average of 500 bp nucleotides for each sequence from each side was read and compared with the databases using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolates with 98% or higher similarity in sequences were identified as the same species.

Phylogenetic Tree Construction—Whole-genome sequencing obtained were then assembled into contiguous sequences (contigs) using DNAStar, about over a hundred genomic contigs were edited use BioEdit and aligned using the Bioedit-ClustalW Multiple Alignment. Phylogenetics tree was made using Mega7 Construct/ Test Neighbor-Join Tree taking 1000 as bootstrap value.

RESULTS AND DISCUSSION

Total Colonies of Lactic Acid Bacteria (BAL)—MRS Agar and MRS Broth are the media that used in this method. The total Colonies of Lactic Acid Bacteria in this sample is 9.4 x 10^8 CFU/mL. The macroscopic colony of BAL showed white small rounded colonies, shiny, slippery and convex edges.

Isolation Genomic DNA and 16S Ribosomal (rRNA) gene amplification with PCR—One of the general methods used to identify Lactic Acid Bacteria is the analysis of 16S rRNA sequence. The amplification's result showed the PCR product was 1500 bp (Figure 2). PCR used 27F and 1525R universal primer of 16s rRNA locus.

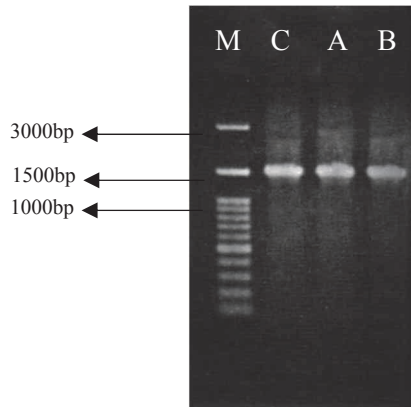

Fig. 1: Electrophoresis PCR Product using 27F and 1525R universal primers of 16S rRNA, M: Marker, C: Positive Control, A and B: Isolates from Virgin Coconut Oil

Phylogenetic Tree Construction—The sequencer was used DNA Sequence used Multi – capillary DNA Sequencer "ABI Prism 3100 / 3130 Genetic Analyzer" (Gifu University, Japan). The sequence from both directions, reverse and forward is contiq by BioEdit and aligned using the Bioedit-ClustalW Multiple Alignment. The sequence was analyzed with data GeneBank NCBI BLAST program. Genomic analysis 16S rRNA gene sequencing confirmed the isolate showed that 99% is similar to *Lactobacillus sakei* and *Lactobacillus plantarum.*

The phylogenetic tree is formed in order to determine the clothes of species based on their similarities and genetic differences with MEGA7 Construct/ Test Neighbor-Join Tree taking 1000 as bootstrap value.

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The effect of Japanese persimmon (*Diospyros kaki*) extract on the prevention of sarcopenia

Nayla Majeda Alfarafisa[1], Kohiji Kitaguchi[2] and Tomio Yabe[2,3]
1. **The United Graduate School of Agricultural Science, Gifu University**
2. **Faculty of Applied Biological Sciences, Gifu University**
3. **G-CHAIN, Gifu University**

INTRODUCTION

Sarcopenia is an age-related syndrome which characterized by progressive loss of mass, strength, and function of skeletal muscle (Budui et al., 2015). People with sarcopenia syndrome usually experienced a progressive and irreversible loss of body weight, followed by muscle wasting or muscular atrophy, decreased muscle stregth, and in severe cases, patient can experienced permanent paralysis and even death. Sarcopenia is a multifactorial syndrome. Several studies suggested that this syndrome can be caused by altered endocrin function, genetic influence, neurodegeneration, disuse, nutrition, oxidative stress, and chronic inflammation (Kim & Choi, 2013). As of now, the exact underlying mechanisms and pathophysiology of sarcopenia remain unclear. At molecular level, the loss of skeletal muscle fibers are resulted from the disturbance of muscle protein turnover, in which muscle protein breakdown rates exceeds muscle protein synthesis (Sandri, 2013). The activation of the PI3K/Akt pathway in skeletal muscle cells is still reliable as a main regulator in muscle protein synthesis through the phosphorylation of mTOR which causes the activation of p70S6 kinase and 4E-BPI, promoting protein translation (Chopard et. al., 2009). On the other hand, ubiquitin-proteasome pathway appears as a main catalyst for the majority of protein breakdown in mammalian cells, including muscle cells (Lecker et. al., 1999). This pathway involves the sequential action of three ubiquitin enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-protein ligase enzyme (Chopard et al., 2009).

Nowadays, the use of natural bioactive compound from plant extract as therapeutic agent had gained much attention. Various botanical compound proved to be effective in maintaining muscle health. Kim et al. (2012) stated that the addition of flavonoid from Korean *C. aurantium* L. can prevent muscular atrophy caused by LPS in L6 skeletal muscle cells through the regulation of inflammatory activity. Flavonoid from Korean *Citrus aurantium* L. has a role as anti-inflammatory agent that can regulate the expression of inflammatory mediators and affecting NF-κB signaling pathway. On the other hand, Wang et al. (2014) also found out that resveratrol, biochemical compound in grape, can reverse C2C12 muscle cell atrophy caused by TNF-α by preventing muscle protein degradation and promote muscle protein sythesis through IGF1-Akt-mTOR signaling pathway. These two papers indicate the potential use of the botanicals compound for the prevention and treatment of muscle function.

Japanese persimmon (kaki fruit persimmon) (*Diospyros kaki*) is one of a well known and highly distributed fruits around the world, especially among east asian countries including Japan. This fruit enriched with many bioactive compounds that have beneficial effect on human health owing to their ability to prevent various ailments (Yaqub et al., 2016). Bioactive compounds which prevalence in persimmon fruit such as phenolics, flavonoids, tannins, proanthocyanidins, terpenoids, carotenoids, and vitamins. It is suggested that bioactive compound in Japanese persimmon also had an effect to pathological condition of sarcopenia.

This study aims to investigate the effect of water soluble compounds from Japanese persimmon extract on skeletal muscle atrophy prevention through muscle protein turnover activity (downstream part), specifically in related to chronic inflammation (upstream part) as the main cause. Muscle protein turnover will be analyzed by evaluating the downstream of PI3K/Akt signal molecules (S6K1, mTOR, and 4E-BP1) and the activity of muscle-specific E3 ubiquitin-ligase enzymes (MAFbx and MuRF-1). On the other hand, the effect of Japanese persimmon-derived water-soluble extract on the regulation of the inflammatory pathway will be analyzed by examining the expression of pro-inflammatory cytokine (iNOS, COX-2, TNF-α, and IL-6) as well as inflammatory signal molecules activity (I-κB, NF-κB and MAPKs). Currently in this research progress, the extraction and fractionation steps have been done in order to isolate water-soluble bioactive compound from Japanese persimmon which may has an effect on muscle atrophy pathophysiological condition.

MATERIALS AND METHODS

Materials

Diospyros kaki Nishimura cultivar was provided by Gifu Prefectural Agricultural Technology Center (Gifu, Japan) and used as a sample. (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid and Trolox (Sigma-Aldrich, St. Lois, USA) were used as a standard for measuring antioxidant activity, and 1,1-Diphenyl-2-picrylhydrazyl (DPPH: Wako Pure Chemical Industry, Osaka, Japan) was used as a stable free radical which turns yellow when scavenged.

Japanese persimmon extraction

After washed and properly handled, the raw material of Japanese persimmons was freezed at –30ºC until it was ready to process. The samples were completely dryed by lyophylization methods. After exclude the calyx and seeds, the remaining parts of the samples were weighted and recorded as dry weight. The samples then crushed using mortar in order to improves the efficiency of extraction by increasing their surface area. Powdered samples (42.5 g) were extracted in 420 mL of 98% aqueous ethanol for 30 min in boiled condition. After three cycles of extractions, the extract was mixed combined and filtered using a Buchner funnel. The solvents were vaporized using rotary evaporator and dried up using lyophylization methods. All crude extract was kept on –30ºC until further analysis.

Japanese persimmon fractionation

Japanese persimmon crude extract was fractionated using different concentrations of ethanol (25, 50, and 75%). The extracts (5.00 g) were dissolved in each solvent (50 mL) and homogenized using magnetic stirrer for 1 h at room temperature. When the crude extract completely dissolved in ethanol solvent, the samples were fractionated again using butanol (1:1) solvent until two different phases were obtained. Solvent were vaporized using rotary evaporator and dried up using lyophylization methods. All samples were kept on –30ºC until further analysis.

DPPH radical scavenging activity

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH. Gradient concentration (3.00-11.00 mg/mL) of Japanese persimmon fraction solutions in water was prepared. On the other hand, trolox solution was prepared by dissolving trolox in ethanol. Gradient concentration of trolox were used as a standard curve. 30µL of Japanese persimmon fraction and trolox solutions were added to 96-well plate, followed by 240µL of methanol. Water was used as a blank. 1mM solution of DPPH in ethanol was prepared and added to each well of the samples. After 30 min of incubation in dark condition, the absorbance was measured at 540nm. The assay was performed in triplicate. Percentage of inhibition of free radical DPPH was calculated by using equation bellows:

$$\text{DPPH Scavenging Activity (\%)} = \frac{(\text{Ab} - \text{As})}{\text{Ab}} \text{ x } 100$$

Ab = Absorbance of the blank
As = Absorbance of the sample

RESULTS AND DISCUSSION

Japanese persimmon extraction

The main focus on this research is Japanese persimmon-derived water-soluble bioactive compounds. In compared with lipid soluble bioactive compounds, water soluble bioactive compounds had several advantages. Recent study suggested that hydrophilic compounds had more simple mechanism of absorption in digestive tract (Rein et al., 2013). In addition, researches that focused on this bioactive compound are still rarely to be found. Table 1 showed an extraction yield of hot ethanol extraction from Japanese persimmon. Theoretically, the quantity of extractive yield was directly affected by the type of extraction methods/solvents and their extraction efficiency (Sharma & Cannoo, 2016). In this research, we conducted three cycles for each extraction step. Relatively high number of yield percentage from extraction step indicated the method of extraction which was chosen can be said sufficiently effective to isolate water soluble bioactive compound from Japanese persimmon. Several studies using hazelnut also suggested that hot ethanol extraction (80^{o} C, 30 min) was the most prominent method for phenolic extraction because it showed the highest extraction yield and total phenolic content (Shahidi et al., 2007).

Table 1. Japanese persimmon extraction yield

Dry Sample (g)	Crude Extract (g)	Yield (%)
42.5	27.8	65.3

Japanese persimmon fractionation

After underwent an extraction step, Japanese persimmon crude extract was fractionated using different concentration of ethanol. There are 25, 50, and 75% (v/v) of ethanol solutons which were used as a solvent for fractionation. Table 2 showed total yield from Japanese perimmon fractionation using 25% of ethanol. After dissolved in 25% of ethanol, the crude extract of Japanese persimmon were completely dissolved in the solvent, indicating that 25% of ethanol was not an effective solvent for separating bioactive compound. Two different phases were obtained after the solution was extracted again using 1-butanol. In total, from 5.00 g of sample, we have obtained 57.0% of butanol phase, and 35.0% of 25% ethanol phase.

Table 2. Total yield from 25% ethanol fractionation and butanol separation

Crude Extract	25% Ethanol Phase	Butanol Phase
5.00 g	1.75 g	2.85 g

Fractionation using 50% of ethanol also dissolved Japanese persimmon crude extract completely. Therefore, the sample needs to be extracted again using 1-butanol to separate bioactive compound based on polarity. In summary, 50% ethanol fractionation and butanol separation method can obtained 18.4% of butanol phase and 70.6% of 50% ethanol phase (Table 3).

Table 3. Total yield from 50% ethanol fractionation and butanol separation

Crude Extract	50% Ethanol Phase	Butanol Phase
5.00 g	3.53 g	0.92 g

On the other hand, slightly different results are obtained from fractionation using 75% ethanol. The solvent could separate Japanese persimmon crude extract into two different phase, there are 12.8% of filtrate phase and 86.0% of 75% ethanol phase (Table 4). In total, fractionation of Japanese persimmon crude extract using graded concentration of ethanol could produce six different fractions.

Table 4. Total yield from 75% ethanol fractionation

Crude Extract	75% Ethanol Phase	Filtrate Phase
5.00 g	4.30 g	0.64 g

According to Visht and Chaturvedi (2012), a number of analytical procedures must be carried out to evaluate the extraction and fractionation methods of plant bioactive compounds. Three types of investigation after extraction and fractionation step can be cathegorized as:

1. Qualitative chemical analysis: including the determination of bioactive compound chemical properties and their nature constituents
2. Quantitative chemical analysis: including the determination of the purity and concentration of an isolated substance
3. Bioassay: including the determination of the biological or pharmacological activity of substances in dosage dependent manners.

Antioxidant activity

Until now, we still doing antioxidant activity analysis using DPPH method to evaluate the biological activity on each fraction. For the future plan, we propose to carry out all the investigation methods mentioned above to evaluate all fraction that have been obtained. As from the investigation results, we can conclude about which fraction have the highest potential on biological activity and which fraction have the potential effect to overcome the pathophysiological condition of muscular atrophy.

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Novel synthesis of haloalkyl nucleobase substituted at 3'-overhang of modified small interfering RNAs to enhance RNAi activity and exonuclease resistance

Akash Chandela[1], Taeko Watanabe[2], Kenji Yamagishi[3] and Yoshihito Ueno[1,3]

1. **The United Graduate School of Agricultural Science, Gifu University, Japan**
2. **Department of Chemical Biology and Applied Chemistry, College of Engineering, Nihon University, Japan**
3. **Course of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, Japan**

INTRODUCTION

RNA interference (RNAi) is a conserved self-defense mechanism found in most eukaryotes where small double-stranded RNA (dsRNA) triggers a series of biochemical events and results in sequence-specific suppression of gene expression (Elbashir et al., 2001; Fire et al., 1998). These small interfering RNAs (siRNAs) are short (21-23 nucleotides in length), double-stranded regulatory RNAs that contain two signature characteristics: a two nucleotide 3'-end overhang and a phosphorylated 5'-end (Sharp, 2001). The functional strand, referred as guide strand, interacts directly with the multi domain AGO proteins to form RNA induced silencing complex (RISC). The RISC recognizes messenger RNAs that contain sequences complementary to the guide strand and cleaves the mRNA. This eventually leads to inhibition of protein expression of the target mRNA.

AGO protein, a key molecule, has four domains: the N-terminal (N), Piwi/Argonaute/Zwille (PAZ), middle (MID), and P-element-induced wimpy testis (PIWI) domains (Elkayam et al., 2012; Schirle and MacRae, 2012). The PAZ domain recognizes the 3'-end of guide strand whereas 5'-end is recognized by MID domain. The PIWI domain possesses endonuclease activity which helps in cleaving the mRNA. The PAZ domain has also been reported to harbor the nucleotides of the 3'-dangling end in its hydrophobic pocket (Lingel et al., 2004; Ma et al., 2004), which forms basis of our research.

Preceding reports have stated that enhanced binding with PAZ domain leads to low RNAi activity, whereas H-bonding seems to do the opposite (Kandeel and Kitade, 2013; Somoza et al., 2010; Valenzuela et al., 2016; Xu et al., 2015). Our group has also reported several 3'-end modifications exhibiting resistance to nuclease as well as mitigating the RNAi activity (Inada et al., 2015; Ueno et al., 2009). In order to evaluate the effect of electrostatic interaction as well as H-binding associated binding with PAZ domain to change silencing activity, we selected haloalkyl nucleobase modification at the 3'-overhang (Fig. 1).

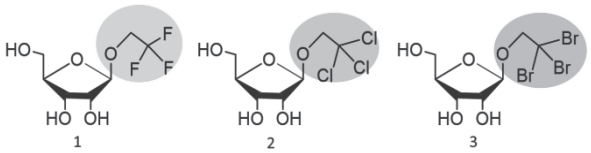


Fig.1. Structure of nucleoside analogs.

Here, in this study, we report the novel and facile synthesis of haloalkyl nucleobases whose incorporation at 3'-overhang improve binding to the PAZ domain and lead to enhanced silencing activity. Furthermore, these modifications also increased resistance against exonuclease. The augmented activity provided substantial evidence for correlating PAZ domain binding to the silencing activity with the association of hydrophobic moieties.

METHODS

Thermal denaturation study

The thermally induced transition of each mixture was monitored at 260 nm on UV-Vis spectrophotometer fitted with a temperature controller in quartz cuvettes with a path length of 1.0 cm and a 3.0 μM duplex concentration. The sample temperature was increased by 0.5˚C/min.

Dual-luciferase reporter assay

Twenty-four hours before transfection, HeLa cells expressing luciferase genes (8 x 10^4/mL) were transferred to a 96-well plate (100 μL/well). Cells in each well were transfected with the indicated amounts of siRNA, and Lipofectamine RNAimax (1.5 μL) in Opti-MEM I Reduced-Serum Medium (Invitrogen), and incubated at 37˚C. Transfection without siRNA was used as a control. After 1 h, DMEM (50 μL) containing 10% BS was added to each well, and the whole was incubated at 37˚C. After 24 h, solution in each well was removed and the plate was incubated at -80˚C. After more than 4 h, activity of firefly and Renilla luciferases in cell were determined with a dual-luciferase assay system (Promega) according to a manufacturer's protocol. The results were confirmed by at least three independent transfection experiments.

Partial digestion of ONs by SVPD

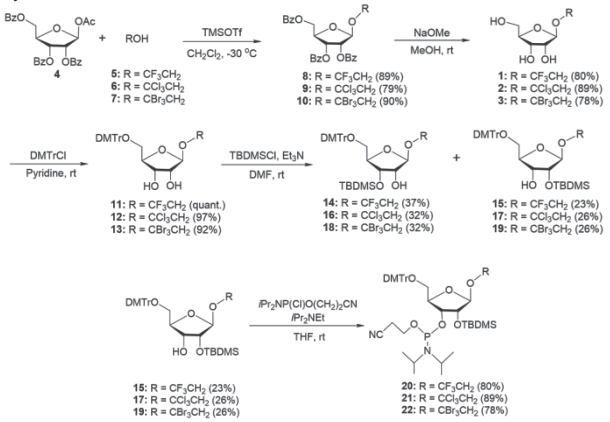
Each ON (600 pmol) labeled with fluorescein at the 5'-end was incubated with SVPD (0.075 unit) in a buffer (150 μL) comprising 0.1 M Tris-HCl (pH 8.0) and 20 mM $MgCl_2$ at 37˚C. After 0, 1, 5, 10, 30, 60, 120, or 240 min, an aliquot (5 μL) of the reaction mixture was mixed with the loading buffer (15 μL), comprising Tris-borate-EDTA (TBE) buffer and formamide. Each sample was analyzed by 20% denaturing PAGE at room temperature for 2 h at 20 mA. The gel was visualized by use of a Luminescent Image analyzer LAS-4000 (Fujifilm).

Molecular modeling for binding affinity

PAZ domain-siRNA complex from the Protein Data Bank (PDB); the entry 1SI3 was used for preparing the structure. The PAZ domain-siRNA complex prepared for calculation consists of PAZ domain (Mse224-Gln295, Val302-Arg349) and a 9-mer RNA (5'-CGUGACUCU-3'). Three-dimensional data for 3' end modified siRNA were constructed by replacing native siRNA. The ab initio Fragment Molecular Orbital (FMO) calculations were adopted to evaluate the specific interactions and binding affinity between PAZ domain and siRNA. All FMO calculations were performed with the PAICS program, where we employed the second-order Møller-Plesset (MP2) perturbation methods with the basis set of 6-31G**.

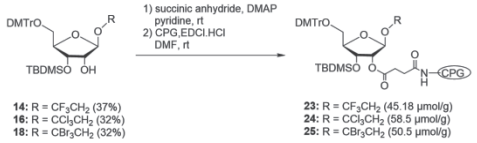
RESULTS

Synthesis



Scheme 1. Synthesis of phosphoramidites for haloalkyl nucleobases.

The synthetic route used to synthesize phosphoramidites **20-22** of haloalkyl analogs is shown in Scheme 1. This simple approach was followed to eventually obtain phosphoramidites and solid supports (Scheme 2) of haloalkyl nucleobases.



Scheme 2. CPG-solid support synthesis

Oligonucleotide Synthesis and Thermal stability of siRNAs

Oligonucleotides (ONs) were synthesized by standard phospharamidite method. siRNAs **1-4** were prepared with the synthesized ONs to incorporate modification. All modified siRNAs exhibited slightly diminished thermal stability. Melting temperature (T_m) and change in melting temperature with respect to native (ΔT_m) has been indicated in Table 1.

Table 1. Sequences of siRNAs, Tm values and gene silencing

siRNA	Sequence	T_m (°C)	ΔT_m (°C)	Upper: 10 nM Lower: 1 nM
siRNA 1	Sense strand 5'-GGCCUUUCACUACUCCUAC**UU**-3' 3'-**UU**CCGGAAAGUGAUGAGGAUG-5' Antisense strand	78.5±0.2	--	17.1±2.0 23.5±2.7
siRNA 2	5'-GGCCUUUCACUACUCCUAC**11**-3' 3'-**11**CCGGAAAGUGAUGAGGAUG-5'	77.1±0.1	-1.4	14.1±1.7 29.8±1.6
siRNA 3	5'-GGCCUUUCACUACUCCUAC**22**-3' 3'-**22**CCGGAAAGUGAUGAGGAUG-5'	78.0±0.3	-0.8	10±1.7 32.6±1.8
siRNA 4	5'-GGCCUUUCACUACUCCUAC**33**-3' 3'-**33**CCGGAAAGUGAUGAGGAUG-5'	76.4±0.2	-2.3	14.4±4.6 30.5±2.4

Silencing activities of siRNAs and binding affinity

Trichloroethyl modified siRNA yielded best silencing activity and also, best binding to PAZ domain. Also, other analogues showed activity corresponding to their binding interaction (Table 1).

Nuclease resistance

3'-exonuclease resistance was found to be best sustained with tribromoethyl analogue modified ON. Other analogues also showed resistance better than the native. The half-life increased to around 9 folds.

DISCUSSION

This study was directed to exploit the interaction of 3'-overhang of siRNA with the PAZ domain to mitigate the RNAi activity. And, with the incorporation of hydrophobic nucleobases, the binding as well as activity was found to increase in a similar trend. Also, the binding studies showed improved interaction with basic residues in the PAZ pocket. All these observations strongly support our hypothesis for augmented silencing activity with enhanced electrostatic interaction between the overhang and the PAZ domain. In addition, these modifications also proved beneficial in improved resistance against 3'-exonuclease, with molecular size and flexibility of the nucleobase being supposed for such a behavior. Hence, we could successfully report the design and synthesis of these novel siRNAs with enhanced resistance and silencing activity. Further studies with these moieties could add more dynamics to the ongoing research in the field.

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LtR, a candidate of transcriptional regulator of major virulence factor of *Dickeya dadantii* 3937

Dina Istiqomah[1] , Naoto Ogawa[2] , Shinji Tokuyama[2] and Masafumi Shimizu[3]

1. **Science of Biological Resources, The United Graduate School of Agricultural Sciences-Gifu University, Japan**
2. **The United Graduate School of Agricultural Sciences-Gifu University; Faculty of Agriculture-Shizuoka University, Japan**
3. **The United Graduate School of Agricultural Sciences-Gifu University; Faculty of Agriculture-Gifu University, Japan**

INTRODUCTION

The pathogenecity of *D. dadantii* is determined by their secreted molecules to colonize plant tissue. The soft rot symptom is mainly caused by the production of pectate lyases (Pels) a plant cell wall degradative enzyme (Reverchon et al. 2013). Hence, *pel* genes encoding pectinases are the major virulence genes in *D. dadantii* (Collimer et al. 1986; Duprey et al. 2016).

In the bacterial adaptation, there is a complex transcriptional control system coordinating the expression of virulence genes ensures efficient expression. Initially, the signals feed into the transcriptional regulatory system of the bacterial cells, then continue to lead the physiological and morphological alterations which facilitate bacterial adaptation and affective survival. There are some regulators potentially involved in bacterial survival under particular stress conditions.

The expression of genes is controlled by complex regulatory systems. Several regulators are reported to regulate *pel* genes in response to various signals (KdgR, Pir, PecS, PecT, Fur, MfbR, VfmE). In addition, it was shown that the sugar catabolism regulator complex cAMP-CRP acts via a direct mechanism as the main activator of the *pel* genes (Nasser 1997; Reverchon 1997). Previous study revealed that there was a gene which involved in the expression of virulent factors and pathogenicity of *D. dadantii* in negative manner, namely *ltR* (LacI transcriptional repressor). Analyzing the structure and physical function of the LtR protein by its interaction with the virulence gene is important to understanding the mechanism of transcriptional regulation in biological components.

MATERIALS AND METHODS

***D. dadantii* strain and culture**

D.dadantii 3937 No. 253 (wild type) was grown in LB Agar and LB liquid medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast, 10 g/L NaCl (1.5% agar in addition for LB Agar)) ; M63 medium pH 7.0 (120 g/L (NH4)2.SO4, 136 g/L KH2PO4, 5 mg/L FeSO4.7H2O (0.2% glycerol and 1% potato extract in addition)) ; M63 medium pH 7.0 + 0.2% glycerol + 0.2% Polygalacturonic Acid (PGA) + 1% potato extract, incubation at 28ºC for ± 24 hours.

RNA Extraction

The liquid culture of *D. dadantii* 3937 which have 0.3-0.5 absorbance in OD600 were extracted by Qiagen RNAEasy Mini Kit. The RNA concentration measured in A260, A280 and A320. RNA Electrophoresis using 1.2% agarose (2 ml 10x FA gel buffer+ 17.64 ml RNAse free dH_2O + 360 µl 37% formaldehyde), 1 µl 5x RNA loading buffer + 4 µl RNA were load in 100 volt, 30 minutes.

Reverse Transcription

The primers which amplify the candidate genes which could regulate the virulence genes (*ltR*) and the regulated virulence genes (*pelA*, *pelE* and *pelD*) and also other genes or reference genes i.e *lpxC, rpoD* and *gyrB* which are conserved and stable to normalizing RT-PCR were designed (Hommais, et al., 2011; Radonie, et al., 2003).

Table 1. Primers for Reverse Transcription

Gene	Primers	Sequence	Function
ltR	ltRfor2	ATCGACGACATCCACCTGCT	Transcriptional regulator
	ltRrev2	GCGGCCAGAAAATCAGTGAC	
pelA	pelAfor2	AATGGGACGGCATGAACATC	Involved in maceration and soft-rotting of plant tissue
	pelArev2	GATATCCAGTGCGCCATCGT	
pelD	pelDfor1	CGACGTGAAAAACAGCGTCT	Pectate lyase activity
	pelDrev1	GCCATCAATGCTCTTCAGGT	
pelE	pelEfor2	CAAAGAATGCAGCGTGGTCA	Maceration and soft-rotting of plant tissues
	pelErev2	GTATGCGCTGAAGCCAAAGC	
lpxC	lpxCfor1	GCTGGATTTCTCCGCTGATG	UDP-3-O-acyl N-acetylglucosamine deacetylase
	lpxCrev1	CATCCACCACGATCGCACTA	
rpoD	rpoDfor1	ACCGACCATACGCTGGAAGA	sigma factor
	rpoDrev1	GCTCGGGTGACGCAGTTTAC	
gyrB	gyrBfor1	GGCCTTATCGAAGACGATGC	DNA gyrase
	gyrBrev1	CTCGCCCAGACCTTTGTAGC	

Reverse transcription using 2.0 µl 5x PrimeScript Buffer, 0.5 µl PrimeScript RT Enzyme Mix I, 1 pmol, 5 pmol and 10 pmol gene specific primers, 200 ng and 500 ng of total RNA and RNAse free H_2O until 10.0 µl total volume (TaKaRa). (-)RNA and (-) RT enzyme are the control. The reaction was 42ºC ; 15 minutes (*Reverse Transcription Process*), 85ºC ; 5 second (*RT Enzyme inactivation*), 4ºC ~.

cDNA PCR

Reaction mixture 5.0 µl of 10x Buffer ExTaq, 4.0 µl of dNTP mix, 1.0 µl of 10 pmol forward primer, 1.0 µl of 10 pmol reverse primer, 2.0 µl of reverse transcription reaction, 0.25 µl of Ex Taq Enzyme and 36.75 µl of dH2O (50.0 µl total volume) (TaKaRa). PCR reaction with hot start PCR, 98ºC ; 10 second for denaturation, 60ºC ; 10 second for annealing, 72ºC ; 15 second for extention (40 cycles) (MyCycler; Biorad). Agarose gel electrophoresis used 1.5% agarose gel (1x TAE buffer). 5.0 µl PCR product + 5.0 µl dH2O + 2 µl 6x DNA loading buffer were loaded in 100 volt for 30 minutes.

Real-Time PCR

Gene expression analysis was carried out using SYBR Green I and run in Roche Lightcycler 480 (Bio-Rad).

Gel Shift Assay

To study the function of *ltR,* we conducted some experiments including cloning of the gene, express the protein and then purify the protein. By electrophoretic mobility shift assay we analyzed the binding of LtR protein with promoter region of related virulence genes of *D. dadantii* (*pelA, pelE* and *pelD*) including the adjacent gene next to *ltR* (*lfaA* gene). The reaction mixture consist of 4.3 µl sucrose, 4.0 µl binding buffer, 1.5 µl BSA, 1 µl of 40 pg labeled promoter DNA of virulence and adjacent genes, 0.2 mM cAMP, 0.5 µg poly d(I-c) and 1 µg protein (Ryu et al. 1995). Electrophoresis method using 5% BIS-polyacrylamide gel with 0.5 TBE run in 90 volt

for 90 minutes. Blotting was carried out in 0.4 A for 30 minutes.

RESULTS AND DISCUSSION

The growth ability of culture of bacteria in three different medium were different (Fig.2.). The culture were grown rapidly in LB medium, M63 medium pH 7.0 + 0.2% glycerol + 0.2% Polygalacturonic Acid (PGA) + 1% potato extract, and M63 medium pH 7.0+ 0.2% glycerol+ 1% potato extract respectively. The addition of PGA in M63 medium could increase the exponential phase of bacterial growth. PGA induces the synthesis of pectinases, which are the essential virulence factors of *D. dadantii*.

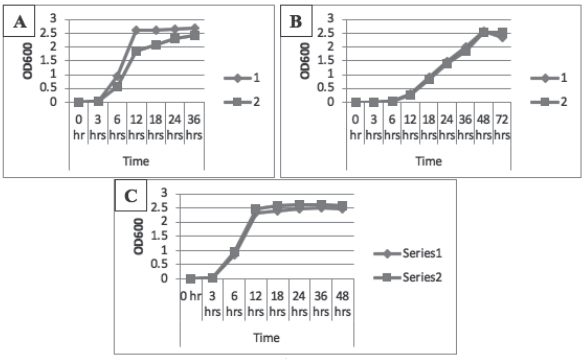

Fig. 1: Growth scale of *D. dadantii* in three different medium. A. LB medium; B. M63 medium pH 7.0+ 0.2% glycerol+ 1% potato extract; C. M63 medium pH 7.0 + 0.2% glycerol + 0.2% Polygalacturonic Acid (PGA) + 1% potato extract.

To detect the target genes expressed at low levels, PCR was performed on cDNA from at high sensitivity conditions using high purity of 200 ng total RNA extracted (Table 2.). From all RNA extracted, the cDNA provides only using RNA extracted from M63 medium pH 7.0 + 0.2% glycerol + 0.2% Polygalacturonic Acid (PGA) + 1% potato extract. (Fig.2.). We assumed that this is due to the PGA and potato extract induce the expression more effective than the other medium. The synthesis of *pel* genes depend on environmental or metabolic signals, such as the presence of pectic compounds (Jiang et al. 2016).

Table 2. Concentration of RNA were used to reverse transcription reaction.

No.	OD600	A260	A280	A320	differences	absorbance ratio	concentration 1µl RNA (ng)	purity
1.	0.468	2.866	1.711	0.050	1.155	1.683	1146.4	1.675
2.	0.441	2.589	1.337	0.031	1.251	1.958	1035.6	1.936
3.	0.437	2.493	1.401	0.042	1.092	1.779	997.2	1.803
4.	0.481	1.833	0.915	0.109	0.918	2.003	733.2	2.138

**1 and 2 were extracted from LB medium; no.3 extracted from M63 medium pH 7.0+ 0.2% glycerol+ 1% potatoextract and no.4 extracted from M63 medium pH 7.0 + 0.2% glycerol + 0.2% Polygalacturonic Acid (PGA) + 1% potato extract.*

The particular importance is the appearance of the targete genes, i.e. *ltR*, *pel* genes (*pelAED*) and reference genes (*lpxC, rpoD* and *gyrB*, because, under standard agarose gel electrophoresis conditions, this complex has the same migration rate as the amplification product. Morover, the bands are appear only in targete genes with reverse transcription enzyme (o), while the non transcription enzyme lane, tha bands are not appear (x). This means the reverse transcription process was succesful (Fig.2.).

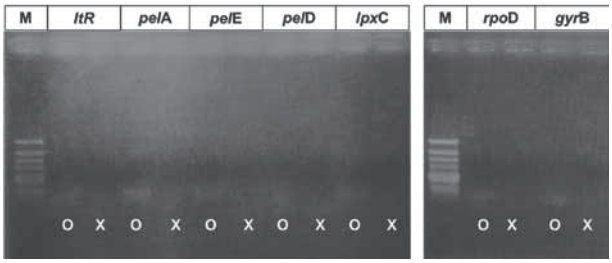

Fig. 2: cDNA PCR of *ltR*, *pel* genes (*pelAED*) and reference genes (*lpxC, rpoD* and *gyrB*).

Further experiments to understand the ability of LtR protein (LtR and LtRplus) bind specifc region of promoter DNA, the gel shift assay was conducted using the method of Ryu et al. which was applied for FruR, a member of LacI family repressor protein.

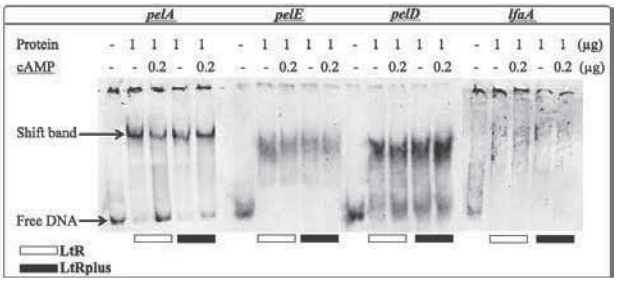

Fig. 3: Gel Shift Assay of the 300 bp of *pelA* promoter, 276 bp of *pelE* promoter, 251 bp *pelD* promoter and 302 bp of *lfaA* promoter with LtR and LtRplus protein. Method provide by Ryu et al., 1995 with small modifications.

The result showed that we got enough clear shift bands in pelA with two kind proteins as well. But in *pelE* and *pelD* the bands rather smear. Also for the adjacent gene, *lfaA* seems there is no binding, but the control negative section shows a migration of free DNA than the targeted section. This due to the affinity binding by LtR is different towards four promoter region. Although there still some tricky situation, we assume that the results indicated that LtR protein has a potential to be a transcriptional regulator for the three genes.

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Effect of human activity on ecology of long-tailed macaque (*Macaca fascicularis*) in One Ecotourism Site, Indonesia

Dwi Nur Setyowati, [1], Kanthi Arum Widayati[1] and Yamato Tsuji[2]
1. **Department of Biology, Bogor Agricultural University**
2. **Primate Research Institute, Kyoto University**

INTRODUCTION

Majority of temporal changes in primate behavior is affected by differences in dietary availability, quality, and distribution (Hanya 2004). The same holds for provisioned primates (Sha and Hanya 2013b; Ménard et al. 2013). For instance, the number of tourists (and amount of provisioning foods) changes seasonally, which in turn affects activity, diet composition, and degree of aggressive behavior of the monkeys; semi-provisioned baboons (*Papio cynocephalus*), spent less time for feeding and more for resting than wild troop (Altmann and Muruthi 1988). Semi-provisioned Barbary macaques (*Macaca sylvanus*) spent less time for feeding and moving, and more for resting, while wild troops spent more time for foraging and moving, and less for resting. And, mean daily travel distance of the former was significantly shorter than the latter (El Alami et al. 2012).

If primates rely much on provisioning foods, they would adjust their activities in response to tourists' activity in given time. In food scarce season, for example, monkey-human conflicts might become severer than food rich season. This study examined effects of seasonal variation in provisioning on 1) activity budgets, 2) dietary composition, 3) daily travel distances, and 4) degree of aggressive behavior of long-tailed macaque (*Macaca fascicularis*) in Pangandaran Nature Reserve, West Java, Indonesia. Specifically, we compared macaque behavior among three different types of days in which number of tourists seemed different: 1) weekday, 2) weekend, and 3) Ramadhan, a month of fasting.

We conducted observation on weekdays (between Monday and Friday), weekends (Saturday and Sunday) and Ramadhan to compare the effects of tourist number on activity budget, dietary composition, daily travel distances, and level of interaction with tourists. We predicted number of tourists influenced the difference feeding strategy, in weekend moving of long-tailed macaque shorter then weekday and Ramadhan, but more feeding time. Equally, on weekend the macaque spent most feeding time on provisioned than natural food. On weekday, long-tailed showed more aggressive behavior toward tourists as amount of provisioned food decreased. While on Ramadhan the daily distance of *M. fascicularis* was larger and approaching urban area to obtain more provisioned food.

MATERIALS AND METHODS

The observation was conducted in Pangandaran Nature Reserve (PNR), Pangandaran District, West Java, Indonesia. Subject animal of this study was one troop of long-tailed macaques, called Pantai Barat troop.

Activity Budget. We employed instantaneous scan sampling every 10 minute to record activity budgets of the macaques, and recorded activity of all visible animals. The behavioral categories of the macaques were classified into moving, resting, feeding, social activity, human junk handling and others.

Interaction between the macaque and tourist. We employed ad libitum sampling to record interactions between the macaques and tourists. We defined the interaction when at least one macaque and one human oriented toward each other and exchange behavior (Fuentes et al. 2008). We classified the interaction into provisioning and four levels of aggression; Level 0 (The macaques showed submissive behavior or no responses to tourist), Level 1 (The macaques approached to and/or taught tourists, but not caused injury), Level 2 (The macaques demonstrated threatening or frightening behavior with short distance (< 3 m), included alarming, showing canine and lifting their tails) and Level 3 (The macaques showed aggressive behavior, including bitting, chasing, stealing and damaged tourists' belongings).

Travel distance. During the behavioral observation, we recorded own locations (represented as location of subject troop) by a handy-type GPS every 10 minutes, from which we estimated the travel distance (m) of the macaques.

RESULTS AND DISCUSSION

Activity Budget. The highest activity was resting (32-36%, across the day types), followed by moving (27-31%) and social behavior (22-25%). During the study, all the activities, except for moving, of the macaques were relatively stable during the study. Number of tourists affected percentages of some activities; when the number of tourists increased, percentage of feeding increased, while of human junk handling activities decreased. It would be speculated that the macaques increased their foraging efforts to obtain more provisioning foods, and as consequently, time spent for junk handling (which is considered as searching of provisioning foods). Previous studies have showed that provisioned long-tailed macaques spent much time for moving and feeding, and less time for resting (Son 2004; Hambali et al. 2012).

Diet Activities. Across the day types, the macaques fed on provisioned foods (17-22%), leaves (27-41%), and fruits and seeds (29-41%). There were no significant differences in percentage among the day types. However we found that percentage of provisioned foods increased with number of tourists, while percentage of fruits and seeds and other natural food. This implies that tourists had potential effects on dietary composition of the macaques. There have been several studies found that provisioning by tourists changed the dietary composition of the long-tailed macaques (Sha and Hanya 2013a). Nila et al. (2014), reported that the long-tailed macaques at Telaga Warna Bogor fed mainly on fruits and seeds on Ramadhan season, while feeding of provisioning food decreased. The reason why the macaques spent much time for provisioned foods is likely due to its higher nutrition (Wheatley 1989).

Travel Distance. The mean travel distances of the macaques was 578 ± 336m, and showed no significant differences among the day types. We found no significant relationship with number of tourists. As to ranging inside the home range, however, we found unique characteristics: on weekdays the

long-tailed macaques showed activities in forest, on weekend in tourist area, and in the urban area on Ramadhan days. Similar finding has been reported from Kuala Selangor Nature Park; the macaques adapted to direct provisioning, the daily activity mostly at the edge of the park which adjacent with residential area (Hambali et al. 2012). This study confirmed that a pattern of range use, but not travel distances, is affected by provisioned foods.

Interaction Macaque-Tourist.

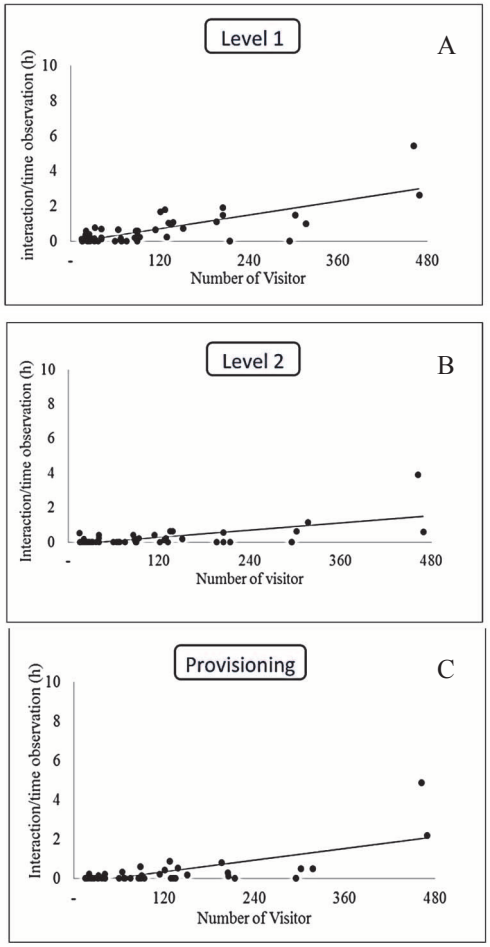

Fig. 1: Relationships between number of tourists and tourist-macaque interactions. A (Level 1), B (Level 2), C (Provisioning).

Majority of interactions with human were tolerant, but sometimes aggressive interactions were observed. Frequency of the provisioning was higher in weekend, while frequencies of other interactions were stable among the other day types. And, degree of level 1 and level 2 increased when number of tourists increased. That is, majority of macaque-tourist interactions were tolerant. (Fig 1). The number of tourists had positive relationships with degree of Level 1 and Level 2 interactions. Sha et al. (2009) found that two-thirds of human-macaque interaction of *M. fascicularis* in Singapore occurred when a human carried food or food cues, which was similar to this study. However, we also found that tourists' presence sometimes threatened the macaques; the macaques showed threatening or frightening behavior (in terms of alarming, showing canine and lifting the tail) (Level 2). Thus, degree of dependence on provisioning in PNR would be relatively lower than other heavy provisioned sites, such as at Padangtegal Monkey Forest in Bali, Indonesia, and the Upper Rock Nature Reserve in Gibraltar (Fuentes 2006). In PNR, interaction level 3 occurred when tourists did not provide foods to the macaques. This implies that the degree of aggressiveness becomes higher when availability of the provisioned foods decreased.

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Current situation, research, and conservation on bonobos in Mbali (Democratic Republic of the Congo)

Yuta Shintaku
Wildlife Research Center, Kyoto University; Japan Monkey Centre

INTRODUCTION

The bonobo (Fig. 1. *Pan paniscus*, Primates; Hominidae) is a great ape species, and one of the closest species to humans. Bonobos are endemic to the Democratic Republic of the Congo (DRC), and their distribution is limited to the left bank of the Congo River. The population size of the bonobos is unclear because of limited field surveys. Fruth et al. (2016) has estimated that the minimum population size is 15,000–20,000 individuals. However, their population size is decreasing owing to illegal hunting, deforestation, and disease. The IUCN Red List categorizes bonobos as Endangered (EN).

In 2005, western population of bonobos was re-discovered in the Lac Tumba Landscape, western DRC (Ignowabini 2007). The estimated population density was higher than that in other regions; thus, this western population is important for the conservation of bonobos.

The Mbali area (Fig. 2, Territoire du Bolobo, Province du Mai-Ndombe) is located in the Lac Tumba Landscape and is the southwestern edge of the bonobo distribution range. Most of the habitat in the distribution range of the bonobos is humid tropical forest. However, the Mbali area has a much drier forest-savanna mosaic habitat. The Mbali area is a unique environment as bonobo habitat and important to understand bonobo adaptation to drier habitats. For example, in the Mbali area, savanna use by bonobos for traveling and feeding was observed.

In the Mbali area, a local NGO, Mbou Mon Tour (MMT), started the habituation of several groups of bonobos with support from the World Wide Fund for Nature (WWF) in 2005. This habituation is aimed at developing eco-tourism focused on wild bonobos and local environment. That is, the aim is to develop the local community through conservation and sustainable use of the local environment as tourism resources. In addition to habituation, MMT has started a local environment conservation program through the prohibition of hunting using guns and traps in the forest, and slash-and-burn management. Conservation efforts by local NGO have been summarized by Narat et al. (2015).

Fig. 1. Young bonobos in the Mbali area (Nkala group).

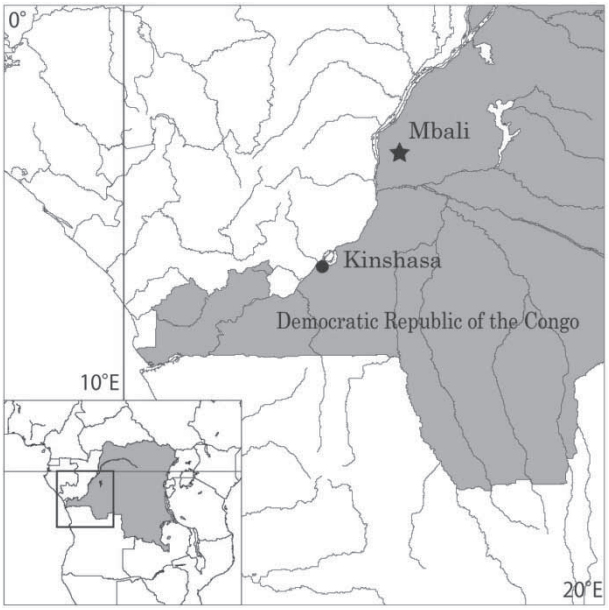

Fig. 2. Location of the Mbali area.

RESEARCH

Since 2015, Japanese research teams have conducted field surveys of one bonobo group habituated by MMT and WWF DRC in Mbali (named Nkala group). According to the progress of habituation, researchers can observe bonobos from much shorter distances for longer time. In future, long-term observational data will provide important information on the socio-ecology of bonobos in drier environments.

HEALTH MONITORING

Diseases, especially those transmitted from humans to the great apes, are one of the important problems for the conservation. Many cases of such zoonosis have been reported (Dunay, 2018). Especially, eco-tourism results in close contact between humans and the great apes, thereby increasing the risk of disease transmission from humans to great apes. In many eco-tourism sites of the great apes, health conditions are monitored.

In 2014 and 2015, respiratory disease outbreaks occurred among the bonobos in the Mbali area. At least four individuals died in both the years and more individuals disappeared. The outbreak in 2015 was occurred in the Nkala group. Grützmacher et al. (2018) reported that the causative agents were human respiratory syncytial virus (HRSV) and Streptococcus pneumoniae. Owing to the outbreaks, the group size of Nkala decreased to around 15 individuals. For the conservation of the Mbali bonobos, zoonosis monitoring is important. As such, since May 2016, I have conducted health monitoring of the bonobos based on non-invasive sampling.

During direct observations of the bonobos in the forest, I collected feces, urine, and food remains (Fig. 3) as samples. Using these samples, I checked human respiratory diseases, such as Influenza virus, Rotavirus, and Streptococcus pneumoniae, using conventional detection kits. In addition, I

also surveyed the intestinal parasites using the direct smear method of fecal samples.

Based on direct observation, use of test kits, and parasite analyses, new outbreaks were not diagnosed in the Nkala group of bonobos. However, continuous monitoring of health conditions is required for sustainable eco-tourism.

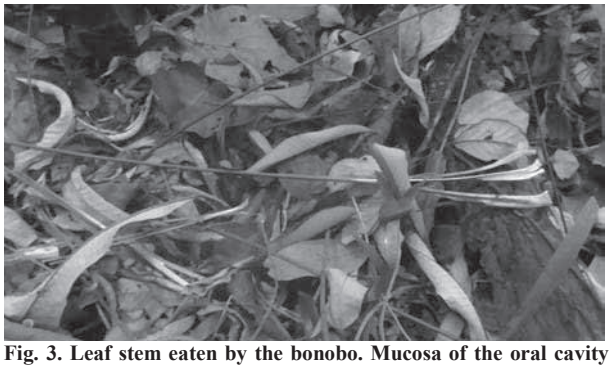

Fig. 3. Leaf stem eaten by the bonobo. Mucosa of the oral cavity from this sample was tested using conventional detection kits.

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Population dynamics of geographically isolated population of stump-tailed macaques in Thailand - from the viewpoint of conservation

Tamaki Maruhashi[1], Aru Toyoda[2], Yuzuru Hamada[2], and Suchinda Malaivijitnond[3,4]

1. Department of Human Cultures, Musashi University, Tokyo, Japan
2. Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan
3. Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand
4. National Primate Research Center of Thailand, Chulalongkorn University, Saraburi, Thailand

INTRODUCTION

The stump-tailed macaque (*Macaca arctoides*) is a species of the genus *Maccaca*, family Cercopithecidae. This species can be found across Asian continent, from north-eastern India to south-western China and downward to Vietnam, Cambodia, Myanmar, Thailand, and Peninsular Malaysia (Fooden 1990). Internationally, this species is categorized in VULNERABLE on The IUCN Red List of Threatened Species, and also listed under Appendix II in CITES. The species is protected in national wildlife acts of Lao PDR, Viet Nam, Thailand, and Myanmar.

Our study site, Khao Krapuk Khao Taomor Non-Hunting Area, is located in Phetchaburi province, the central part of Thailand. Here is the only study site of stump-tailed macaques where long-term continuation study has been conducted based on individual identification. Direct observation including population census has been conducted since 2005 by Tamaki Maruhashi (TM) and Aru Toyoda (AT). An important and urgent conservation issue here is a rapid growth in the population and a destruction of the habitat. Historically, this population was discovered in 1984 and the population size at that time was only 22 monkeys (7 adult males, 5 adult females, 6 juveniles and 4 infants) (Theesucon 1988). However, at present, population is increased up to 391 individuals. Together with the increase in population, a part of their habitat was recently invaded by a construction of the photovoltaic power plant which could be a threat to this population because their foraging area was shrunken. In this poster, we report the detailed data of population census, changes in ranging areas after a habitat destruction, and the latest activities towards the protection of this local population.

MATERIALS AND METHODS

Khao Krapuk Khao Taomor Non-Hunting Area located at 99°44' E, 12°48' N, and the total area was about 3.5–4 km^2. There were 5 groups of stump-tailed macaques, namely Ting, Nadam, Third, Fourth and Wngklm group. This site consisted primarily of secondary forest, including stands of bamboo, and agricultural areas. From our long-term survey, no natural predators to the stump-tailed macaques were observed. The macaques also visited the adjacent areas such as temple, cassava and pineapple plantations, and human settlements on their daily roaming. They were occasionally fed by humans, both locals and tourists, at the temple grounds or along the roadside.

We recorded the group activities by a video camera, counted the number of individuals and categorized their age and sexes. Ranging area was recorded by GPS devise (GARMIN GPSmap 62s) which was carried by observers when followed the monkeys.

Photo. 1: Stump-tailed macaques passing across the road in the forest of Khao Krapuk Khao Taomor Non-Hunting Area. This photograph was taken from the same angle as the video camera. Photo by AT.

RESULTS

Population size –Although the population was composed of only 22 monkeys in 1988 (Theesucon 1988), the latest counting by AT in 2016-2017 was 391 individuals which were divided into 5 groups (Toyoda and Malaivijitnond 2018).

Group fission - On November 10th, 2015, AT found a small group containing only 37 individuals. According to the individual identification list, some males and females were members of the Third group, thus we concluded that this small group was separated from the Third group and named "Wngklm group". This group size became 43 by the end of 2017.

Provisioning – Food provisioning to wildlife including macaques is common in Thailand regarding the principle of their religion of Buddhism. Unexceptionally, food provisioning to these stump-tailed macaques by passerby was usual Although we did not measure the amount and the frequency of foods provided, the high nutritional fruits such as bananas and mangoes with high amount were frequently provided.

Habitat destruction – In 2015, northern part of their home range was deforested and a huge solar power plant was built. As a consequence, the bamboo forest which was one of their main feeding sites was cleared up.

Activities for conservation –Department of the National Parks, Wildlife and Plant Conservation of Thailand (DNPT) was recently actively involved in conserving these monkeys. Thanks to their activities, signboard telling about monkeys crossing the road has been posted, and a provisioning along the road which can cause a traffic accident and death to monkeys is prohibited.

Supernumerary nipples — Although the relationship between a rapid increase in population and food provisioning is not tested, a supernumerary (>2) nipples was confirmed in this population. Of the 332 monkeys counted, the percentage of supernumerary nipples was 22% (Toyoda and Malaivijitnond 2018).

Photo. 2: Stump-tailed macaques feeding a plentiful egg-plant provided by local farmer. The amount of provided foods is often excessive, which is certainly one of the major factors in a rapid population growth. Photo by AT.

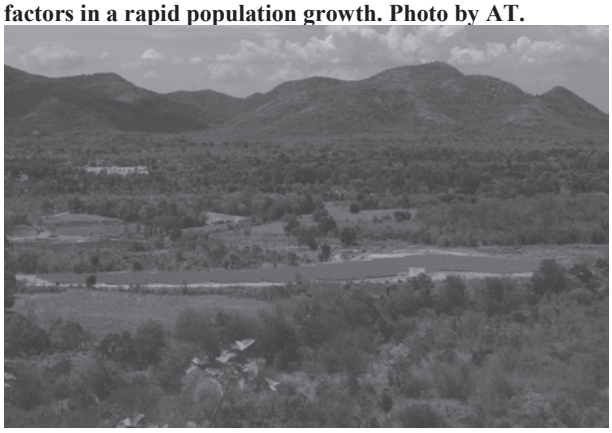

Photo. 3: Solar panels set in the area of photovoltaic power plant. Due to the construction of this power plant, the monkeys lost the major ranging area of the northern part. Photo by AT.

Photo. 4: Local people giving pineapples on the roadside next to the signboard saying "DO NOT FEED WILD ANIMALS". Photo by AT.

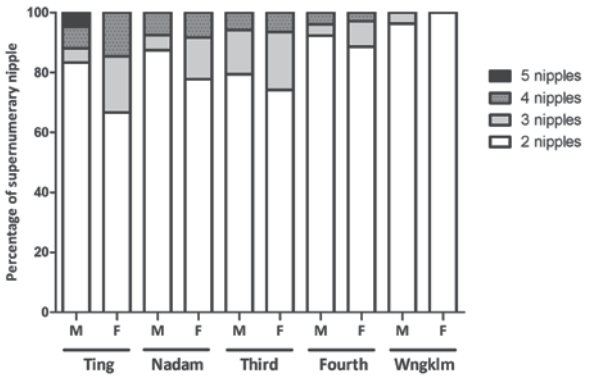

Figure. 1: Percent occurrence of supernumerary nipples in the Ting, Nadam, Third, Fourth, and Wngklm groups (Toyoda and Malaivijitnond 2018).

DISCUSSION

While the global trend of the population of stump-tailed macaques is decreased, in our study site, it is reversed. Since they have been successfully protected and a population is rapidly grown, without natural predators, the existing habitat might not be able to hold them. Thus, they started to forage into and damage to the surrounding agricultural areas which will finally lead to a conflict with humans in the vicinity.

Since this population is geographically isolated from other conspecific populations (Malaivijitnond et al., 2005), a gene flow between populations has been impossible. Thus, if the population is kept increasing, an inbreeding depression and a decline in genetic diversity could be occurred.

In the future, a multifaceted perspective, including genetic analysis, and more effort on conservation in cooperation with DNPT will be incorporated into our study.

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The United Graduate School of Agricultural Science, Gifu University
1-1 Yanagido, Gifu 501-1193, Japan
Tel: +81-58-293-2984 (or, 2985)
E-mail: renno@gifu-u.ac.jp
Home Page: http://www.ugsas.gifu-u.ac.jp/eng/

Promotion Office of Gifu University Rearing Program
for Basin Water Environmental Leaders, Gifu University
1-1 Yanagido, Gifu 501-1193, Japan
Tel: +81-58-293-2085
E-mail: bwel@green.gifu-u.ac.jp
Home Page: http://www.green.gifu-u.ac.jp/BWEL/eng/index.html

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