# Improving Smallholder and Industrial Livestock Production For Enhancing Food Security, Environment and Human Welfare

# The Proceeding of

## Human-Chicken Multi-Relationships (HCMR) Symposium

The Human-Chicken Multi-Relationships Research Project,
H.I.H. Prince Akishinonomiya Fumihito's Research
under the Royal Patronage of H.R.H. Princess Maha Chakri Sirindhorn

Human-Chicken Multi-Relationships (HCMR) Symposium
A satellite symposium organized in conjunction with
the 15th AAAP Animal Science Congress
27 November 2012

The 15th AAAP Animal Science Congress
26-30 November 2012, Thailand

**Improving Smallholder and Industrial Livestock Production For Enhancing Food Security, Environment and Human Welfare**

**The 15th AAAP Animal Science Congress**

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## Human-Chicken Multi-Relationships (HCMR) Symposium

**The Human-Chicken Multi-Relationships Research Project, H.I.H. Prince Akishinonomiya Fumihito's Research under the Royal Patronage of H.R.H. Princess Maha Chakri Sirindhorn**

Editors: Pairash Thajchayapong, Akaki Osamu, Hayashi Yoshihiro and Chanin Tirawattanawanich

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# 15 AAAP PRESIDENT'S REPORT

Sawatdee krup!

Greetings to all 15 AAAP attendants:

It's our great honor and pleasure to welcome you to the 15 AAAP Animal Science Congress, being held during 26-30 November 2012 at the Rangsit campus of Thammasat University, Bangkok, Thailand. The AHAT (Animal Husbandry Association of Thailand under the Royal Patronage of H.R.H. Princess Maha Chakri Sirindhorn), as the official host of the 15 AAAP Congress, has collaborated with the other three significant government agencies as the co-hosts of this Congress, these are the Department of Livestock Development (Ministry of Agriculture and Agricultural Cooperatives), Kasetsart University (KU), and Thammasat University (TU). The Rangsit campus of TU, situated in the northern outskirt of Bangkok, which is under the jurisdiction of Pathum Thani province, is the beautiful venue of this Congress.

The 15 AAAP animal Science Congress programs consist of scientific and technical programs and social and cultural activities. The scientific and technical programs offer 4 enlightening plenary sessions, 9 significant symposia, one-day mid-Congress study tours, and numerous scientific sessions (both oral and poster presentations), as well as other scientific meetings. The most significant symposium is "The Human-Chicken Multi-relationships Based on H.I.H Prince Akishino Research Project under the Royal Patronage of H.R.H. Princess Maha Chakri Sirindhorn".

It is expected that around 1,200 scientists, livestock producers, development personnel, as well as graduate and undergraduate students from 38 countries will attend 15 AAAP Congress; and more than 700 research papers in all fields of animal production and related fields will be presented and discussed at the Congress. Very interesting and exciting one-day mid-Congress tours on 28 November 2012, consisting 7 alternative routes at your preferred choice, are offered for learning experiences which could be useful for future technical understandings.

The social and cultural programs of the 15 AAAP Congress are as important as the scientific and technical programs since the promotion of friendship and future scientific cooperation are also central to this AAAP Congress. Reception party and opening ceremony will offer selected exciting Thai cultural shows from all regions of the Kingdom. On 28 November 2012, immediately after the mid-Congress tour, the Loy Kratong Festival, a very significant annual festivity of Thailand as being organized by TU, will allow all participants to join this Thai traditional activity, which is full of fun and excitement.

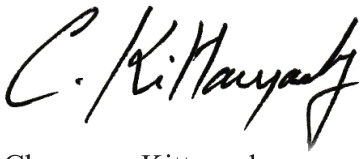
The fantastic farewell party will be offered on the night of 29 November 2012. Participants from each and every country will have a chance to enjoy traditional and cultural exchange in order to strengthen friendship and future cooperation. We do hope that you will not miss this opportunity.

Beside all these colorful programs, spouse programs and other recreational and sports activities are made available for your pleasure at your own convenience.

You can be assured that, in our hospitable Thai way, we will try our best to make your brief visit to our country a very pleasant and memorable one.

Wish you all a very happy and most enjoyable stay in Thailand.

Sawatdee krup,



Chayanon Kittayachaweng
President
Asian-Australasian Association of
Animal Production Societies

## REMARK FROM CHAIRMAN OF 15th AAAP NOC-TEP

Dear Distinguished Scientists and Ladies and Gentlemen,

It is overwhelmingly heart-felt impressive to receive all attention and high interest from all scientists and friends from all over the world to participate in this important the 15th AAAP Congress being organized in Bangkok, Thailand. This is a good indication of the great concern and interest of the animal scientists to share and learn experiences among each others to help solve the problems in animal production as well as for future research and development collaborations.

You can also agree with me that the Congress is accommodating with high quality of the invited plenary papers, invited lead-papers as well as all short oral and poster presentations. Furthermore, the many Symposia encompassing very important and hot issues dealing with animal production and development are being held by a number of organizations who have been experiencing in the respective fields and by the eminent scientists around the world. It is the great forum for all participants to learn and enrich as well as to interact among one another.

One high-light is to observe the high attendance of the participants especially by the young scientists which is the imperative for them to interact and to link-up for the future research collaborations.

The Congress is not to avail all participants to enrich the high standard of research merits, but also open up the scenario for all to enjoy the social and cultural environment during the Congress. The highly successful and fruitful outcomes of the programs are of concerted efforts contributed by all parts and organizations including from the government, private sector, all participants and with the continuous hard work of the Scientific Committee Members. The special contributions from the Symposia Organizers and their supporters are highly acknowledged and appreciated.

Finally, may I on behalf of the Scientific Committee Members and all associates, wish all the participants to highly achieve the participation expectations and successful in your deliberations as well as to mutually enjoy and interact with all scientist fellows during the Congress.

The great support from all sectors especially from the Congress site host, the Thammasat University, Rangsit Campus, is gratefully acknowledged for their concern, close cooperation and for the available facilities for the Congress.

Special thanks to my Scientific Committee and the Advisory Board Members especially the Vice Chairs and the Secretary of the NOC-TEP of the 15th AAAP Congress are sincerely thankful for their great contribution to make the program the high-caliber one.

Looking forward to meeting all participants in the future Congresses to continue.

With best wishes and warm regards,

Professor Dr. Metha Wanapat
Chairman, National Organizing Committee for Technical Programs (NOC-TEP)
The 15th AAAP Animal Science Congress

# Welcome Remarks

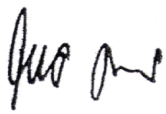
Your Imperial Highness, distinguished guests, and colleagues,

It has been a privilege and great honor for all of us to have the opportunity to be part of such enthusiastic research projects initiated by H.I.H. Prince Akishinonomiya Fumihito of Japan on deciphering human-chicken multi-relationships and on tracing the origin of the world's domestic chickens. The Human-Chicken Multi-Relationships (HCMR) research project has been established under a supervision of His Imperial Highness and the royal patronage of H.R.H. Princess Maha Chakri Sirindhorn. This HCMR symposium represents a long-standing friendship among HCMR researchers and a continuation of collaborations on various multi-disciplinary research projects. Both Japanese and Thai scientists from the fields of Biology and Biotechnology, Humanities and Linguistics, Ecology, Economics and Zoo-archeology have been joining the studies in every project. Key research questions, hypotheses and study protocols have been carefully identified and formulated. The projects' findings have led us to understand more and more of the untold history of chicken influences on mankind and vice versa.

On behalf of the organizing committee and Thai HCMR researchers, let me take this opportunity to express a humble gratitude and deep admiration for His Imperial Highness and for his advice given to us on every occasion. I additionally would like to extend my sincere thanks to all Japanese members of the HCMR group for their valuable friendships and collaborations. My heartfelt thanks as well go to participants of HCMR symposium and to all supporting organizations.

I sincerely hope that all participants will find the symposium both enjoyable and educational.

Best regards,



Prof. Dr. Pairash Thajchayapong
Thai Representative of HCMR Project
Senior Advisor
National Science and Technology Development Agency (NSTDA)

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| Time | Keynote Speech | Speaker |
|---|---|---|
| 13:30-14:00 | **Overview of Human-Chicken Multi-Relationships and Future Prospects for Further Research of Domestication and Breeding of Varieties** | **H.I.H. Prince Akishinonomiya Fumihito** |
| **Time** | **Scientific Presentations** | **Speaker** |
| 14:00-14:15 | **Domestic Animals in Prehistoric Thailand** | **Dr. Amphan Kijngam**<br>Fine Arts Department, Silpakorn University, Thailand |
| 14:15-14:30 | **New Dimension in HCMR: A Case of Zooarchaeological Approach-Pig Domestication in Japan** | **Dr. Akira Matsui**<br>Nara National Research Institute for Cultural Properties, Japan |
| 14:30-14:40 | **Q&A** | |
| 14:40-14:55 | **Phylogeography and Demography of the Red Junglefowl and Its Domestication Process Revealed by Mitochondrial DNA Sequences** | **Dr. Takeshi SASAKI**<br>Tokyo University of Agriculture, Japan |
| 14:55-15:10 | **Comparative Genomics Among Red Junglefowl, Thai Native Chicken and Commercial Line** | **Assoc. Prof. Dr. Monchai Duangjinda**<br>Khon Kean University, Thailand |
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| 16:50-17:00 | **Closing remarks** | Prof. Dr. Pairash Thajchayapong<br>National Science and Technology Development Agency |

# Keynote Speech

by H.I.H. Akishinonomiya Fumihito

# Overview of Human-Chicken Multi-Relationships and Future Prospects for Further Research of Domestication and Breeding of Varieties (The 15th AAAP Animal Science Congress) 121124v.

## INTRODUCTION

According to statistics from the United Nations Food and Agriculture Organization (FAO), over 18 billion chickens are being reared in the world today, an exceptional number by comparison with other livestock such as oxen or swines. As the numbers are partly a function of the size of the animals, they may not be the single measure of the intensity of the relationship between humans and animals. However, we can at least say that chickens are one of the most frequently used animals in our daily lives, for economic motives and for rituals, entertainment, and appreciation.

Though they are in intimate contact with humans, little seems to be known about chickens. For example, when and where, and why or how, were chickens domesticated by mankind, and which wild species of junglefowl, genus *Gallus*, was the real ancestor of present- day chickens? Fortunately, the origin of chickens and the locations of their domestication are gradually becoming clearer as a result of recent advances in molecular biological studies. However, there are still unanswered questions of when, why, and how, related not only to domestication but also to the breeding of varieties after chickens were domesticated from junglefowl.

In elucidating these subjects, multidisciplinary approaches are considered quite useful, so I have implemented them by organizing the "Human-Chicken Multi-Relationships Research Project" in Thailand from 2004 to 2008 under the Royal Patronage of Her Royal Highness Princess Maha Chakri Sirindhorn. In this paper, I would like to introduce the outline of the project, and also propose and note some possible ideas for future studies of the domestication and breeding of varieties of chickens, based on my experience of travels throughout Asia.

## OVERVIEW OF HUMAN-CHICKEN MULTI-RELATIONSHIPS RESEARCH PROJECT

The Human-Chicken Multi-Relationships Research Project, or HCMR for short, started in 2004 using the model region of Chiang Rai Province in northern Thailand, with domestication as the keyword. In starting the project, four major research areas were specified, namely humanities, biology, economics, and geography because chickens are not merely living organisms, but are also cultural creatures bred by humans. Throughout the project, Japanese and Thai researchers from each of these fields worked together and implemented joint surveys in several places in Chiang Rai to obtain data to use in researching and anticipating the process of domestication and the breeding of varieties.

As a result of many joint surveys, we gained quite a lot of information on the subject of human-chicken multi-relationships, and the book entitled "Chickens and Humans in Thailand: Their Multiple Relationships and Domestication" was published by The Siam Society in 2010.

The contents of this book roughly consist of four parts, following a preface by Her Royal Highness Princess Maha Chakri Sirindhorn and my general remarks. The four parts are as follows;

Part 1: "From Forest To Village"
In this part, the early stage of the domestication process from junglefowl to chickens is explored by investigating the nature of the bird alongside human activities such as hunting, taming, rearing, and breeding.

Part 2: "Spiritual Interactions between Chickens and Humans"
Here we explore human intervention in the domestication process and in the "breeding of varieties" of chickens as domestic animals that live in intimate contact with humans. Examples of cultural roles in rituals, divination, and cockfighting are investigated, and traditional customs practiced among various ethnic groups are observed by the choice of chickens in beliefs and myths.

Part 3: "Consumption and Utilization of Chickens"
This part elucidates the new relationship between humans and chickens by investigating the actual conditions of production, distribution, and consumption of chickens, because chickens are still offer significant economic benefits to the present-day society of northern Thailand.

Part 4: "Future Studies in the Biology of Junglefowl and Chickens"
In this part, we provide fundamental information about the biological features of junglefowl and chickens such as their morphology, physiology, and molecular biology. As you may notice, the biological perspective is one of the effective approaches for clarifying the nature of junglefowl and chickens, and elucidating the domestication process.

Although we conducted research on a lot of matters related to domestication and the breeding of varieties, the research project has barely touched on molecular biological research, and zooarchaeological research has yet to start. These two subjects can contribute greatly to future domestication studies.

## THE FUTURE OUTLOOK

From a different perspective than molecular biology and zooarchaeology, I would like to note three research themes for future studies based on my personal research interests in investigating the domestication and breeding of varieties of chickens, and their multiple relationships, namely selection by color, cultural clustering of chickens using questionnaire-based surveys, and design based on geographic and cultural barriers.

**1. Selection by color.**

In Southeast Asia, one key to the study of the domestication and breeding of varieties is probably coloration, such as that of the plumage, shanks, and meat. The results of the research conducted above in Sipsongpanna, Yunnan, China, and Laos, reveal that there is a clear distinction between good color and bad color that is related to whether the chickens are edible (good for health) or unpalatable (cause illness).

For instance, chicken shanks can be broadly divided into two colors: black and yellow. From my experience, most of villagers avoid eating yellow-shanked chickens because yellow may evoke illnesses such as fever, headache, diarrhea, back pain, and so on. On the other hand, black-shanked chickens are preferred for health, being considered good for the sick, helpful in overcoming dizziness, etc., and this kind of chicken is also used for rituals as well as for food.

These preferences have been developed based on customs and beliefs among the ethnic groups in Sipsongpanna and Laos including the Tai Lu, Lao, Jino, Hani, Lahu, Hmong and Khmu. I think it is worth considering these beliefs and customs that may contribute to creating chickens with uniform characters. However, the problem with this theory is that if these traditions are phased out as a result of modernization or urbanization of the villages, this selection process may come to a halt.

**2. Cultural clustering from questionnaire-based surveys.**

Along with biological data, it is possible to say that quantitative cultural data can also be useful to clarify the cultural similarities of chickens. In this context, I once implemented cultural clustering by conducting questionnaire-based surveys consisting of more than 110 questions on the exploitation of chickens and the livelihoods of more than 40 villages that rear chickens in Yunnan.

The phenogram shown from this questionnaire is based on the idea that chickens reared in various villages represent the culture of those villages. In other words, it aims to compare chickens as cultural creatures, not as living organisms, by comparing the uses of chickens, and the livelihoods, between different villages.

Research that applies this kind of cultural clustering or organizes its results into this kind of phenogram is not necessarily viewed positively by present ethnology. However, visualizing relationships in this way may provide clues to understanding the breeding of varieties and their dispersal patterns as a cultural process.

**3. Design based on Geographic and Cultural Barriers.**

It might also be useful to view the forms and color schemes as man-made designs and to investigate the ideas behind their creation in particular ethnic groups or areas. There are often geographical barriers such as mountains or rivers present where there are differences among ethnic groups or areas. And this also means cultural segregation. By applying this idea to breeding of varieties of local chickens, it is quite understandable that small differences exist among the same or similar types of chickens.

## THE ULTIMATE GOALS FOR HUMAN-CHICKEN MULTI-RELATIONSHIP STUDIES

Finally, I would like to propose the ultimate goals for human-chicken multi -relationship studies. One is to construct a model of domestication and the breeding of varieties, and the other proposal is to preserve chicken breeds.

1. **Constructing a model of domestication and the breeding of varieties.**

One of the final goals of human-chicken multi-relationship studies is to construct a model of domestication and the breeding of varieties.The model that I wish to propose covers the entire process from what led to human encounters with junglefowl, as the progenitor of chickens, and why humans started to domesticate junglefowl, up to the factors that have led to the variations observed today through artificial selection by form and color. Constructing this kind of model would be helpful in providing insights into domestication and the breeding of varieties as a process. It may also apply to domesticated animals in general.

2. **Preserving chicken breeds as a living cultural heritage.**

My second proposal is to preserve chicken breeds that inhabit various areas in their existing or preserved forms. According to FAO statistics, of the close to 8,000 registered varieties of livestock in general, around 20 percent are more or less endangered varieties. There are also many varieties that have already become extinct. This is also true of chicken breeds. I think that the existence of diverse varieties of chickens enables the breeding of varieties that suit local conditions and are disease resistant and tolerant.

In that sense, it should be emphasized that chickens, like other domestic animals, are human creations - a living cultural heritage - and I believe that we humans have a responsibility to preserve our creations. However, sometimes when it is difficult to preserve them in living form, conserving carcasses and DNA as preserved specimens would at least enable a proper understanding of what kind of varieties exist at present. I also believe that these specimens would play an extremely significant role in future studies.

As research of this issue is quite complicated, it will take considerable time and effort to clarify these matters. In this paper, I have pointed out some possible proposals for further research activities. However, further research subjects may need to cover areas other than those I have noted here. In order to carry out investigations on these subjects, various views related to surrounding areas of research need to be presented to enable deeper discussions. I hope this occasion will offer one of the opportunities for advancing research into domestication and the breeding of varieties of chickens.

# Scientific Presentations

# Domestic Animals in Prehistoric Thailand

**A. Kijngam**
Fine Arts Department, Silpakorn University, Thailand

## ABSTRACT

Thailand has an area of 513,000 square kilometers. The geography of Thailand has been divided into six principal regions: North, West, Central, Northeast, Peninsular and the East Coast. The archaeological evidence from these areas has revealed five prehistoric periods: Palaeolithic, Mesolithic, Neolithic, Bronze Age and Iron Age, covering the time from c. 40,000 BP. to 1,500 BP. The analysis of animal bones from various archaeological sites by comparison from the modern specimens has suggested that many species were hunted and collected during the Palaeolithic and Mesolithic periods, while some were domesticated from the Neolithic period at the sites of Ban Chiang and Ban Non Wat, Northeast Thailand. The domestic animals in question are cattle, water buffalo, pig, dog and chicken.

**Keywords** : Thailand, domestication, Ban Chiang, Ban Non Wat, cattle, water buffalo, dog, pig, chicken

# INTRODUCTION

Thailand has an area of 513,000 square kilometers. The geography of Thailand has been divided into six principal regions (Pongsabutra 1991) (Figure 1). These are:

1. North Thailand is geographically defined by the area drained by the upper courses of the rivers, Ping, Wang, Yom and Nan, which are tributaries of the Chao Praya River. The area is partly mountainous and is covered by dense forests. The principal prehistoric sites in this region comprise rock shelters containing evidence for Hoabinhian occupation. These date between 35,000 BC and 800 AD. Animal bones from these contexts come from a wide variety of hunted species, while the plant remains evidence use as stimulants, gums for the manufacture of composite hunting tools, and for subsistence. The rice remains from Banyan Valley cave have been shown to come from a wild species.

2. West Thailand is characterized by ranges of hills and mountains from the north to peninsular. Archaeological research has identified sites such as Sai Yok, Ban Kao and Ongbah that cover all five periods.

3. Central Thailand is characterized by the Chao Phraya Plain ; a wide river valley, covering some 10,000 square kilometers. Two famous ancient kingdoms, Sukhothai in the north and Ayudhaya in the South, predecessor of Bangkok, were established in the plain. Lopburi, which was also once a capital of the Khmer, was founded in the middle of central Thailand. In addition prehistoric sites from Neolithic have been found in Lopburi area such as Tha Kae and Non Pa Wai, and more recent archaeological research projects in the upper tributary basin of Lopburi have identified the remains of Neolithic, Bronze and Iron age occupation dating between 2,000 B.C. – 200 A.D.

4. Northeast Thailand is by far the largest of the regions, and the least favourable to agriculture. Although the mean rainfall is similar to that in the other regions, the soil does not hold water long enough to sustain certain staple food plants. The region has, however, become of great interest to international field archaeologists following the recovery of early agricultural and domesticated animals from sites of Non Nok Tha in Khon Kaen province and more Ban Chiang in Udorn Thani Province. Current dating of these sites strongly suggests that northeast Thailand developed an early agriculture and domestication by 1700 B.C. The more recently site, Ban Non Wat in Nakhon Ratchasima province, has been excavated and revealed the same evidence as Non Nok Tha and Ban Chiang.

5. Peninsular Thailand is characterized by ranges of hills and mountains. The west coast consists of narrow terraces and plains; only on the east coast are the terraces and plains wide enough to permit agricultural use and provide harbour facilities. A number of historical towns and communities were located on ancient shorelines in the provinces of Nakhon Si Thammarat, Suratthani (Chaiya) and Songkhla. Nevertheless, some Palaeolithic and Mesolithic sites have been found such as Lang Rongrien rockshelter and Mor Khiew Cave.

6. The East coast of Thailand is comparatively less known archaeologically, except for a few chance finds such as prehistoric cord-marked pottery and polished stone axes. One extensive excavation at Khok Phanom Di in Chonburi province, a prehistoric site dating to between 2000 – 0 B.C. has thus far been carried out in this region.

From the investigations and excavations on the areas mentioned above, all the archaeological evidence reveals the following chronological framework employing European technology (Anderson 1990,1997 ; Bayard 1971 ; Gorman 1971 ; Gorman and Charoenvongsa 1976; Higham and Thosarat 1998; Pookajorn 1992, 1994; Shoocondej 2004, 2008).

1. Palaeolithic, characterized by crude stone tools dating 40,000 – 10,000 BP.
2. Mesolithic, with bifacial core or Hoabinhian tools dating 10,000 –4,000 BP.
3. Neolithic characterized by polished stone axes dating 4,000 – 3,000 BP.
4. Bronze Age characterized by bronze artifacts dating 3,000 – 2,500 BP.
5. Iron Age characterized by iron artifacts dating 2,500 – 1,500 BP.

A new terminology in Thai prehistory has been suggested, based on economic change, which is generally accepted by Thai archeologists, as follows: (Kijngam 2010)

**1. Hunting and food gathering society**

1.1 First Period or Palaeolithic dating 40,000 – 10,000 years ago.

Small communities, temporarily living in caves or rockshelters, hunting and food gathering were the basis of subsistene. Flaked and crudely fashioned tools were used.

1.2 Second Period or Mesolithic dating 10,000 – 4,000 years ago.

Small communities, temporarily living in cave or rockshelters, food gathering and hunting were undertaken. Stone tools dominated by unifacial discoids (Hoabinhian culture). Between 7,000 – 4,000 years ago, polished stone axes and pottery were probably used in some communities.

**2. Agricultural Society**

2.1 First Period or Neolithic dating 4,000 – 3,000 years ago.

Small communities (about 100 – 150 persons), living on plains near the streams, growing rice, domestication of pigs, dogs and cattle, hunting and fishing continued. Polished stone tools were used. There is much evidence for long distance trade. The burial rites involved extended inhumation with a variety of mortuary offerings.

2.2 Second Period or Bronze Age dating 3,000 – 2,500 years ago.

Population expansion (about 250 persons/ 1 site), growing rice, maintaining domestic stock, hunting and fishing continued. Bronze tools were used. There was increased trade and evidence for some very rich burials in term of grave goods.

2.3 Third Period or Iron Age dating 2,500 – 1,500 BP years ago.

More population expansion (about 500-2,000 persons/ 1 site) on different locations, (plains and highlands), growing rice, domestication and hunting were found. Fishing decreased. Iron tools were used. Expanded external trade links with India and China brought precious stone and glass beads to Thailand. Salt was a major resource. A more formal burial rite developed and we find evidence for elite leaders.

In Thailand the most interesting area is the Khorat Plateau of Northeast Thailand, which is divided into two regions: the Sakon Nakorn basin in the north and Khorat basin in the south. Research has thus far concentrated on both basins. Palaeolithic and Mesolithic assemblages have found along the banks of Mekong River. Habitation sites have not been discovered in either of the northeast regions. However, there is evidence of a Neolithic, bronze and iron assemblage at Udonthani province in Sakon Nakorn basin and at Khon Kaen and Nakornrachasima province in Khorat basin. A number of these sites are located on low land plain in both areas. The well-known sites are Non Nok Tha and Ban Chiang (Bayard 1984 ; White 1982). Both sites have produced evidence of Neolithic culture with early domestic animals. The recent extensive excavation at Ban Non Wat, at the upper Mun River

in Khorat basin, have produced evidence of Neolithic culture at least 1700 B.C. together with evidence for domestic cattle, pig, dog, water buffalo and chicken as well as rice cultivation dating from the same period as at Non Nok Tha and Ban Chiang (Higham and Kijngam 2010) (Figure 2).

The initial objective of this paper was to represent the domestic animals in the prehistoric Thailand. Thus the bones from the excavations of Ban Chiang and Ban Non Wat in Northeast Thailand have been studied due to the extensive excavations in AD 1974-1975 and 2002 – 2008, respectively.

## MATERIALS AND METHODS

All bone fragments from the sites of Ban Chiang and Ban Non Wat came from occupation layers and burial contexts. They were initially cleaned and labeled according to provenance by square and layer. They were then assigned to genus and where possible, species. In some cases, ascription was only feasible at the family level.

With the identification of all possible bone fragments, the analysis continued by calculating the number of individual animals per species in each of the excavated layers in each square. The results of this analysis form the basis of the faunal spectrum which is used to interpret the economy and environment at the site. It was then decided to pool all bones of the same species and anatomical type, so that measuring of particular bones allowed a comparison with other sites. Mortality frequences were also estimated on the basis of tooth eruption and wear patterns (Kijngam 1979, 2008)

## RESULTS

The two sites, Ban Chiang and Ban Non Wat, provided considerable numbers of fragmentary bones which were designated to genus, species or family. The preliminary results of the domestic animals found are described below.

**BOVIDAE;** (*Bibos* sp. , *Bubalus bubalis*)

The basic problem of distinguishing between the bones of water buffalo (*Bubalus bubalis*) and cattle (*Bibos* sp.) has largely been resolved (Kijngam 1979). For several anatomical bones, differences in the morphology have been identified which marks separation such as 1st fore phalanx, 2nd fore phalanx, magnum, metacarpal, metatarsal, etc. There remains the problem of distinguishing between wild and domestic cattle.

*Bibos* sp.

There are three species of indigenous bibos in Thailand. Their habitations are slightly different (Lekakul and McNeely 1977). The analysis of the Ban Chiang and Ban Non Wat *Bibos* remains relied upon size differences. The absence of comparative samples for the three indigenous cattle (*Bibos gaurus*, *Bibos javanicus* and *Novibos sauveli*) rules out distinctions between them on the basis of prehistoric of bone samples. Nevertheless from the measuring of bone sizes in each anatomical types suggested that the smallest were probably domestic and the largest, wild (Figure 3). In addition, the mortality frequences were made on the basis of tooth eruption and wear patterns, although it is at present impossible to distinguish between the teeth of cattle (*Bibos*) and water buffalo (*Bubalus*), or between wild and domestic animals unless the whole adult mandibles or crania are available. When the bovid teeth from Ban Chiang and Ban Non Wat were analysed, it was found that the great majority of bovids were

of adult body size at death. This may suggest that the bovid teeth come from both domestic and wild animals.

*Bubalus bubalis*

The wild water buffalo is indigenous to Southeast Asia, though it is now very rare. Lekagul and Mc Neely (1977) report a small herd survives in Uthai Thani province. A large bull can stand nearly two metres in height and weight 1,200 kg. It inhabits low-lying well watered terrain. Kijngam (1979) has described the principal distinctions between the bones of water buffalo and cattle. Water buffalo bones are found in the initial occupation at the site of Ban Chiang and Ban Non Wat. Measurable buffalo bones at both sites come from and animals matching in size with the present domestic stock, while some were similar to wild animals (Figure 4). The evidence of burnt bones suggests that the meat was removed for consumption.

**SUIDAE**: *Sus scrofa*

The basic problem in considering pig bones from the sites in Northeast Thai prehistoric contexts, is that separating wild and domestic animals on the basis of immature bones is impossible. Moreover, the lower size range for bones from *Sus scrofa jubatus*, the indigenous wild pig, is not known. Nevertheless, the presence of a prehistoric domestic breed in Northeast Thailand has been demonstrated from the complete pig crania from burials contexts of Non Nok Tha, the prehistoric site in Khon Kaen province, Northeast Thailand. In discussing the Non Nok Tha pig bones, Higham (1975) describes two almost complete mandibles. They display considerable shortening relative to mandibles from wild pig. Thus, Higham concluded that these short mandibles probably derived from domestic stock.

When we turn to the prehistoric pig mandibles found at Ban Chiang and Ban Non Wat, some are similar to the pig mandibles from prehistoric site at Non Nok Tha. This suggests that the prehistoric pig mandibles at both Ban Chiang and Ban Non Wat are also domestic. The mortality frequences of pig dentitions from both sites suggest that most of the pig teeth came from young or immature animals, but some came from adults (Figure 5). Thus we can conclude that the prehistoric pigs in both sites could come from both domestic and wild animals.

**CANIDAE:** *Canis Familiaris*

A detailed consideration of the Ban Chiang canid bones has been undertaken, in conjunction with specimens from related Northeast Thai sites and wild species of wolf, jackal and cuon (Higham, Kingam and Manly 1980). Both multivariate analysis of cranial measurements and distinct morphological differences revealed that the Ban Chiang dogs were domestic, and descended from the wolf. Since there are no native wolves in Thailand, the dog must have been introduced. The size of the Ban Non Wat dog bones match those from Ban Chiang as does their butchering treatment. They were smashed and charred in the same manner as cattle, pig and deer bones. The age at death of pattern of tooth eruption and wear from Ban Non Wat teeth are as followings.

The main age stages of tooth eruption and wear are:

1. Very young dogs, with unworn deciduous teeth.
2. Young dogs, with erupting permanent teeth.
3. Sub adult dogs, with permanent teeth erupted, but unworn.
4. Adult dogs, with permanent erupted and slightly worn.
5. Old dogs, with well-worn permanent teeth.
6. Mandibles without teeth in place, but which are at least subadult and possibly older.

The mortality data are as follows:

| | Ban Non Wat |
|---|---|
| Very young | 2 |
| Young | 2 |
| Sub adult | 4 |
| Adult | 6 |
| Old | 1 |
| At least ubadult | 6 |

There are 21 dog mandibles from Ban Non Wat, 17 of which are at least subadult. This result suggested that the dogs from Ban Non Wat were killed when they were full body size at death (Figure 6).

From the reasons above, it is concluded that Ban Non Wat dogs were domestic, descended from the wolf and were raised at least for food, as were the Ban Chiang Dogs.

### *Gallus* sp.

Chicken bones were commonly rare at Ban Chiang and Ban Non Wat but appeared from the initial occupation to uppermost layers, Neolithic to Iron Age. The identification even to family is ruled out due to the lack of the modern comparative samples. Nevertheless the chicken bones identified were carefully measured and found usually larger than the comparative specimen, a wild jungle fowl from Northern Thailand (Figure 7). The following dimensions of the tarsometatarsal and tibiotarsus bones were obtained.

| Bone | No. | GGL | BBD | SSC | Bp |
|---|---|---|---|---|---|
| tarsometatarsal | 1 | - | - | - | 14.30 mm |
| " | 2 | - | - | - | 15.10 mm |
| " | 3 | - | - | - | 13.90 mm |
| " | Wild specimen | - | - | - | 12.20 mm |

| Bone | No. | Dip | GL | Bd |
|---|---|---|---|---|
| tibiotarsus | 1 | - | - | 13.40 mm |
| " | 2 | - | - | 12.80 mm |
| " | 3 | - | - | 12.90 mm |
| " | 4 | - | - | 11.90 mm |
| " | 5 | - | - | 12.10 mm |
| " | Wild specimen | - | - | 10.05 mm |

## DISCUSSION

The animal remains from the sites of Ban Chiang and Ban Non Wat contrast markedly with those from Palaeolithic and Mesolithic sites in Thailand which have been ascribed to hunting and gathering society (Gorman 1971; Higham 1977, Anderson 1990). Ban Chiang and Ban Non Wat, the cemetery sites, are situated on the agricultural plains under dipterocarp forest. Most of the animal bones from both sites came from occupation layers and burial contexts. The analysis of bone size suggested that cattle, water buffalo and pig were smaller than wild specimens, while dog and chicken bones are bigger. The mortality frequences for cattle, pig and dog show that most come subadult and young animals. Thus the animal bones from Ban Chiang and Ban Non Wat represent domesticated cattle, water buffalo, pig, dog and chicken, although some bones might have been wild one. The study of DNA in tracking the origins and the spread of these domestic animals is now work in progress.

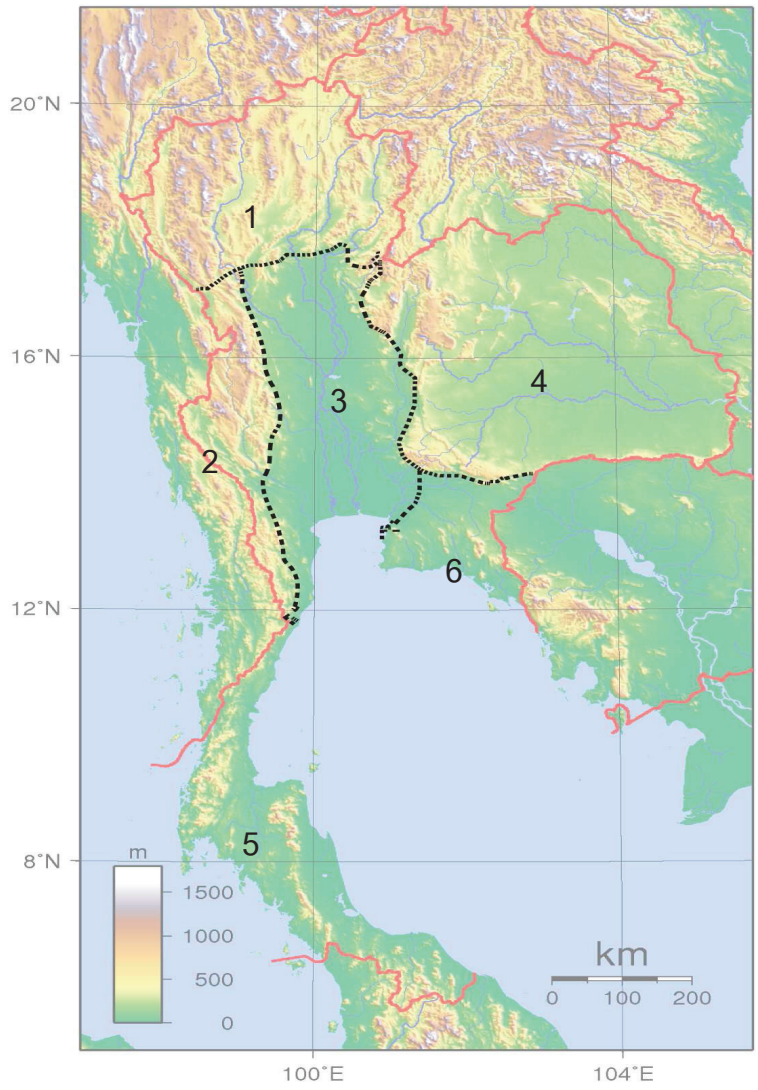



**Figure 1:** The six principal regions of Thailand

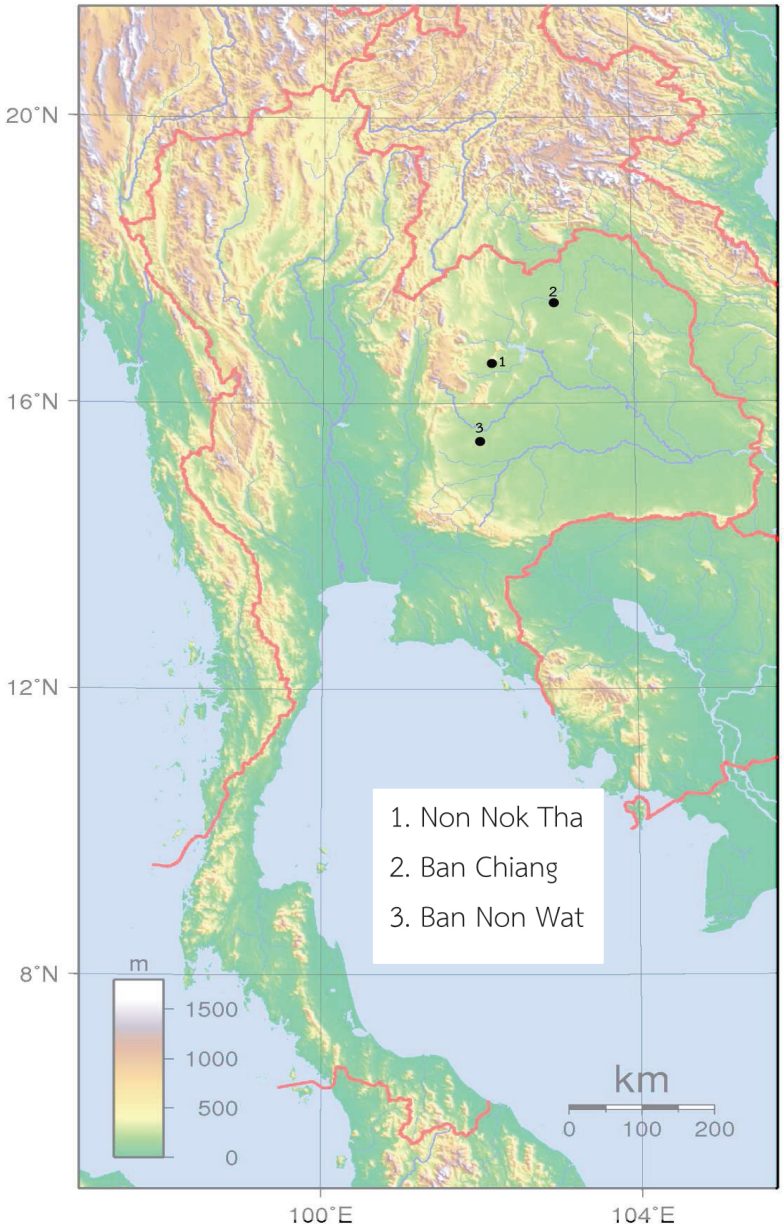
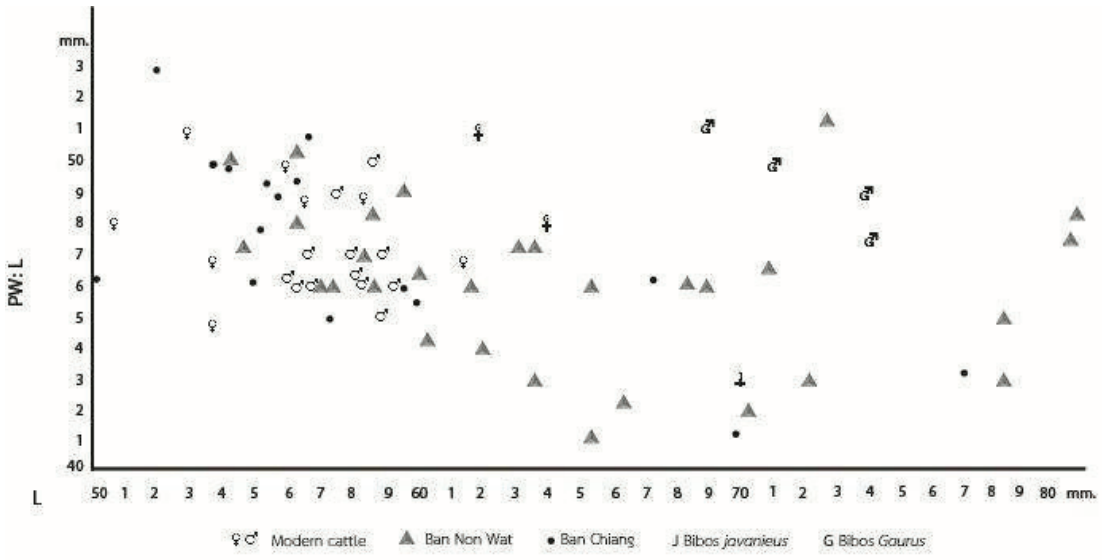



**Figure 2:** The location of the prehistoric sites in Northeast Thailand




**Figture 3 :** The dimensions of the first phalanges of cattle from Ban Chiang Ban Non Wat, wild cattle (Jand G) and modern domestic animals (Length/pw : L)

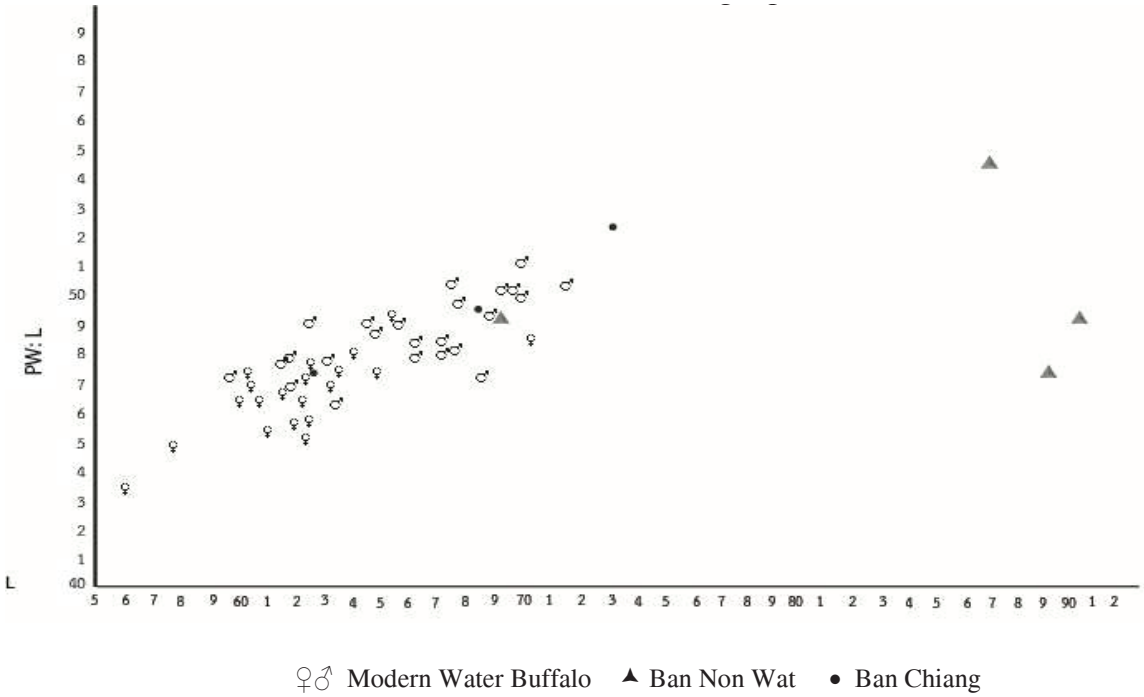



**Figure 4:** The dimensions of the first phalanges of water buffalo from Ban Chiang, Ban Non Wat and the modern domestic animals (Length/PW :)

**Figure 5:** The prehistoric pig manibles showing the tooth eruption.

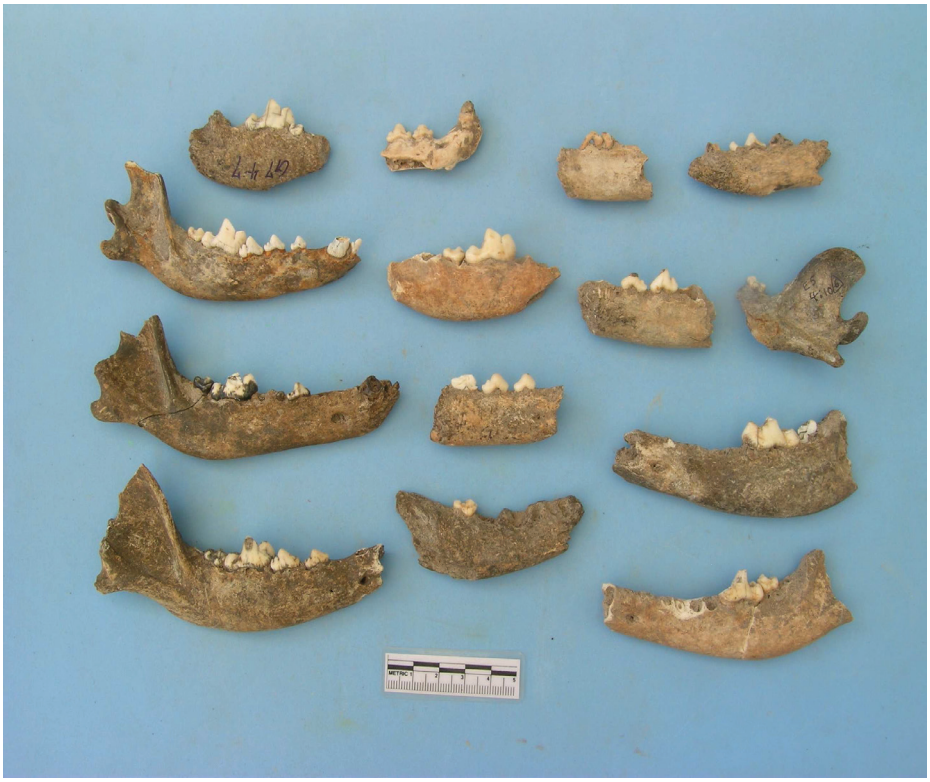


**Figure 6:** Dog mandibles at Ban Non Wat showing tooth eruption and wear pattern

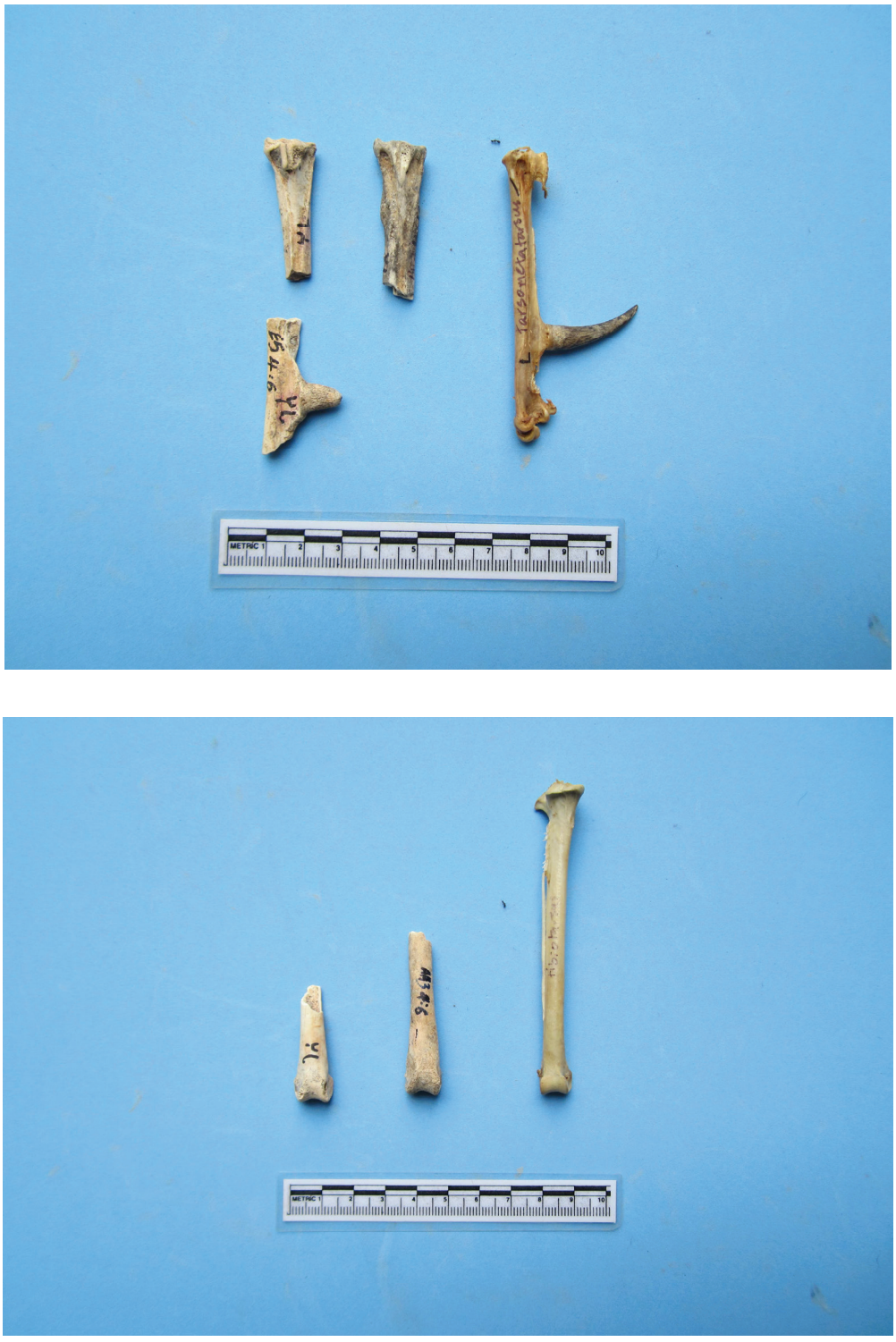


**Figure 7:** The chicken bones, tarsometatarsal (above) and tibiotarsus (below), Compare with the modern wild chicken.

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# New Dimension in HCMR: A Case of Zooarchaeological Approach- Pig Domestication in Japan

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## ABSTRACT

The domestic pig, *Sus scrofa*, is one of the hallmark species representing the global manipulation of wild animals by humans. It is now clear that unraveling the zoological nomenclature of the genus *Sus* in Europe and Asia (there are currently 15 subspecies designations) requires more than the use of traditional methods detailing cranial morphological criteria. One of the fundamentally important issues in zooarchaeology in East Asia and Japan, including the Ryukyu (Okinawa) archipelago, has been the timing and nature of the domestication of the pig from its wild cousin, the wild boar research on this problem in Japan dates to before the 1930's. Therefore, we applied mtDNA and stable carbon and nitrogen isotope analysis in order to separate the domestic pigs from the wild boars in bone samples recovered from the Japanese mainland, Ryukyu archipelago, Korea, and Vietnam. The results demonstrate that the domestic pig and wild boar can be distinguished by these methods. Carbon and nitrogen isotope analysis shows that two feeding patterns developed during domestication, one based on consumption of human leftovers and human excrements (high $^{15}N$ content) and the other utilizing cultivated C4 plants. We conclude that the isotopic signatures of *Sus* remains from archaeological sites in East Asia form three clusters. Two clusters reflect two different methods of raising pigs in Korea. In addition, wild boars consuming solely natural C3 plants show features similar to sika deer. Genetic relationships were examined using 574-bp mitochondrial DNA control region sequences for prehistoric *Sus* bones on the Ryukyu Islands, domestic pigs on the Ryukyu archipelago and the Asian continent, and modern wild boars. Some of the *Sus* samples from Yayoi sites (ca. 2,000 cal. BP) and the Premodern sites (ca. 16-19th c. AD) clustered phylogenetically with East Asian domesticated pigs. This indicates that domestic pigs were bred on Okinawa during the Yayoi Period, 2000 years ago. Furthermore, it is apparent that the *Sus* sp., although we may not call them domestic pigs, that had been bred appeared by at least 7000 BP on Okinawa.

**Key words:** animal domestication, stable isotopic analysis, ancient DNA, D-loop, diet, pig, wild boar *Sus scrofa,* zooarchaeology

**A myth of not eating habit in Japanese history**

Before the end of the Jomon period that was flourished since 15000 BP depending on the regions of the Japanese archipelago, meat was commonly consumed by people who made their livelihood by hunting, gathering and fishing with some manipulated plants. Once rice paddy cultivation was diffused in the Yayoi period from China via Korea probably 7$^{th}$ to 5$^{th}$ BC., the ratio of meat eating habit dropped and the archetypal Japanese-style diet with rice and a main dish was established. As the Buddhist tenet against killing was introduced in the middle of sixth century AD., and the concept of *kegare* (uncleanness) of beasts or meat eating developed in the Early Heian era, that is 9-11 AD. Japanese people began avoiding meat eating and took animal protein mainly from seafood until in the closing days of the Tokugawa Shogunate or the end of the Edo period in 1868 and people began to eat land mammals again since the Meiji period being under the influence of European culture.

The above may be the story that the majority of most Japanese believe about the history of their eating habits, or their dietary culture. However, through my research on bones and other faunal remains found at various sites though various periods in Japan, I have become aware of numerous animal bones with artificial damage marks apparently made by edged tools when chopped or when the flesh was stripped, showing that the animals were eaten. I began to believe that throughout all periods people in Japan have been eating meat regularly not only for some specific classes but for the ruling classes and holy classes, like monks and the Shinto priests (Matsui, 1994[1]). Of course, the word "people in Japan" here includes various social classes from Buddhist monks and Shinto priests to aristocrats and warriors who handed down heritages of the ancient to the medieval and Pre-modern periods , that is called the Shogunate or Edo era. Although rural areas and impoverished neighborhoods in urban marginal areas where prominent relics rarely remain are unfortunately not often targeted for excavation, it seems certain that meat eating had been rooted deeply in the lives of the Daimyo, or senior samurai in Edo period and urban residents (Matsui, 2011).

Then, what is the reason for this discrepancy? My assumption is that the conventional view of anti-meat-eating history is mainly based on literary documents and folkloric narrative researches with an influence of *kokugaku（the Japanese classics）* in the Edo period which emphasized the uniqueness of Japan. However, written records scarcely include "facts" that writers did not wish people of the same and later periods to know. Interview-based folkloric researches may also involve similar hesitation by the informers. Some information that were difficult to mention, such as the realities regarding sex and meat eating, are less likely to be recorded either in written or oral forms.

**Zooarchaeology revealed the meat eating habit in Japanese History**

Archaeology, on the other hand, has the advantage of being able to reveal "facts" people in the past attempted to hide away through excavation. In particular, food-related information which is rarely recorded in written documents may be brought to light by excavating middens at premises, where people of the time buried the evidences of meat eating in their backyards.

Archaeological excavations have unearthed wild boar bones at Shimotakabora site in Izu Oshima in the Initial Jomon: 10000-7000 BP., and other island sites where no wild boar naturally inhabited. Likewise, in Hokkaido across the Tsugaru Straits where no wild boar lived, wild boar bones have been found at the sites of the Late or the Final Jomon settlements, approximately dated around 2000 BP. and later, as well as Epi-Jomon settlements of the same period as Yayoi mainly in the Oshima Peninsula of southern Hokkaido. There are several Jomon sites in Honshu where a number of mandible bones of infant wild boar alone are buried; such "special charred" conditions also suggest the possibility of wild boar keeping. Furthermore, it has been known since before World War II that the Okhotsk people that prospered in East Hokkaido from the 6th to 12th centuries had kept many Sakhalin domesticated pigs.

Since 1989 when Toyohiro Nishimoto of the National Museum of Japanese History reported the existence of domesticated pigs at Shimogori-Kuwanae Site in Oita City (Early Yayoi), domesticated pigs in Yayoi period became a major topic of archaeology. The evidence presented by Nishimoto (1991[2]) included pyorrhea typically seen when kept by humans, as well as morphological changes such as undeveloped jaws due to soft food, short nose, and wider face.

I myself have reported that among small Rukyu wild boar *(Sus scrofa riukiuanus)* remains found in Gushibaru Shell Midden in Ie Island, Okinawa (parallel Yayoi), there were remains of individuals as large as the Japanese wild boar *(Sus scrofa leucomystax)* in 1997 (Matsui, 1997). After a long deliberation and comparison with other specimen, I proposed that the individuals were domesticated pigs from outside Okinawa. I received no particular response from the academic society, anticlimactically.

Because it is easy to distinguish domesticated pigs from wild boars with their flesh and skin but is very difficult to differentiate fractured bones, many researchers disagreed with Nishimoto's Yayoi domesticated pig theory.

**Controversy of pigs or wild boar by ancient mtDNA**

Actually, in the same issue of a journal titled *Quartenery Journal of Archaeology*, two researchers concluded that there were no domesticated pigs in the Yayoi period; archaeologist Makoto Watanabe from an archaeological point of view (Watanabe 2004), and geneticist Tomoo Ozawa (Ozawa 2004) based on a mitochondrial DNA lineage analysis. Their clear denial in the journal seemed to be easily accepted by many archaeologists who do not understand genetic principles. One of many journals annually featuring the trends of the academics summed up the controversy over the existence of Yayoi domesticated pig saying that it had been conclusively proven that domesticated pigs did not exist during that period. However, Watanabe's theory hardly seemed (at least to me) logically convincing, and our group found out that Ozawa's conclusion was based on the amplification and comparison of a mitochondrial control region which is not necessarily effective for distinguishing domesticated pig from wild boar. In fact, with efforts of Naotaka Ishiguro of Gifu University and others(Morii 2002), we adopted a method to divide a sequence too long to amplify at once into three fragments, then amplify and connect them together. We constructed a phylogenetic tree consisting of Japanese wild boar, Ryukyu wild boar, Northeastern Asian wild boar, native Chinese domesticated pig, and other extant *Sus* species, put amplified genes from *Sus* bones from archaeological sites, and succeeded to determine each of their genealogical positions.

As a result, we figured out that the Ryukyu wild boar belongs to the strain of Southeast Asian wild boars, contrary to conventional assumption to categorize it as a variety of Japanese wild boar, and judging from unique base in unique position, *Sus* remains from Shimizu Shell Midden (Parallel Yayoi) in Kume Island are also likely to be of Southern origin as well, while having similarities to the Ryukyu wild boar. Having examined many specimens we collected from Miyashita Shell Midden in Goto Islands, Nagasaki (Early Jomon), Agata Shell Midden in Imabari, Ehime (Early Yayoi), and the Kitasaya Site of Miyamae-gawa Sites in Matsuyama, Ehime (the end of the Yayoi or the beginning of the Kofun), we also found the existence of individuals having genes common among modern East Asian indigenous domesticated pigs and not seen in Japanese wild boar. This means that domesticated pigs from the continent had been brought into the Japanese Archipelago during the Yayoi period or earlier, although specimens from Miyashita Shell Midden, which is dated as old as the Late Jomon, need to be reexamined including the dating of the bones.

**Application of stable isotopic analysis on pig bones**

In order to prove the existence of domesticated pigs more convincingly, we further

studied the diet. Animal bodies including ours are made of what they have been eating since their birth. Thus there is a method to find out what an animal had eaten from the stable isotope ratio of carbon or nitrogen in animal bone. Due to the photosynthetic mechanism of plants, the stable isotope ratios of $^{12}C$ and $^{13}C$ differ between C3 plants such as acorns, rice, and wheat, and C4 plants such as millet. In addition, the isotope ratios of $^{14}N$ and $^{15}N$ tend to be larger in higher positions of the food chain. In North American anthropological studies this method has been established as a measure to clearly prove the shift from hunting and gathering economy where people focused on C3 plants, to a C4 plant corn growing economy by comparing stable carbon and nitrogen isotope ratios found in human bones. I asked Masao Minagawa, an expert of this method whom I have known for years, if it was possible to apply the method for distinguishing wild boars from domesticated pigs. That was where our joint study started (Minagawa et al. 2004). Seeking specimens for analysis, I traveled South Korea and Okinawa, collected bones identified as wild boars as well as deer and human bones for comparison from sites of Yayoi period in Okinawa, Kyushu, Chugoku, Shikoku, and Kinki regions, in addition to major archaeological sites in the Korean Peninsula of the same period. We were the pioneers of this method in the world, and the results exceeded our expectation. We analyzed isotope ratios of bones that have been understood to be of wild boars. The isotope ratios of wild boar remains from Jomon shell middens in the Kanto region turned out to be almost the same as those of plant-eating deer bones, showing a high "wildness." On the other hand, bones from sites in Okinawa or the Korean Peninsula of the period parallel to the Yayoi included a high rate of those with high $^{13}C$ from millets, those with high $^{15}N$ from fish and other marine products, and those showing values similar to humans, suggesting human food scraps and manure (Matsui et al. 2005).

To our surprise, samples from Noguni B Shell Midden of the Early Jomon around 7900-6500 BP. in Yomitan Village, Okinawa showed high nitrogen isotope ratios, contrary to our expectations that they were typical wild boar. The values are probably the way they are because of the consumption of human food scraps, manure, or marine products. This site is dated from the initial stage of the Early Jomon and a radiocarbon dated to approximately 7900 years BP. It is suggested that wild boars found at a further earlier site, the aforementioned Shimotakabora Site (Initial Jomon) in Izu Oshima, Tokyo, may had been brought in by the Jomon people. The same phenomena are pointed out for Mediterranean islands such as Cyprus and Crete Island where wild boars, goats, sheep, and cattle are unearthed from sites dated 9000 BP at earliest, which is earlier than the period when wild boars in West Asia becomes domesticated as domesticated pigs. Remains of *Dama dama* and *Cervus elaphus* which have never been domesticated were also excavated from the same sites, suggesting that

the Ceramic or Neolithic people brought wild animals to the islands and released them. It seems the further research advances, the more difficult it becomes to draw a clear boundary between wild and domesticated animals.

## CONCLUSION

Aside from individual domesticated pigs, the technique of domestication may have been introduced from West Asia and China and was applied to wild boars widely distributed in every region of the Eurasian Continent. In that case, it would be difficult to prove domestication from genetic lineage analysis. Besides, considering the number of offspring a female domesticated pig can produce during her life, it is much more efficient to introduce a superior sire to cross with local female wild boars than bringing a breeding pair. In fact, in Ie Island in Okinawa where I have been researching, large male wild boar bones apparently of a sire were found. Because mitochondrial genes are matroclinous, i.e., inherited only from the mother, it will as well be impossible to genetically prove the ancestry of domesticated pigs.

We are expanding our field further to Russian coastal oblasts, South Korea, Southern China, Taiwan, Vietnam, and Cambodia. Our plan is to conduct a comparative study on our achievements which are fragmentary at present; we would like to take a panoramic view covering whole East Asia.

We know too well that there still remain many questions to be addressed in order to prove the existence of domesticated pigs. For me it took more than 10 years to discuss the existence of domesticated pigs, as described above. The origin of our endeavor is inspiration we got when examining archaeological remains in our hands one by one. Seeking ways to prove this hypothesis led by inspiration, we looked for new researching methods and pursued new "facts" through joint studies with researchers from various fields. In moments when I realize I'm reaching such facts, I pause to appreciate my luck at having decided to be a researcher.

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# Phylogeography and Demography of the Red Junglefowl and Its Domestication Process Revealed by Mitochondrial DNA Sequences

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## ABSTRACT

Red jungle fowl (RJF) inhabits in South Asia and Southeast Asia, and is divided into five subspecies (*Ggallus gallus gallus, G. g. spadiceus, G. g. jabouillei, G. g. murghi* and *G. g. bankiva*). It is well known that this species is the wild progenitor of domestic chickens. To reveal the origin of chicken domestication is a topic of considerable interest to humanity. However, there are two hypotheses on the origin of domestication: a single origin situated in Thailand and its adjacent regions, or multiple origins in South and Southeast Asia. In this study, we determined mitochondrial D-loop sequences of 40 RJF specimens and 43 domestic chickens. We first estimated phylogenetic relationships among RJF (40 our own specimens and 87 Genbank data). In this analysis, RJF specimens, except for *G. g. bankiva* and Sumatran *G. g. gallus,* formed a continental super clade (CSC), in which we detected four clades (clades 1 to 4). Whereas clades 2 and 4 were composed of *G. g. murghi* only, clades 1 and 3 did not reflect taxonomic status of subspecies. Taken together, these phylogenetic relationships and estimated ancestral population sizes suggested that genetic differentiation of *G. g. bankiva*, *G. g. spadiceus*, and *G. g. gallus* were caused by i) the vicariance of their distribution area coincident with marine introgression-regression cycles in the Middle to Late Pleistocene, and ii) differentiation between *G. g. murghi* and other RJF caused by dispersal events in the Late Pleistocene. Finally, to estimate the origins of domestication, we constructed a comprehensive phylogenetic tree of RJF and domestic chickens. Our results suggest that chicken domestication occurred multiple times in South and Southeast Asia. However, Sumatran *G. g. gallus* lineage and one *G. g. murghi* lineage (clade 4) do not appear to have been involved in domestication events.

**Keywords:** chicken, red junglefowl, mitochondrial DNA, domestication, phylogeny

## INTROCUTION

Elucidating the origins and history of domesticated animals is a subject of considerable interest to humanity, because it is closely related with development of human culture (Diamond, 2002). The domestic chicken is widely farmed around the world for purposes as diverse as food, ornamental bird, gamecock and religious affiliation. It is thought that wild progenitor of the domestic chicken is red jungle fowl (*Gallus gallus*), which is distributed in South Asia and Southeast Asia (Nishida et al., 1985, 1992) and its domestication dates back to approximately 5400 BC (Underhill, 1997). Wild populations of red jungle fowl (RJF) are morphologically divided into five subspecies; *G. g. bankiva* distributed in Java, Bali, and Lombok in Indonesia; *G. g. gallus, G. g. spadiceus* and *G. g. jabouillei* distributed in Southeast Asia; and *G. g. murghi* distributed in South Asia (Fig. 1).

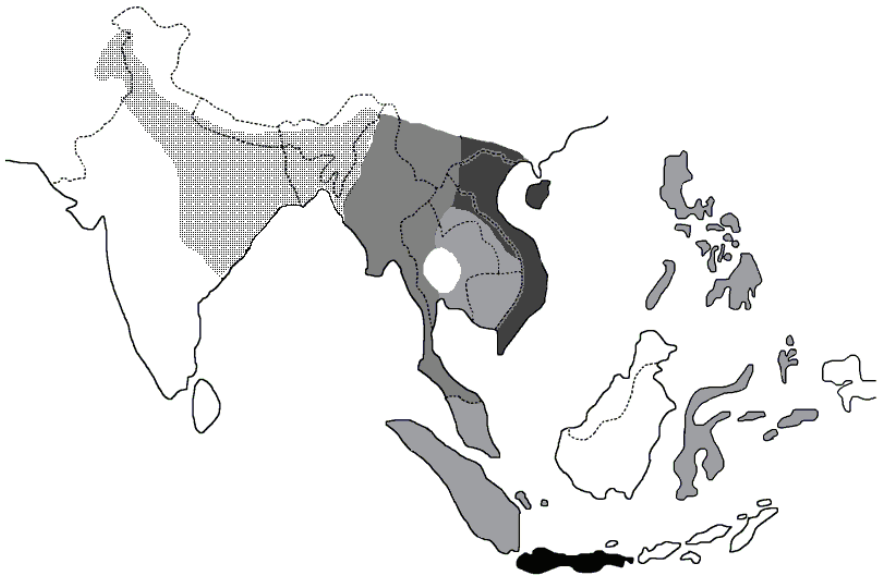

Figure 1. Distribution of subspecies of red jungle fowl (RJF) in South and Southeast Asia.

However, with regard to location and wild population, the domestication origin of chickens remains unclear, and there is still intense debate surrounding this subject (Fumihito et al., 1996; Liu et al., 2006; Kanginakudru et al., 2008). To date, two hypotheses regarding the domestication origin of the chicken have been proposed by molecular phylogenetic studies. Fumihito et al. (1996) suggested that the domestic chicken originated from a single lineage of continental RJF, which was distributed in Thailand and its adjacent regions. On the other hand, Liu et al. (2006) and Kanginakudru et al. (2008) suggested that there were multiple domestication events in South and Southeast Asia. Both studies lacked comprehensive taxon sampling for their phylogenetic analyses (for both wild populations of RJF and domestic chicken breeds). Thus, these two opposite hypotheses remain to be verified.

Mitochondrial DNA (mtDNA) is (mostly) maternally inherited and has a rapid evolutionary rate (Brown et al., 1979). Therefore, this gene is one of the most powerful genetic markers for assessing genetic differentiation within a relatively short evolutionary

time. In this study, we intended to elucidate the following subjects; 1) phylogenetic relationships among RJF subspecies, 2) phylogeographic, evolutionary and demographic history of RJF and 3) single or multiple origins of chicken domestication.

## MATERIALS AND METHODS

### DNA samples and sequence determination

Total genomic DNA samples were extracted from whole blood samples by Invisorb Spin Blood Mini Kit (Stratec Molecular, Germany). In this study, we analyzed D-loop sequences of mtDNA. PCR primers for amplification of D-loop were derived from Oka et al. (2007). The PCR amplification profile consisted of 35 cycles of denaturation at 94°C for 45 sec, annealing 55°C for 45 sec, and extension at 72°C for 45 sec. The PCR reaction mixture contained 2.5 units Ex *Taq* polymerase (Takara), 1×Ex *Taq* buffer, 0.2mM dNTPs, 1μM each primers, and 100 ng of genomic DNA, in a final volume 25 μl. To verify the amplified DNA fragment, we confirmed by electrophoresis in a 1.0% agarose gel (Wako) and stained with ethidium bromide for fragment characterization via ultraviolet transillumination. To remove excess primers and nucleotides, PCR products were treated with isopropyl alcohol precipitation. The precipitation mixture contained 20 μl of isopropyl alcohol and 250 mM NaCl for 20 μl of PCR product. The internal primers for sequencing were F3 (5'-GGT TCT CAA CTA CGG GAA C-3'), F2 (5'-TGG TTC CTC GGT CAG GCA CAT CC-3'), R3 (5'-CAG TGC CAT GCT TTG TGG GT-3') and R2 (5'-CGC AAC GCA GGT GTA GTC-3'). Sequence determination was performed by a sequencing service company (Macrogen Japan).

### Phylogenetic and demographic analyses

The nucleotide sequence data determined in this study and those retrieved from previous studies (Fumihito et al. 1996, Liu et al. 2004, Oka et al. 2007, Kanginakudru et al. 2008, Silva et al. 2009, Berthouly-Salazar et al. 2010) were automatically aligned using Clustal W (Thompson et al. 1997) and carefully verified by eye. All gaps were retained and treated as missing data (total length of the alignment was 1229 bp). Phylogenetic trees were inferred by RAxML ver. 7.2.6 (Stamatakis et al. 2008) with the GTR+I+□ model. The confidences of the internal branches were evaluated by the rapid bootstrap method (Stamatakis et al. 2008) with 1000 replications.

Ancestral population sizes were estimated by the Bayesian Skyline Plot method with BEAST ver. 1.7.4 (Drummond and Rambaut 2007), using an HKY+□ nucleotide substitution model. All gaps were eliminated, and an alignment of 350 bp in length was retained for these analyses. A strict clock model with a substitution rate of 7.0 x $10^{-8}$ /site/year was assumed (Sasaki et al. unpublished). The MCMC was conducted under the following conditions: The total length was 100,000,000 generations, with trees and parameters sampled every 1000 generations. The first 10,000,000 generations were discarded as burn-in. Verification of MCMC convergence and summarization of posterior parameters were carried out with TRACER ver. 1.5 (http://evolve.zoo.ox.ac.uk/software/ 2003).

Table 1. Specimens used in this study.

| Breeds/Species | No. of individuals | Locality/Source |
|---|---|---|
| Ayam | 2 | Republic of Indonesia |
| Bangkok | | |
| Ayam Kate | 2 | Republic of Indonesia |
| Brahma | 7 | United States of America*1 |
| Cochin | 3 | United States of America*1 |
| Cornish | 4 | United States of America*1 |
| Jersey | 8 | United States of America*1 |
| Giant | | |
| Langshan | 6 | United States of America*1 |
| Spanish | 5 | United States of America*1 |
| Wyandotte | 6 | United States of America*1 |
| *Gallus* | 1 | Thailand |
| *gallus* sp.*2 | | |
| *Gallus* | 1 | Unknown |
| *gallus* sp.*2 | | |
| *Gallus* | 38 | Bangladesh |
| *gallus* sp.*2 | | |

*1=Murray McMurray Hutchery. *2=subspecies name is unknown.

## RESULTS AND DISCUSSION

**Phylogenetic relationships among red jungle fowls**

Figure 2 shows the unrooted maximum likelihood (ML) tree among the specimens of RJF based on mitochondrial D-loop sequences. *G. g. bankiva* was relatively far from the other RJFs in genetic relationship as demonstrated previously (Fumihito et al., 1996; Liu et al., 2006). We also confirmed that the three *G. g. gallus* lineages derived from South Sumatra of Indonesia formed a distinct, independent clade with relatively distant position from the continental specimens as mentioned by Fumihito et al. (1996) (Fig. 2). The phylogenetic tree indicated a difference in geographical distribution of RJFs between islands and the continent. Therefore, we designated the clade constituted by continental RJFs as the "continental super clade" (CSC) (Fig. 2). In the CSC, four major clades of continental RJFs could be recognized, except for one Bangladeshi RJF and one Yunnan *G. g. spadiceus*. In addition, the clade 1 was subdivided into four subclades (Fig. 2; Table 2 shows a summary of the clade classification). Previously, Fumihito et al. (1996) raised questions about taxonomic status of *G. g. gallus* and *G. g. spadiceus* subspecies in particular. Our phylogenetic tree also indicated nested and highly intermingled phylogenetic relationships with respect to subspecies classification (see clade 1 and 3 in Fig. 2 and Table 2). However, it appeared that some populations showed evidence of genetic differentiation, as shown in *G. g. murghi* single clades (see clade 2 and 4 in Fig. 2 and Table 2).

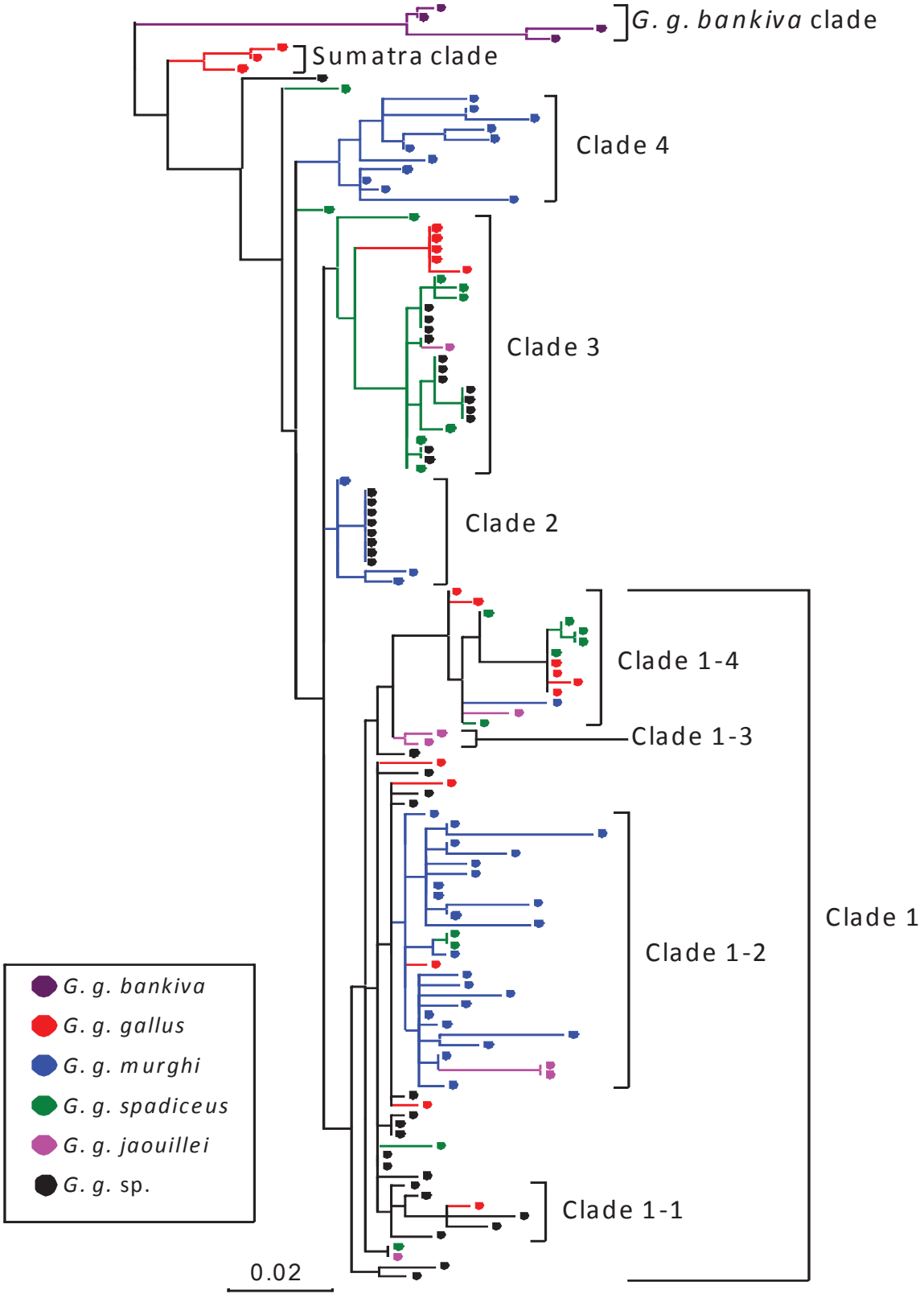


Figure 2. Unrooted maximum likelihood tree among RJFs. See text for details of estimation.

Table 2. Taxonomy, locality and number of individual contents of each clade in Fig.2.

| Clade-subclade | Subspecies | Locality | No. of individuals |
|---|---|---|---|
| 1-1 | *G. g. gallus* | Thailand | 1 |
| | *Gallus gallus* sp.*1 | Bangladesh | 5 |
| 1-2 | *G. g. gallus* | Thailand | 2 |
| | *G. g. spadiceus* | Yunnan, China | 1 |
| | | Myanmar | 1 |
| | *G. g. jaouillei* | Unknown | 1 |
| | *G. g. murghi* | India | 23 |
| 1-3 | *G. g. jaouillei* | Hainan, China | 2 |
| 1-4 | *G. g. gallus* | Thailand | 4 |
| | | Vietnam | 2 |
| | *G. g. spadiceus* | Yunnan, China | 3 |
| | | Myanmar | 3 |
| | *G. g. murghi* | India | 1 |
| | *G. g. murghi* | India | 2 |
| | | Nepal | 1 |
| | *Gallus gallus* sp.*1 | Bangladesh | 8 |
| 3 | *G. g. gallus* | Vietnam | 6 |
| | *G. g. spadiceus* | Yunnan, China | 5 |
| | | Myanmar | 2 |
| | *Gallus gallus* sp.*1 | Bangladesh | 13 |
| 4 | *G. g. murghi* | India | 11 |

*1=subspecies name is unknown spcimen.

**Estimated ancestral demography of the red jungle fowl**

Our estimated fluctuations in the ancestral population sizes of the each subspecies of RJFs are also shown in Figure 2. The population sizes of *G. g. gallus*, *G. g. spadiceus,* and *G. g. bankiva* (Fig. 3a, b, c, respectively) have been basically stable, or shown slight increase or decline from the TMRCA (time of the most recent common ancestor). By contrast, that of *G. g. murghi* (Fig. 2 d) shows rapid population expansion beginning around 50,000 years ago. The significant negative Tajima's D of this subspecies also supported an ancestral recent population expansion event (-1.834: P-value < 0.05).

We also estimated fluctuations of the ancestral population size of RJF as a whole, excluding the subspecies *G. g. murghi* (Fig. 3e). Interestingly, while the population sizes of each subspecies have been stable, those of the whole RJF clade show moderate increase. Taking these demographic analyses and phylogenetic relationships together, we have constructed the following evolutionary scenario for RJF.

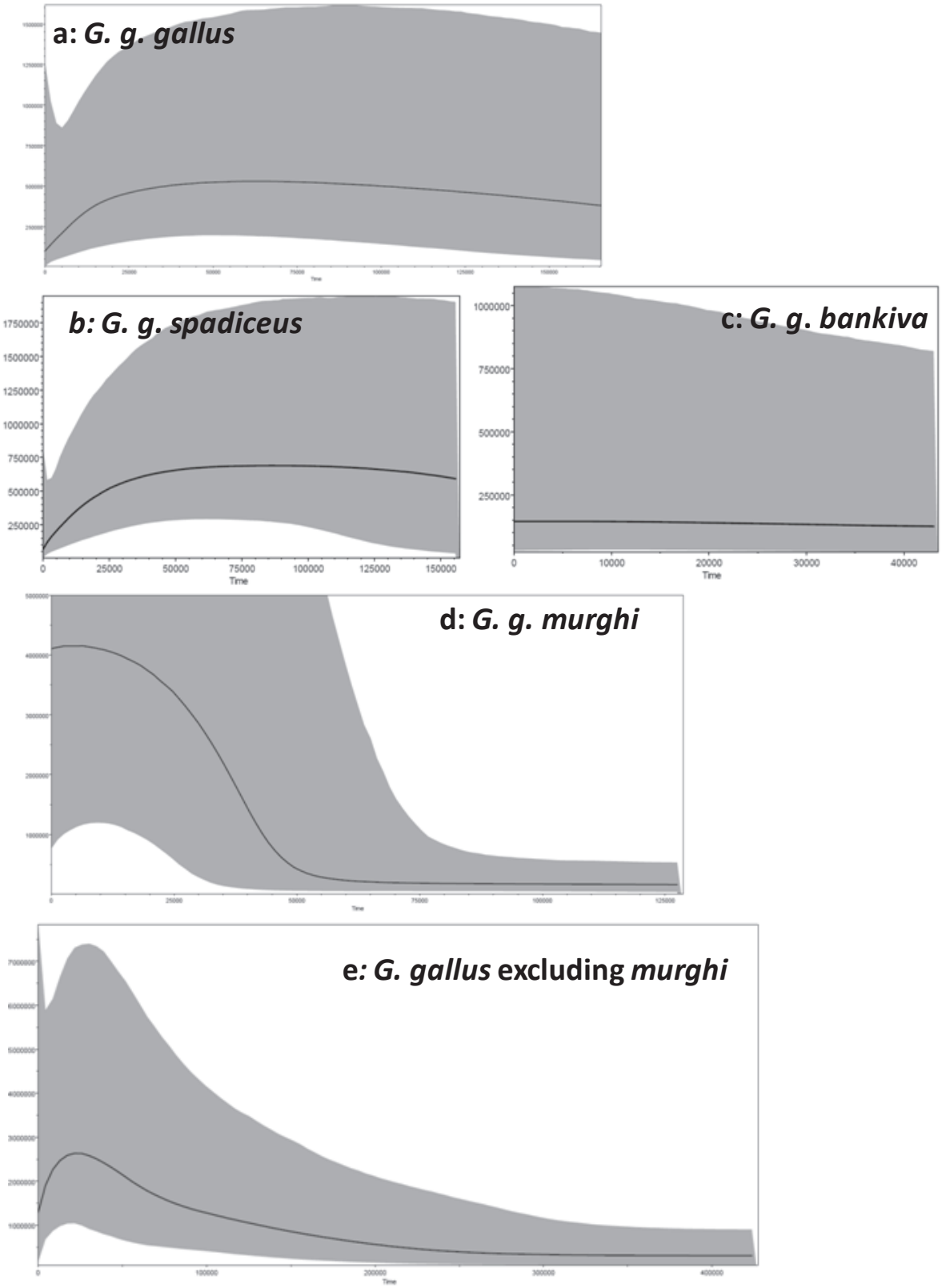


Figure 3. Bayesian Skyline Plot of each RJF subspecies. The X axis represents time (years before present) and Y axis indicates the effective female population size multiplied by generation interval. Blue belt indicates range of 95% confidence intervals.

i) The subspecies *bankiva* branched earliest from other subspecies. The divergence time between *G. g. bankiva* and other subspecies was estimated to be about 625 Ka (kilo annum or thousands of years), assuming a substitution rate $7.0*10^{-8}$/site/year (data not shown). This would have been during the transition between Glacial C (the glacial period) and Cromerian V (the inter-glacial period) in the Middle Pleistocene.

ii) After the divergence of *bankiva*, the total population size of other RJF shows a moderate increase. As we discuss below, the geographical distribution area of the ancestral RJF seems to be almost the same as that of extant *G. g. spadiceus*. This increase in ancestral population size accelerated beginning around 100,000 years ago (Fig. 3 e), and its timing is almost coeval with a marine regression that occurred during the Wisconsinan period (the last glacial period). It is well known that "Sundaland" formed during this period. Sundaland was a broad area encompassing the present-day Sunda shelf and Asian continental shelf, and was exposed by marine regression coincident with the glacial period. The inferred increase in population size during this time may be related to the expansion of their distribution area caused by the formation of Sundaland. The stable or even declining population size of *G. g. spadiceus* may suggest that this subspecies has maintained its ancestral distribution area, and has not expanded its range.

iii) By contrast, even though it was very moderate, the increasing population size of *G. g. gallus* in this period may imply that this subspecies is descended from the pioneers that colonized the modern distribution area of RJF. Taking into account the distribution area of this subspecies, expansion first occurred eastward and then southward. Finally, the ancestor of *G. g. gallus* expanded throughout the whole of Sundaland. The last glacial maximum was 20000 years ago. Afterward, sea-level rose due to global warming, and resulted in the reduction of the land mass of Sundaland, leading naturally to a reduction of the distribution area of *G. g. gallus.*

Interestingly, their population size also turned to decline. During this process, the populations of Sumatra, Java, and the islands of Philippines should have become geographically isolated. The maximum marine introgression was 7000 years ago. During this period, the sea-level was about 10 m higher than today, and both the Kura Isthmus that connects the Asian continent and Malay Peninsula, as well as a broad area of the Chaophraya River plain, were both submerged undersea. This process would have led to the isolation of *G. g. gallus* populations of the Malay Peninsula from those of the area comprising modern Thailand, Laos, and Vietnam. The genetic differentiation of *G. g. gallus* and *G. g. spadiceus* might be caused by these "vicariance" events accompanying marine introgression. Since these events should be very recent, genetic differentiation between *G. g. gallus* and *G. g. spadiceus* remains incomplete; for this reason, the ancestral polymorphisms of mitochondrial DNA can be still observed.

iv) The genetic differentiation of *G. g. murghi* and others may result from a different process. The original ancestral population size of *G. g. murghi* (Indian population) was small and then increased rapidly from 50000 Ka (the Late Pleistocene). However, the population size of the RJF from Bangladesh was stable. A geological feature such as the Ganges River could have served as a geographical barrier, restricting the migration of the RJF. We hypothesize that relatively small populations of the ancestral RJF occasionally moved westward across this barrier, and then rapidly expanded in this new territory. This small population evolved to *G. g. murghi.* Thus, genetic differentiation of *G. g. murghi* seems to

have been be caused by a dispersal event. Concerning the origin and evolution of *G. g. jabouillei*, available data remains too limited to construct an hypothesis of their evolutionary process.

**Multiple origins of domestic chickens**

To examine the origin of domestic chickens, we analyzed phylogenetic relationships among RJF and 262 chicken specimens (Fig. 4). Domestic chickens were found to be widely distributed in clades that were defined by the RJF phylogenetic tree (Fig. 2). Liu et al. (2006) had suggested previously that domestic chickens occurred in multiple continental RJF lineages based on their phylogenetic tree constructed from mtDNA D-loop sequences.

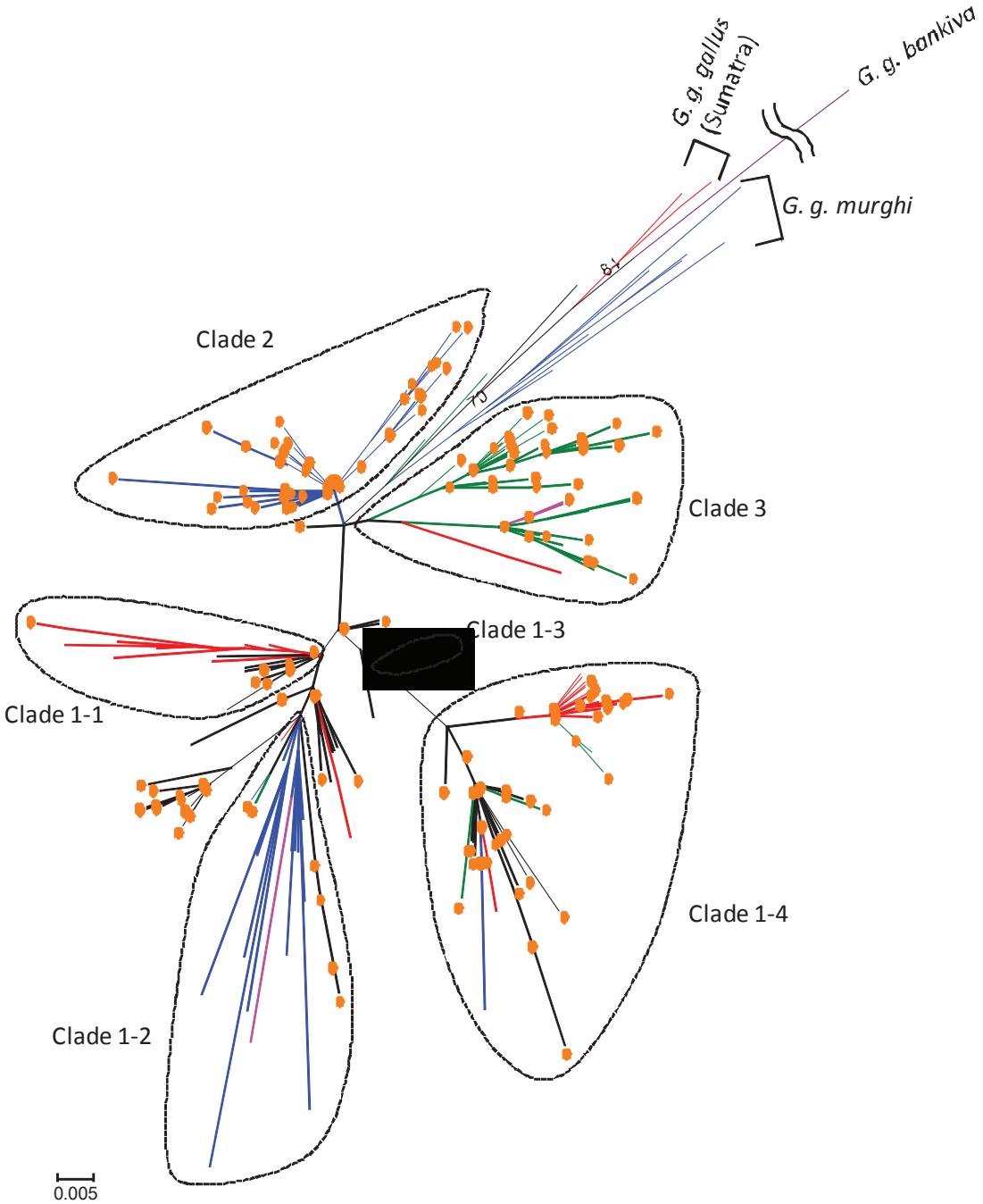


Figure 4. Unrooted maximum likelihood tree among RJF and domestic chickens.

Phylogenetic positions of each domestic chicken are indicated by orange circles.

The present study's clade composed of Sumatran *G. g. gallus,* seemingly corresponds to their clade H. It is probable that the RJFs in clade A have never been domesticated in their history, as suggested previously (Fumihito et al., 1996; Liu et al., 2006). Notably, Clade 4 was also constituted by RJF (*G. g. murghi*) only. This clade was newly discovered as a nondomesticated lineage in the present study. Our phylogenetic tree indicated that chicken domestication occurred in three of four clades composed of continental RJF, namely clades 1 (except for clade 1-3), 2 and 3. Clade 1-3 did not contain a cluster with domestic chickens. This phylogeny may indicate that clade 1-3 was not involved in chicken domestication. The RJF belonging to clades 1 and 3 were distributed in South and Southeast Asia, whereas the RJF belonging to clade 2 were exclusively distributed in South Asia (Table 2). This result indicates that chicken domestication occurred multiple times in various areas in South and Southeast Asia. In conclusion, we suggest that chicken domestication occurred multiply in a broad area of South Asia and Southeast Asia. Our study supported the hypothesis suggested by Liu et al. (2006).


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# Comparative Genomics Among Red Junglefowl, Thai Native Chicken and Commercial Line

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## ABSTRACT

The objectives of this study were to investigate the genetic variation of Red junglefowl (RJF), indigenous chicken, commercial broiler and layers using microsatellites and to compare microsatellites and functional genes for genetic assessment before utilizing the chicken genetic resources with efficiency. For the first objective, four Thai indigenous strains and three commercial lines were genotyped genetic variability and divergence using twenty microsatellite loci of which sixteen are recommended by Food and Agriculture Organization. The highest (0.81) and lowest (0.77) average of expected heterozygosities were observed in Indigenous chicken (Dang; DG) and commercial layer (Isa Brown; IB), respectively. Four genetic clusters were detected: first group consisted of layers (IB and White Leghorn, WL); second group was broiler; third group consisted of non-black feather indigenous chicken (Chee; CH, DG and Leung Hang Khoa; LK); and the fourth group was black feather indigenous chicken (Pradu Hang Dam; PD). This study also revealed that PD is suitable to be developed as a meat type chicken due to lower genetic distance between PD and broiler. Moreover, eighteen microsatellites revealed Bhutanese native chickens; Yuebjha Narp (Black plumage chicken) represented the lowest genetic variability. A Neighbor-Joining tree was constructed to show genetic relationship while principal component analysis plot revealed Bhutanese native chickens should be prioritized for conservation because of their genetic distinctiveness. When, we compared the efficiency of genetic characterization of chicken populations that had been under different intensities of selection using selective functional gene versus microsatellite marker analyses. A neighbor-joining tree from Nei's genetic distance was constructed to show genetic relationships. A similar pattern was found in both functional genes and microsatellites: three groups were formed, consisting BR and WH separated into two groups and the third group was RJF and TIC. We tried to confirm tree by a principal component plot based on individual similarity using Dice's coefficient based on functional gene analysis also gave three clusters. However, a different result was found between the cluster from neighbor-joining and principal component analysis when using microsatellite. According to, neighbor-joining showed BR separated from GG but principal component formed BR and GG in the same group. Thus, we showed that genetic characterization with functional genes is superior compared to microsatellites, especially when a different genetic makeup among populations under selection.

**Key words:** functional genes, genome comparisons; genetic variability**,** microsatellites

# INTRODUCTION

Genetic diversity refers to the existence of genetic variants among genomes of individuals, families, strains and populations. Rich genetic resources must be maintained because it will provide for unforeseen breeding requirements to satisfy both farmer and consumer demands in the future. Indigenous chicken may be regarded as much diversified populations due to long-term adaptation from their ancestor (**RJF**) with response to varied agro-ecological zones. Moreover, Thai indigenous chicken (**TIC**) are generally preferred for the quality of the meat (Teltathum and Mekchay 2009), especially as healthy food because of lower triglyceride and cholesterol compared to exotic breeds (Jaturasitha et al. 2008) consensus**,** Bhutanese native chickens have socio-cultural and economic importance to the livelihood of many rural populations. For instance, they are slaughtered to please local deities, entertain guests, and sustain the health of women during pregnancy and after birth through egg and meat production (Nidup *et al.,* 2005) while the commercial lines are superior in terms of growth or egg production. However, under evolution or genetic selection, this may cause native chicken had change in genetic makeup, and even the repair or loss of genes associated with specific characteristics. Consistency, many genetic studies reported the decrease in genetic diversity of native chicken populations. It is because the unique and valuable genotypes and traits of native populations are at greater risk of being lost, with consequent threat to food security (Nassiri *et al.,* 2007). Thus, an assessment of genetic variations and genetic distances among original indigenous and commercial strains is essential**.** The objectives of this study were to investigate the genetic variation of RJF, indigenous chicken, commercial broiler and layers using microsatellites and to compare microsatellites and functional genes for genetic assessment before utilizing the chicken genetic resources with efficiency.

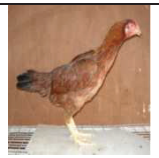
# MATERIALS AND METHODS

**Animals and DNA isolation**

Chicken with no genetic relationships (no common ancestors) were randomly selected. The minimum sample size suggested by Tadano *et al*. (2007) has been considered in this study. one ml of blood samples were drawn from ulnar vein in a microtube containing 0.5 M EDTA from birds two subspecies of RJF, *Gallus gallus gallus* (**GG**) and *Gallus gallus spadiceus* (**GS**) from the Thailand National Park, Wildlife and Plant Conservation Department in collaboration with the Wildlife Conservation Office; TIC names are based on male plumage (Table 1); Pradu Hang Dam (**PD**) from Research and Development Network Center for Animal Breeding (Native chickens) of Khon Kaen University, Leung Hang Khao (**LK**), Chee (**CH**) and Dang (**DG**) from Department of Livestock, Bhutanese native chickens (Seim, Yuebjha Narp, Khuilay and Phulom) and three commercial lines (Isa Brown, **IB**; Broiler, **BR** and White Leg Horn, **WL**) from private Thai company. The DNA was extracted from whole blood by Guanidium Hydrocloride protocol as described in Goodwin et al. (2007). Spectrophotometer was used to adjust the genomic DNA concentration to 50ng/µl.

**Table 1**. Characteristics of Red Junglefowl, Thai and Bhutanese indigenous chicken, subspecies of used in this study

| Population | Male | Female | Distribution | Morphology features | | |
|---|---|---|---|---|---|---|
| | | | | Comb type | Plumage | Shank and beak |
| *G. g. gallus*[1,2] |  |  | Northeast of Thailand | Single | Red jungle fowl like, greenish feathers tailed and sickle shaped, male are golden brown, sometimes reddish brown saddle; female are brownish red with dark-greenish strip following at each feather. | Black, yellowish |
| *G. g. spadiceus* [3,4] |  |  | North of Thailand | Single | Male has uniform golden yellow covered neck to lower back; tail feathers are greenish black with white patches; female are dark brown, yellowish plumage designed for camouflage, red ear lobe. | grey, yellowish |
| Pradu Hang Dam |  |  | Northeast | Pea | • Both adult are completely black<br>• Male has dark-brown fringed feather on the saddle | Black, Black |
| Leung Hang Khoa |  |  | East and Central | Pea | • Males are mainly black on ventral part while dorsal plumage is yellowish.<br>• Rarely primary wing is coloured on the web.<br>• Female are usually black with whitish dorsal plumage. | |
| Chee |  |  | Central | Pea | • Both adult entire plumage is white | Yellowish, Yellowish |

| Population | Male | Female | Distribution | Morphology features | | |
|---|---|---|---|---|---|---|
| | | | | Comb type | Plumage | Shank and beak |
| Dang |  |  | South | Pea | • Male are reddish brown.<br>• Female has blackish plumage around the neck. | Yellowish, Yellowish |
| Seim |  |  | Throughout Bhutan | Rose, pea, single | • Red jungle fowl like, greenish feathers tailed and sickle shaped, male are golden brown, sometimes reddish brown saddle; female are brownish red with dark-greenish strip following at each feather. | Black, yellowish |
| Yuebjha Narp |  |  | Southwest, West of Bhutan | Rose, pea | • Both sexes are entirely black; name derived from morphology. | Blackish, slate |
| Khuilay (Naked neck) |  |  | South, Southwest of Bhutan | Rose, pea, single | • Generally soft-feather red, diverse plumage color occurs (such white, partridge), featherless at neck. | Yellowish, whitish |
| Phulom (Frizzle) |  |  | Southwest, South of Bhutan | Rose, pea | • Feathers faced outwards (various colour such as Seim, black). | Yellowish, black |

[1]*Gallus gallus gallus* male (Bao, 2009); [2]*Gallus gallus gallus* female (Tsai, 2010); [3]*Gallus gallus spadiceus* male; [4]*Gallus gallus spadiceus* female (Somchai, 2009)

**Microsatellites and functional genes genotyping**

The microsatellite markers were selected based on having more than four alleles (Nassiri et al. 2007; Nassiry et al. 2009). Twenty microsatellites loci were genotyped to compare genetic variation among four TICs and three commercial lines. Microsatellites eighteen loci were used to classify two RJF, two TIC (PD and CH), Bhutanese native chickens and broiler. Eighteen microsatellites were versus with five function genes (six loci) in two RJF, PD, broiler and WL.

**Statistical analysis approach**

The alleles were computed and analyzed to examine mean number of alleles (MNA), observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$).

A Neighbor-Joining method (Saitou and Nei, 1987) of Numerical Taxonomy System (NTSYSpc) Version 2.10 package was used to construct a phylogenetic tree based on Nei (1978) unbiased genetic distance. Principal component analysis (SAS, 1998) based on individual Dice genetic distance was employed to visualize genetic relationships and detect geographical clines that may not be apparent from the phylogenetic tree.

## RESULTS AND DISCUSSION

**Genetic diversity between Thai indigenous and commercial chickens**

The regular parameters used to assess population variations are Mean number allele (MNA), Observed (direct count) heterozygosity ($H_O$) and expected heterozygosity ($H_E$) (Tadano et al. 2007). MNA per locus was 11.35 for seven populations and 14.17 for ten populations. Genetic variability for every microsatellite loci were analyzed and summarized in Table 2 and 3.

The results of genetic diversity for seven populations are summarized in Table 2. The MNA examined minimum and maximum for IB (7.60) and CH (8.80), respectively. The MNA and $H_E$ for all Thai chicken populations were greater than the commercial lines except for PD. Among the Thai chicken populations, CH and DG exhibited for superior $H_E$ (CH: $H_E$ = 0.80; DG: $H_E$ = 0.81). On contrary, IB was inferior in $H_E$ (0.77) though $H_O$ (0.71) was the highest. This study showed considerable genetic diversity in the populations. MNA is another form of reporting genetic diversity (Toro et al. 2009; Nassiry et al. 2009) intended for conservation. The MNA value is determined by sample sizes (Toro et al. 2009), hence, $H_E$ and $H_O$ are fundamental parameters extended to infer the population diversity (Nassiry et al. 2009; Toro et al. 2009). Compared to the commercial lines, the TIC populations had greater $H_E$. This implies that random mating is frequent within population and also with the wild RJF. The higher $H_O$:$H_E$ ratio in commercial lines, particularly in IB, depicted that the population size was relatively small at the beginning (Tadano et al. 2007). Among the TIC populations, the highest heterozygosity was found in CH and DG, represented the greater genetic diversity. Conversely, PD exhibited lower MNA and heterozygosity which showed that slight selection pressure might occur. The $H_E$ (~0.8) in TIC populations was higher than $H_E$ (0.58) found in *Mazandaran* chicken populations using same 20 microsatellite loci (Nassiri et al. 2007), reflecting that the Thai indigenous chickens retained the rich of genetic diversity.

**Table 2.** Estimation of average number of alleles and genetic variation using twenty microsatellite primers for seven populations (four TICs and three commercial lines)

| Primer | Allele | PD | | DG | | CH | | LK | | BR | | IB | | WH | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $H_O$ | $H_E$ | $H_O$ | $H_E$ | $H_O$ | $H_E$ | $H_O$ | $H_E$ | $H_O$ | $H_E$ | $H_O$ | $H_E$ | $H_O$ | $H_E$ |
| MCW 14 | 12 | 0.73 | 0.84 | 0.90 | 0.85 | 0.88 | 0.88 | 0.60 | 0.81 | 0.37 | 0.87 | 0.73 | 0.84 | 0.50 | 0.81 |
| MCW 34 | 13 | 0.87 | 0.87 | 1.00 | 0.83 | 0.88 | 0.89 | 0.90 | 0.87 | 0.67 | 0.85 | 0.90 | 0.90 | 0.93 | 0.89 |
| MCW 37 | 11 | 0.47 | 0.69 | 0.62 | 0.73 | 0.72 | 0.80 | 0.83 | 0.81 | 0.57 | 0.58 | 1.00 | 0.84 | 0.43 | 0.80 |
| MCW 69 | 9 | 0.33 | 0.75 | 0.90 | 0.80 | 0.50 | 0.80 | 0.53 | 0.80 | 0.20 | 0.58 | 0.87 | 0.79 | 0.80 | 0.81 |
| MCW 81 | 10 | 0.87 | 0.82 | 0.75 | 0.88 | 0.72 | 0.81 | 0.83 | 0.82 | 0.50 | 0.80 | 0.90 | 0.75 | 0.60 | 0.78 |
| MCW 104 | 15 | 0.23 | 0.83 | 0.79 | 0.88 | 0.69 | 0.87 | 0.77 | 0.88 | 0.67 | 0.86 | 0.93 | 0.80 | 0.20 | 0.70 |
| MCW 111 | 6 | 0.30 | 0.68 | 0.55 | 0.66 | 0.44 | 0.64 | 0.53 | 0.65 | 0.47 | 0.69 | 0.17 | 0.61 | 0.27 | 0.68 |
| MCW 123 | 8 | 0.47 | 0.76 | 0.72 | 0.82 | 0.63 | 0.80 | 0.70 | 0.80 | 0.43 | 0.73 | 1.00 | 0.67 | 0.57 | 0.76 |
| MCW 183 | 16 | 0.37 | 0.85 | 0.48 | 0.92 | 0.34 | 0.88 | 0.47 | 0.87 | 0.57 | 0.87 | 0.50 | 0.83 | 0.60 | 0.91 |
| MCW 222 | 14 | 0.83 | 0.86 | 0.59 | 0.85 | 0.75 | 0.89 | 0.87 | 0.89 | 0.77 | 0.89 | 0.43 | 0.64 | 0.43 | 0.82 |
| MCW 248 | 12 | 0.43 | 0.77 | 0.14 | 0.79 | 0.25 | 0.80 | 0.37 | 0.72 | 0.10 | 0.83 | 0.40 | 0.78 | 0.03 | 0.84 |
| MCW 295 | 8 | 0.13 | 0.72 | 0.48 | 0.77 | 0.31 | 0.76 | 0.40 | 0.77 | 0.47 | 0.81 | 0.43 | 0.68 | 0.37 | 0.56 |
| ALD 112 | 13 | 0.73 | 0.78 | 0.24 | 0.79 | 0.22 | 0.69 | 0.23 | 0.77 | 0.40 | 0.77 | 0.80 | 0.78 | 0.60 | 0.73 |
| ADL 123 | 10 | 0.17 | 0.72 | 0.38 | 0.62 | 0.38 | 0.56 | 0.57 | 0.69 | 0.47 | 0.84 | 0.70 | 0.75 | 0.53 | 0.88 |
| ADL 127 | 9 | 0.90 | 0.77 | 0.45 | 0.77 | 0.16 | 0.84 | 0.47 | 0.77 | 0.53 | 0.80 | 0.77 | 0.78 | 0.50 | 0.81 |
| ADL 147 | 12 | 0.87 | 0.86 | 0.69 | 0.89 | 0.63 | 0.86 | 0.77 | 0.87 | 0.60 | 0.86 | 0.97 | 0.85 | 0.43 | 0.80 |
| ADL 268 | 8 | 0.50 | 0.77 | 0.69 | 0.74 | 0.38 | 0.66 | 0.13 | 0.67 | 0.33 | 0.73 | 0.73 | 0.83 | 0.50 | 0.77 |
| ADL 372 | 10 | 0.77 | 0.73 | 0.62 | 0.87 | 0.34 | 0.83 | 0.67 | 0.85 | 0.47 | 0.77 | 0.03 | 0.63 | 0.73 | 0.72 |
| LEI 94 | 15 | 0.97 | 0.91 | 0.69 | 0.81 | 0.94 | 0.87 | 0.80 | 0.85 | 0.90 | 0.87 | 0.93 | 0.78 | 0.90 | 0.80 |
| LEI 166 | 16 | 0.70 | 0.84 | 0.86 | 0.90 | 0.78 | 0.88 | 0.60 | 0.88 | 0.53 | 0.86 | 0.93 | 0.79 | 0.60 | 0.85 |
| **Mean** | 11.35 | 0.58 | 0.79 | 0.63 | 0.81 | 0.55 | 0.80 | 0.60 | 0.80 | 0.50 | 0.79 | 0.71 | 0.77 | 0.53 | 0.79 |

Note: PD = Pradu Hang Dam; DG = Dang; CH = Chee; LK = Leunghangkhoa; BR = Broiler; IB = Isa Brown; WH = White Leg Horn. $H_O$ and $H_E$ = observed and expected heterozygosity respectively.

**Table 3**. Genetic variability estimates (mean ± SD) from eighteen microsatellite loci for 10 chicken populations

| Population | MNA ± SD[a] | $H_O$ ± SD[b] | $H_E$ ± SD[c] |
|---|---|---|---|
| *Gallus gallus spadiceus* | 9.28 ± 0.66 | 0.47 ± 0.06 | 0.81 ± 0.02 |
| *Gallus gallus gallus* | 9.50 ± 0.59 | 0.52 ± 0.06 | 0.82 ± 0.01 |
| Seim | 9.33 ± 0.72 | 0.51 ± 0.06 | 0.82 ± 0.01 |
| Yuebjha Narp (Black chicken) | 7.94 ± 0.40 | 0.44 ± 0.05 | 0.79 ± 0.02 |
| Khuilay (Naked neck) | 9.50 ± 0.68 | 0.49 ± 0.05 | 0.83 ± 0.02 |
| Phulom (Frizzle) | 8.50 ± 0.57 | 0.55 ± 0.04 | 0.81 ± 0.01 |
| Pradhu Hang Dam (Black chicken) | 9.78 ± 0.69 | 0.59 ± 0.06 | 0.83 ± 0.02 |
| Chee (White chicken) | 10.83 ± 0.85 | 0.58 ± 0.04 | 0.84 ± 0.02 |
| Broiler | 9.28 ± 0.77 | 0.49 ± 0.06 | 0.82 ± 0.02 |
| White Leghorn | 8.67 ± 0.82 | 0.45 ± 0.06 | 0.78 ± 0.02 |

[a] Mean number of alleles per locus, [b] observed heterozygosity, [c] expected heterozygosity

**Genetic diversity among Red Junglefowl, Thai indigenous, Bhutanese indigenous and commercial chickens**

The levels of genetic variations across ten populations were assessed (Table 3). They were greater for CH (MNA, 10.83 ± 0.85; $H_O$, 0.58 ± 0.04; $H_E$, 0.84 ± 0.02) and Khuilay (MNA, 9.50 ± 0.68; $H_O$, 0.49 ± 0.04; $H_E$, 0.83 ± 0.02). By contrast, Phulom (MNA, 8.50 ± 0.57; $H_O$, 0.55 ± 0.04; $H_E$, 0.81 ± 0.01) and Yuebjha Narp (MNA, 7.94 ± 0.40; $H_O$, 0.44 ± 0.05; $H_E$, 0.79 ± 0.02) tends to contain lower genetic variations compared to the control populations. For all loci, the mean $H_E$ was higher than mean $H_O$ describing the sampling biasness or possibly inbreeding mating system. Low observed heterozygosity may lead to positive assortment or a situation of high homozygosity.

Evidently, data regarding the breeds and their specific adaptations, distinct phenotypes, performance level, demography (includes effective population size, local or transboundary, geographical distribution, level of enlargement), and description databases are also required to assess decision on the breeds for conservation and breeding programs (Groeneveld *et al.,* 2010). Nevertheless, the genetic data is a fundamental method to indicate the existence of biodiversity (Nassiri *et al.,* 2007; Semik and Krawczyk, 2011).

The environmental influences on individual and geographical barrier possibly explain the presences of very high number of alleles at various loci but also fairly high $F_{IS}$ values. Though mean $F_{IS}$ value was high, the test for HWE indicated non-significant deviation from HWE in native chickens and Junglefowl chickens. On the other hand, eight loci (Broiler) and two loci (WL) deviating HWE informs commercial populations were intensively selected decades for morphology and production, genetic subdivision then occur. It was possible that some loci might be associated with genes that might be lost due to genetic drift this could explain for a few loci with a strong genetic differentiation and others slightly. However, mean $F_{ST}$ value indicates that subpopulation division is moderate and 8.4% of the total genetic variation is caused by population differences while 91.6% corresponds to differences within populations.

Comparable population variations were observed for Seim and Khuilay with original and ancestor fowl populations. Strain Seim is commonly reared by Bhutanese farmers while Khuilay has highly diversified plumage colour (soft-red, white, black, partridge, and speckled) and possible gene flow from Indian Naked neck populations. The major issue of

concern is for Yuebjha Narp population which has low variations. The possible reasons could be the least diversified morphology and finite population sizes (approximated average 20 to 25 individuals per village). As expected, the $H_E$ for the two subspecies of RJF across the loci, was higher than the WH, even more than those obtained by Hillel *et al.* (2003) and Granevitze *et al.* (2007). The present study shows that the wild progenitor of the domestic chickens contains considerable genetic variation as reported in RJF of Northern India (Mukesh *et al.,* 2011). The wild ancestors of major livestock species considered to be genetic diversity reservoirs are either extinct or low in numbers (Hanotte and Jianlin, 2005). Therefore, putative wild ancestors of our present-day chickens must be conserved because they are threatened to extinction by the habitat loss, fragmentation, and poaching. On contrary, commercial lines were developed from few breeds. Thus, the commercial lines has low genetic base and in other words lower genetic variations than the native and Junglefowl populations. Interestingly the result revealed substantial genetic variation content was observed similarly as reported that enable further genetic progress (Pirany *et al.,* 2007).

**Phylogenetic relationships**

A phylogenetic tree was reconstructed exclusively based on Nei's unbiased genetic distance (Figure 1), four Thai indigenous chickens and three commercial lines split into four clusters (two clusters each represented by Thai chicken populations and commercial lines. IB and WL branched together to form an egg layer and commercial broiler represented another group. Among the Thai chicken populations, PD clustered separate from CH, DG and LK. It confirmed that the high pressure on selection for meat (broiler) or egg (IB and WL) could differentiate the genetic structure from the unselected TIC (*G. domesticus*). This result was in agreement with Tadano et al. (2007a) for 12 commercial lines. Among Thai chicken populations, PD formed different cluster from the others and it might be related with special characteristics of black plumage, shank and beak while the other TIC were white, yellow or red plumage with yellow shank and beak. The tree from this study also revealed that based on genetic clustering, the group of CH, LK, and DG were closer to the group of layers. On the contrary, the relative genetic distance between PD was closer to commercial broiler (0.044) than commercial layers (0.055). These result suggested that PD could justify to be improved for meat type while the others TIC should consider for egg type.

Moreover, the genetic classification of RJF, TIC, Bhutanese chicken, broiler and layer chicken, illustrated that one Khuilay (Bhutanese naked neck) was most closely related to to PD (Thai native black). The other three Bhutanese strains, Seim (RJF like), Yuebjha (black feather), and Phulom (frizzle) were in a separate group with a node connect to PD. According to the results, Bhutanese native chickens should be classified genetically close to Southeast Asian domestic chicken. This study also showed that Bhutanese native chicken and TIC (*Gallus gallus domesticus*) were related to *Gallus gallus spadiceus*, the red earlobe RJF (Figure 2). The relatedness of Khuilay and PD, and separate genetic group of the other Bhutanese native chicken were confirmed in the PCA plot, however, the result from phylogenetic tree and PCA showed a silent difference (the data not shown).

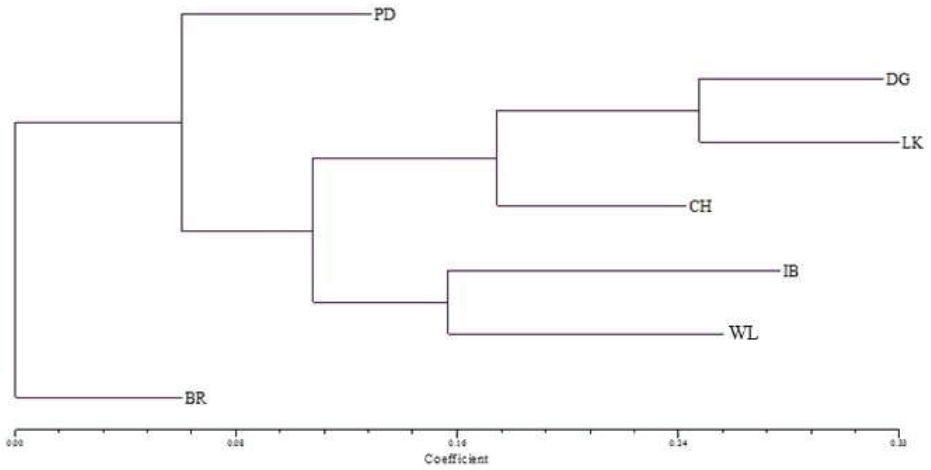



**Figure 1.** A phylogenetic tree among seven subpopulations (four TICs and three commercial lines) based on Nei's unbiased genetic distance method. (PD = Pradu Hang Dam; DG = Dang; CH = Chee; LK = Leunghangkhoa; BR = Broiler; IB = Isa Brown; WL = White Leg Horn)

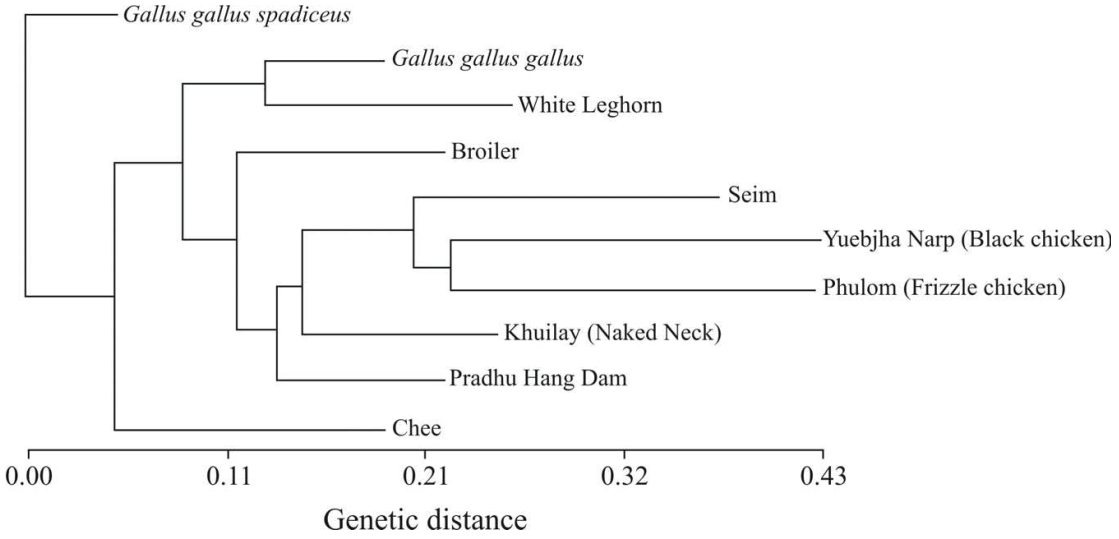
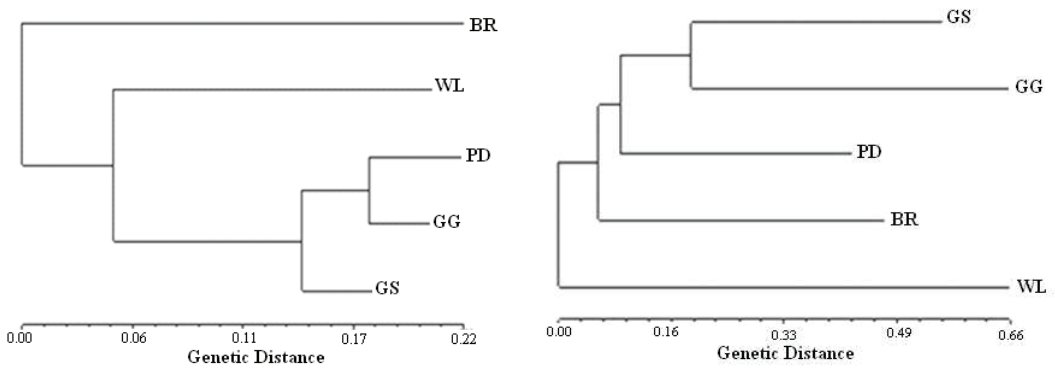


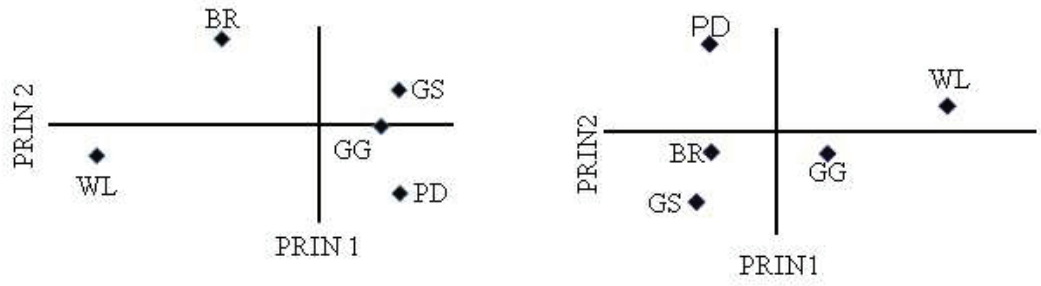

**Figure 2.** A phylogenetic tree based on Nei's genetic distance (Nei, 1978) for ten chicken populations

In addition, genetic comparison between microsatellites and functional genes in RJF, TIC and two commercial chickens revealed phylogenetic tree and PCA plot derived from microsatellites and functional genes were similar (Figure 3 and 4). Overall, the genetic comparison for RJF, PD and commercials line with functional genes was highly efficient in detecting genetic differences between populations. Thus, the appropriate set of functional genes may be regarded as useful tools, taking into consideration populations that are under different degrees of selection.




**(a)** **(b)**

**Figure 3.** A phylogenetic tree based on Nei's Unbiased distance from six loci of functional genes (a), and eighteen microsatellite markers (b) for *G. gallus gallus* (GG), *G. gallus spadiceus* (GS), Pradu Hang Dam (PD), Broiler (BR) and White Leghorn (WL)




**(a)** **(b)**

**Figure 4.** Two-dimention principal components plot among 5 populations based on Dice's genetic similarity of six loci of functional genes (a), and eighteen microsatellite markers (b) for *G. gallus gallus* (GG), *G. gallus spadiceus* (GS), Pradu Hang Dam (PD), Broiler (BR) and White Leghorn (WL)

## CONCLUSIONS

Despite the bias in comparing with previous report we may conclude that Thai indigenous chicken seems to have good genetic diversity with DG showing the highest variations followed by CH and LK. If we consider the relatively small genetic distance between PD with broiler, it is suitable for PD to be developed as a meat type. The other Thai indigenous chickens might be developed as an egg type due to closely genetic clustering. Principal component analysis plot revealed Bhutanese native chickens should be prioritized for conservation because of their genetic distinctiveness. The comparison between microsatellites and functional genes showed appropriate set of functional genes may be regarded as distinguished alternative tools for consideration populations that are under different degrees of selection.

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# Analysis of the Spatiotemporal Behavior of Red Junglefowl and Free-Range Chickens using a WiFi Positioning System

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## ABSTRACT

This paper describes two experimental studies that analyzed the spatiotemporal behavior of red junglefowl and free-range chickens using a WiFi (wireless fidelity) positioning system. The major findings obtained from the experimental study of junglefowl were: the experimental field was mostly dominated by one junglefowl, but the replacement of the dominant junglefowl occurred once in the experimental period, and this replacement took place over two days; junglefowl were temporally separated in the experiment field, and the closest distance between two junglefowl at a given point in time was 108 m; the home ranges of junglefowl overlapped to some extent (8%); the lower bound of the home range of a junglefowl was 2.1 ha; junglefowl change roosting places at nights; one junglefowl was found to have a walking speed of 32 m/min and a flying speed of 415 m/min. The major findings obtained from the experimental study of free-range chickens were: the shapes of the home ranges of three cocks were different and they overlapped to some extent, and the overlap was larger than that of the junglefowl; the home ranges of some hens were almost the same as those of their cocks; the home ranges of young hens were different from those of the cocks; the home ranges of hens with chicks were different from those of the cocks; the chickens that formed a group in daytime did not always roost together at the same site at night; chickens did not always sleep at the houses where they were kept; and the home range expanded when food was not given, but the extent of expansion differed from chicken to chicken. In particular, the expansion rate of the young hens was the largest. These empirical finding demonstrates that the WiFi positioning system was very useful for continuously observing animal behavior over space and time, although it has a few limitations.

**Key Words:** red junglefowl, free-range chicken, WiFi positioning systems, spatiotemporal behavior, home range

# INTRODUCTION

Understanding animal behavior over time and space is one of the major subjects in animal science. To achieve this understanding, the trajectory data of animals in an area over a certain length of time, at least a few days, are indispensable. To acquire such data, two methods are commonly used in animal ecology: a telemetry system (Millsoaugh and Marzluff, 2001) and a global positioning system (GPS) (El-Rabbany, 2006). These methods each have their own advantages and disadvantages, depending on the environments in which the animals will be studied, as well as the animals' characteristics themselves.

An advantage of a telemetry system is that the weight of a tag is light, e.g., one of the lightest tags is 0.5 g. A disadvantage, on the other hand, is that a researcher has to operate the antenna constantly during any observation period; therefore, the researcher has logistical difficulties when attempting to observe many animals over day-and-night periods for several days.

In contrast, an advantage of a GPS is that once a GPS logger (tag) is fixed on an animal, trajectory data are continuously stored in the logger as long as its battery lasts. However, to obtain the data, the animal must be recaptured. If recapture fails, we cannot obtain the data from the GPS logger. Another difficulty is that the weight of a GPS tag is heavier than that of a telemetry tag. In 2005 when our experiment was carried out, the lightest one was 65 g (a tag of around 25 g is currently available, but the battery lasts at most a day). According to Ando and Osawa (1970), the weight of a device that does not disturb the free movement of an animal is less than 5% of its weight. Therefore, the weight of an animal to which a 65 g GPS tag is attached should be more than 1.3 kg. Consequently, we could not fix GPS loggers on junglefowl. To overcome those difficulties, we employed a WiFi positioning system.

The studies presented in this paper were performed under the Human–Chicken Multi-Relationship Research Project. This project pays special attention to chickens, and has studied their domestication in Thailand for several years. In this project, we carried out two experimental applications of a WiFi positioning system: the first was applied to red junglefowl living near the Wildlife Research Station in Khao Ang Rue Nai Wildlife Sanctuary, Thailand, and the second to free-range chickens kept in the Chiang Rai Livestock Technology Transfer Center in Thailand.

This paper first describes the methods for applying a WiFi positioning system to acquiring the trajectories of junglefowl and chickens. Next, the procedures of the two experiments are detailed and the resulting data are shown. Last, some characteristics of the spatiotemporal behavior of the animals are discussed, together with the advantages and disadvantages of the WiFi positioning system.

# MATERIALS AND METHODS

**WiFi positioning system**

We employed a WiFi positioning system developed by AeroScout (Redwood City, CA). The system consists of tags, activators, receivers (with antennas), power-over-Ethernet hubs, WiFi access points, and a managing engine. Tags are fixed on animals and send the

position data (x–y coordinates) by radio waves. The weight of a tag is 35 g, and its dimensions are 62×40×17 mm. Receivers receive transmitted data from tags through an antenna and send the data to a managing engine by wire. The managing engine processes the position data through access points, and displays the positions of animals on its display in real time.

These devices were configured according to the environmental conditions of the experimental areas. Prior to the experiments reported in this paper, we tested the WiFi positioning system in the Koishikawa Botanical Garden in Tokyo to see what area a single receiver could cover in a tree-covered area. We found that the maximum distance between two receivers was 75 m.

The WiFi position system represents a position by a discrete point on a one-meter grid. Therefore, the precision of a position cannot be better than one meter. The time interval for transmitting two successive positions was set to one second. Therefore, a trajectory of a chicken is represented by the sequence of discrete time points on a discrete grid. The time interval may however be longer than one second, depending on environmental conditions in an experimental field, because transmission may fail.

In the following two subsections, we describe the materials and methods adopted in the experimental studies on junglefowl and on free-range chickens, respectively. Because Okabe *et al*. (2009) described the latter experiment in detail, we focus on the former, outlining the latter.

**Materials and methods of the experimental study of junglefowl**

The experimental field for the study of junglefowl was a 150×300 m area densely covered with trees and bushes near the Wildlife Research Center in Khao Ang Rue Nai Wildlife Sanctuary, Thailand (N13°24′32″–13°24′42″ and E101°52′37″– 101°52′42″). The experimental field was not enclosed and the natural environment outside the experimental field was similar. A 6 m wide road (the gray stripe in Figure 1) ran through this area. The configuration of the WiFi devices in the field is illustrated in Figure 1.

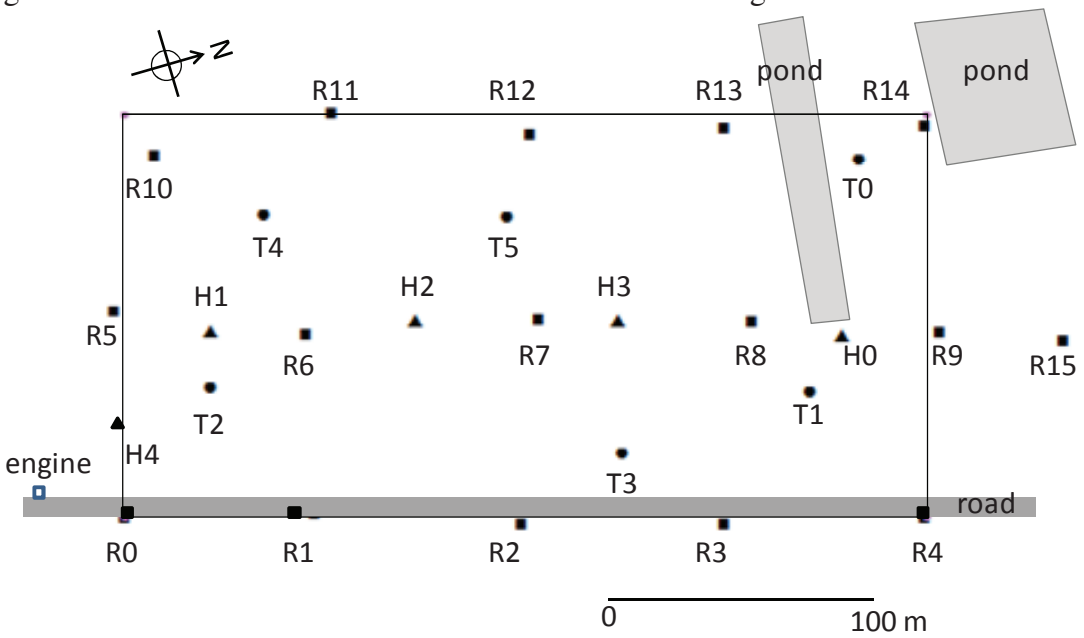


**Figure 1** The configuration of the WiFi devices in the experiment field near the Wildlife Research Center in Khao Ang Rue Nai Wildlife Sanctuary, Thailand (R1–R15: receivers, H0–H4: hubs, T0–T5: fixed tags).

The experiment was carried out from February 12 to March 10, 2008 including installing and uninstalling the WiFi positioning system. Prior to this experiment, we observed junglefowl around the Wildlife Research Center (including the experimental field) over a year, and noticed that there were five junglefowl staying in or stepping into the experimental field. We trapped one male junglefowl (referred to as RJF1) on February 12; one male (RJF2) and one female (RJF3) on February 14; two males (RJF1 and RJF4) on February 16 (RJF1 was trapped again); and one male (RJF5) on March 4. We released them soon after fixing tags on their backs. The WiFi positioning system was able to record the positions of junglefowl (provided that they were within the experimental field) from February 22 to March 10.

**Materials and methods of the experimental study of free-range chickens**

The experimental field for the study of the free-range chickens was a cleared $200 \times 200$ m area surrounded by bushes (being naturally enclosed, this contrasted with the junglefowl case) and eight small concrete block one-storied houses (a few people lived in two houses (HH1 and HH2 in Figure 2) who worked outside during the daytime, while the other houses were empty) in the Chiang Rai Livestock Technology Transfer Center in Thailand (N $19^{\circ}59'58'' - 20^{\circ}00'04''$ and E $99^{\circ}49'50'' - 99^{\circ}49'58''$). The configuration of the WiFi devices and houses for humans and chickens in the experimental field is illustrated in Figure 2.

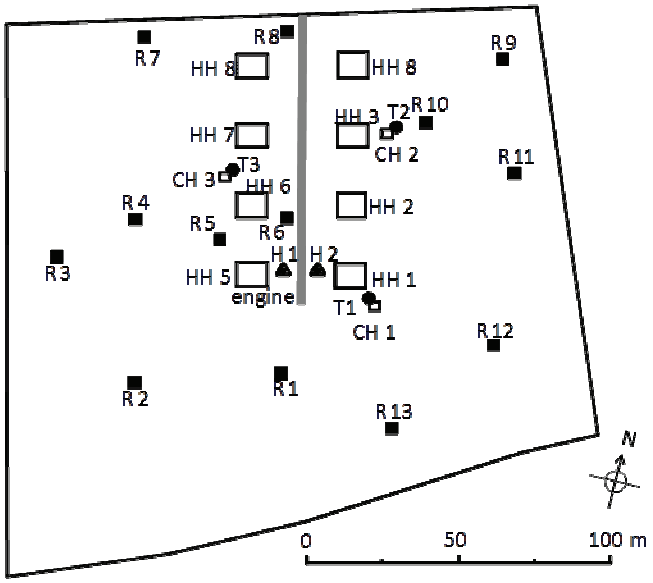


**Figure 2** The configuration of the WiFi devices in the experimental field in the Chiang Rai Livestock Technology Transfer Center in Thailand (R1–R13: receivers, H1, H2: hubs, HH1–HH8: houses for humans, CH1–CH3: houses for chickens, T1–T3: fixed tags).

The subjects were eighteen chickens, called *Kai Chon* in Thai, and were moved to the chicken houses (CH1, CH2, and CH3 in Figure 2) on October 5, 2005. One 12-month-old cock (referred to as CH*i*_C1, where *i* corresponds to the *i*-th chicken house in Figure 2) and five hens of varying ages were kept in each chicken house CH*i*. Specifically, the composition of each family was as follows.

Chicken house CH1 housed one cock, denoted by CH1_C1, five 6–7-month-old hens with no baby chickens (chicks), CH1_H1, CH1_H2, …, CH1_H5;

Chicken house CH2 housed one cock CH2_C1, one 6–7-month-old hen with chicks, CH2_H1_M, two 2–3-month-old hens (young hens), CH2_H2_Y, CH2_H3_Y, and two 6–7-month-old hens without chicks, CH2_H4, CH2_H5;

Chicken house CH3 housed one cock CH3_C1, four 6–7-month-old hens without chicks, CH3_H1, CH3_H2, CH3_H3, CH3_H5, and one 6–7-month-old hen with chicks, CH3_H4_M.

The experiment was carried out from November 2 to 9, 2005. On November 3, we opened the doors of the chicken houses and let the chickens free. We continuously obtained the data from November 4 to November 9. Food was given once in the morning and once in the afternoon until November 5, but not thereafter although food that was given on November 5 remained available until November 6.

## RESULTS AND DISCUSSION

We now discuss the spatiotemporal behavior of junglefowl and of free-range chickens, respectively, analyzing the data obtained from the two experiments.

**The results of the experimental study of junglefowl and discussion**

Because the results are heavily dependent on the quality of the data, we begin by discussing data quality, which was mainly determined by the stability of signal reception from the tags fixed on junglefowl moving in the experimental field. Unfortunately, the stability was less than we expected. There were at least four reasons. First was trouble with the receivers. During the experimental period, several receivers sometimes failed; specifically, receiver R3 (see Figure 1) on February 28 and 29, March 1, 2, and 8 for a few hours; receiver R4 on February 27 and 28 for a few hours; receiver R13 on March 8 for an hour; receivers R14 and R15 from February 29 to March 8 for three hours. It was difficult to maintain the large-scale WiFi positioning system for a month.

Second, the density of bushes was higher than that in the Koishikawa Botanical Garden. We realized that the bushes in the experimental field were a more obstructive factor than we had anticipated (as junglefowl walk on the ground).

Third, the experimental field looked flat, but it was actually slightly rolling. As a result, the radio waves transmitted from a junglefowl on the ground were interrupted by small hills.

To see the influence of these factors, we analyzed the position data of the fixed tags. In theory, the position of a fixed tag is supposed to remain the same, but in practice the coordinates of the fixed tags were distributed around the center with a directional bias (Figure 3); the average distance from the center was 3.62 m with a standard deviation 2.71 m, meaning 95% of points were within 6.63 m of the mean position. The position data were transmitted every second from the tags on junglefowl, but the received data had an average time interval of 7 seconds, with a standard deviation of 5.76 seconds. Many position data were lost during transmission and the time interval was very unstable.

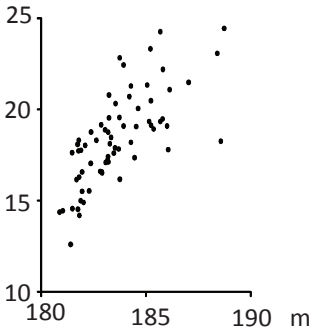


**Figure 3** The coordinates of a fixed tag, T3 in Figure 1, from 07:59–09:04 on March 4while junglefowl RJF1 and RJF5 stayed in the experimental field. The origin is the left lower corner of the experimental field in Figure 1.

Finally, during nights when a junglefowl perched at about 10 m high on a roosting tree (we estimated the height from its droppings), the position data showed systematic large fluctuations. Assuming that the junglefowl did not move at night, we discarded those data. We also discarded position data from outside the experimental field because the WiFi system was imprecise outside the experimental field (although we acquired outside position data to some extent).

Together, those factors produced unstable position data. This instability made our analysis very hard and the implications are not always decisive. However, considering that this study is one of the earliest studies (probably the first) applying a WiFi positioning system to junglefowl, we describe our analysis and its implications, hoping that they are helpful for further studies on junglefowl.

As mentioned in the preceding section, we fixed tags on five junglefowl. However, only three junglefowl, RJF1, RJF4, and RJF5, went in and out of the experimental field. The periods over which those junglefowl stayed in the experimental field are shown in Figure 4 (the numbers along the line segments indicate the minutes of stay and those on the right margin indicate the total minutes in each day). Inspecting these time and position data, we identified the following eight findings.

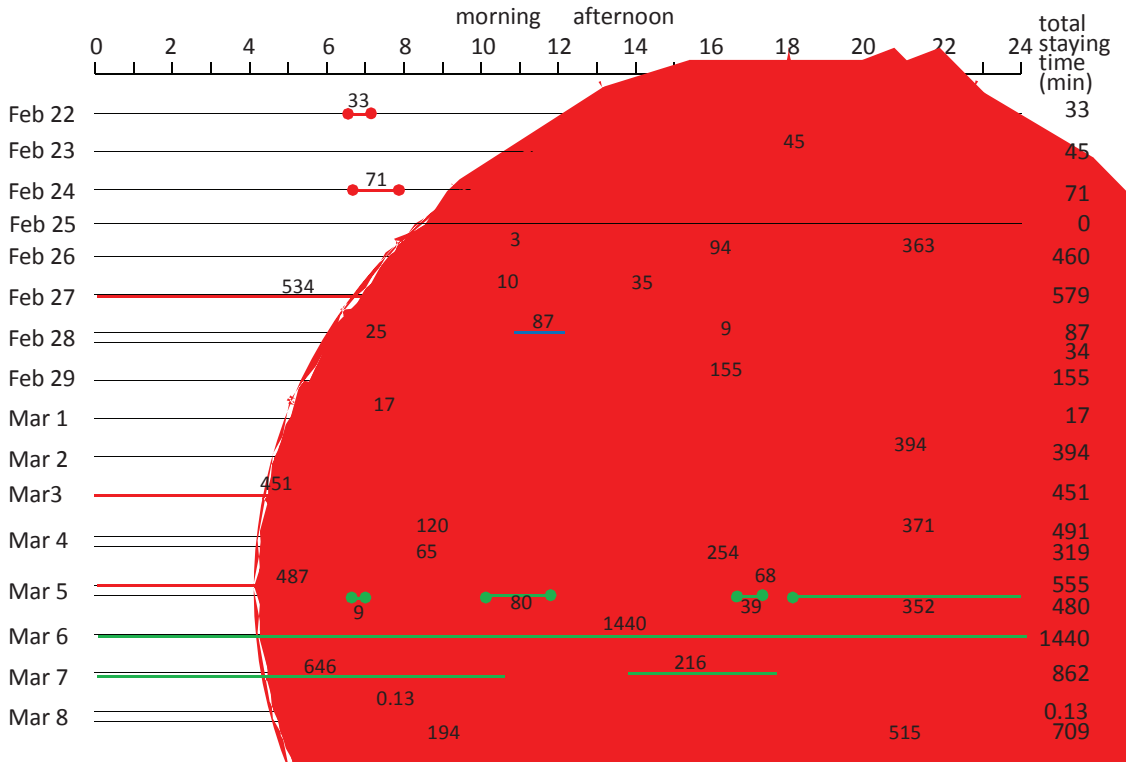


**Figure 4** The time periods in which junglefowl RJF1 (red), RJF4 (blue), and RJF5 (green) stayed in the experimental field during February 22–March 8, 2008.

First, RJF1 dominated the experimental field in the period of February 22–March 3, 2008; RJF5 dominated in the period of March 6–8, 2008. The transition in dominance occurred during March 4–5. This implies that a 150×300 m area is dominated by one junglefowl and the replacement of the dominant junglefowl takes place over a few days (two days in this experiment).

Second, the periods in which two junglefowl stayed in the experimental field at the same time were very short except in the transition period. In fact, RJF1 and RJF5 were both in the experimental field for only eight seconds on one day (March 8). This implies that junglefowl are temporally separated in a 150×300 m area. In the transition period, however, both RJF1 and RJF5 stayed in the same field for 79 minutes on March 4 and for 39 minutes on March 5. The colocation on the second day (March 5) was much shorter than that in the first day (March 4) (also see Figures 5 and 6).

Figures 5 and 6 show the spatial relationship between RJF1 and RJF5 in the transition period. Inspecting these figures, we obtained the third finding: when both were in the experimental field at the same time, they were separated spatially, and the minimum distance between them was 121 m on March 4 and 108 m on March 5. When two junglefowl do not occupy the same place at the same time (i.e., the two do not meet at all), we call such a separation *temporal separation*.

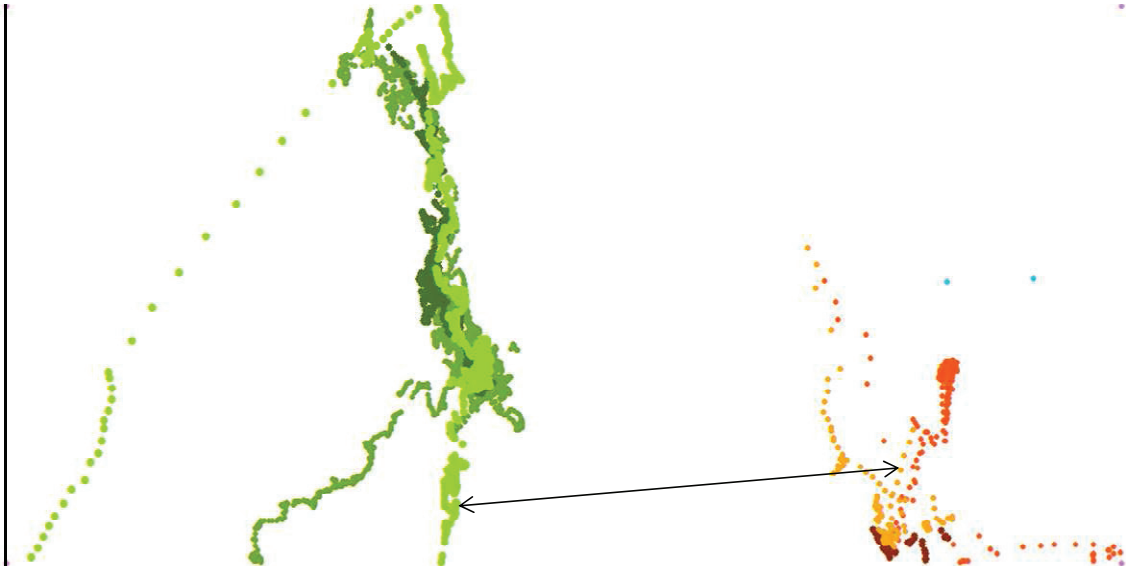

**Figure 5** The positions of junglefowl RJF1 and RJF5 on March 4. The light-brown and light-green dots indicate the positions of RJF1 and RJF5, respectively, during 07:59–09:04; the dark-brown and dark-green dots indicate those during 18:04–18:18 (in these two periods, RJF1 and RJF5 coexisted in this field); the medium-brown and green dots indicate their positions during the rest of the time periods while the junglefowl stayed in this experimental field. Note that some of those dots overlap. The line segment indicates the minimum distance, which was observed at 09:02.

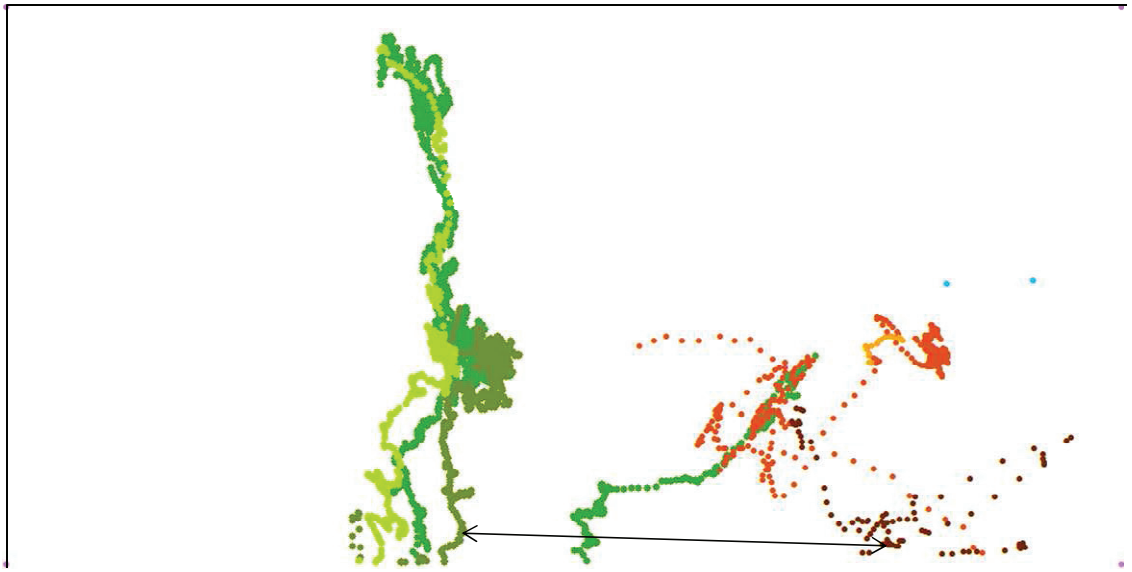

**Figure 6** The positions of junglefowl RJF1 and RJF5 on March 5 (see the caption in Figure 5 except the time periods are 06:42–06:51 and 16:44–17:14). The line segment indicates the minimum distance, which was observed at 17:09.

Having noticed the temporal separation of junglefowl, we now question whether or not a junglefowl, say RJF5, walks in the area where another junglefowl, RJF1, once stayed but is now out of the area. That is to say, may two junglefowl visit the same place at a different time? To discuss this spatial relation, we introduce the concept of *home range*, which is defined as the minimum convex area that includes all the positions that a junglefowl visits at least once during a long period of time. The above question is then stated as: can two

home ranges overlap? If not, we say such a separation is *spatial separation*. Note that spatial separation implies temporal separation, but the converse is not always true. To examine spatial separation, we created Figure 7, where the red, blue, and green dots indicate the positions of junglefowl RJF1, RJF4, and RJF5, respectively. The brown and green broken-line polygons in Figure 7 indicate the home rages of RJF1 and RJF5, respectively (note that the green dots outside the green broken-line polygon were the trajectory of a researcher carrying RJF5 into the area).

This figure shows the fourth finding: their home ranges overlapped and the overlapping area of RJF5 was 8% of its home range; sometimes a junglefowl stepped into the home range of another as if the junglefowl made a reconnaissance visit to that area.

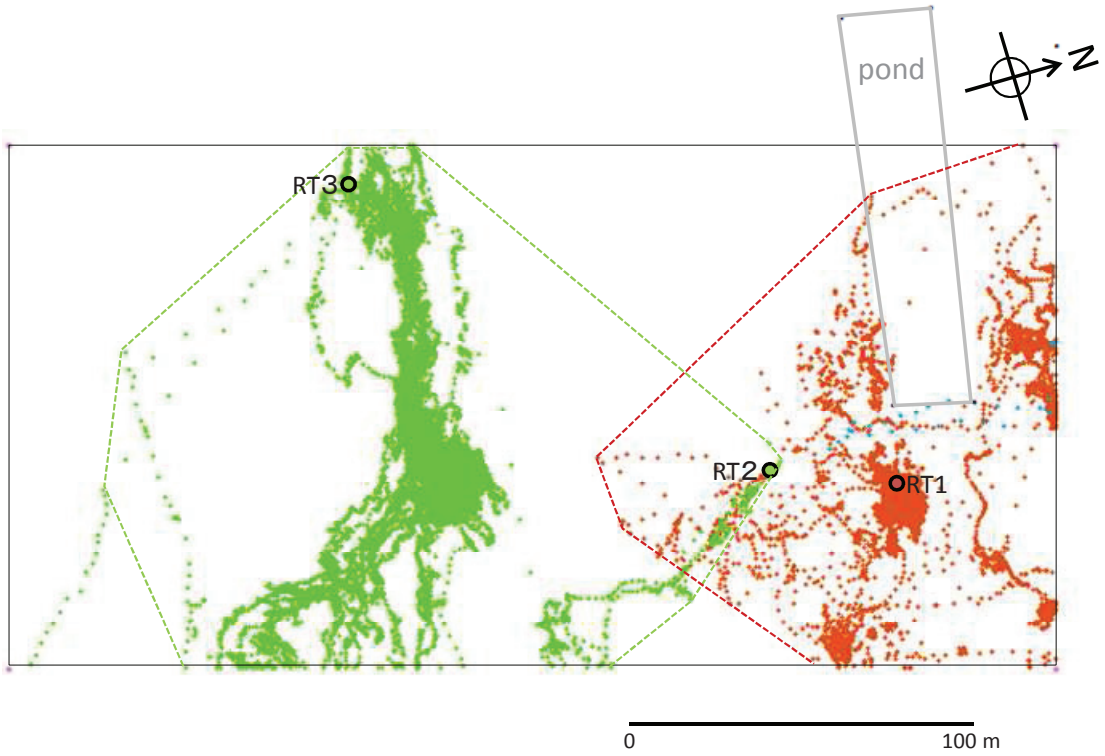


**Figure 7** The positions of junglefowl RJF1 (red), RJF4 (blue), and RJF5 (green) in the experimental field during February 22–March 8, 2008. The black circles indicate roosting trees.

Both RJF1 and RJF5 repeatedly left and entered the experimental field. Therefore, it is difficult to estimate the area of the home range of a junglefowl, but RJF5 remained in the field almost all day on March 6. This is the fifth finding: the lower bound of RJF5's home range was 20.6 ha.

The sixth finding is about roosting places. As noted from Figure 4, RJF1 roosted at RT1 in Figure 7 on February 26, March 2, and March 4; RJF5 roosted at RT2 on March 5 and at RT3 on March 6 and 8. These facts imply that junglefowl do not always roost on the same place; they have a few favorite roosting trees and they wander from roosting tree to roosting tree.

The seventh finding is that the average walking speed of RJF5 was 32 m/min (1.92 km/h). It should be noted that its variance was so large (the standard deviation was 47 m/min)

that the average speed might be a misleading value. This partly resulted from the variance in time intervals available for estimating speed. As noted earlier, the time intervals at which data was received by the WiFi system were not constant, although tags transmitted data every second. The time interval was sometimes one second but sometimes ten minutes. This variation gives different meanings of speed; for instance, a 32 m walk during one minute may be different from a 1.92 km walk in one hour (although they are mathematically the same speed), because a junglefowl may stop walking for a few minutes during the one hour. We may call the troublesome problem resulting from differences in time units (intervals) the *modifiable time unit problem*, which corresponds to the *modifiable area unit problem*, a notorious problem known in spatial analysis (Openshaw, 1984).

While the last finding is uncertain, it may be worth noting for further studies. We observed that junglefowl flew; in fact, when we released junglefowl, some of them flew away. They were soon out of sight in trees and it was difficult to see where they landed. In inspecting the data we found, fortunately, that RJF1 crossed a pond twice (see Figure 7), implying that RJF1 must have flown across the pond twice. We estimated from those data that in the case of a 12 m flight, the flying speed was 45 m/min (2.7 km/h); in the case of 52 m flight, the flying speed was 415 m/min (24.92 km/h). We were bewildered by this great difference, but recalling that the spatial resolution of the system was 3.62 m, we consider the latter value to be more reliable than the former, because of the much longer flight distance. In addition, we observed in a well-controlled experiment carried out in a park in Tokyo on February 17, 2007 that the maximum speed (almost flying) of a white leghorn was 367 m/min (22 km/h). Alternatively, it may be that the junglefowl hovered during the 12 m flight, because, as seen in Figure 7, the flight was not straight, but crooked. Further experimental studies are necessary to confirm the flying speed of a junglefowl.

The WiFi positioning system was actually useful for obtaining the above results, but installing and uninstalling the system in a bushy area required much labor and cost. In addition, the installation of the system possibly caused some junglefowl to leave the experimental field; in fact, we never received data from RJF2 and RJF3. We wanted to continue the experiment to stabilize the experimental environment, but our budget did not allow further continuation.

**The results of the experimental study of free-range chickens and discussion**

Like the study of junglefowl in the preceding subsection, position data were also unstable in this experiment but their stability was better. The fixed tags placed on the three chicken houses (Figure 2) showed that the positions of each fixed tag were dispersed over time around the center and that 95% points were within 5.63 m, which was shorter than in the junglefowl case by one meter. This better accuracy resulted from the fact that the field was mainly cleared ground with a few trees. With this accuracy in mind, we analyzed the spatiotemporal behavior of free-range chickens.

“Free”, as in free-range chickens, does not imply that chickens can walk freely around a field; their walking ranges are spatially restricted to some extent because of the interaction between chickens. We estimated the home ranges of the chickens by the kernel density estimation method (Sliverman, 1998). According to Okabe *et al*. (2009), the major findings are as follows.

First, the shapes of the home ranges of the three cocks were different, although they overlapped to some extent. This overlap was larger than that of junglefowl.

Second, there existed hens whose home rages were almost the same as their cocks (some hens followed their cocks). CH1_H1–H5 and CH2_H4 followed CH1_C1; CH2_H5 followed CH2_C1; and finally CH3_H1–H3 and CH3_H5 followed CH3_C1.

Third, the home ranges of the young hens CH2_H2_Y and CH2_H3_Y were different from those of the cocks.

Fourth, the home ranges of the hens with chicks were different from those of the cocks. The home range of the hen CH_H3_M with chicks was similar to those of the young hens during November 6–7, but then it was different on November 8.

Fifth, the groups formed in daytime were different from the groups as originally kept in the chicken houses. After being freed, the groups were: CH1_C1, CH1_H1–H5, CH2_H4; CH2_C1, CH2_H5; CH3_C1, CH3_H1–H4; CH2_H2_Y, CH2_H3_Y; CH1_H1_M; and CH3_H4_M.

Sixth, the chickens that form a group in daytime did not always sleep at the same site at night. The sleeping site of CH2_H5 was different from that of CH2_C1 and the sleeping site of CH2_H4 was different from that of CH1_C1, CH1_H1-H5. Chickens that belong to different groups in daytime may sleep at the same site. CH3_H4_M and CH2_H2_Y, CH2_H3_Y slept at the same site, the house CH2

Seventh, the chickens did not always sleep at the houses where they were originally kept. We observed that CH2_C1 slept under the floor of house H5; CH2_H4 and CH2_H5 slept in a tree near H5 and a tree in the east of the field (triangle in Figure 2), respectively; CH1_H1 was evicted from CH1 and slept on the roof of CH1.

Last, the home range expanded when food was not given, but the extent of expansion differed from chicken to chicken. In particular, the expansion rate of the young hens was large.

Having found the above behavioral characteristics of free-range chickens as well as those of junglefowl, we consider that the WiFi positioning system was very useful for continuously observing animal behavior over space and time. However, we noticed a few disadvantages of this system. First, WiFi devices are expensive. Second, the installation of the system requires several days with much labor. Third, the system cannot be employed in areas that humans cannot access. The WiFi positioning system, a new information technology, has these shortcomings, but with the rapid recent progress of information technologies, we expect that a new positioning system will be invented in near future to overcome those disadvantages and reveal the detailed spatiotemporal behavior of animals.

## ACKNOWLEDGEMENTS

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# The Taming Process of Red Junglefowl

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## ABSTRACT

The objective of this paper is to depict how local farmers tame junglefowl in the distribution area of junglefowl. Specifically, we conducted research about the capturing and taming of live junglefowl in northern Thailand. We found four junglefowl raised in northern Thailand. The junglefowl raisers had in common that they were all male and hunted junglefowl during the agricultural off-season. The places where they captured junglefowl were of two types, forest or orchard. They encountered the junglefowl accidentally and failed to capture the parent junglefowl but captured the junglefowl chicks relatively easily. The methods of raising junglefowl were that they fed them maize and rice. The raising places, for example raising in a cage, on a tree branch or free range, differed according to the junglefowl's age.It is known that junglefowl are very cautious and nervous birds but our research found some local farmers succeeded in raising junglefowl using a cage or basket. There is a high possibility that, although the junglefowl on the basis of local resident's recognition were the same, different individuals had different natures and characteristics.

**Key Words**: junglefowl, taming process, chicks, hunting, semi-domestication

## INTRODUCTION

It is known that Red Junglefowl (*Gallus gallus*, hereinafter called junglefowl) are distributed roughly in humid tropical Asia. We have found several Red Junglefowl raised in the remote areas of Thailand, Laos, Vietnam, Burma and Bangladesh etc.(Photo1). The objective of this paper is to indicate how local farmers tame Red Junglefowl in the distribution area of junglefowl in humid tropical Asia. Specifically, we elucidate how they capture junglefowl, the process of taming them and the methods of rearing and the reason why they raise junglefowl.

At the moment there is not enough basic information concerning the taming process of chickens. We attempt to present basic information concerning the taming process of chickens in northern Thailand on the basis of the direct observation, interview and collecting documents. In this paper we focus on some case studies in terms of the relationship between Red Junglefowl and hillside farmers. We have checked the morphological and body colour changes of captive junglefowl and have observed the methods of raising junglefowl and its variations through observation. We conducted this study at a Hmong village in Nan Province and two Mien (Yao) villages in Phayao Province in northern Thailand and employed an anthropological approach.

The people in humid tropical Asia have long had a close relationship with junglefowl, for example hunting them for food (Ikeya et al. 2008). Although it is said that taming junglefowl is difficult since they are very cautious and nervous birds (Okamoto, 2001), it has been partly reported that local residents have raised captured junglefowl and junglefowl crossed with native chickens (Nishida et al. 2000).

## ECOLOGY OF JUNGLEFOWL AND LOCAL PEOPLE'S PERSEPTION

Junglefowl hunting has been conducted in Thailand, Laos and southern China (Ikeya et al. 2008). The hunters of junglefowl have an in-depth knowledge of the ecology of junglefowl and recognise that junglefowl are very nervous birds (Takada and Oshima 2008:181). They believe it is difficult to bring junglefowl under their control. The male junglefowl makes a mating call in the breeding season so hunters can easily find them, so junglefowl hunting takes place in this season. Junglefowl, especially males, establish their territory in the breeding season. If a hunter brings a male domestic chicken into their territory, the male junglefowl will approach the male domestic chicken and attack it, in some cases because the male junglefowl thinks that its territory is being invaded. There is a report on the ecology of junglefowl in Chiang Rai Province which suggests that junglefowl have a different ecological behavior in the rainy season (June-October) and the dry season (November-May). In the breeding season (February-April), they make a breeding flock which consists of one male and several females. The home range of junglefowl in the dry season is smaller than in the rainy season. Calling at dawn is done by male flock leader (Ohshima, Takada and Kawashima, 2006).

Several ethnic minority groups, so-called hill tribe people, for example the Karen, Akha (E-ko), Lahu (Musser), Hmong and Mien (Yao) live in the hillside areas of northern Thailand. Principally, we focused on the Hmong and Mien people and conducted a field study in three villages, one village in Nan Province which consisted of Hmong people mainly and two villages, in Phayao Province consisting of Mien peoples. These two ethnic groups originated in South China and migrated to northern Thailand in the nineteenth century.

The main subsistence and economic activity in the villages studied is agriculture. They grow upland rice, maize and lychee, etc. As far as livestock raising in the study village is concerned, chickens and pigs are very common in all villages and some villagers raise cattle (Nakai 2009).

We will start by explaining the local name for Red Junglefowl. Hmong people called chickens *Qaib* in the Hmong language and junglefowl *Qaib qus* or *Qaib teb*. The word *qus* means a thing that runs away when it meets people, so *Qaib qus* means a chicken that so runs away in literal translation. The word *teb* means agricultural field, so *Qaib teb* means a chicken that lives in an agricultural field, in literal translation. The male chicken and female chicken are called *Hlau qaib* and *Poj qaib*, respectively. The junglefowl is similarly named, for example, the male junglefowl is called *Hlau qaib qus*. In addition, a decoy chicken is called *Qaib dib*.

On the other hand, Mien people called chickens *Jai* in the Mien language. Junglefowl are usually called *nx'g-Jai* and also called *hiad-Jai*. The word *nx'g* means a bird and the word *hiad* means wild or not tamed. So in literal translation, *nx'g-Jai* and *hiad-Jai* mean wild chicken and not tamed chicken, respectively (Masuno, 2006). The male chicken and female chicken are called *Jai-kxvj* and *Jai-Eeid*, respectively. Lastly, *Kai paa* in Thai word show pure wild chicken and the domestic chicken entered into the forest from the settlement (Takada and Oshima 2008:184). As the result, we have to be take care of the genetic aspect of junglefowl.

As described above, Hmong and Mien hillside farmers in northern Thailand recognize junglefowl as 'the chicken that runs away when it meets people', 'the chicken that lives in an agricultural field' and 'the chicken that is not tamed' and they maintain their knowledge about the ecology of junglefowl by hunting junglefowl.

## RESULTS

**The methods of taming Jinglefowl**

**1. The situation when people captured junglefowl**

**Case 1:** We could find two junglefowl called *Kai paa* (one male and one female), raised by Mr.X living in the Village B, Nan Province. In May 2005, Mr. X was looking at his cattle grazing the grass under lychee trees and found junglefowl in the lychee orchard. He captured five chicks but failed to capture their parents. He brought the chicks back home to raise them but three chicks (sex unknown) died immediately. The remaining two chicks (one male and one female) survived.

**Case 2:** In May 2005, Mr. K captured Junglefowl in the longan orchard of his Village (Photo2). He found Junglefowl , which was not a chick but already grown, and chased it. Junglefowl ran into some dense thickets of *Mimosa sp.*, which was viny with hooked thorns and became trapped in the thicket, so Mr. K was able to catch it with his hands.

**Case 3:** In 2002, when Mr. S was roaming the forests alone hunting but not seeking any particular animal, he happened to meet several junglefowl chicks and a female parent junglefowl. The female parent junglefowl flew away but the chicks remained. He caught one male and one female chick with his hands. Mr. S brought these two chicks home to raise them. Although Mr. S was able to keep the female chick alive for a while, it died before growing to adulthood.

**2. The process of taming junglefowl**

**Case 1:** Firstly we focus on the rearing environment of the junglefowl. On 25 April 2006, two Junglefowl were kept together in a box type cage woven from bamboo and placed 50 cm above the ground at Mr. X's house. Both junglefowl jumped in the cage so their wings were damaged. These junglefowl were not tethered in the cage but when we asked Mr. X to show us his junglefowl, he tied the junglefowl's leg with string to bring it out of its cage. When we revisited in October 2007, the same cage was used to raise these junglefowl.

Secondly we focused on the feed of the junglefowl. The junglefowl were fed in the cage which has two small plastic holders, one for water and the other for feed. We studied the frequency of feeding and the variety of feed for seven days from 13 to 19 September 2006 by direct observation (Table 1). The junglefowl were fed once per day for five days, two times per day for one day and they were not fed on 17 September. The feeding times were five times in the morning, one time at noon and one time in the evening. Mr. X fed the junglefowl two times and his mother fed them five times. We observed four kinds of feed, unhusked rice, milled rice, boiled rice and maize. The maize was fed after they ground it by using a millstone. They fed unhusked rice on two occasions, milled rice two times, boiled rice one time and hybrid maize two times. Thus, they fed rice or maize to their junglefowl once a day usually.

**Case 2:** In November 2005, Junglefowl was raised in a basket made of bamboo in the backyard (Photo3). Junglefowl and the cage were tied with string. Mr. K use maize, unhusked rice and boiled rice as food stuff. In March 2006, Junglefowl was raised on a tree branch near Mr. K's house. Its right leg and tree branch were secured with string. In May 2006, Junglefowl was raised in a cage made of wood in the backyard. In March 2008, Junglefowl was raised under the floor of the hut which is on stilts. Under the planks Junglefowl and domestic chickens were raised together and cross bred with each other.

**Case 3:** Mr. S succeeded in raising one male junglefowl and continued to raise it. Junglefowl was raised with Mr. S's domestic chickens and kept free range throughout the day. Mr. S fed maize and milled rice to Junglefowl and his domestic chickens. Although Mr. S raised Junglefowl for more than three years, Junglefowl kept a certain distance at all times from people, including Mr. S. When sensing danger, Junglefowl flew away. Mr. S told us that Junglefowl was different from his domestic chickens in the difficulty of getting close to it and its flying off when alarmed.

Mr. S also told us that if he forced Junglefowl to live in a cage, Junglefowl would not eat any feed so he had no other choice but to raise Junglefowl free range. In the daytime, Junglefowl moved around the village, the fields and elsewhere but in the evenings Junglefowl came back to its sleeping place and stayed there the night. Mr. S told us that Junglefowl had slept in the tree every night but when he chased the junglefowl with a stick to move it to another place, Junglefowl spent the night in the gap between the chicken house and its roof.

**3. The purpose of raising junglefowl**

**Case 1:** During our research from April 2006 to December 2006, Mr. X told us that he would cross his junglefowl with his domestic chickens but the crossing had still not succeeded during our research in October 2007.

**Case 2:** In November 2005, Mr. K told us that he tried to cross the junglefowl and a

domestic chicken but had still not succeeded. In our research in August 2007 and March 2008, he had succeeded in cross-breeding. At first, he obtained five crossed chickens of which two fowls died and one fowl was used as gift, so two fowls remained. In the next case he obtained five crossed chickens of which one fowl died so four fowls remained. Mr. K told us that he would choose a good one and use it as a decoy chicken in junglefowl hunting.

**Case3:** More than three years have passed since the Junglefowl was raised and there have been several clutches of crossed chickens between Junglefowl and domestic chickens (hereinafter called crossed chickens). Junglefowl crossed with the domestic chickens spontaneously because they were raised free range. In November 2005, Mr. S kept three crossed chickens and we were able to confirm two chickens in his garden. Mr. S told us that the crossed chickens flew less well than Junglefowl and that a male crossed chicken could be used as a decoy chicken in junglefowl hunting. But in November 2005, the crossed chickens in fact had still not been used as decoy chickens.

## DISCUSSION

The objective of this paper is to depict how local farmers tame junglefowl in the distribution area of junglefowl. Specifically, we conducted research about the capturing and taming of live junglefowl in northern Thailand had raised junglefowl. The results can be summarized as follows.

1. We found four junglefowl raised in northern Thailand. The junglefowl raisers had in common that they were all male and hunted junglefowl during the agricultural off-season.

2. The places where they captured junglefowl were of two types, forest or orchard (longan or lychee). They encountered the junglefowl accidentally and failed to capture the parent junglefowl but captured the junglefowl chicks relatively easily.

3. The methods of raising junglefowl were that they fed them maize and rice. The raising places, for example raising in a cage, on a tree branch or free range, differed according to the junglefowl's age (one month to three years). It is known that junglefowl are very cautious and nervous birds but our research found some local farmers succeeded in raising junglefowl using a cage or basket. There is a high possibility that, although the junglefowl on the basis of local resident's recognition were the same, different individuals had different natures and characteristics.

4. One of the important purposes of taming and raising junglefowl is to make decoy chickens for use in junglefowl hunting. The local farmers think that a decoy chicken whose body shape, wing colour and call resembles the junglefowl is suitable for junglefowl hunting, and cross-breeding between junglefowl and domestic chickens can produce good decoy chickens.

As stated at the beginning, the taming process from junglefowl to domestic chicken is not a simple process. It has been said that once humankind developed a close relationship with junglefowl, they developed semi-domesticated chicken and then domestic chickens were created from the repeated process between semi-domesticated chickens and escaped chickens (Akishinonomiya 2008, 2010). In this paper we could clarify the methods that local farmers use for taming junglefowl.

Figure 1. The junglefowl raised in the cage in Vietnam

Figure 2. The lychee orchard where junglefowl were captured in Thailand

Figure 3. The junglefowl called *Kai paa* in Thai word are raised in a baboon cage

Table 1. Feeding activities and feed stuff of reared junglefowl in September 2006.

Source: Ikeya K.et al.(2010:84)

| Date | Time | Feeding Number | Variety of feed | | | | Feeding person | |
|---|---|---|---|---|---|---|---|---|
| | | | Maize | Unhusked rice | Milled rice | Boiled rice | Mr.X | Mother |
| 13 | Morning<br>Noon<br>Evening | 1 | | X | | | | X |
| 14 | Morning<br>Noon<br>Evening | 2 | X | | | | | X |
| 15 | Morning<br>Noon<br>Evening | 3<br><br>4 | X | | | <br><br>X | X | <br><br>X |
| 16 | Morning<br>Noon<br>Evening | 5 | | | X | | | X |
| 17 | Morning<br>Noon<br>Evening | | | | | | | |
| 18 | Morning<br>Noon<br>Evening | 6 | | X | | | | X |
| 19 | Morning<br>Noon<br>Evening | 7 | | | X | | X | |

Source: Direct observation by the author.
Note: (X) indicated fed.

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# Conservation of Red Junglefowl Biodiversity by Primordial Germ Cell Cryopreservation

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## ABSTRACT

Primordial germ cell (PGC) can be cryopreserved and used as a potential tool for the conservation of avian biodiversity. By transplantation of donor PGCs to recipient embryos to generate germline chimeras, the PGCs can migrate to the developing gonads where germ cells are produced thereby enabling a reproduction of the offsprings derived from the donor PGCs through mating of these germline chimeras. From the cryopreserved PGCs, *in vitro* propagation is required to gain sufficient active PGCs for further transplantation therefore a culture condition for Red junglefowl PGCs was studied and herewith reported. A co-culture system was employed, using feeder cells derived from a quail embryo and Kav-1 media containing 5% each of fetal bovine and chicken serum. The cultured PGCs grew in colonies of two distinct forms either composed of aggregated round cells or spreading flattened cells. These cultured PGCs were positive to PAS staining and SSEA-1 indirect immunofluorescent detection, suggesting that PGC characteristics are retained. A biological analysis of both distinctive colonies remains to be explored. The culture condition used in this study can generally support Red junglefowl PGC survival and growth which could be employed in the protocol for Red junglefowl conservation by cryopreserved PGCs.

**Key words:** primordial germ cell**,** culture, red junglefowl, cryopreservation

# INTRODUCTION

Red Junglefowl has been suggested for being the ancestor of world domestic chickens (Fumihito et al., 1996) of which a rich biodiversity can offer a vast resource for the development of subspecies, breeds and lines of descendants including ornamental chickens and commercial poultry. Our previous studies suggested that domestication process partly involving man-made genetic selection resulted in the reduction of anti-oxidant capacity. This could be a risk factor for survivability of domestic chickens living under stressful conditions such as high stocking density in industrialized poultry farming. The healthy population of Red Junglefowl could therefore serve as a gene bank to ensure the continuation of chicken species.

Although the Red Junglefowl biodiversity seems critically essential, it is at risk of decline. Effective and timely implementation of conservation program is therefore crucial for ecological homeostasis. The maintenance of living stocks as a traditional conservation approach could be hampered by various threats i.e. limited food sources and habitats, poaching and illegal trade, climate changes, and emerging diseases. In avoidance of the trouble of maintaining the living stocks, focused technology for preservation of avian genetics has been on cryopreserved semen (Blesbois, 2007) by which homozygous recessive female characters could hardly be recovered. In addition, the problem of low fertilizing ability of frozen/thawed avian semen remains to be corrected (Long, 1996; Makhafola et al., 2009). Some promising tools for conservation of mammalian species such as embryo and oocyte cryopreservation (Prentice and Anzar, 2011) and cloning (Marshall, 2000; Shimozawa et al, 2002) are not yet possible in avian. Effective means to conserve and restore the avifauna biodiversity has therefore been in search of science.

Biotechnology as a promising tool to create newborn from genetic materials could be potentially applied in part of the restoration protocol; however, current available technology such as cloning has been unsuccessfully tried in avian species. The later development of germline stem cell technology has been introduced as an effective alternative. From cryopreserved primordial germ cells (PGCs), chimeric birds can be produced by transplantation of cultured PGCs to recipients of either the same or different species (Naito et al., 1994). Donor-derived offspring are then produced by breeding of these germline chimeras (Wernery et al., 2010).

Germline chimera technology has been successfully developed with varied efficiency among reports (Macdonald et al., 2010; Naito et al., 2010; Park, 2003; van de Lavoir et al., 2006; Tajima et al., 1993; Wernery et al., 2010). PGC propagation *in vitro* appears to be indispensable to obtain sufficient number of PGCs for transplantation and so to increase the success rate of PGC homing to the developing gonads. The reported culture protocols are mostly based on chicken PGC experiment, which might need adjustments for culturing PGCs derived from other species.

This study therefore aimed to establish an in vitro culture protocol for the propagation of PGCs collected from Red Junglefowl and to cryopreserve the cultured PGCs for future creation of germline chimera.

## MATERIALS AND METHODS

*Embryos and PGC isolation*

Ten fertilized eggs of white ear-lobed Red Junglefowl (*Gallus gallus gallus*) were used for PGC collection. The PGCs were collected from blood circulation of the embryos at 13-15 developmental stage, stage identification was according to Hamburger & Hamilton (1951). To obtain the embryos of 13-15 staged development, the eggs were subjected for 50-55 hour incubation provided the conditions of 38.5 °C, 55% relative humidity and 1-hour interval rotation. Under a stereoscope (Olympus, SZ51), a fine glass micropipette was inserted into the vitelline vein through a small window on the shell priorly made. The aspirated blood was mixed with Kav-1 media containing 5% each of fetal bovine serum and chicken serum before transferring onto a double layer of 5.5% and 11% nycodenz in a centrifuge tube, which was subsequently centrifuged at 4 °C with 400 g force for 30 min. After centrifugation, the fluid between the two layers of nycodenz suspended the PGCs was aspirated and transferred to another tube for further pelleting. The pellet was washed thrice, and then re-suspended in 200 µL of Kav-1 media containing 5% each of fetal bovine serum and chicken serum.

The cell suspension, consisting of a mixed population of PGC and blood cells was examined under a phase-contrast inverted microscope (Olympus, CK40) for further purification of PGCs by manual aspiration using a glass micropipette. The PGCs were differentiated from blood cells by morphology (Zhao and Kuwana, 2003).

*Feeder cells*

Feeder cells were employed in this study to support the culture of wild chicken PGC using a protocol reported by Kuwana et al. (1996). The feeder cells were priorly screened for the capacity to support PGC survival and growth, using commercial broiler chicken PGCs for screening. Each feeder tested was derived from a quail embryo of 13-15 developmental stage. A hind gut sample from the last somite to the distal end of the embryo was excised under a stereoscope (Olympus, SZ51) and transferred to a culture dish containing Kav-1 plus 5% each of fetal bovine serum and chicken serum. The sample was cut into tiny pieces before being transferred into a 12.5 $cm^2$ tissue culture flask (BD Falcon, USA) that was later incubated at 38 °C. Propagation of the tissue sample derived cells was continued with the media changed at every 3 days until reaching confluency. Subpassage was performed at a splitting ratio of 1:3, using 0.1% Trypsin-EDTA to detach the cells from the flask. The $10^{th}$ and higher passages of these embryonic fibroblast-like cells were used for PGC culture.

*Preparation of mitotically inactivated quail embryonic fibroblast-like cells*

The $10^{th}$ and higher passages of quail embryonic fibroblast-like cells were grown in 25 $cm^2$ tissue culture flask (Corning, USA) to confluency. Cellular mitotic activity was inactivated by applying 10 µg/mL Mitomycin C (Sigma, USA) treatment at 38 °C. The duration of treatment was varied from 1, 2, 3 and 4 hours to find the optimum condition justified by a completed inactivation of mitotic activity with the least cell death. The mitotically inactivated cells were subsequently treated with 0.1% Trypsin-EDTA until the cells detached from the flask. After washing the cells thrice with and re-suspending in Kav-1 plus 5% each of fetal bovine serum and chicken serum, the cell suspension was counted and approximately 4 $x10^4$ cells were loaded in each well of a 96-well plate pre-coated with rat tail type-I collagen, and then were incubated at 38 °C for 2 hours for them to attach to the

surface. The cells were washed with Kav-1 plus 5% each of fetal bovine serum and chicken serum and observed under a phase-contrast inverted microscope. A mono-layer completely covering the well surface was anticipated.

*PGC culture*

The PGCs were loaded on the feeder cell layer in Kav-1 media containing 5% each of fetal bovine and chicken serum at a density of 100-200 cells/well before being incubated at 38 °C, half of the media was changed every other day. Subculture was performed when the proliferative PGCs aggregated in large colony of more than 30 cells per colony. The PGC colonies were detached from the surface and disaggregated by gently blowing and pipetting, respectively. Trypsinization with 0.1% trypsin-EDTA was applied to detach the tightly adherent colony if present.

*Characterization of cultured PGC*

The cultured cells were examined for PGC characteristics, including positive stage specific embryonic antigen 1, SSEA-1 expression (D'Costa and Petitte, 1999) and Periodic Acid-Schiff, PAS staining (Meyer, 1964). Briefly, the cultured cells were fixed by applying freshly prepared 4% paraformaldehyde. After 10 min of incubation at room temperature, the cells were washed thrice with PBS, pH 7.4 for subsequent examination of SSEA-1 expression or PAS staining.

For SSEA-1 detection, indirect immunofluorescent assay was used. Briefly, the cells were incubated with 1:50 dilution of monoclonal anti-mouse SSEA1 (Santa Cruz Biotechnology, USA) for 2 hr at room temperature then washed twice with PBS, pH 7.4. Following washing, 1:200 dilution of secondary antibody, goat anti-mouse IgG-FITC (SantaCruz Biotechnology, USA) was applied onto the cells and incubated for 1 hr at room temperature, and then repeated washing. The cells were subjected for nuclear counterstaining using 5µg/mL Hoechst 33342 (Sigma, USA) before being observed under a phase-contrast inverted fluorescent microscope (Olympus IX71).

PAS staining was performed by incubating the cell with periodic acid for 10 min then thoroughly washing with PBS, pH 7.4. The cells were subsequently incubated with freshly prepared shift reagent for 30 min then were washed with PBS, pH 7.4. The PAS stained cells were observed under an inverted microscope (Olympus, CK40).

*Cryopreservation*

The cultured PGCs were cryopreserved in liquid nitrogen using fetal bovine serum containing 10% DMSO as a freezing media. Briefly, the cultured PGCs were harvested, washed in PBS, pH 7.4, resuspended in the freezing media before being transferred into a cryo-tube and then cooled down to -80 °C at the rate of approximately 1 °C/min using a Bicell bio-freezing vessel (Nihon Freezer Co., Ltd, Japan). The frozen sample was then stored in liquid nitrogen.

## RESULTS AND DISCUSSION

*PGC isolation and purification*

In this study, the embryos at developmental stages of 13-15 based on Hamburger and Hamilton scores (1951) were used for collection of the blood circulating PGCs. Avian PGCs transmigrate from germinal crescent into the blood circulation at stage 11 and remain in the circulation until the embryo reaches stage 17 when the PGCs again transmigrate into the developing gonads (Fujimoto *et al*., 1976; Kuwana, 1998). PGC collection from the

embryo younger than stage 13 was fairly difficult due to very small blood vessel. Furthermore, less numbers of PGCs were present in the circulation. For the embryo older than stage 15, a decrease in the numbers of isolated PGCs as compared to those collected from 13-15 staged embryos was experienced. This is in line with the report by Tajima et al. (1999) in which the number of circulating PGCs was found at maximum at stage 14-15 then declining due to the migration to the germinal ridges at stage 15-16. We also noticed that more blood was channeled to supply the developing organs causing a difficulty in aspirating the total blood completely from the vitelline vein.

In the whole blood sample collected from the embryo, PGC can be distinguished from blood cells by different morphology under a phase contrast inverted microscope (fig 1a). The PGC is round in shape and larger in size with large nucleus that reflects the oblique illumination observing under a dark-field inverted microscope. The population of PGCs in the whole blood sample was found relatively small. After the purification with nycodenz density gradient, a significant number of blood cells were removed giving much higher proportion of the PGCs (fig 1b) in the semi-purified sample. The final purification by manual aspiration of each microscopically identified PGC yielded absolute purified PGCs (fig 1c) for further used in *in vitro* cultivation.

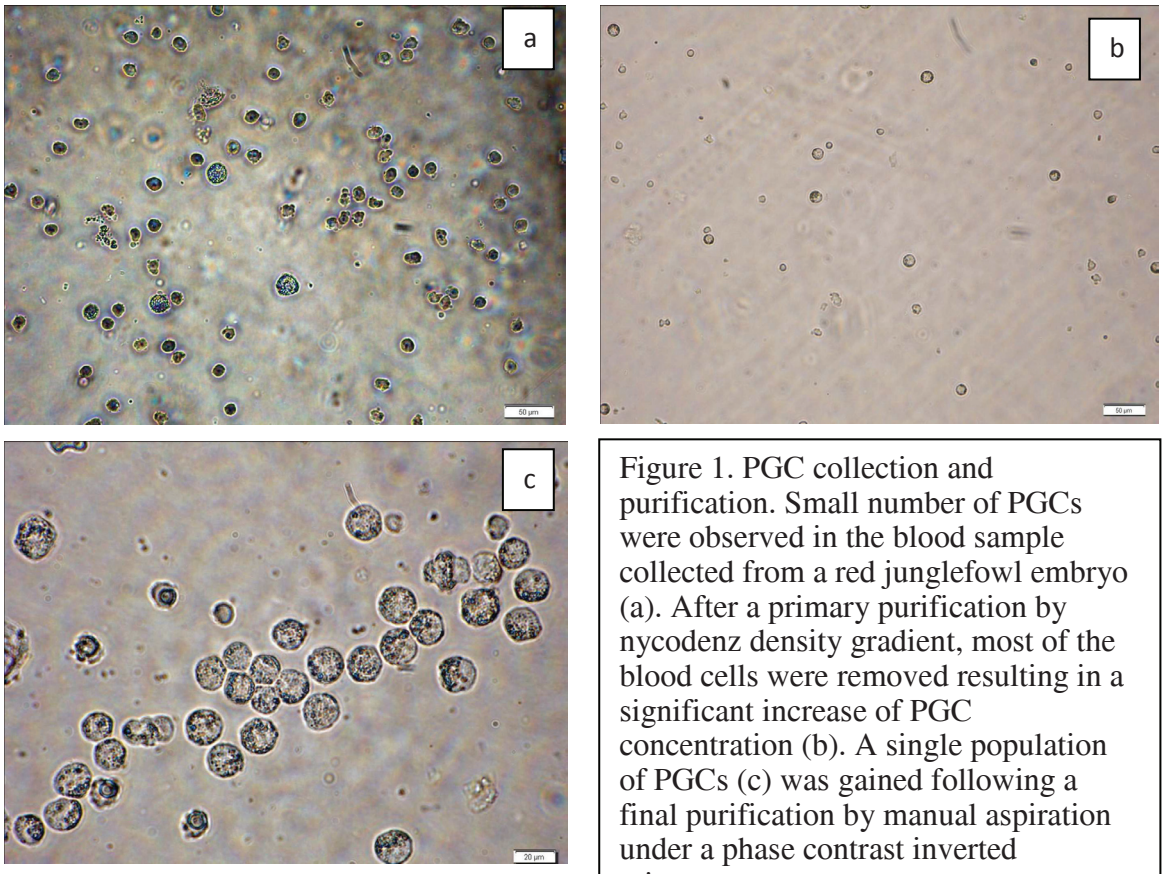


Figure 1. PGC collection and purification. Small number of PGCs were observed in the blood sample collected from a red junglefowl embryo (a). After a primary purification by nycodenz density gradient, most of the blood cells were removed resulting in a significant increase of PGC concentration (b). A single population of PGCs (c) was gained following a final purification by manual aspiration under a phase contrast inverted microscope.

The number of PGCs purified from the whole blood sample of each embryo was in a range of 4-20 cells, which is relatively small compared to a range of 100-200 PGCs isolated from each commercial broiler chicken embryo.

*Feeder cell preparation*

The embryonic hindgut sample attached to the surface of the tissue culture flask after 24 hours of incubation in Kav-1 media containing 5% each of fetal bovine serum and chicken serum at 38 °C. A propagation of fibroblast-like cells was initially observed after day 2 of culture. Starting from the edge of the tissue, the fibroblast-like cells continued on growing to form a dense patch of mono-layer surrounding the tissue sample (fig 2) which took approximately 10 days.

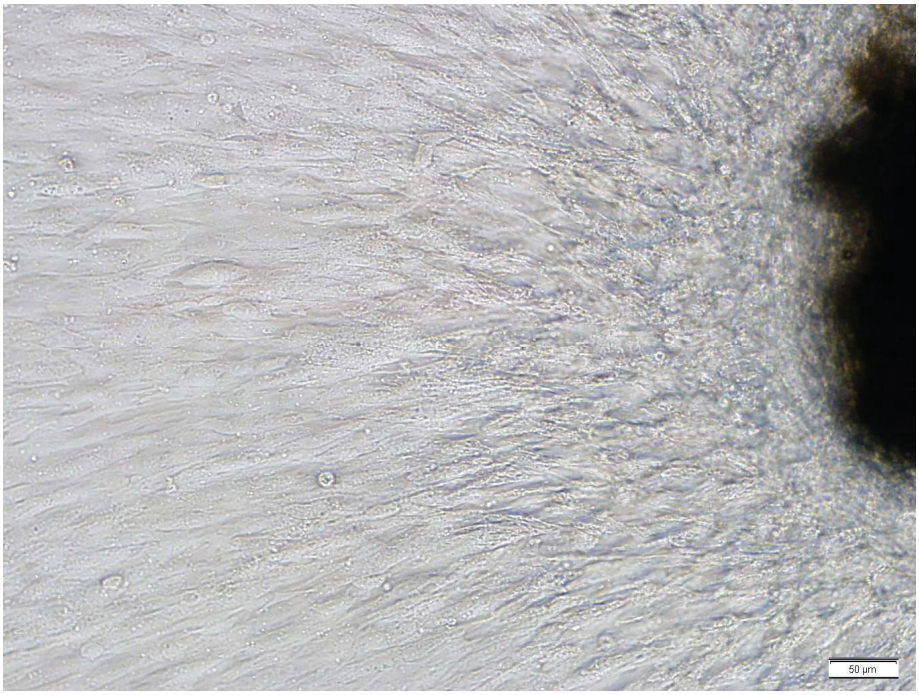


Figure 2. Fibroblast-like cells continued on growing to form a dense patch of mono-layer surrounding the tissue sample

Subculture using 0.1% Trypsin-EDTA yielded a nice single cell suspension. The early passages appeared morphologically non-homogeneous with different average doubling times between the passages. This might suggest that the culture contained a mixed population. By passaging, the uniformity of the cultured cells was gradually increased. The passage containing a morphologically homogeneous cell population with a constant doubling time for 3 consecutive passages was used for PGC culture. In this study, the $34^{th}$- $40^{th}$ passages were used as feeder for the culture of Red Junglefowl PGCs.

The optimum duration of Mytomycin C treatment for inactivating the mitotic activity of quail embryonic fibroblast-like cells found in this study was 4 hours. Incomplete inactivation was presented in the trials using the shorter treatment durations while a significant increase of cell death was observed in the trial with the longer duration.

*Culture of PGCs*

The PGCs loosely attached to the feeder layer after 24 hours of incubation in Kav-1 media containing 5% each of fetal bovine serum and chicken serum at 38 °C. After a few days, the PGCs aggregated in small islets consisting of 4-7 cells each (fig 3).

Slow proliferation was observed in the first weeks of culture, subsequently the proliferation rate was significantly increased. By the end of week 2, some large colonies

consisting of more than 30 cells each were grown. Interestingly, the propagated colonies appeared in 2 distinct forms, one comprised tightly aggregated round cells (fig 4a) and the other consisted of spreading flattened cells (fig 4b). Biological differences between the two forms remain to be explored.

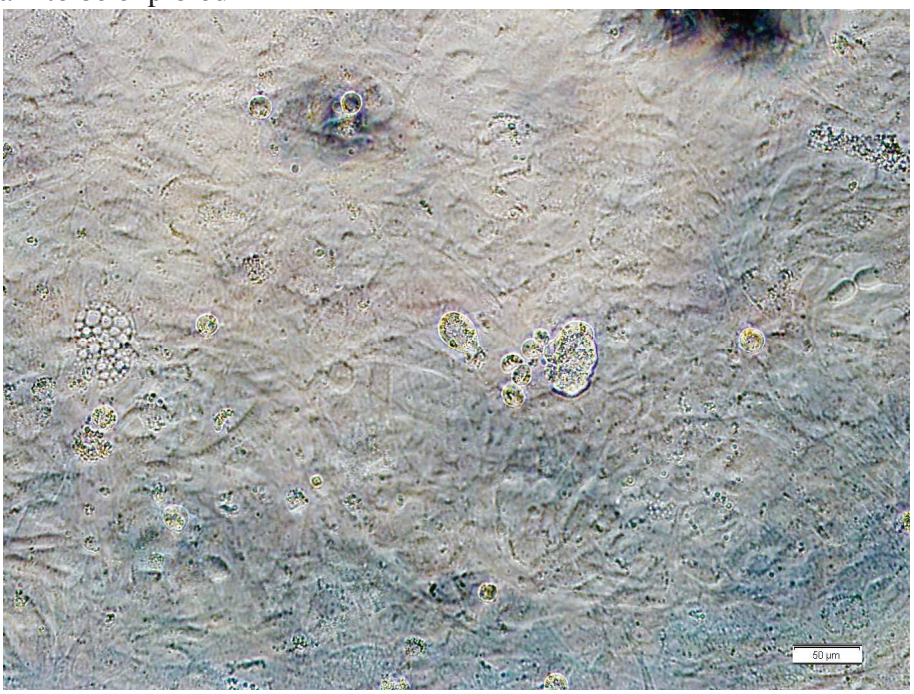


Figure 3. PGCs aggregated in small islets consisting of 4-7 cells/colony

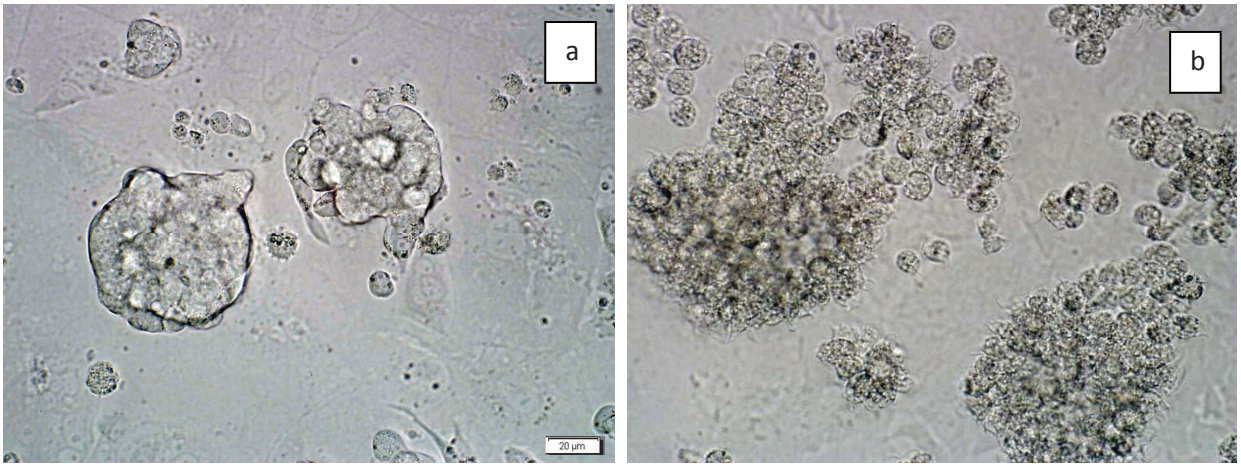


Figure 4. PGC co-culture with feeder cells: Two distinct forms of colonies composed of aggregated round cells (a) or spreading flattened cells (b) were observed.

The tightly aggregated round cell composing colonies attached loosely to the feeder layer; therefore, subculture was achieved by simply blowing and pipetting. Trypsin-EDTA was used to detach the other form of colony which bound tighter to the feeder layer making it difficult to be dispersed by blowing and pipetting. In this study, the culture and subpassage of Red Junglefowl PGCs can be maintained to the $8^{th}$ passage which accounted for a total culture period of 3 months.

*Characterization of cultured PGCs*

The cultured PGCs were stained pink while the feeder cells were negative to PAS staining (fig 5). PGC has been characterized as a PAS positive cell due to large accumulation of glycogen in the cytosol (Macdonald *et al.*, 2010). The PAS positive staining therefore suggested that the general PGC character was retained in the newly proliferated cells under the culture conditions used in this study.

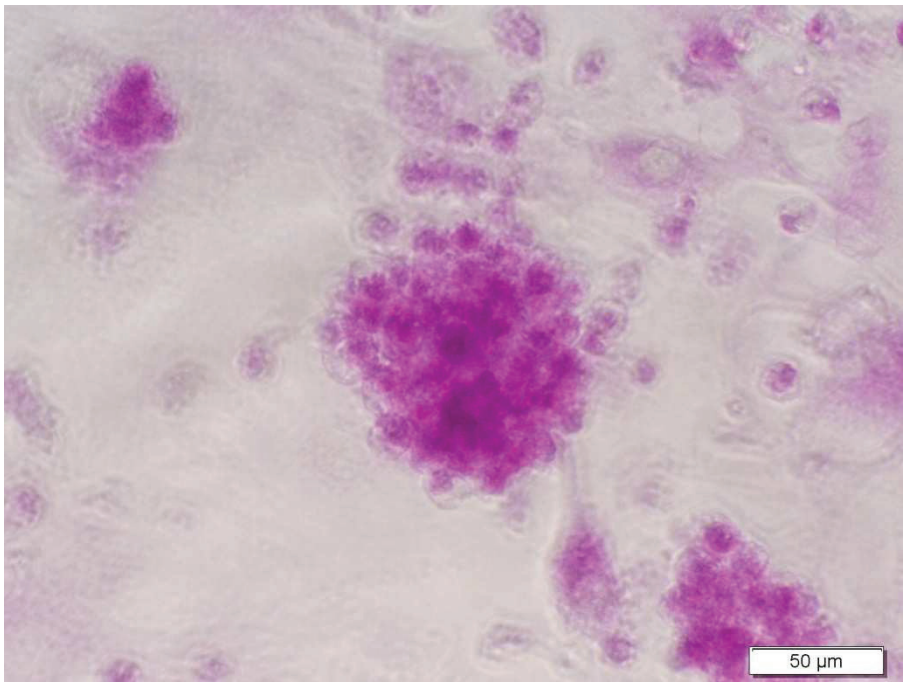


Figure 5. Positive PAS staining of cultured PGC colonies.

The culture cells were confirmed for their stem cell marker, SSEA-1 by immunofluorescent assay (fig 6) using anti-mouse SSEA1 (mouse monoclonal) and FITC conjugated goat anti-mouse IgG as the primary and secondary antibody, respectively.

A positive result as presented by green fluorescent on the cultured cells together with the PAS positive staining suggested that these grown cells are PGCs. It can be derived from this study that Red junglefow PGCs can be propagated *in vitro* by using Kav-1 medium containing 5% each of fetal bovine and chicken serum and embryonic Japanese quail derived feeder cells.

Although the feeder cells can support avian PGC survival and proliferation in this study as well as in other reports (Choi et al., 2010; Naito et al., 2010; Tang et al., 2007), the main obstacle found is the consistency of the feeder prepared for each culture. As being primary embryonic fibroblast-like cells, feeder characteristics including morphology, growth behavior and response to mytomycin C treatment as well as the capacity in supporting PGC culture were subjected to differences even among passages within line of the cells prepared from each embryo. Establishment of a novel cell line having consistent characters and being capable of promoting avian PGC growth is therefore worthwhile.

A feeder-free culture system is another approach to avoid the above mentioned problem. More importantly, this could avoid a possible contamination of the feeder cells in the PGC harvest to be used for transplantation in the production of chimeric birds.

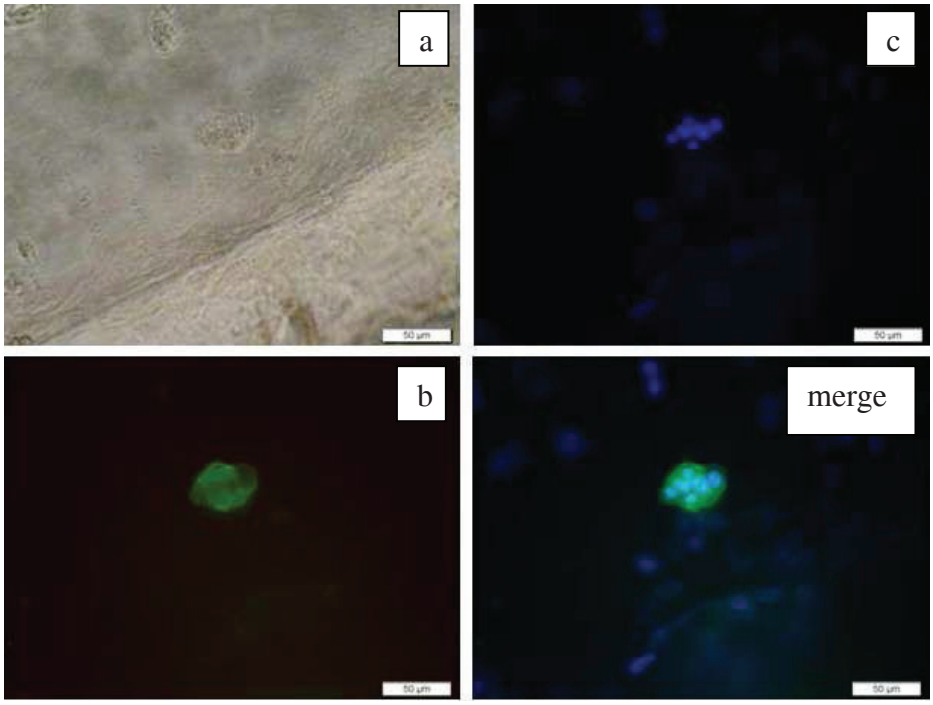


Figure 6. SSEA-1 expression on the cultured PGCs. The PGC colony identified by phase-contrast microscopy (a) was positive to SSEA-1 immunofluorescent staining (b). Nuclear counter staining with Hoechst 33342 confirmed the SSEA-1 localization on the cell surface (c, merge).

Conditioning medium and several survival and growth factors i.e. LIF, bFGF have been experimented (Shiue et al., 2009; Choi et al., 2010); however, the PGC culture performance was apparently inferior to the co-culture system using an embryo derived feeder cell.

The cultured PGCs were cryopreserved in liquid nitrogen using serum containing 10% DMSO as a freezing media. The cryopreserved cells were highly viable (>90 % viability) suggesting that this cryopreservation protocol is acceptable for preservation of Red junglefowl PGCs. Biodiversity of the collected samples will be explored and used for conservation and sustainable utilization planning.

## ACKNOWLEDGEMENT

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# Habitat Utilization of the White Ear-lobed Red Junglefowl (*Gallus gallus gallus*) In the Khao Ang Rue Nai Wildlife Sanctuary, Chachoengsao Province, Eastern Thailand

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## ABSTRACT

The habitat utilization of white ear-lobed red junglefowl (weRJF), *Gallus gallus gallus*, in Khao Ang Rue Nai Wildlife Sanctuary, Chachoengsao province, Eastern Thailand was studied during March 2011 to April 2012. Three types of habitat were classified as the roosting, foraging and nesting sites, with 40, 20 and six different sites, respectively, studied. All habitats were located in dry evergreen forest. The roosting habitats were in open areas with a low ground vegetation density and at most sites (9/10) females roosted in the same roosting branch as the male, with only one case where females roosted separate from the male was found. There was no significant difference in all the measured physical and biological traits for the roosting habitat between the breeding season and the non-breeding season except for the tree density and humidity. The roosting habitat of males showed a significant difference in the perch-to-trunk distance between male and female. Most foraging locations (12 / 20) were close to the roosting habitats. There was no significant difference between male and female foraging habitats or between the breeding and non-breeding seasons. With respect to the roosting and foraging habitats of male and female RJFs in the breeding season, no significant difference in all the physical and biological traits measured was found except for the temperature. In the breeding season, males and females foraged in flocks of 5 to 15 individuals, the largest flock being comprised of two males and 13 females, while in the non-breeding season males and females foraged individually. Nesting sites were located under tree stubs and surrounded by ground vegetation with an average height of 39.8 ± 7.1 cm above ground level, which they likely use as a shelter. Nests were oval in shape and bedded with dry leaves. The foraging and nesting habitats of females in the breeding season used significantly different tree sizes and depth of ground leaf litter, but were not significantly different for all the other measured physical and biological variables.

**Key Words:** habitat utilization, white ear-lobed red junglefowl, Khao Ang Rue Nai wildlife sanctuary

## INTRODUCTION

In Thailand, there are two subspecies of Red jungle fowl (RJF): the red ear-lobed *Gallus gallus spadiceus*, which is distributed from the North to the South of Thailand, and the white ear-lobed RJF (weRJF), *Gallus gallus gallus*, which is distributed in a much narrower range, being found only in parts of Eastern Thailand, such as in Khao Ang Rue Nai Wildlife Sanctuary (KARNWS), Chachoengsao and Chanthaburi province and overlaps with the red ear-lobed RJF subspecies at Pak Thong Chai district northwards to Khao Yai National Park (Meckvichai, 2009). Wanghongsa reported a large weRJF population of some 149 individuals in the KARNWS over the 1079 $km^2$ area (Wanghongsa, 2009). Previously, the KARNWS was subject to degradation or destruction by trespassers using it for logging and farming but at present there are no more human activities in this lowland forest. The KARNWS is composed of more than 90% dry evergreen forest with smaller patches of residual plantations and farmland. There are 666 recorded species of wild animals, including a large population of elephants (Wanghongsa *et al*., 2008). The breeding season of the weRJF is annual and in this area it starts in November and continues until May with the non-breeding season then being from June to October. The ranging area of males was reported to be 9.81 ha. (within 34 days) and for females was 10.2 ha. (in 18 days) (Wanghongsa, 2009), but these previous studies have not included any data on the foraging habitat and nesting sites of weRJFs. In addition, there are Siamese firebacks (*Lophura diardi*) living in the KARNWS as well and they may use the same niche as the weRJF. Such potential competition and the lack of any comprehensive database on the ecology of weRJFs are the principal reasons for the requirement for further research on weRJFs in this area. Although the weRJFs can be productive and breed in captivity, the pure wild breed is quite rare and has possibly cross bred with domestic chickens, while the natural wild population nowadays is still decreasing due to over hunting, and habitat loss and fragmentations. Moreover, more comprehensive ecological information of their habitat utilization is required for any effective conservation management and their continued survival in the wild. The aims of this research were to study the habitat utilization of weRJF, including the roosting and foraging habitats in the breeding and non-breeding seasons and the nesting site in the breeding season. The results of this study will provide the basic data for help in decision making in wildlife management and conservation of KARNWS.

## MATERIALS AND METHODS

### Study area

The study area was located in the Northern part of the KARNWS, Klongtaglao district, Chachoengsao province in Eastern Thailand (Fig. 1) and focused on the area around the Chachoengsao Wildlife Research Station.

The habitat used by the weRJF was surveyed each month, for a week per month, all year round and so included the breeding season (November-May) and the non-breeding season (June-October). The habitat type and microhabitats used be weRJF for roosting, foraging and nesting were recorded.

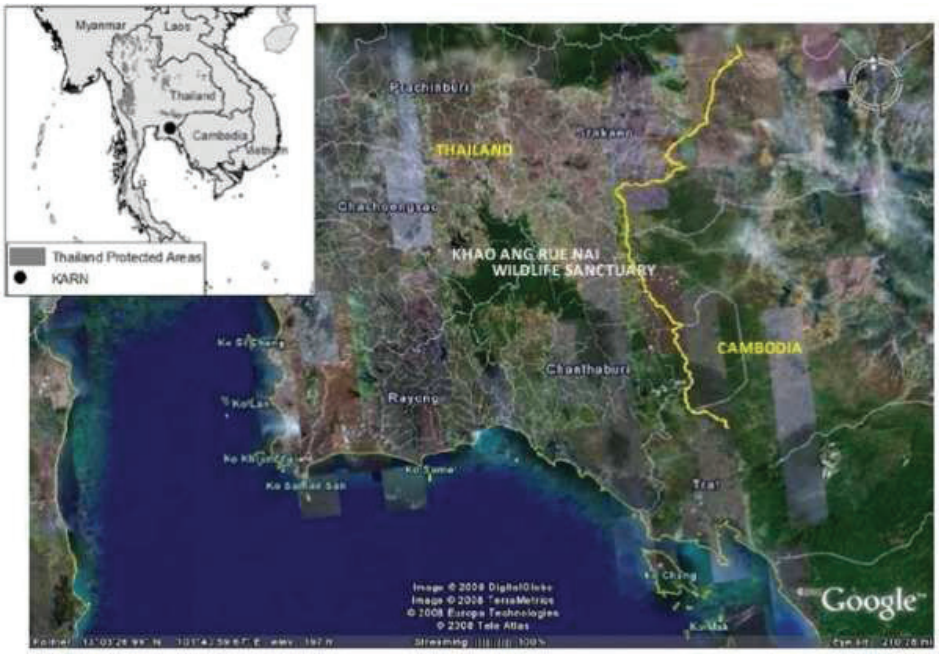



**Figure 1.** Study area: Khao Ang Rue Nai Wildlife Sanctuary (KARNWS)

**Roosting habitat**

The roosting habitat was separated into the physical and biological factors, following Wanghongsa (2009), and measured and recorded accordingly. The physical factors were the air temperature (thermometer), relative humidity (hydrometer) light intensity (light meter) and time of the morning that weRJF descended from their roosting point to the ground. The altitude above mean sea level (amsl) and monthly precipitation were also noted. The roosting trees were recorded in the Universal Transverse Mercator (UTM) coordination system with GPS.

The biological factors studied were the characteristics of the roosting tree, in terms of the tree species, roosting tree height, tree DBH (diameter at breast height, measured at 1.3 m above the ground), and those of the perching branch in terms of the branch height above the ground, diameter and distance from the trunk of perching branch. In addition, the roosting flock size and gender were recorded.

Roosting trees were located as previously reported (Collias and Collias, 1967; Wanghongsa, 2009). Briefly, the roosting site was initially located by the position of the crowing male in the early morning before they descended from roosting tree. In addition, in the non-breeding season, weRJFs usually roost together in the same tree, which then allows the observation of a pellet-pile under the roosting point. Females always roost in the same tree, and typically the same branch as the male weRJF. The behavior of weRJFs at each roosting site was also recorded.

The tree and ground vegetation densities at the roosting habitat were estimated using the point centered quarter method (Bonhum, 1989 in Wanghongsa, 2009). When the pellet pile was

located it was used as a core center. The nearest tree in each quadrate was measured (so at least four trees at the center were estimated). In addition, every tree in each quadrate at distance of 20 m from the center was recorded.

The vertical density of the roosting habitat was estimated using a 20-cm color code graduated 2-m long PVC pole. Holding the PVC pole vertically at 0, 5, 10 and 20 m distance from the center point in each quadrate, the number of 20-cm colored bars that were covered by plants was visually determined and recorded, and then used to calculate the proportion height as a percentage prior to average the vertical density (Rabinowitz, 1999).

The canopy cover was estimated using a 6-cm mirror with 25 grid intersections, holding the mirror at the center point, and visually noting the number of intersections that were covered by the canopy in the four directions. From this the average canopy cover (%) was derived.

**Foraging habitats**

The foraging habitat was separated into the same physical and biological factors, and measured and recorded accordingly, as that for the roosting habitats outlined above. In addition, for the biological factors we studied the diversity and abundance of the potential food species available. The seeding plants and grasses were determined by estimating the total ground area covered by them, whilst small vertebrate and invertebrates were evaluated from four replicated random sampling plots of 1 x 1 m, where the percentage of vertebrate and invertebrate food abundance were estimated as the number of individuals per total plot area. The depth of the litter was measured at the same places that the soil fauna was sampled and so was recorded as the average depth from four random sampled plots. The foraging behavior of weRJF's was also observed by direct observation and recorded.

We located the foraging habitat by direct visual observation in addition to detection of the digging or scratching holes and foot prints of RJF. In the breeding season we followed male weRJFs by their crowing, whilst females were followed by direct observation to find the foraging area in both the breeding and non-breeding seasons.

**Nesting habitat**

The nesting habitat was studied by direct observation and focusing on open areas to find the nests and so their habitat. The nesting habitat was separated into the same physical and biological factors and measured and recorded accordingly, as that for the foraging habitats outlined above. The size, position, and construction material of the nest were recorded as well as the clutch size and any evidence of predation or predators, such as footprints.

**Human disturbance**

The level of human disturbance was studied by measuring the distant from any human settlements to each respective weRJF nest site and the frequency of human activities within 15 m from the nest. We graded human activities into the four categories of (i) no human activity, (ii) humans walk past, (iii) cars and / or motorcycles pass, and (iv) weRJFs are subjected to hunting.

**Statistical analysis**

Data are presented as the mean ± standard deviation (SD), derived from the indicated number of individuals or categories. The statistical significance of difference between means was tested using the Mann-Whitney U-test, accepting a p-value of equal to or less than 0.05 as significant. Specifically, differences between the different physical and biological factors characterized in the roosting and foraging habitats in the breeding and non-breeding season between males and females and between the non-breeding and breeding seasons, and between the foraging and nesting habitat of females in the breeding season.

## RESULTS

The study site is located at the Northern part of the KARNWS at coordinates N13$^0$24' and E101$^0$52'. During March 2010 to March 2011, 40 roosting habitats (male = 30, female = 10), 20 foraging habitats (male = 14, female = 6) and six nesting habitats were defined and studied. All habitats were located in dry evergreen forest, at an altitude of 86 to 123 amsl. The mean annual temperature was 27.55 ± 1.37 $^o$C, average humidity was 91.6 ± 4.9% and a mean of rainfall of 4.66 ± 4.03 mm per month (Chachoengsao Wildlife Research Station, 2011).

**Roosting Habitat**

From direct observation, male weRJF calls (crows), sought and claimed a suitable roosting site and jumped to get on it. Before they alighted from the roosting site and glided down to the ground they would crow thoroughly in several directions, and this was especially marked in the breeding season, although sometimes they jumped to another branch(es) before descending so as to choose a better point to land. The data for the roosting habitat of weRJF in this study is based upon the 40 roosting sites found (male = 30, female = 10). At all bar one roosting site (9/10) females roosted in the same roosting branch as the male but in one case females were found separate from the male roosting tree. Typical images of a roosting habitat, tree and a pellet on the ground below a roosting site are shown in Figure 2.

(a) (b) (c)

**Figure 2.** The roosting habitat of weRJFs. Images show a typical (a) roosting habitat, (b) roosting tree (weRJF shown in red circle), and (c) pullet.

The roosting sites of male and female weRJFs showed no significant difference in the mean tree height (9.01 ± 3.78 m and 10.17 ± 4.50 m, respectively), tree DBH (0.13± 0.05 m and 0.12 ± 0.05 m, respectively, perching branch height (4.48 ± 1.26 m and 4.25 ± 1.59 m), perch

branch diameter (5.85 ± 1.59 cm and 5.21 ± 1.98 cm) or perch to trunk distance (4.45 ± 2.9 m and 6.93 ± 3.32 m).

When comparing the physical and biological factors of the roosting habitat of weRJF between the breeding and non-breeding seasons (Table 1), all the factors were found to not be significantly different except for the tree density ($p = 0.001$) and relative humidity ($p = 0.006$). When comparing the male and female roost sites for all the measured factors across the year, no significant difference was noted for all of them except for the perch-to-trunk distance ($p = 0.042$). In the breeding season, none of the physical and biological factors measured were significantly different between males and females.

**Table 1.** The physical and biological factors (mean ± 1 SD) of the roosting habitat of *G. g. gallus* (weRJF) in the breeding and non-breeding seasons

| Variable | Breeding season (n = 33) | Non-breeding season (n = 7) |
|---|---|---|
| *Tree density (tree/$m^2$) | 0.10 ± 0.34 | 0.14 ± 0.29 |
| DBH (m) | 0.13 ± 0.06 | 0.10 ± 0.02 |
| Ground vegetation density (tree/$m^2$) | 0.09 ± 0.53 | 0.10 ± 0.42 |
| Canopy cover (%) | 75.3 ± 13.9 | 77.9 ± 7.21 |
| Vertical density (%) | 53.6 ± 17.2 | 49.9 ± 18.2 |
| Perching branch height above ground (m) | 4.34 ± 1.32 | 4.67 ± 1.34 |
| Perch branch diameter (cm) | 5.91 ± 1.62 | 4.78 ± 1.70 |
| Perch to-trunk distance (m) | 5.30 ± 3.23 | 4.08 ± 2.54 |
| Temperature ($^oC$) | 25.6 ± 1.28 | 25.6 ± 0.55 |
| *Humidity (%) | 80.0 ± 7.9 | 89.0 ± 4.94 |
| Precipitation (mm/ month) | 2.11 ± 2.65 | 5.87 ± 4.63 |
| Elevation (m amsl) | 104.5 ± 8.0 | 109.8 ± 5.34 |

*Significantly different between the two seasons.

**Foraging habitat**

The foraging behavior of male and female RJFs was observed visually. After crowing, the cock was seen to glide down from the roosting tree and walk around foraging for food near the roosting tree, and then stop for a while and crow before continuing to look for food. This forage-crow-forage cycle was repeated several times, especially in the breeding season. In contrast, the female just walked around silently and looked for food. The foraging habitat of weRJFs in this study is based upon the 20 found foraging sites (male = 14, female = 6). Most foraging sites (12/20) were located close to the roosting habitats, the exceptions being that in late March, we found two couples of weRJFs eating Jumbul seeds (*Syzygium cumini*) at the garage near the Chachoengsao Wildlife Research Station office quarters. Typical images of a foraging habitat and foraging groups are shown in Figure 3.

(a) (b) (c)

**Figure 3.** The typical foraging habitat of weRJFs. Images show a typical (a) foraging habitat, (b) foraging group (1 male and 1 female weRJF on the road in Chachoengsao Wildlife Research Station, marked by red circles), and (c) a flock of foraging weRJFs.

With respect to the foraging sites, there was no significant difference between the mean depth of ground litter (5.83 ± 1.58 cm and 5.52 ± 1.43 cm), ground seed ground cover (49.7 ± 9.2% and 51.3 ± 9.8%) or grass ground cover (53.5 ± 8.0% and 45.5 ± 6.7%) between male and female sites. A diverse array of both vertebrate and invertebrate fauna that could act as food sources was found in both the male and female foraging habitats, being comprised of 66.52 ± 65.78 and 83.04 ± 33.85 numbers/$m^2$ from 15 and 17 taxonomic orders, respectively. Overall, the major invertebrate group was Insecta (82.5%), followed by Cladocera (11.7%) and Arachnida (3.6%). The niche breadth of male and female RJFs were 0.69 and 0.67 respectively, with a niche overlap between males and females of 0.88. With respect to the foraging habitat of male and female RJFs, there was no significant difference for all the physical and biological variables measured between the breeding and non-breeding seasons (Table 2), and between males and females in the breeding season except for the air groups of about 5 to 15 individuals of mixed gender, the largest flock being comprised of two males and 13 females, but in the non-breeding season they were only found foraging as individual males and females.

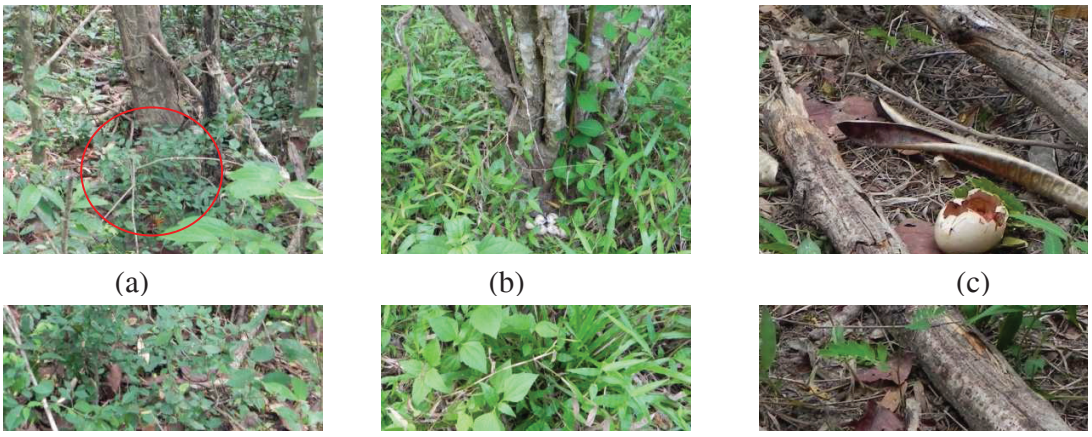
**Nesting habitat.**

Six nests were found, all at an altitude of 105 to 112 m amsl. Nest sites were located under tree stubs and surrounded by ground vegetation with an average height of 39.8 ± 7.1 cm above ground level, which might be used as a protective shelter. Nests were built in an oval to round shape of approximately 22.9 ± 3.8 cm width, 23.9 ± 3.7 cm length and 3.73 ± 2.39 cm depth, and were lined (bedded) with dry leaves. The clutch size varied from 3 to 7 eggs (average 4.5 ± 1.5 eggs per nest). The average depth of ground leaf litter around the nest sites was 9.0 ± 3.0 cm. The average seed and grass ground cover was 63.8 ± 11.9% and 76.5 ± 13.7%, respectively, within which invertebrates of 19 different orders were found. Insects were the major group (89.2%) followed by Arachnida (5.9%) and Cladocera (3.1%).

**Table 2.** The physical and biological factors (mean ± 1 SD) of the foraging habitat of *G. g. gallus* (weRJF) in the breeding and non-breeding seasons

| Variable | Breeding season (n = 18) | Non-breeding season (n = 2) |
|---|---|---|
| Tree density (tree/$m^2$) | 0.12 ± 0.68 | 0.10 ± 0.91 |
| DBH (m) | 0.11 ± 0.06 | 0.10 ± 0.03 |
| Ground vegetation density (tree/$m^2$) | 0.76 ± 0.96 | 0.86 ± 0.11 |
| Canopy cover (%) | 66.4 ± 19.0 | 74.3 ± 12.4 |
| Vertical density (%) | 36.3 ± 31.4 | 46.9 ± 8.0 |
| Depth of ground litter (cm) | 5.93 ± 1.26 | 3.50 ± 2.12 |
| Total basal ground cover (seed) (%) | 49.2 ± 9.0 | 59.5 ± 6.4 |
| Total basal ground cover (grasses) (%) | 50.2 ± 7.7 | 59.0 ± 12.4 |
| Temperature ($^oC$) | 29.7 ± 3.2 | 33.3 ± 1.1 |
| Humidity (%) | 84.0 ± 12.5 | 94.3 ± 2.6 |
| Precipitation (mm/month) | 2.70 ± 1.66 | 6.95 ± 6.90 |
| Elevation (m amsl) | 107.2 ± 9.3 | 108.5 ± 2.1 |

Typical nest site, clutch and evidence of nest predation are shown in Figure 3.



(a) (b) (c)

Figure 3. Nesting habitats of weRJFs. Images show a typical (a) hen brooding eggs (in red circle), (b) nest, and (c) destroyed egg near the nest that might have been eaten by a predator

With respect to the foraging and nesting habitats of females in the breeding season, these were only significantly different for the tree DBH ($p = 0.045$) and depth of the ground leaf litter ($p = 0.016$) (Table 3).

**Table 3.** The physical and biological factors (mean ± 1 SD) of the nesting foraging habitats of female weRJFs in the breeding season

| Variable | Nest (n = 6) | Foraging habitat |
|---|---|---|
| Tree density (tree/$m^2$) | 0.13 ± 0.89 | 0.15 ± 0.62 |
| *DBH (m) | 0.08 ± 0.02 | 0.13 ± 0.04 |
| Ground vegetation density (tree/$m^2$) | 0.13 ± 0.18 | 0.6 ± 0.96 |
| Canopy cover (%) | 63.7 ± 19.4 | 66.8 ± 19.2 |
| Vertical density (%) | 53.9 ± 12.6 | 38.5 ± 27.4 |
| *Depth of ground litter (cm) | 9.03 ± 2.99 | 5.52 ± 1.43 |
| Total basal ground cover (seed) (%) | 63.8 ± 11.9 | 51.3 ± 9.8 |
| Total basal ground cover (grasses) | 76.5 ± 13.7 | 45.5 ± 6.7 |
| (%) | | |
| Temperature ($^oC$) | 28.9 ± 3.0 | 29.7 ± 2.1 |
| Humidity (%) | 75.3 ± 18.3 | 85.8 ± 8.7 |
| Precipitation (mm/month) | 2.48 ± 2.19 | 2.76 ± 0.25 |
| Elevation (m amsl) | 109.5 ± 4.2 | 108.7 ± 6.8 |
| Height of screen (cm) | | - |
| Distance from human settlement | 39.8 ± 7.1 | - |
| (m) | 252.3 ± 387.9 | |

*Significantly different between the nesting and foraging habitats of female RJF in the breeding season

We found evidence of potential nest predator activity at 50% (3/6) of the nest sites. The distance from human settlements to each nest was highly variable (range 12 to 1031 m; 252.3 ± 387.9 m), and accordingly human disturbance was fairly common with human activity (21.7%) near the nest site being exceeded by the frequency of nests by humans walking past (43.3%) or cars or motorcycle passing (35%), but no hunting was found in this area. The relationship between all the evaluated physical factors and nests was not significant.

## DISCUSSION

### Roosting habitat

In this study, all of the weRJF roosting sites overlapped with their foraging habitat all year round, and were in open ground with a low tree density (0.1 tree/$m^2$) and high canopy cover (77.3%). The average height of the roosting branch above ground (4.42 m) and its diameter (5.7 cm), as well as the distance from the perch to the tree trunk (5.0 m), were all similar to that reported before in the same location (Wanghongsa, 2009). These are good roosting sites for RJFs because when predators attack along the roosting branch they can be detected by the shaking giving the RJFs enough time to escape. The height above ground of each roosting branch was more than 4 m, a height that is possibly safe from disturbance from elephants (Wanghongsa, 2009), given that there are plenty of elephants roaming around the study site in the KARNWS. In this study, significant differences in the tree density, humidity and precipitation levels were noted between the breeding and non-breeding seasons. The latter two reflect the general seasonal

climatic changes since the non-breeding season of weRJF is in the rainy season and so the humidity and precipitation were higher than in the dry season. Also, in the non-breeding season male weRJFs do not defend their territory, and so there are more choices for roosting sites that are comfortable for them. Only the perch-to-trunk distance was significantly different between male and female weRJFs. Females used a larger perch-to-trunk distance than males because they are lighter than males and so giving the female more time to escape from predators.

**Foraging habitat**

WeRJFs were found to forage in areas of low tree densities (0.13 trees/$m^2$), low ground vegetation density (0.08 trees/$m^2$) and a low vertical density (37.3%), which is likely to be the case as whilst they are searching for food they must be aware of predators. If the ground vegetation is too dense or the vertical density is too high, then they could not see approaching predators, like the foraging habitat of Hume's Pheasant (*Syrmaticus humiae*) in northern Thailand (Iamsiri and Gale, 2008). However, the results of this study for weRJFs here is different to that reported for Grey jungle Fowls (*Gallus sonneratii*) in India that use a higher density ground cover for foraging, perhaps because these areas have a higher leaf litter content that contains more insects (Subramanian *et al.,* 2008). There were no significant differences between the roosting and foraging habitats of the weRJFs because the roosting and foraging sites overlapped. When birds descended from the roosting site they typically started to look for food near the roosting site, which in terms of availability was principally comprised of insects (89.2%), but the seed (50.2%) and grass (51.1%) coverage by area was also high. Thus, whilst the actual diet composition of these weRJFs remains to be evaluated, the potential diet items of weRJFs in this study are similar to those reported for the RJF (Wanghongsa, 2009) and the green peafowl in Hua Khakheng Wildlife Sanctuary (Pinthong, 2009). However, it is in contrast with that reported for RJFs in Malaysia and India, where their major group was plant seeds (Arshad *et al*., 2000), probably caused by the limited food resources in that habitat. Collias and Saichuae (1967) and Arsirapoj (2008) reported that red ear-lobed RJFs in Western Thailand were omnivores and can eat both seeds and vertebrate or invertebrate animals. In this study site at KARNWS they have a diverse choice, and so would be likely to choose the abundant high quality food items, such as invertebrates.

**Nesting Habitat**

Nest sites were first recorded from only one nest in late of February, two nests in each of March, April and one in May, the latter of which was late in the weRJF breeding season being into the dry season. These results contrast with those from the related Siamese fireback (*L. diari*), where the nesting period is from April to June (Sukumal *et al*., 2010) in the dry season. Here the potentially reduced invertebrate levels is compensated for by the lower precipitation level and warmer temperature that is more suitable for egg brooding. In this study, all of the weRJF nests were found near the weRJFs' trail on a tree stub and faced with ground plant coverage, such as *Tiliacora triandra*, which provide a good shelter to protect the nests from predators. This is similar to that reported for the Hume's Pheasant (*Syrmaticus humiae*) in northern Thailand (Iamsiri and Gale, 2008). However, the footprints of a monitor lizard and palm civet were found near one nest and evidence of hunting (destroyed egg) near the nest suggests it might have been

eaten by a monitor lizard. Nevertheless, no evidence of any nest being destroyed by elephants was seen, albeit at this low sample size of just six nest sites. A significant difference in the tree DBH and depth of ground leaf litter was found between the foraging and resting habitat of females, which is likely to be because the area with a higher level of ground leaf litter may supply more was are used as food for young chicks. In this study the major food item of weRJF was likely to be termites based on that they were the most abundant invertebrates found around the nesting habitats, are easily caught and are edible. Although this requires confirmation, if correct this is similar to that reported previously for RJF in western Thailand (Collias and Saichuae, 1967), where a lot of termites were found in the crop of five downy red ear-lobed RJFs. Although the weRJFs of this study site nested near human settlements, these were the forest ranger and officials of the wildlife research station and so these sites are protected from hunting and other human activities.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