Doctoral Thesis

Population genetic structure and genetic diversity of the Japanese lentic salamanders for conservation of endangered *Hynobius*

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Abstract

Salamanders are expected to differentiate genetically among local populations because they have low dispersal ability. Thus, they are potentially susceptible to loss of genetic diversity if the populations are isolated by habitat fragmentation. In addition to these factors, the urban neighborhood-dwelling species can be strongly affected by several human activities, and an immediate conservation is needed. In conservation of these species in the wild, there are three problems to solve before conservation activities: (1) taxonomic, (2) ecological, and (3) genetic problems. Especially, to clarify the four genetic matters is a great help in appropriate conservation planning: (1) genetic monitoring, (2) management unit, (3) genetic diversity, and (4) genetic pollution. In the present study, the analyses of population genetic structure including the four genetic matters are performed using both mitochondrial and microsatellite DNAs for the future conservation of the urban neighborhood-dwelling salamanders, *Hynobius tokyoensis* (mainly distributed in the Kanto District) and *H. dunni* (mainly distributed in the eastern Kyusyu).

Japanese lentic *Hynobius* species may contain cryptic diversity and most species of this genus are generally difficult to identify without information on sample locality because of their morphological similarities. In conservation of these species, many inappropriate conservation decisions can be made if the taxonomic status is incorrect. To solve the problems, phylogenetic relationships of them were examined for 55 populations including ten lentic *Hynobius* species using mitochondrial 16S rRNA (1103-bp) and cytochrome b (630-bp) genes. As a result, populations were clearly separated into eastern Japan group and western Japan group with high bootstrap values. *H. nebulosus* may be polyphyletic species despite it may be monophyletic species based on morphological characters, but monophyly of *H. tokyoensis* is supported by some previous studies and the present study. Also, Kyushu populations of *H. dunni* may be monophyletic group, but Kochi population of *H. dunni* was genetically different from them. Thus, taxonomic and phylogenetic reassessment of *H. nebulosus* and Kochi population of *H. dunni* using high variable nuclear markers are needed.

The salamander *Hynobius tokyoensis* is a lowland lentic breeder and endemic to the narrow area of central Japan. In this urban area, their habitats are extensively fragmented and several populations are threatened with extinction. Genetic management of this salamander is now needed, but information on genetic divergence and loss of genetic diversity is little available. So, mitochondrial cytochrome b (650-bp) gene and microsatellite (five loci) DNA analyses were made for 815 individuals from 46

populations in the 12 regions across their entire distribution range. As a result, populations were clearly separated into northern and southern groups, and genetic differentiation among 12 regions was also evident. Regional genetic differentiation seems to be affected by complicated geographical history, but genetic diversity of each population may be affected by recent habitat fragmentation. Some populations have lost genetic diversity in both mitochondrial and microsatellite DNAs because a positive correlation was detected between the mitochondrial and microsatellite DNA diversities. However, female-biased bottleneck effects were also evident in several populations in which mitochondrial DNA diversity was more reduced than microsatellite DNA diversity. Even if we need recovery of the genetic diversity in a small population by transferring other individuals, particularly the females, we must pay attention to avoid genetic pollution.

Oita salamander *Hynobius dunni* Tago, 1931, endemic to eastern Kyushu and western Shikoku of southwestern Japan, is a lowland lentic breeder and has declined its distribution range. To contribute to the future conservation of this salamander, current population genetic structures and genetic diversities were examined for 12 populations of eastern Kyushu, by using a mitochondrial cytochrome b (569-bp) gene and three microsatellite loci. As a result, populations were genetically separated into northern and southern groups, and there were some genetic differences even in the northern regions based on microsatellite analysis. The southern group was restricted to the narrow area and had low genetic diversity in both mitochondrial and microsatellite DNAs. In the northern group, the mitochondrial and microsatellite DNA diversities were also low in some peripheral populations. Toward the accurate genetic management of this species, we must pay more attention to such genetic differentiation and diversity in a fine scale.

The two salamander species have larger genetic differences among breeding sites and these populations are expected to conserve separately. However, closely related populations may justify management as single unit. Genetic diversities of the two species tended to decrease around the periphery of distribution range and completely isolated populations. The genetic pollution should be carefully examining when introduction to the inbred populations from other non-inbred populations for the resurrection of their genetic diversity.

1. General introduction

The biological diversity of the earth is being rapidly decreased as a direct or indirect consequence of human activities (Frankham et al. 2002). A large number of species are already extinct and many other species have also reduced population sizes (World Conservation Monitoring Centre 1992). Currently, many species of the world require appropriate human intervention to improve their management (Frankham et al. 1999). According to Leakey and Lewin (1995), current extinction problem has been called the 'sixth extinction', as its magnitude compares with other five mass extinctions. Extinction is a natural evolutionary process (e.g. mass extinction at the end of Cretaceous 65 million years ago), but the sixth extinction is different because species are being lost rapidly that outruns the birth of new species (Frankham et al. 2002). In response to these situations, International Union for Conservation of Nature and Natural Resources (IUCN) recognizes the need for conservation of three diversity levels (i.e. species diversity, ecological diversity, and genetic diversity) (IUCN 2014). According to research conducted by IUCN (2014), threatened species of animals fall into one of the three categories (i.e., critically endangered (CR), endangered (EN), and vulnerable (VU)), and classified more than 10% of species in every one of the vertebrate taxa: Mammalia = 21.7%, Aves = 13.2%, Reptilia = 21%, Amphibia = 30.5%, fishes (Pisces) = 17.8%. Especially, class Amphibia has over 30% threatened taxon and is declining and disappearing worldwide at an increasing rate as compared to pre-1980 decades, even in protected areas (Blaustein and Wake 1990; Stuart et al. 2004). Basically, amphibians are susceptible to environmental change derives from some basic amphibian characteristics: (1) relatively small body size, (2) ectothermic physiology, (3) limited capacity for migration, (4) highly permeable skin, and (5) dependence on aquatic or moist habitats (Murphy et al. 2000). However, many declines cannot be explained by only these characteristics. Scientists have hypothesized six major threats: (1) habitat modification and destruction, (2) commercial over-exploitation, (3) introduced species, (4) environmental contaminants, (5) global climate change, and (6) emerging infectious diseases (e.g. *Batrachochytrium dendrobatidis*) (Collins and Storfer 2003). Most agree the primary threat is (1) (i.e., habitat modification and destruction) among six major hypotheses (Crump 2010).

Japanese *Hynobius* species can be divided into still-water breeding type and running water breeding type (Sato, 1943). The species of former type mainly inhabit the lowland areas, and also found around human dwellings. The urban neighborhood-dwelling species, *H. tokyoensis* and *H. dunni*, can be strongly affected by

human activities, and an immediate conservation is needed. In conservation of these species, we should resolve the major three problems before conservation activities. First, we need to resolve the taxonomic problems. Incorrect ‘lumping’ of several distinct species into one recognized species or ‘splitting’ of one species into two or more recognized taxa may lead to erroneous conservation decisions (Frankham et al. 2002). Therefore, we need to reveal the taxonomic status and phylogenetic relationships among populations. Second, we also need to resolve the ecological problems. Limited life history and long-term population monitoring data exist for most wild populations, so to predict the population viability may not be possible (Frankham et al. 2002). To conduct the effective conservation, information of species life histories and evidence of decreasing the population size are essential. Third, genetic problems are also important for conservation of wild populations. Genetically differentiated populations within species should be managed separately (Moritz 1995). To conduct the management, we should understand the fine-scale population genetic structure (e.g., phylogenetic relationships among populations, gene flow among populations, genetic diversity, etc.).

Genetics is involved directly in the first of these and is a crucial factor in species conservation (Frankham et al. 2002). Four primary questions are asked when seeking to conserve the endangered species. First, genetic monitoring using some genetic markers (e.g. mitochondrial or microsatellite DNAs) is important to clarify the demography or more complex evolutionary and ecological processes (Schwartz et al. 2007). Schwartz et al. (2007) separate the genetic monitoring into two categories. Category I includes the spatial population monitoring through the identification of individuals, populations, species, and other taxonomic levels, whereas category II includes the temporal population monitoring with several genetic parameters. Currently, many studies of the fine-scale spatial genetic monitoring using variable microsatellite markers have been conducted on salamander species around the world (e.g., Pabijan and Babik 2006; Mullen et al. 2010; Chen et al. 2012; Blank et al. 2013; Unger et al. 2013; Sunny et al. 2014). In Japanese salamander species, there are several studies on population genetic structure using mitochondrial DNA (e.g., Matsui et al. 2008; Sakamoto et al. 2009; Aoki et al. 2013), but investigations of fine-scale population genetic structure using variable microsatellite markers are absent. Second, populations within a species may justify management as separate units if they have a unique genetic structure (Moritz 1995). Also, to avoid the outbreeding depression, defining management units within species or species is essential (Moritz 1995). The estimations of management unit on the Japanese salamander species have been conducted by the mitochondrial or nuclear DNAs (e.g., Honda et al. 2012; Tominaga et al. 2013). However, the information may be insufficient

to estimate the management unit within species, because genetic variations of mitochondrial and nuclear DNAs are usually not enough. Third, environmental change is a continuous process and genetic diversity is essential for populations to evolve to adapt against several environmental fluctuations (Frankham et al. 2002). Furthermore, loss of genetic diversity is often associated with inbreeding and reduction in reproductive fitness (Frankham et al. 2002). Thus, assessment of genetic diversity is a primary objective in the management of threatened species (Frankham et al. 2002). In Japanese lentic *Hynobius* species, assessment of genetic diversity on local populations was also conducted using several mitochondrial DNA genes (e.g., Yamane and Nishida 2010; Azuma et al. 2013). Forth, genetic pollution is also important factor for conservation, and it is flow of genes from one species (sub-species or population) to another (Ellstrand 2001; Potts et al. 2003). Typically, hybridizations occur when humans introduce exotic populations (or species) into the range of threatened populations (Ellstrand et al. 1999). This phenomenon is a threat to the genetic integrity on several species (Rhymer and Simberloff 1996). In Japan, there are no evidences of genetic pollution on the urban neighborhood-dwelling salamanders. However, urban neighborhood areas have a high potential for occurrence of genetic pollution. Finally, the aim of this study is to answer of these four genetic matters (i.e., (1) inference of spatial population genetic structure using the method of genetic monitoring with mitochondrial and microsatellite markers, (2) estimation of management unit, (3) assessment of genetic diversity, (4) detection of genetic pollution) on two urban neighborhood-dwelling salamander species, *H. tokyoensis* and *H. dunni*, for their future conservation.

2. Study species

2-1. Tokyo salamander

The Tokyo salamander, *Hynobius tokyoensis* (Tago, 1931) (Fig. 2-1), is endemic to Japan: the pacific coast of Fukushima Prefecture and thoughout the Kanto District, with the exception of Gunma Prefecture (Kusano et al. 2014). This species has a total length of 80–130 mm, generally 12 costal grooves, and shorter limbs (when the forelimb and hindlimb are adpressed to the flank, the toes are separated by the space of about 1 costal groove) (Takada and Ootani 2011). Dorsal color varies widely from yellowish brown to blackish brown, with dark brown individuals predominating and the flanks, cheeks, and limbs are densely speckled with light blue flecks (Takada and Ootani 2011). Two morphologically similar species inhabit around the distribution range of *H. tokyoensis*. First, *Hynobius lichenatus* is morphologically very similar to *H. tokyoensis*, but has 11 costal grooves (sometimes 12), and relatively longer limbs (toes and fore- and hindfeet make contact when the limbs are adpressed to the body) (Takada and Ootani 2011). Second, *Hynobius nigrescens* is also morphologically very similar to *H. tokyoensis*, but has large body size (120–190 mm), 11 costal grooves (sometimes 12), and much longer limbs (when adpressed to the flank, the toes of the fore- and hindlimbs overlap by the space of 1–3 costal grooves) (Takada and Ootani 2011). Larvae of these three species are difficult to identify because have similar morphological characters. In this case identification can be made from characters of egg sacs (Fig. 2-2), which may differ tremendously, despite the morphological similarities among adults.

Breeding behavior may start while the water temperature is still cold (3–5°C). In warmer distribution areas, such as the Boso Peninsula, egg sacs can be observed in December or January, while in colder areas, such as the outskirts of Tokyo, egg sacs do not begin to appear until February (Sparreboom 2014). Breeding may occur in fresh water: rice paddies, seepage pools, roadside ditches (Goris and Maeda, 2004). Males arrive at the breeding ponds first and hind in the mud or under debris in the daytime (Goris and Maeda, 2004). After that, males prowl and select appropriate breeding points for egg laying (mainly at night), which are defended against other males (Goris and Maeda, 2004). When female was reached near the breeding points, males wag their tails aggressively and begin to congregate at the preselected breeding sites (Goris and Maeda, 2004). After that, they group and swim about in clusters while female walks around slowly (Goris and Maeda, 2004). Finally, female select and join one of the clusters and oviposits pair of egg sacs, and attaches them to a suitable twig, leaf, or stem of grass

(Sparreboom 2014). Each pair of egg sacs contains a total of 15–192 eggs, and larger females produce more and larger eggs (Sparreboom 2014). In addition, the female produces a small clutch of large eggs under cool climatic conditions, and a large clutch of small eggs under warm conditions (Kusano et al. 2014). After egg laying, the males grasp the egg sacs and eject their sperm, and embryogenesis occur after fertilization (Goris and Maeda, 2004). The larvae of lentic *Hynobius* species have usually one pair of balancers on the cheeks after hatched (Fig. 2-1), and the balancers have been resorbed with the growth of larvae. They feed on plankton, *Daphnia*, *Tubifex*, blood worms, or eat each other (Goris and Maeda, 2004). Adults after breeding and juveniles after metamorphosis scatter widely in the forested areas around the breeding site (Kusano et al. 2014). They spend their lives in the maze of tunnels dug by earthworms, moles, or beetle larvae and they feed on earthworms, spiders, and other arthropods living in the litter layer (Goris and Maeda, 2004). They are especially active in autumn because are prepared for hibernation (Goris and Maeda, 2004). Sexual maturity is reached after 4–5 years, in males a little sooner than in females (Kusano et al. 2014).

2-2. Oita salamander

The Oita salamander, *Hynobius dunni* (Tago, 1931) (Fig. 2-3), is endemic to Japan and found in eastern Kyusyu and south western Kochi (Sato 1998). This species has a total length of 100–165 mm, 12 costal grooves (sometimes 11 or 13), and much longer limbs (toes and fore- and hindfeet make contact when the limbs are adpressed to the body) (Takada and Ootani 2011). The dorsal color is greenish brown to blackish brown with small darkish flecks, while the ventral color is bluish gray (Takada and Ootani 2011). During the breeding season, males have a Y-shaped cloaca (Sparreboom 2014). Furthermore, head width, tail height and hind legs of males become enlarged, and at this time coloration also becomes more subdued in the males (Fig. 2-3) (Sparreboom 2014). However, it is difficult to distinguish the sexes after the breeding season (Sparreboom 2014). One morphologically similar species, *H. nebulosus*, inhabit around the distribution range of *H. dunni*, but has stouter body, V-shaped vomero-palatine teeth series, yellow edges to the tail, shorter tail, 13 coastal grooves (sometimes 12), and relatively shorter limbs (toes do not touch when the fore- and hindlimbs are adpressed to the flank) (Sparreboom 2014). Despite the morphological similarities when the periods of adults and larvae, characters of egg sacs are differ tremendously (Fig. 2-4). Each pair of egg sacs of *H. dunni* contains a total of 87–143 eggs (Sato 1998). Basically, life history and breeding behavior are similar to *H. tokyoensis*.

Fig. 2-1. (a) Breeding pond of *Hynobius tokyoensis*; (b) larva and (c) adult of *Hynobius tokyoensis*.

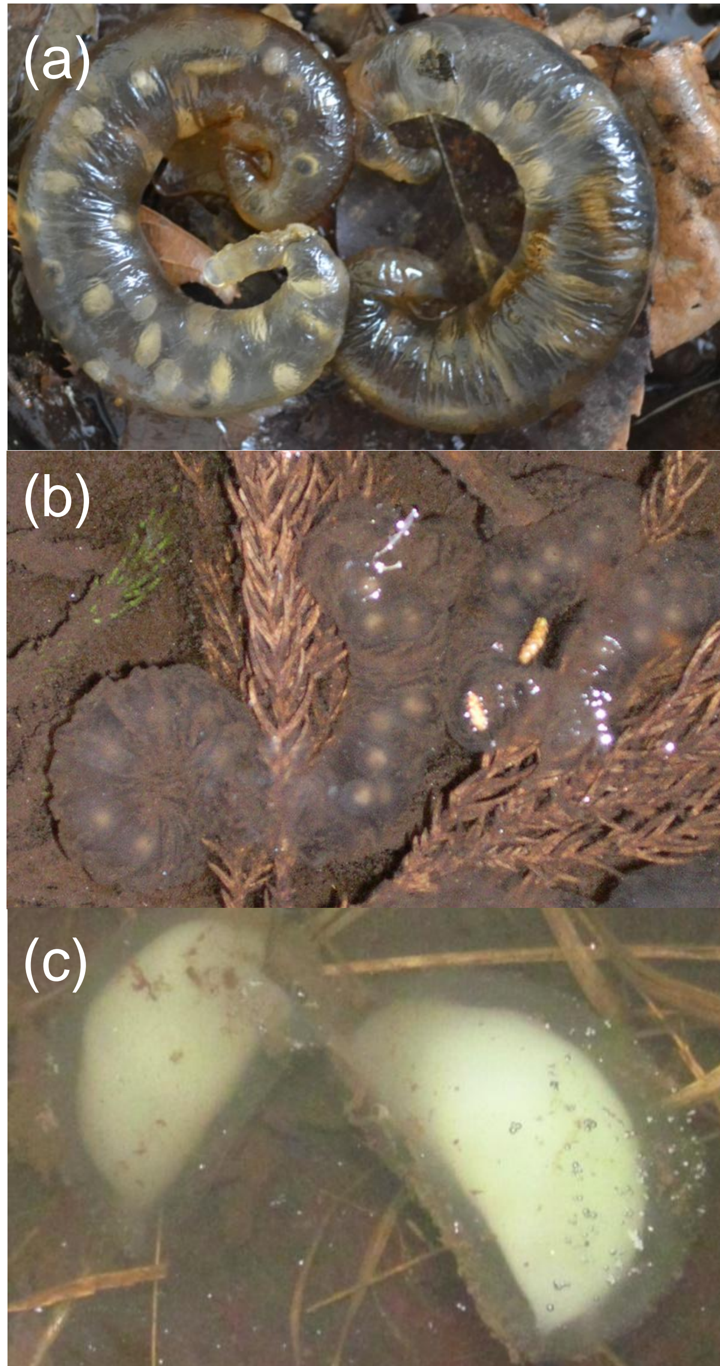

Fig. 2-2. Egg sacs of three morphologically similar species: (a) banana-shaped egg sacs of *Hynobius tokyoensis*, (b) coil-shaped egg sacs of *Hynobius lichenatus*, and (c) akebi-shaped egg sacs of *Hynobius nigrescens*.

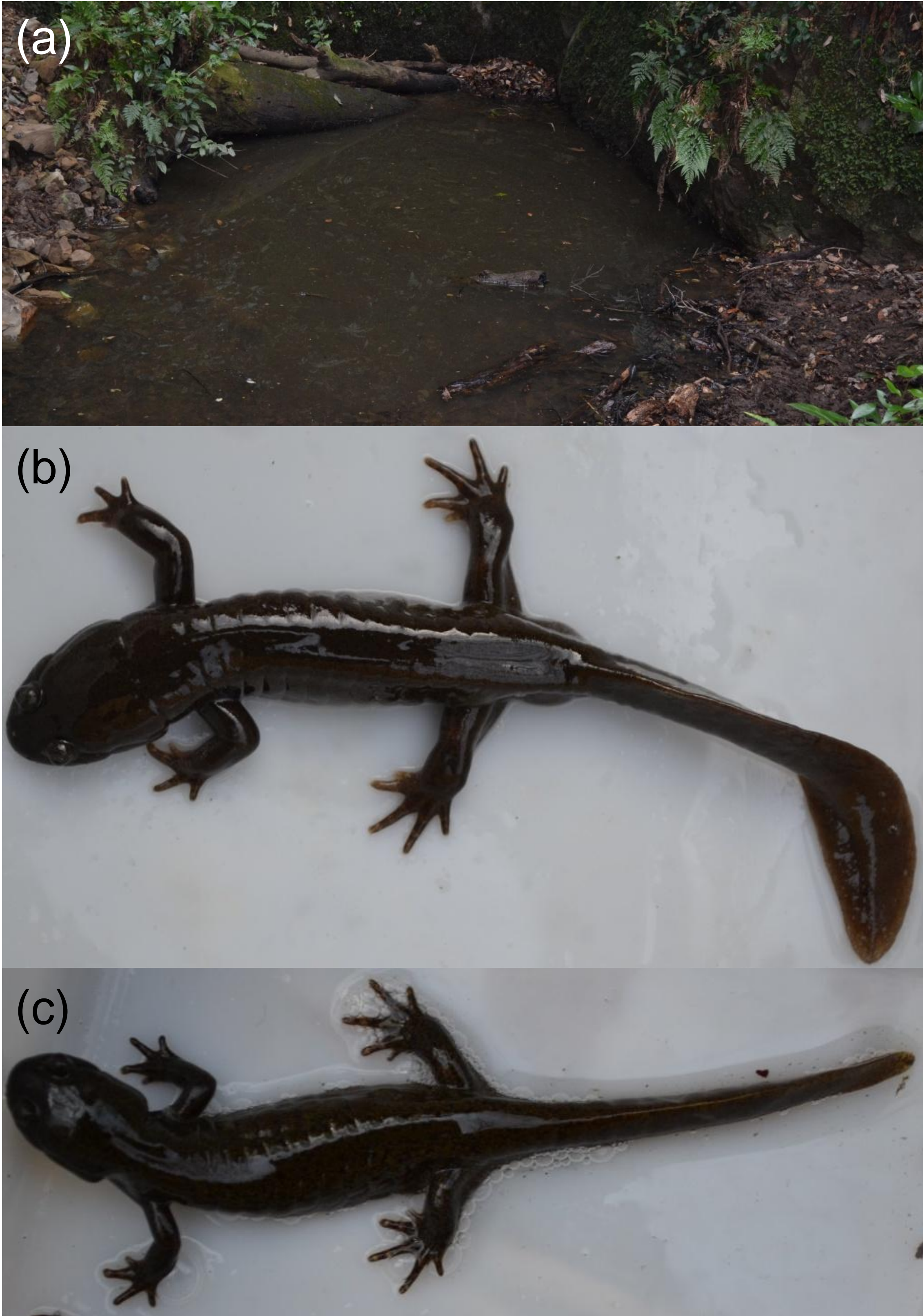

Fig. 2-3. (a) The breeding site of *H. dunni*. (b) Male and (c) female individuals of *H. dunni*.

Fig. 2-4. Egg sacs of two morphologically similar species: (a) banana-shaped egg sacs of *Hynobius dunni* and (b) non banana-shaped egg sacs of *Hynobius nebulosus* (populations of Oita Prefecture).

3. Molecular phylogenetic analysis of Japanese lentic *Hynobius*

3-1. Introduction

Increasing worldwide destruction and disturbance of natural ecosystems are precipitating catastrophic extinctions of species (Brook et al. 2006). In conservation of endangered species, many erroneous decisions may result if the taxonomic status of populations or species is not correctly assigned: (1) unrecognized endangered species may be allowed to become extinct; (2) incorrectly diagnosed species may be hybridized with other species; (3) resulting in reduced reproductive fitness; (4) resources may be wasted on abundant species or hybrid populations; (5) populations that could be used to improve the fitness of inbred populations may be overlooked; (6) endangered species may be denied legal protection while populations of common species may be granted protection (Frankham et al. 2002). Many species descriptions trace to limited information on the geographic distribution of a small number of traits (usually morphological) without genetic basis can lead to taxonomic problems (Avise 1996). To avoid the erroneous conservation activities, molecular techniques are important for protection of endangered species. Currently, the increasing use of molecular phylogenetic techniques has highlighted the prevalence of cryptic species, which are morphologically similar species with long independent evolutionary histories (Kozak et al. 2006).

The genus *Hynobius* is comprised of a diverse assemblage of over 30 species distributed in eastern Asia (AmphibiaWeb 2015). More than half of the species in this genus occur in Japan and approximately half of Japanese *Hynobius* species are designated as an endangered species by the International Union for Conservation of Nature and Natural Resources (IUCN) (International Union for Conservation of Nature and Natural Resources 2014). According to Sato (1943), Japanese species of *Hynobius* are split into the still-water breeding type (lentic breeders) and stream breeding type (lotic breeders). Lotic breeders mainly found in mountain areas. On the other hand, some lentic species are distributed in urban neighborhood. These species are clearly more susceptible to human activities than the other species and urgent conservation measures are essential. However, Japanese *Hynobius* may contain cryptic diversity (Matsui et al. 2006) and most species of *Hynobius* are generally difficult to identify without information on sample locality because of their morphological similarities (Matsui et al. 2002). In conservation of these species, many inappropriate conservation decisions can be made if the taxonomic status is incorrect (Frankham et al. 2002). This

study presents the phylogenetic relationship of Japanese lentic *Hynobius* and the taxonomic status of urban neighborhood-dwelling salamanders, *H. tokyoensis* and *H. dunni* based on molecular analysis.

3-2. Materials and methods

3-2-1. Sampling

From February to July in 2007, 2008, 2010, 2013 and 2014, 56 individuals (include ten species) were sampled from 55 populations across its entire distribution range (Fig. 3-1, Table 3-1). When breeding season of salamanders (February to May), a single tailbud embryo was removed from each paired egg sac and preserved in 99.5% ethanol. On the other hand, clipped caudal extremities were sampled from larvae or adults and preserved in 99.5% ethanol when non-breeding season of salamanders. Individuals of *Hynobius kimurae* and *H. boulengeri* collected at Tokyo and Wakayama Prefecture, respectively, were used as outgroup (Table 3-1; Fig. 3-1).

3-2-2. Mitochondrial DNA analysis

Total genomic DNA was extracted from the tail tips or single tailbud embryo and preserved in 99.5% ethanol, using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). For all 56 individuals of lentic *Hynobius* species and individuals of two outgroup species (*H. kimurae* and *H. boulengeri*), the 1103-bp fragment of 16S rRNA and 630-bp fragment of cytochrome b (cyt b) genes were amplified using Ex Taq® (TaKaRa, Tokyo, Japan) with primers 16SF (5′-GTCGTAACATGGTAAGTTTACCGGA-3′) and 16SR (5′-GGATCAATTATGTTAAATATTTTAT-3′) (in this study), and L14010 (5′-TAHGGWGAHGGATTWGAWGCMACWGC-3′) and H14778 (5′-AARTAYGGGTGRAADGRRAYTTTRTCT-3′) (Matsui et al., 2007). The PCR reaction mix (total volume 10 µl) contained 1.0 µl 10× Ex Taq Buffer, 0.8 µl 25 mM dNTP mix, 0.5 µl each of the forward and reverse primers (10 pM), 0.05 µl Taq polymerase, 6.15 µl distilled deionized water, and 1.0 µl template DNA. Using a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA), the PCR protocol is as follows: an initial 10-min denaturing step at 95°C, 30 cycles of 60 s at 95°C, 60 s at 53°C, and 120 s at 72°C, with a final 10-min extension at 72°C. The PCR products were purified with Illustra™ ExoStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) and

sequenced using BigDye® Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Direct sequencing data were aligned using MEGA5 (Tamura et al. 2011). Phylogenetic analyses of the aligned sequences were performed with the neighbor-joining (NJ) method based on p-distance and the maximum likelihood (ML) estimation based on Tamura-Nei model using MEGA5 (Tamura et al. 2011). The best-fit nucleotide substitution model was estimated based on the Bayesian Information Criterion (BIC: Schwarz, 1978) using MEGA5 (Tamura et al., 2011).

3-3. Results

The phylogenetic trees were basically same between NJ and ML estimates (Fig. 3-2). In lentic *Hynobius* salamanders, two major groups were clearly distinguished with high bootstrap values (Fig. 3-2). The boundary of two groups was in Chugoku and Shikoku Districts (Fig. 3-1). Six species *H. nigrescens*, *H. lichenatus*, *H. tokyoensis*, *H. takedai*, *H. nebulosus*, and *H.* sp. were included in eastern Japan group (Aomori to eastern part of Tottori, Okayama, Kagawa, and Tokushima Prefectures) (Fig. 3-1). On the other hand, four species *H. okiensis*, *H. tsuensis*, *H. nebulosus*, and *H. dunni* were included in western Japan group (western part of Tottori, Hiroshima, Ehime, and Kochi Prefectures to Nagasaki, Kumamoto, and Miyazaki Prefectures with the exception of *H. hidamontanus*) (Fig. 3-1). Although *H. hidamontanus* inhabit eastern Japan, it belonged to the western Japan group (Figs. 3-1 and 3-2). Seven species (*H. tokyoensis*, *H. lichenatus*, *H. takedai*, *H.* sp., *H. nigrescens*, *H. tsuensis*, and *H. okiensis*) were monophyletic species, but *H. nebulosus* and *H. dunni* were polyphyletic species (Fig. 3-2). Especially, despite the same species based on morphological characters, populations of *H. nebulosus* were included in both groups.

3-4. Discussion

Molecular phylogenetic analysis using mitochondrial DNA detected two groups with high bootstrap values. Thus, Japanese lentic *Hynobius* may be divided into two groups: eastern Japan group (east from eastern part of Tottori, Okayama, Kagawa, and Tokushima Prefectures) and western Japan group (west from western part of Tottori, Hiroshima, Ehime, and Kochi Prefectures). However, it is not known exactly why two groups were genetically separated at this area despite no remarkable geographical barriers. The Hakuba salamander *H. hidamontanus* may belong to western Japan group,

but the distribution range is clearly located in eastern Japan. Thus, a part of species belonging to the western Japan group may have expanded their distribution range to eastern Japan in the past. To clarify the phylogenetic position of *H. hidamontanus*, further analyses based on more individuals, localities (e.g. populations of Niigata and Nagano), and molecular markers (e.g. other mitochondrial and nuclear markers) are essential.

Monophyly of *H. nebulosus* is supported by several morphological characters (Sato 1943), and previous study using allozyme is also supported this opinion (Matsui et al. 2006). According to Matsui et al. (2006), four groups (i.e. the western, eastern, montane, and Chugoku groups) in *H. nebulosus* are recognized based on the three trees obtained and the result of the MDS analysis. However, these results have some phylogenetic problems. For example, the western group (Kyushu to westernmost Honshu) was distinct in all the analyses performed, although its support in the trees was weak (bootstrap value = 57). Additionally, the distinctness of the Chugoku group was ambiguous. On the other hand, current molecular phylogenetic study using mitochondrial DNA suggests polyphyly of *H. nebulosus* (Zheng et al. 2012). Phylogenetic analysis of the present study also indicates the polyphyly of *H. nebulosus*. Thus, taxonomic reassessment with additional morphological analysis and phylogenetic reassessment of *H. nebulosus* using high variable region of nuclear DNA are needed.

The monophyly of *H. tokyoensis* is supported by some previous studies (Yoshizawa et al. 2005; Hayashi and Kusano 2006; Matsui et al. 2007). Also the results of this study, *H. tokyoensis* may be monophyletic group based on both phylogenetic analysis (bootstrap value = 100) (Fig. 3-2). Monophyly of *H. dunni* is supported by several morphological characters and crossing experiment (Sato 1943; Sato and Seto 1993) and previous study using mitochondrial DNA also supported this opinion (Michigoshi 2000). According to Michigoshi (2000), two major groups (i.e. Oita and Miyazaki-Kochi groups) in *H. dunni* are recognized based on the mitochondrial DNA analysis. However, phylogenetic analysis of this study did not include the Kyushu populations of *H. nebulosus* and used the distantly related species (i.e. *H. boulengeri*) as outgroup. In the present study, Kyushu populations of *H. dunni* may be monophyletic group based on both phylogenetic analysis (bootstrap value ≥ 95%), but Kochi population of *H. dunni* was genetically different from Kyushu populations (Fig. 3-2). Thus, taxonomic reassessment with additional morphological analysis and phylogenetic reassessment of Kochi population using high variable nuclear markers are needed.

Table 3-1. A list of analyzed samples in this study. Asterisks show the out group species.

Species	Sampling locality
**Hynobius boulengeri*	Tanabe-shi, Wakayama
Hynobius dunni	Usa-shi, Oita
Hynobius dunni	Oita-shi, Oita
Hynobius dunni	Saiki-shi, Oita
Hynobius dunni	Bungoono-shi, Oita
Hynobius dunni	Takamori-machi, Kumamoto
Hynobius dunni	Miyazaki-shi, Miyazaki
Hynobius dunni	Tosashimizu-shi, Kochi (01)
Hynobius dunni	Tosashimizu-shi, Kochi (02)
Hynobius hidamontanus	Toyama-shi, Toyama
**Hynobius kimurae*	Hinohara-mura, Tokyo
Hynobius lichenatus	Hachimantai-shi, Iwate
Hynobius lichenatus	Akita-shi, Akita
Hynobius lichenatus	Kooriyama-shi, Fukushima
Hynobius lichenatus	Minamiuonuma-shi, Niigata
Hynobius lichenatus	Nasushiobara-shi, Tochigi
Hynobius nebulosus	Goto-shi, Nagasaki
Hynobius nebulosus	Iki-shi, Nagasaki
Hynobius nebulosus	Hirado-shi, Nagasaki
Hynobius nebulosus	Amakusa-shi, Kumamoto
Hynobius nebulosus	Kikuchi-shi, Kumamoto
Hynobius nebulosus	Munakata-shi, Fukuoka
Hynobius nebulosus	Usa-shi, Oita
Hynobius nebulosus	Nagato-shi, Yamaguchi
Hynobius nebulosus	Izumo-shi, Shimane
Hynobius nebulosus	Higashihiroshima-shi, Hiroshima
Hynobius nebulosus	Kurashiki-shi, Okayama
Hynobius nebulosus	Kitahiroshima-cho, Hiroshima
Hynobius nebulosus	Kobe-shi, Hyogo
Hynobius nebulosus	Tsu-shi, Mie
Hynobius nebulosus	Tanabe-shi, Wakayama
Hynobius nebulosus	Takamatsu-shi, Kagawa
Hynobius nebulosus	Mugi-cho, Tokushima
Hynobius nebulosus	Imabari-shi, Ehime
Hynobius nebulosus	Nichinan-cho, Tottori
Hynobius nebulosus	Tahara-shi, Aichi
Hynobius nebulosus	Tottori-shi, Tottori
Hynobius nigrescens	Aomori-shi, Aomori
Hynobius nigrescens	Takaoka-shi, Toyama
Hynobius nigrescens	Katashina-mura, Gunma
Hynobius nigrescens	Wajima-shi, Ishikawa
Hynobius nigrescens	Nasushiobara-shi, Tochigi
Hynobius okienisis	Okinoshima-cho, Shimane
Hynobius takedai	Wajima-shi, Ishikawa
Hynobius takedai	Shiga-machi, Ishikawa
Hynobius tokyoensis	Iwaki-shi, Fukushima
Hynobius tokyoensis	Hitachiota-shi, Ibaraki
Hynobius tokyoensis	Sano-shi, Tochigi
Hynobius tokyoensis	Yokoze-machi, Saitama
Hynobius tokyoensis	Musashimurayama-shi, Tokyo
Hynobius tokyoensis	Oume-shi (Kaji Hill), Tokyo
Hynobius tokyoensis	Oume-shi (Kusabana Hill), Tokyo
Hynobius tokyoensis	Akiruno-shi, Tokyo
Hynobius tokyoensis	Hachioji-shi, Tokyo
Hynobius tokyoensis	Sousa-shi, Chiba
Hynobius tokyoensis	Tateyama-shi, Chiba
Hynobius tsuensis	Tsushima-shi, Nagasaki
Hynobius sp.	Echizen-machi, Fukui

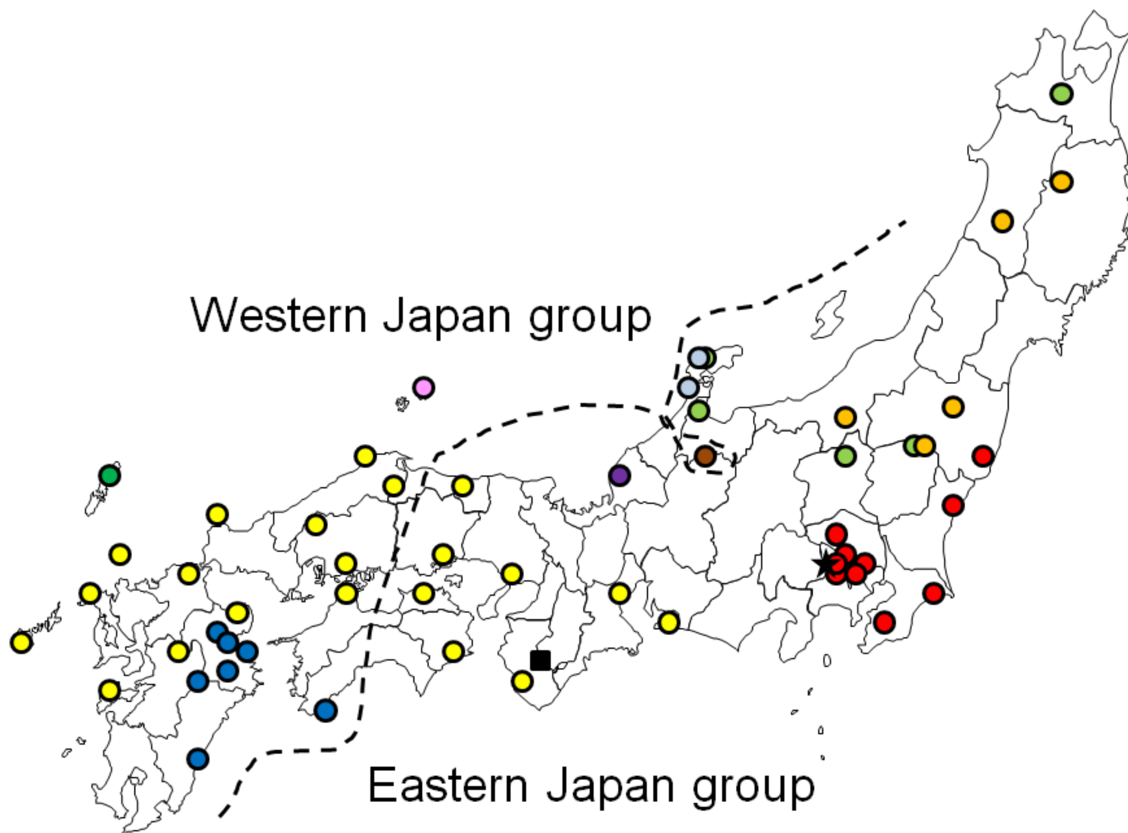

Fig. 3-1. Sampled localities of lentic *Hynobius* used in the present study. Circles of yellowish green, orage, red, light blue, yellow, purple, pink, green, and blue indicate the sampled locations of *H. nigrescens*, *H. lichenatus*, *H. tokyoensis*, *H. takedai*, *H. nebulosus*, *H.* sp., *H. okiensis*, *H. tsuensis*, and *H. dunni*, respectively. The closed star and closed square indicate sampled localities of outgroup species *H. kimurae* and *H. boulengeri*, respectively. For the sample localities, see Table 3-1.

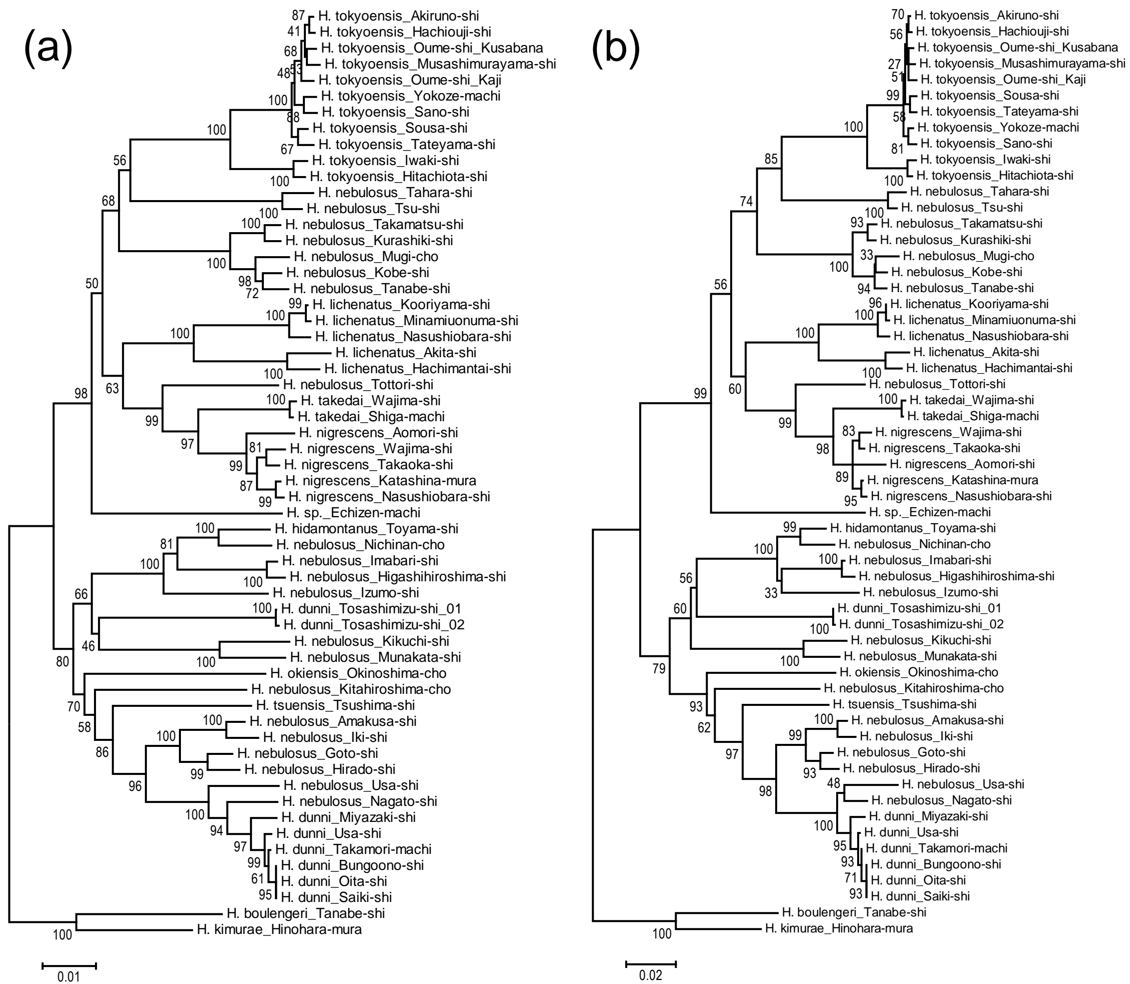

Fig. 3-2. NJ (a) and ML (b) phylogenetic trees based on 1103-bp16S rRNA and 630-bp cytochrome b sequences. The scale bar indicates the genetic distance (substitutions per site) of each tree. Numerals indicated near branches are bootstrap probabilities with 1,000 replications. For the sample names and localities, see Table 3-1.

4. Population genetic structure and genetic diversity of Tokyo salamander

4-1. Introduction

Animals with shorter dispersal distances are able to differentiate at smaller spatial scales than those with longer dispersal distances, leading to genetic divergence within a given area (Kisel and Barraclough 2010). On the other hand, animals with shorter dispersal distances are potentially susceptible to loss of genetic diversity in the single population, which is caused by local bottleneck and/or founder effects (Alexandrino et al. 2000; Chen et al. 2012). Molecular analyses can provide important insights on genetic factors that contribute to such patterns of genetic divergence and diversity loss (Avise 2000). From a conservation perspective, these analyses will prove invaluable for defining conservation units for species management (Moritz 1994) and for estimating indirect dispersal abilities, reproductive strategies, and population demography to help in planning of conservation strategies (e.g., Jarne 1995; Miller et al. 2002; Mahoney 2004; Miller et al. 2006).

Habitat fragmentation is one of the major factors of species extinction (Wilcox and Murphy 1985; Andrén 1994; Fahrig and Merriam 1994). After fragmentation, the populations are smaller and more susceptible to greater spatial and temporal variation in population size, which increases their extinction probabilities (Reed and Hobbs 2004). The events also caused the breakdown of effective metapopulation dispersal rates (Gonzalez et al. 1998) and decreasing the probability of regional population persistence (Sjögren 1991). Furthermore, it can influence regional genetic diversity and increase susceptibility to other threats such as disease transmission, inbreeding and local extirpation, or decrease the potential for adaptation to local environments (Pearman and Garner 2006; Spear et al. 2006).

Distributions of salamanders and frogs depend on aquatic or moist conditions and often have patchy distributions due to habitat specificity and restricted physiological requirements (Stebbins and Cohen 1995). Habitat destruction or fragmentation poses the greatest threat to them, because they have low dispersal ability and low tolerance to environmental changes (Green 1997; Dodd and Smith 2003; Blaustein and Bancroft 2007). Many studies have dealt with fragmentation caused by industrial activities, and especially agricultural or logging activities have a large impact on the increase of fragmented areas (e.g. Kolozsvary and Swihart 1999; Vos et al. 2001). Urban development has also been a major factor of habitat fragmentation (Miller and Hobbs 2002). Urbanization has emerged as a substantial cause of the decline of a lot of amphibians (Gibbs et al. 2005; Hamer and McDonnell 2008). In fragmented or small populations, bottlenecks lead to inbreeding and it has critically negative effects on genetic variability in small populations, and populations may experience loss of rare alleles and a diminution of heterozygosity through genetic drift and inbreeding after bottlenecks (Newman and Pilson 1997). These processes contribute to the erosion of genetic

diversity and can lead to fitness reduction (Reed and Frankham, 2003). Reduced genetic diversity can inhibit the ability of a population to respond to rapid environmental changes (Young et al. 1996).

The Tokyo salamander *Hynobius tokyoensis*, originally described from Tokyo by Tago (1931), is a lowland lentic breeder (Sato 1943) and is distributed in the narrow area of central Japan where their habitats are heavily fragmented by urban development around Tokyo metropolis (Fig. 4-1). During the past few decades, the population has been declining abruptly due to habitat loss and destruction by land development (Kusano et al. 2014), and increased predation pressures from invasive predators such as the crayfish *Procambarus clarkii* and the raccoon *Procyon lotor* may also cause the population decline and extinction (Hayama et al. 2006, Takeuchi et al. 2011; Kaneda et al. 2012). This salamander has been listed as a class B1 vulnerable species in the IUCN Red List of Threatened Species (International Union for Conservation of Nature 2014), and urgent conservation is needed. According to the previous studies, monophyly of *H. tokyoensis* is strongly supported by molecular phylogenetic analyses of mitochondrial D-loop and/or cytochrome b sequences (Yoshizawa et al. 2005; Hayashi and Kusano 2006; Matsui et al. 2007) and allozymic comparisons (Matsui et al. 2001). However, fine-scale population genetic structure is still unclear. In this study, we examined the genetic divergence and diversity of *H. tokyoensis* across its entire distribution range using both mitochondrial and microsatellite DNA analyses, and based on the results, we attempted to detect the important genetic factors for its future conservation and genetic management.

4-2. Materials and methods

4-2-1. Sampling

From February to May in 2013 and 2014, 815 individuals were sampled from 46 populations in the 12 regions across its entire distribution range (Fig. 4-1, Table 4-1). Each population (1-46) means the individuals found in a single wetland including small ponds, marshes, and streams. The current distribution range of *H. tokyoensis* is separated into 12 regions (A-L) (Narita 1978; Inaba 2000, Aoyagi and Hayashi 2004; Ogano et al. 2007; Hasegawa 2011; Odaya et al. 2011; Kusano et al. 2014; H. Fujita, personal communication). Because a female deposits a pair of egg sacs in a single breeding season, one tailbud embryo was collected from only one of each pair of egg sacs and preserved in 99.5% ethanol. To avoid sampling of individuals from the same female, we never took DNA-samples from larvae, juveniles, and adults. The number of individuals examined in each population ranged from 5 to 56 (Table 4-1). Two individuals of *H. lichenatus* collected at Kooriyama, Fukushima Prefecture, and Nasushiobara, Tochigi prefecture, were used as the outgroup (Fig. 4-1).

4-2-2. Mitochondrial DNA analysis

Total genomic DNA was extracted from these embryos preserved in 99.5% ethanol, using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). For all 815 *H. tokyoensis* and two *H. lichenatus* samples, a 650-bp fragment of the mitochondrial cytochrome b (cyt b) gene was amplified using Ex Taq® (TaKaRa, Tokyo, Japan) with primers L14010 (5′-TAHGGWGAHGGATTWGAWGCMACWGC-3′) and H14778 (5′-AARTAYGGGTGRAADGRRAYTTTRTCT-3′) (Matsui et al., 2007). The PCR reaction mix (total volume 10 μl) contained 1.0 μl 10× Ex Taq Buffer, 0.8 μl 25 mM dNTP mix, 0.5 μl each of the forward and reverse primers (10 pM), 0.05 μl Taq polymerase, 6.15 μl distilled deionized water, and 1.0 μl template DNA. Using a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA), the PCR protocol is as follows: an initial 10-min denaturing step at 95°C, 30 cycles of 60 s at 95°C, 60 s at 53°C, and 120 s at 72°C, with a final 10-min extension at 72°C. The PCR products were purified with Illustra™ ExoStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) and sequenced using BigDye® Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Direct sequencing data were aligned using MEGA 5 (Tamura et al. 2011). Phylogenetic analyses of the aligned sequences were performed with the neighbor-joining (NJ) method based on p-distance and the maximum likelihood (ML) estimation based on Hasegawa-Kishino-Yano model using MEGA 5 (Tamura et al. 2011). The best-fit nucleotide substitution model was estimated based on the Bayesian Information Criterion (BIC: Schwarz, 1978) using MEGA5 (Tamura et al., 2011). The genetic variations among and within regions or among and within populations were subjected to analysis of molecular variance (AMOVA) using Arlequin ver. 3.5 (Excoffier and Lischer 2010). The haplotype diversity h and nucleotide diversity π were calculated using Arlequin ver. 3.5 (Excoffier and Lischer, 2010). Significance of the pairwise Φ_{ST} was determined using Arlequin ver. 3.5 (Excoffier and Lischer, 2010).

4-2-3. Microsatellite DNA analysis

We amplified five microsatellite loci of all 815 *H. tokyoensis* samples, using the primer sets developed for *H. nebulosus* by Yoshikawa et al. (2013): the NH002 locus with the repeat motif $(AACTC)_n$ using the primers 5′-ATTTCATCTGGCCAACCCG-3′ and 5′-TCATTCCTCCAAGGCAGGG-3′; HN004 with $(ACTC)_n$ using 5′-GCCGTACCGATGTTGATAGC-3′ and 5′-TCCTGGCCACTCTATTGCC-3′; HN019 with $(AAT)_n$ using 5′-GACAAGGCTTTCTCGGTGC-3′ and 5′-CGTCAAATCTGAGCTCCCTG-3′; HN020 with $(AAT)_n$ using

5′-AGACATGACGTGTGGAGGC-3′ and 5′-GGAAAGCCACACTGACTGC-3′; and HN021 with $(AAT)_n$ using 5′-CAGCAGTGACTTGGGAAGC-3′ and 5′-CTGTGAGTGGGCCCTAGAC-3′. The PCR was performed on a T100™ thermal cycler (Bio-Rad) using KOD FX Neo DNA polymerase (Toyobo, Tokyo, Japan) with a thermal profile consisting of 94°C for 120 s, followed by 35 cycles at 94°C for 15 s, 59°C for 30 s, and 68°C for 60 s. The reaction mix (total volume 10 µl) contained 4.8 µl of 2× KOD FX Neo Buffer, 2.0 µl 2 mM dNTPs, 0.5 µl fluorescent (6-FAM or HEX) forward primer (10 pM), 0.5 µl reverse primer (10 pM), 0.1 µl *Taq* polymerase, 1.1 µl distilled deionized water, and 1.0 µl template DNA. The PCR products were then diluted (1:10) and mixed with GeneScan™ LIZ® 500 Size Standard (Applied Biosystems) and Hi-Di Formamide (Applied Biosystems), combining 0.2 µl Liz, 8.8 µl Hi-Di, and 1 µl diluted product. Fragment analysis data were collected using an ABI 3130xl Genetic Analyzer (Applied Biosystems). Allele lengths were scored using Peak Scanner ver. 1.0 (Applied Biosystems).

The effective number of alleles (N_E), observed (H_O) heterozygosity, expected (H_E) heterozygosity, and information index (I) in each population were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012), and the allelic richness (A_R) was calculated using FSTAT ver. 2.9.3.2 (Goudet, 1995). Deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were estimated using Genepop’007 (Rousset 2008). The significance of F_{ST} was determined using FSTAT ver. 2.9.3.2 (Goudet 1995). Tests of significant genetic differentiation among populations were conducted using F-statistics (Weir and Cockerham 1984) with each parameter tested against zero by a bootstrapping method using FSTAT ver. 2.9.3.2. The software POPTREE2 (Takezaki et al. 2010) was used to construct a neighbor joining tree using D_A distances with 1,000 bootstrap replications. The genetic differentiation among regions, among populations within regions, among individuals within populations, and within individuals was subjected to analysis of molecular variance (AMOVA) using Arlequin ver. 3.5 (Excoffier and Lischer 2010). Current genetic structure was assessed using the program STRUCTURE ver. 2.3.3 (Pritchard et al. 2000) with LOCPRIOR model for accurate inferences (Hubisz et al. 2009) and we used the correlated allele frequency models (Falush et al., 2003). The model assumes that the populations all diverged from a common ancestral population at the same time. Ten runs were set with a burn-in length of 50,000 and a Markov chain Monte Carlo (MCMC) run of 50,000 for each K (1 to 10). ΔK was calculated to examine the true K number (Evanno et al. 2005).

4-3. Results

4-3-1. Mitochondrial DNA analysis

Of the 650-bp sequences of 815 *H. tokyoensis*, nucleotide substitution occurred at 82

positions (Table 4-2), producing 72 haplotypes, T1-T72 (GenBank accession numbers LC004030– LC004101) (Table 4-3). We used the corresponding sequences of the outgroup *H. lichenatus*, haplotypes L1 and L2 (GenBank accession numbers LC004028 and LC004029, respectively). The phylogenetic trees were basically same between NJ and ML estimates (Fig. 4-2). In *H. tokyoensis*, the two haplotype-groups were clearly distinguished. The first group is in northern part (haplotypes T1-T8 in populations 1-5 of the regions A and B) and the second group is in southern part (T9-T72 of populations 6-46 in the regions C-L). However, in each group, further grouping of haplotypes was unclear. The most dominant haplotype was T72 (113/815 individuals) which was obtained widely in the regions H, I, J and K (Fig. 4-2). The secondary abundant haplotype was T13 (68/815 individuals), which was obtained from the two distant regions G and L (Fig. 4-2). Haplotype T45 was also detected from the distinct two regions; population 22 in the region G and populations 14 and 15 in the region F (Fig. 4-2). All other haplotypes were found in the narrow regions or population specific; particularly T10 was the only haplotype found from 60 individuals of populations 6-8 in the regions C and D (Fig. 4-2). In AMOVA, the most variance (72.5%) was explained by among regions, and 17.3% and 10.2% were explained by among populations within region and within populations, respectively (Table 4-4). Pairwise Φ_{ST} values between populations differed significantly in most population combinations after the Bonferroni corrections (above diagonal in Table 4-5).

4-3-2. Microsatellite DNA analysis

Microsatellite analysis detected 3, 4, 14, 4, and 4 alleles in HN002, HN004, HN019, HN020, and HN021 loci, respectively (Table 4-6). The observed and expected heterozygosities ranged from 0.000 to 1.000 and 0.000 to 0.820, respectively (Table 4-7). No significant LD was detected in any combinations of loci for 46 populations, and deviations from HWE were identified only in 7 populations (22, 23, 24, 29, 30, 40, and 41) at HN002 locus, after Bonferroni corrections (Table 4-7). In the phylogenetic tree (Fig. 4-3), populations were separated into two groups (1-5 and 6-46), which is the same of northern and southern groups in mitochondrial DNA phylogenies (see Fig. 4-2). In microsatellite analysis, however, populations 41 and 42 in the region K were separable at the bootstrap probability 57% from the other southern populations (Fig. 4-3).

The most variance (51.8%) was explained by within individuals, and 30.4% was by among regions (Table 4-8). Variance among populations within regions and variance among individuals within populations explained only 14.1% and 3.7% of variation, respectively (Table 4-8). The mean F_{ST} calculated for all loci was 0.423 (99% confidence interval 0.362-0.525). Pairwise multilocus F_{ST} between populations revealed that the frequency distributions of alleles at the five loci differed in nearly all combinations of populations after Bonferroni corrections (Table 4-5). The estimated genetic structure by the software

STRUCTURE (Fig. 4-4), in which ΔK calculated for K = 2 to 10 was highest at K = 5 (ΔK = 6.4, SD = 14.5), suggested that (1) populations 1-5 in the regions A and B were similar but clearly distinguished from all other populations; (2) there seemed to be a genetic trend from north to south populations of 6-8 in the regions C and D, 19-29 in the region G, and 30-40 in the regions H-J; (3) another genetic trend was observed from north to south populations of 9-18 in the regions E and F; (4) populations 30-40 were similar to each other in the regions H, I and J, but populations 41and 42 in the region K differed from them; and (5) populations 43-46 in the region L was quite similar to populations 15-18 in the southern part of the region F, despite being separated by the sea.

4-3-3. Genetic diversity

The number of mitochondrial haplotypes (N_H), haplotype diversities (h), and nucleotide diversities (π) within populations ranged from 1 to 8, 0.00 to 0.85, and 0.000 to 0.041, respectively (Table 4-1). The mean number of microsatellite alleles (N_A), mean effective number of alleles (N_E), mean allelic richness (A_R), mean expected heterozygosities (H_E), and mean information indices varied from 1.40 to 3.40, 1.02 to 2.34, 1.09 to 2.62, 0.02 to 0.51, and 0.04 to 0.84, respectively (Table 4-1). There were statistically significant correlations between all combinations of these mitochondrial and microsatellite genetic diversities (Fig. 4-5); N_H and mean N_A ($r = 0.43$, $t = 3.18$, $df = 44$, $P < 0.01$), N_H and mean N_E ($r = 0.38$, $t = 2.71$, $df = 44$, $P < 0.01$), N_H and mean A_R ($r = 0.42$, $t = 3.06$, $df = 44$, $P < 0.01$), N_H and mean H_E ($r = 0.46$, $t = 3.43$, $df = 44$, $P < 0.01$), N_H and mean I ($r = 0.44$, $t = 3.29$, $df = 44$, $P < 0.01$), h and mean N_A ($r = 0.46$, $t = 3.45$, $df = 44$, $P < 0.01$), h and mean N_E ($r = 0.44$, $t = 3.20$, $df = 44$, $P < 0.01$), h and mean A_R ($r = 0.51$, $t = 3.91$, $df = 44$, $P < 0.01$), h and mean H_E ($r = 0.51$, $t = 3.90$, $df = 44$, $P < 0.01$), h and mean I ($r = 0.50$, $t = 3.84$, $df = 44$, $P < 0.01$), π and mean N_A ($r = 0.38$, $t = 2.73$, $df = 44$, $P < 0.01$), π and mean N_E ($r = 0.43$, $t = 3.15$, $df = 44$, $P < 0.01$), π and mean A_R ($r = 0.43$, $t = 3.18$, $df = 44$, $P < 0.01$), π and mean H_E ($r = 0.42$, $t = 3.11$, $df = 44$, $P < 0.01$), π and mean I ($r = 0.43$, $t = 3.14$, $df = 44$, $P < 0.01$). In mitochondrial DNA analysis, populations 2, 3, 4, 6, 7, 8, 18, 19, 41, 42, 43, 44, 45, and 46 had only one haplotype (Table 4-1). In microsatellite analysis, populations 1, 5, 6, 18, 43, 44, 45, and 46 had lower diversity (mean $N_A < 1.5$ and/or mean $H_E < 0.11$) than the other populations (Table 4-1). Thus, several populations restricted to the narrow area had lost genetic diversity in both mitochondrial and microsatellite DNAs, as in population 6 in the region C, population 18 in the southernmost part of the region F, and populations 43, 44, 45 and 46 in the region L. In contrast, populations 2, 3 and 4 in the region B, populations 7 and 8 in the region D, and populations 41 and 42 in the region K had lost only mitochondrial DNA diversity, while population 1 in the region A had a lower microsatellite diversity than mitochondrial diversity.

4-4. Discussion

4-4-1. Local genetic divergence and conservation

Before conservation activities of the threatened species, we must resolve the several problems. First, incorrect lumping of several distinct species into one recognized species or splitting of one species into two or more recognized taxa may lead to erroneous conservation decisions (Frankham et al. 2002). Therefore, we need to reveal the taxonomic status of the target species and phylogenetic relationships with closely related species. Second, genetically differentiated populations within species should be managed separately (Moritz 1995). To do so, we should understand fine-scale genetic structures of the target species at the first time, although for the effective conservation, we ultimately need to understand population dynamics and effects of landscape features, such as fragmentation, permeability and configuration of habitat patches, on gene flow (Gibbs 1998; Guerry and Hunter 2002).

Hynobius tokyoensis dealt with a class B1 vulnerable species in the IUCN Red List of Threatened Species is now distributed in the separated narrow regions (Fig. 1). This is the well-defined species among Japanese *Hynobius* salamanders based on molecular phylogenetic analyses (Yoshizawa et al. 2005; Hayashi and Kusano 2006; Matsui et al. 2001, 2007). Our results of mitochondrial cyt b analysis also showed the clear difference between *H. tokyoensis* and parapatrically distributed *H. lichenatus* (Fig. 4-2). Our analyses of a fine population genetic structure revealed that *H. tokyoensis* is genetically divergent among local populations, separating into northern and southern groups (Figs. 4-2 and 4-3). Moreover, population genetic structure is complicated in the southern group. There may be two genetic trends; one is from north to south in the inland regions C, D, G, H, I, and J, and another is from north to south along the coastal regions E and F (Fig. 4-4). Populations in heavily fragmented areas close to Tokyo metropolis (regions H-K) have specific genetic characteristics, but differ genetically between the regions H-J and the region K (Fig. 4-4). Populations in the region L are similar to the southern populations of the region F despite being separated by the sea (Fig. 4-4), which is also pointed out by Hayashi and Kusano (2006) and Matsui et al. (2007).

Complicated population genetic structure observed for *H. tokyoensis* may be explained partly by geographical history of their distribution area. The closer relationship between the region L and the southern part of F may be concerned to the past connections of these two hill areas when the bay opens to the east (Kaizuka et al. 1977). The same geographical variation as in *H. tokyoensis* is known in the carabid beetle *Carabus insulicola*. In this green colored beetle, the subspecies *C. insulicola nishikawai* with dark red color is found only in the southern part of F and L regions (Ishikawa 1991). By separating these two hill areas and forming the current topography of the region F, gene flow may occur gradually between the southern and northern parts of this region. This results in a genetic cline observed in the

regions E and F (Fig. 4-4).

There was a great genetic variation among local populations of *H. tokyoensis* and thus genetic management of this salamander must be made separately in a fine scale. Artificial transportation even from the neighboring populations may cause a serious genetic disturbance. In our survey, artificial transplantations were guessed. The first example is that populations 43 and 44 in the region L had the single haplotype T13 which was also seen in populations 20, 22, 23, and 24 in the region G (Fig. 4-4), although populations 45 and 46 of the same region L had unique haplotypes T36 and T37. The second example is that the haplotype T45 was detected from the distinct regions; population 22 in the region G and populations 14 and 15 in the region F (Fig. 4-4), suggesting that partial genetic pollution of mitochondrial DNA may occur. In addition, these individuals have similar bar plot rate as other individuals within each population (Fig. 4-4). Thus, these populations have the well-mixed population genetic structure based on nuclear DNA and the genetic admixture events may have occurred a long time ago, because sexual maturity is reached after 4-5 years on salamander species (Kusano et al. 2014).

4-4-2. Genetic diversity and conservation

Populations with low genetic diversity are susceptible to demographic and environmental risks, and information regarding the extent of gene flow among populations is critical to determine whether a species requires translocation of individuals to prevent inbreeding and loss of genetic diversity (Frankham et al. 2002). Recent developments of genetic markers can reveal the genetic diversity of the populations and can provide important information on how populations recover genetic diversity and will contribute to the future genetic management of wild populations including the reintroduction and re-establishing extinct populations.

Our molecular analyses revealed that population 6 in the region C, population 18 in the southernmost part of the region F, and populations 43, 44, 45 and 46 in the region L have lost genetic diversity greatly both in mitochondrial and microsatellite DNAs (Table 4-1). So that, we need to monitor carefully these populations for long time, and if noticed population decline, conservation management must be done. Salamanders have generally a long lifespan. Skeletochronological examinations of the phalanges revealed that the breeding adults of *H. tokyoensis* are 4-21 years old (Kusano et al. 2006). This delays the demographic responses to environmental changes, and so that longterm monitoring is essential for planning conservation.

On the other hand, populations 2, 3 and 4 in the region B, populations 7 and 8 in the region D, and populations 41 and 42 in the region K have lost genetic diversity in mitochondrial DNA, but kept diversity in microsatellite DNA. This suggests strong bottleneck effects on females. This phenomenon may be attributed to male-biased movements among

populations (Macdonald 2008). Male salamanders tend to arrive at breeding ponds earlier and also stay in water after peak of egg-laying period (Nussbaum 2003). The active phase of male is probably longer than female and males have presumably more chance of dispersal, although additional research is needed to resolve the difference of dispersal abilities between the sexes. In contrast, relatively low microsatellite diversity was observed in the region A, but this may be caused by small sample size. In *H. tokyoensis*, their breeding migration is mainly restricted within the range of 100 m from the pond (Kusano and Miyashita 1984). Under the unexpected condition of naturally occurring migration, it may be an efficient option for us to transplant females from the genetically similar populations if the genetic hazard via strong inbreeding appears.

Table 4-1. Number of haplotypes (N_H), haplotype diversities (h), and nucleotide diversities (π) in 650-bp cytochrome b sequences, and the mean number of alleles (N_A), mean effective number of alleles (N_E), mean allelic richness (A_R), mean expected heterozygosities (H_E), and mean information indices (I) in five microsatellite loci of *Hynobius tokyoensis* . N = number of individuals examined. SD = standard deviation. For the localities of 46 populations and 12 regions, see Fig. 4-1.

Region	Population	Locality	N	Cytochrome b					Microsatellite									
				N_H	h	SD	π	SD	Mean N_A	SD	Mean N_E	SD	Mean A_R	SD	Mean H_E	SD	Mean I	SD
A	1	Iwaki	6	3	0.73	0.16	0.024	0.018	1.40	0.45	1.28	0.32	1.40	0.45	0.16	0.18	0.24	0.27
B	2	Hitachiota	10	1	0.00	0.00	0.000	0.000	1.60	0.80	1.50	0.74	1.59	0.79	0.21	0.27	0.33	0.44
	3	Tokai	10	1	0.00	0.00	0.000	0.000	1.60	0.80	1.45	0.69	1.58	0.78	0.19	0.26	0.31	0.42
	4	Kasama	10	1	0.00	0.00	0.000	0.000	1.80	1.60	1.49	0.98	1.65	1.30	0.14	0.28	0.28	0.56
	5	Motegi	20	2	0.34	0.11	0.012	0.010	1.40	0.80	1.06	0.12	1.19	0.38	0.05	0.09	0.09	0.18
C	6	Utsunomiya	20	1	0.00	0.00	0.000	0.000	1.40	0.49	1.24	0.31	1.38	0.46	0.15	0.18	0.22	0.27
D	7	Iwafune	20	1	0.00	0.00	0.000	0.000	1.60	0.49	1.19	0.17	1.49	0.40	0.15	0.12	0.24	0.20
	8	Sano	20	1	0.00	0.00	0.000	0.000	1.60	0.49	1.17	0.20	1.42	0.38	0.13	0.13	0.21	0.20
E	9	Chosi	20	2	0.19	0.11	0.002	0.003	2.20	0.98	1.32	0.34	1.75	0.65	0.20	0.18	0.36	0.32
F	10	Sousa	34	4	0.52	0.08	0.020	0.013	1.60	0.80	1.45	0.69	1.57	0.76	0.19	0.26	0.31	0.42
	11	Yoshiikami	20	2	0.52	0.04	0.032	0.020	2.40	1.20	1.84	0.99	2.08	1.06	0.30	0.30	0.54	0.53
	12	Chosei	20	2	0.44	0.09	0.016	0.012	2.40	1.50	1.90	1.08	2.09	1.16	0.30	0.31	0.54	0.57
	13	Morimiya	20	3	0.63	0.08	0.015	0.011	2.40	1.50	1.87	0.97	2.10	1.09	0.32	0.30	0.56	0.54
	14	Tsukizaki	20	3	0.57	0.09	0.011	0.009	2.20	1.17	1.62	0.74	1.92	0.93	0.26	0.27	0.46	0.48
	15	Tsujimori	20	3	0.70	0.04	0.034	0.021	2.40	1.50	1.48	0.66	1.90	1.03	0.22	0.24	0.42	0.47
	16	Futsu	10	2	0.47	0.13	0.006	0.006	2.20	1.17	1.31	0.28	1.86	0.79	0.20	0.17	0.37	0.33
	17	Kamogawa	20	2	0.44	0.09	0.005	0.006	1.80	0.75	1.54	0.58	1.73	0.70	0.26	0.25	0.41	0.39
	18	Tateyama	20	1	0.00	0.00	0.000	0.000	1.20	0.40	1.02	0.04	1.09	0.18	0.02	0.04	0.04	0.80
G	19	Namekawa	20	1	0.00	0.00	0.000	0.000	1.80	1.17	1.36	0.70	1.51	0.89	0.14	0.25	0.25	0.45
	20	Yoshimi	20	2	0.48	0.07	0.006	0.006	2.20	0.75	1.38	0.45	1.76	0.67	0.21	0.21	0.38	0.35
	21	Yorii	5	2	0.60	0.18	0.022	0.018	2.00	0.89	1.60	0.57	2.00	0.89	0.29	0.25	0.48	0.43
	22	Kinshozan	20	2	0.27	0.11	0.020	0.014	2.80	1.17	2.20	0.97	2.47	0.88	0.45	0.23	0.77	0.42
	23	Ogawa	20	4	0.73	0.07	0.019	0.013	2.40	0.49	1.88	0.56	2.18	0.52	0.42	0.19	0.66	0.29
	24	Ranzan	20	2	0.39	0.10	0.005	0.005	2.40	0.80	1.78	0.77	2.08	0.77	0.35	0.23	0.58	0.39
	25	Higashimatsuyama	10	2	0.53	0.09	0.033	0.021	2.40	0.80	2.01	0.90	2.32	0.78	0.43	0.18	0.69	0.35
	26	Hatoyama	10	4	0.78	0.09	0.018	0.013	2.60	0.80	1.91	0.65	2.46	0.67	0.43	0.14	0.72	0.29
	27	Ogose	10	4	0.78	0.09	0.021	0.015	3.00	2.10	2.34	1.64	2.61	1.59	0.41	0.26	0.74	0.59
	28	Yokoze	20	5	0.66	0.09	0.041	0.024	2.60	1.74	2.06	1.35	2.23	1.26	0.35	0.28	0.62	0.56
	29	Ogano	20	3	0.68	0.05	0.021	0.014	2.60	1.36	1.96	0.79	2.31	0.96	0.39	0.25	0.67	0.46
H	30	Higashimurayama	5	2	0.60	0.18	0.007	0.008	1.60	0.49	1.28	0.28	1.60	0.40	0.18	0.17	0.29	0.25
	31	Musashimurayama	56	8	0.76	0.03	0.023	0.015	2.20	0.98	1.59	0.35	1.83	0.44	0.33	0.18	0.51	0.27
I	32	Kosogi	20	2	0.19	0.11	0.009	0.008	3.20	1.47	1.82	0.96	2.13	0.97	0.32	0.26	0.58	0.48
J	33	Komagino	12	5	0.85	0.06	0.029	0.019	2.40	1.02	1.35	0.23	1.91	0.56	0.23	0.14	0.42	0.25
	34	Tomoda	12	4	0.71	0.11	0.021	0.015	2.20	0.40	1.57	0.19	2.02	0.20	0.35	0.08	0.55	0.12
	35	Onita	20	2	0.48	0.07	0.006	0.006	3.20	1.94	2.15	1.07	2.56	1.08	0.45	0.18	0.79	0.44
	36	Sugou	10	2	0.20	0.15	0.002	0.004	3.00	1.10	2.09	0.36	2.62	0.68	0.51	0.08	0.84	0.23
	37	Yokosawairi	20	6	0.76	0.06	0.012	0.010	2.60	1.20	1.77	0.84	2.10	0.84	0.33	0.23	0.57	0.42
	38	Kowada	25	3	0.29	0.11	0.008	0.007	3.40	2.33	2.04	0.89	2.55	1.08	0.44	0.18	0.78	0.45
	39	Kawaguchi	20	4	0.71	0.05	0.020	0.014	3.20	1.94	2.25	1.24	2.47	1.16	0.45	0.22	0.77	0.48
	40	Tobuki	20	4	0.56	0.12	0.013	0.010	3.00	1.55	2.09	0.83	2.50	0.89	0.46	0.18	0.78	0.39
K	41	Horinouchi	30	1	0.00	0.00	0.000	0.000	2.80	1.17	2.14	1.00	2.47	0.88	0.45	0.19	0.77	0.39
	42	Renshoji	20	1	0.00	0.00	0.000	0.000	1.80	0.75	1.21	0.20	1.54	0.45	0.15	0.14	0.26	0.23
L	43	Yamanaka	10	1	0.00	0.00	0.000	0.000	1.40	0.49	1.29	0.36	1.40	0.49	0.17	0.21	0.24	0.30
	44	Mineyama	10	1	0.00	0.00	0.000	0.000	1.40	0.80	1.31	0.61	1.38	0.76	0.12	0.24	0.20	0.40
	45	Ikegami	20	1	0.00	0.00	0.000	0.000	1.60	0.49	1.14	0.18	1.35	0.36	0.10	0.12	0.18	0.19
	46	Nobi	10	1	0.00	0.00	0.000	0.000	1.40	0.80	1.33	0.65	1.39	0.78	0.12	0.25	0.21	0.41

Table 4-2. Variable nucleotide positions of the mtDNA cytochrome b region of *Hynobius tokyoensis* .

Haplotype	14	18	41	53	54	66	67	87	90	93	99	102	112	113	117	129	132	136	141	147	159	165	177	178	181	183	194	199	210	234	237	241	258	261	276	279	303	321	324	325	330
T1	C	C	T	G	A	C	G	A	T	C	C	C	T	G	A	T	A	A	T	A	G	C	C	A	T	A	C	G	C	A	A	C	C	G	C	C	C	A	T	A	T
T2	-	-	-	C	-	-	C	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T3	-	-	-	C	-	-	C	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T4	T	-	-	C	-	-	C	-	-	-	-	-	-	T	-	-	-	-	-	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T5	T	-	-	C	-	A	C	-	-	-	-	-	-	T	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T6	T	-	-	C	-	-	C	-	-	-	-	-	-	T	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T7	T	-	-	C	-	-	C	-	-	-	-	-	-	T	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T8	T	-	-	C	-	-	C	-	-	-	T	-	-	T	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T9	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T10	T	T	-	C	-	-	C	-	C	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T11	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T12	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T13	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T14	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	-	-	-	C
T15	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	G	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	-	-	-	C
T16	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	G	-	C	-	A	T	-	-	-	-	-	A	-	-	-	-	T	A	-	-	A	-	-	-	C
T17	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T18	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T19	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	C	-	C
T20	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T21	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T22	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	T	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T23	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	G	-	T	A	-	-	A	G	-	T	C
T24	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	G	-	T	A	-	-	A	G	-	-	C
T25	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	-	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T26	T	T	-	C	-	-	C	-	-	G	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T27	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T28	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T29	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T30	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	T	-	-	-	T	A	-	-	A	G	-	-	C
T31	T	T	-	C	-	-	C	-	-	-	-	T	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T32	T	T	-	C	-	-	C	-	-	-	-	T	C	T	-	C	-	-	C	-	A	T	-	C	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T33	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T34	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T35	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	T	T	A	-	T	A	G	-	-	C
T36	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T37	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T38	T	T	-	C	G	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T39	T	T	-	C	G	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T40	T	T	-	C	G	-	C	-	-	-	-	-	C	T	-	C	-	G	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T41	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	-	-	T	A	G	-	-	C
T42	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	-	-	T	A	G	-	-	C
T43	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T44	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T45	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	-	-	-	C
T46	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	-	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T47	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T48	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	T	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T49	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T50	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T51	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	C	G	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T52	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T53	T	T	-	C	C	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	-
T54	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T55	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	T	-	A	G	-	-	C
T56	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T57	T	T	-	C	-	-	C	-	-	-	-	-	C	T	G	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T58	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	T	A	G	-	-	C
T59	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	T	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T60	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	C	-	A	T	T	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T61	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T62	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T63	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	-	G	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T64	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T65	T	T	-	C	-	-	C	-	-	-	-	-	C	T	-	C	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	G	-	-	C
T66	T	T	-	C	-	-	C	G	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	G	-	-	T	A	-	-	A	G	-	-	C
T67	T	T	C	C	-	-	C	G	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	G	-	-	T	A	-	-	A	G	-	-	C
T68	T	T	-	C	-	-	C	G	-	-	-	-	C	T	-	C	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	-	-	-	C
T69	T	T	-	C	-	-	C	G	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	-	C	-	C
T70	T	T	-	C	-	-	C	G	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	-	-	-	C
T71	T	T	-	C	-	-	C	G	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	-	-	-	C
T72	T	T	-	C	-	-	C	G	-	-	-	-	C	T	-	C	-	-	C	-	A	T	-	-	-	-	-	-	-	-	-	-	T	A	-	-	A	-	-	-	C

Nucleotide Position (column headers 14–330)

Table 4-2. (continued)

Haplotype	Nucleotide Position																																								
	357	361	363	420	429	438	444	453	460	462	466	467	468	471	475	480	492	495	501	504	510	516	525	541	543	555	558	561	564	570	576	577	585	591	594	595	609	612	639	642	643
T1	C	C	A	C	G	T	C	C	G	T	T	A	C	T	G	G	A	C	T	A	T	T	A	G	A	A	C	G	C	T	G	A	T	T	C	C	A	C	T	A	A
T2	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T4	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
T5	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
T6	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
T7	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-
T8	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-
T9	-	-	G	-	-	-	T	-	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	-	G	T	-	A	T	C	-	G	C	-	T	-	-	-	C	T	-
T10	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	-	G	T	-	A	T	C	-	G	C	C	T	-	-	-	C	T	-
T11	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	G	T	-	-	C	-	-	-	G	T	-	A	T	C	-	G	C	C	T	-	-	-	C	T	-
T12	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	-	G	T	-	A	T	C	-	G	C	C	T	-	G	-	C	T	-
T13	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	-	G	T	-	A	T	C	-	G	C	C	T	-	-	-	C	T	-
T14	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	-	G	T	-	A	T	C	-	G	C	C	T	-	-	-	C	T	-
T15	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	-	G	T	-	A	T	C	-	G	C	C	T	-	-	-	C	T	-
T16	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	-	G	T	-	A	T	C	-	G	C	C	T	-	-	-	C	T	-
T17	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	C	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	-	-	-	C	T	-
T18	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	C	-	C	-	-	-	G	T	-	A	T	C	-	G	C	C	T	-	-	-	C	T	-
T19	T	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	-	-	-	C	T	-
T20	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	-	-	-	C	T	-
T21	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	-	-	A	C	T	-
T22	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	-	-	-	C	T	-
T23	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	G	A	G	T	-	A	T	C	-	G	C	-	T	-	-	-	C	T	-
T24	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	G	A	G	T	-	A	T	C	-	G	C	-	T	-	-	-	C	T	-
T25	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	-	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T26	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	-	-	G	C	-	T	T	-	-	C	T	G
T27	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	C	-	A	G	T	-	A	T	-	-	G	C	-	T	T	-	-	C	T	G
T28	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	-	-	G	C	-	T	T	-	-	C	T	G
T29	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	-	-	G	C	-	T	T	-	-	C	T	-
T30	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	-	-	G	C	-	T	T	-	-	C	T	-
T31	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T32	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T33	-	-	G	-	-	-	T	T	A	C	-	-	-	C	A	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T34	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T35	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T36	-	-	G	A	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T37	-	-	G	-	-	-	T	T	A	C	C	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T38	-	-	G	-	-	-	T	T	A	C	C	-	-	C	-	-	-	-	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T39	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T40	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T41	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T42	T	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T43	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	G	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T44	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T45	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T46	-	-	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T47	-	-	-	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T48	-	T	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T49	-	T	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	G	T	-	-	C	T	-
T50	-	T	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T51	-	T	G	-	-	-	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T52	-	-	G	-	A	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T53	-	-	G	-	A	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T54	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	A	G	C	-	T	T	-	-	C	T	-
T55	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T56	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T57	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T58	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T59	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T60	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	-	C	-	G	C	-	T	T	-	-	C	T	-
T61	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	A	-	T	-	-	C	-	-	A	G	T	-	A	-	C	-	G	C	-	T	T	-	-	C	T	-
T62	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	-	C	-	G	C	-	T	T	-	-	C	T	-
T63	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T64	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T65	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	C	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T66	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	C	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T67	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T68	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	C	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T69	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T70	-	-	G	-	-	C	T	T	A	C	-	-	-	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T71	-	-	G	-	-	C	T	T	A	C	-	-	T	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-
T72	T	-	G	-	-	C	T	T	A	C	-	-	T	C	-	-	-	T	-	-	C	-	-	A	G	T	-	A	T	C	-	G	C	-	T	T	-	-	C	T	-

Table 4-3. Frequencies of cytochrome b haplotypes (T1-T72) in 46 populations of *Hynobius tokyoensis* . For the localities of 46 populations, see Fig. 4-1.

No.	Population	Haplotype																																			
		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	T19	T20	T21	T22	T23	T24	T25	T26	T27	T28	T29	T30	T31	T32	T33	T34	T35	T36
1	Iwaki	2	1	3																																	
2	Hitachiota				10																																
3	Tokai					10																															
4	Kasama						10																														
5	Motegi							16	4																												
6	Utsunomiya										20																										
7	Iwafune										20																										
8	Sano										20																										
9	Chosi																																				
10	Sousa																																				
11	Yoshiikami																																				
12	Chosei																																				
13	Morimiya																																				
14	Tsukizaki																																				
15	Tsujimori																																		7	6	
16	Futsu																																	3	7		
17	Kamogawa																															14	6				
18	Tateyama																															20					
19	Namekawa												20																								
20	Yoshimi												7	13																							
21	Yorii																				3				2												
22	Kinshozan													17																							
23	Ogawa											5		3	3	9																					
24	Ranzan												5	15																							
25	Higashimatsuyama																		4								6										
26	Hatoyama																										4	2	1		3						
27	Ogose																										2			1							
28	Yokoze																3	4					1	11						1							
29	Ogano									5										9		6															
30	Higashimurayama																																				
31	Musashimurayama																																				
32	Kosogi																									2											
33	Komagino																																				
34	Tomoda																																				
35	Onita																																				
36	Sugou																																				
37	Yokosawairi																																				
38	Kowada																																				
39	Kawaguchi																																				
40	Tobuki																																				
41	Horinouchi																																				
42	Renshoji																																				
43	Yamanaka													10																							
44	Mineyama													10																							
45	Ikegami																																				
46	Nobi																																				10
Total		2	1	3	10	10	10	16	4	5	60	5	32	68	3	9	3	4	4	9	3	6	1	11	2	2	12	2	1	2	3	34	6	3	14	6	10

Table 4-3. (continued)

No.	Population	Haplotype																																		Total
		T37	T38	T39	T40	T41	T42	T43	T44	T45	T46	T47	T48	T49	T50	T51	T52	T53	T54	T55	T56	T57	T58	T59	T60	T61	T62	T63	T64	T65	T66	T67	T68	T69	T70	T71	T72	
1	Iwaki																																					6
2	Hitachiota																																					10
3	Tokai																																					10
4	Kasama																																					10
5	Motegi																																					20
6	Utsunomiya																																					20
7	Iwafune																																					20
8	Sano																																					20
9	Chosi					2	18																															20
10	Sousa		1	9	2											22																						34
11	Yoshiikami								11						9																							20
12	Chosei							14			6																											20
13	Morimiya										4	5		11																								20
14	Tsukizaki									6			12	2																								20
15	Tsujimori									7																												20
16	Futsu																																					10
17	Kamogawa																																					20
18	Tateyama																																					20
19	Namekawa																																					20
20	Yoshimi																																					20
21	Yorii																																					5
22	Kinshozan									3																												20
23	Ogawa																																					20
24	Ranzan																																					20
25	Higashimatsuyama																																					10
26	Hatoyama																																					10
27	Ogose																4		3																			10
28	Yokoze																																					20
29	Ogano																																					20
30	Higashimurayama																													2							3	5
31	Musashimurayama																3	2			1	10							21	15	2						2	56
32	Kosogi																																				18	20
33	Komagino																			2			3									1		3			3	12
34	Tomoda																			3			2											1			6	12
35	Onita																																7				13	20
36	Sugou																																1				9	10
37	Yokosawairi																							6			1	1				3	1				8	20
38	Kowada																								1			21					3					25
39	Kawaguchi																									4								7	1		8	20
40	Tobuki																2									3								2			13	20
41	Horinouchi																																				30	30
42	Renshoji																																			20		20
43	Yamanaka																																					10
44	Mineyama																																					10
45	Ikegami	20																																				20
46	Nobi																																					10
Total		20	1	9	2	2	18	14	11	16	10	5	12	13	9	22	9	2	3	5	1	10	5	6	1	7	1	22	21	17	2	4	12	13	1	20	113	815

Table 4-4. Analysis of molecular variance (AMOVA) of 650-bp mitochondrial cytochrome b sequences in 46 populations of *Hynobius tokyoensis* in 12 regions.

Source of variation	Sum of squares	Variance components	Percentage variation (%)	P-value
Among regions	2671.63	3.56	72.5	<0.001
Among populations within regions	496.33	0.85	17.3	<0.001
Within populations	382.84	0.50	10.2	<0.001
Total	3550.80	4.91		

Table 4-5. Pairwise genetic distances (above diagonal Φ_{ST}, based on mtDNA and below diagonal F_{ST}, based on microsatellite DNA) among 46 populations of *Hynobius tokyoensis* (no asterisks P>0.05; * P<0.05; ** P<0.01). For the localities of 46 populations, see Fig. 4-1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	-	0.901**	0.901**	0.913**	0.863**	0.988**	0.988**	0.988**	0.983**	0.955**	0.933**	0.959**	0.961**	0.969**	0.927**	0.973**	0.979**	0.988**	0.987**	0.975**	0.944**	0.949**	0.949**
2	0.294	-	0.000	1.000**	0.649**	1.000**	1.000**	1.000**	0.996**	0.961**	0.946**	0.971**	0.973**	0.981**	0.941**	0.993**	0.990**	1.000**	1.000**	0.988**	0.981**	0.962**	0.963**
3	0.320	-0.031	-	1.000**	0.649**	1.000**	1.000**	1.000**	0.996**	0.961**	0.946**	0.971**	0.974**	0.981**	0.941**	0.993**	0.990**	1.000**	1.000**	0.988**	0.981**	0.962**	0.963**
4	0.353	0.262**	0.267*	-	0.494**	1.000**	1.000**	1.000**	0.996**	0.963**	0.948**	0.972**	0.974**	0.982**	0.943**	0.993**	0.991**	1.000**	1.000**	0.989**	0.982**	0.963**	0.964**
5	0.672**	0.528**	0.539**	0.484**	-	0.984**	0.984**	0.984**	0.981**	0.959**	0.947**	0.965**	0.967**	0.972**	0.943**	0.976**	0.978**	0.985**	0.982**	0.975**	0.963**	0.957**	0.957**
6	0.784**	0.761**	0.772**	0.808**	0.856**	-	0.000	0.000	0.991**	0.899**	0.874**	0.932**	0.930**	0.951**	0.849**	0.984**	0.973**	1.000**	1.000**	0.910**	0.946**	0.794**	0.745**
7	0.786**	0.771**	0.781**	0.805**	0.851**	0.448**	-	0.000	0.991**	0.899**	0.874**	0.932**	0.930**	0.951**	0.849**	0.984**	0.973**	1.000**	1.000**	0.910**	0.946**	0.794**	0.745**
8	0.807**	0.788**	0.798**	0.823**	0.869**	0.493**	0.200	-	0.991**	0.899**	0.874**	0.932**	0.930**	0.951**	0.849**	0.984**	0.973**	1.000**	1.000**	0.910**	0.946**	0.794**	0.745**
9	0.739**	0.734**	0.745**	0.758**	0.851**	0.437**	0.588**	0.609**	-	0.738**	0.734**	0.763**	0.815**	0.862**	0.695**	0.955**	0.939**	0.981**	0.989**	0.959**	0.920**	0.877**	0.902**
10	0.687**	0.725**	0.735**	0.771**	0.828**	0.471**	0.543**	0.556**	0.409**	-	0.299**	0.570**	0.291**	0.307**	0.489**	0.747**	0.721**	0.744**	0.873**	0.837**	0.683**	0.740**	0.798**
11	0.612**	0.633**	0.645**	0.693**	0.771**	0.276**	0.383**	0.402**	0.304**	0.098**	-	0.401**	0.324**	0.359**	0.454**	0.679**	0.672**	0.701**	0.844**	0.800**	0.586**	0.688**	0.752**
12	0.645**	0.640**	0.652**	0.693**	0.785**	0.365**	0.370**	0.378**	0.305**	0.299**	0.142**	-	0.500**	0.591**	0.520**	0.795**	0.779**	0.821**	0.914**	0.872**	0.717**	0.759**	0.814**
13	0.614**	0.625**	0.636**	0.681**	0.777**	0.243**	0.383**	0.396**	0.204**	0.209**	0.091**	0.108**	-	0.131*	0.437**	0.774**	0.743**	0.791**	0.911**	0.864**	0.684**	0.735**	0.801**
14	0.671**	0.663**	0.674**	0.710**	0.807**	0.398**	0.357**	0.360**	0.278**	0.350**	0.193**	0.105**	0.074**	-	0.457**	0.828**	0.799**	0.853**	0.937**	0.893**	0.750**	0.765**	0.827**
15	0.718**	0.708**	0.718**	0.758**	0.834**	0.499**	0.372**	0.368**	0.483**	0.428**	0.294**	0.093**	0.228**	0.184**	-	0.307*	0.295**	0.300**	0.808**	0.781**	0.580**	0.680**	0.739**
16	0.731	0.724*	0.738*	0.781*	0.875**	0.529**	0.438**	0.449**	0.501**	0.449**	0.316**	0.253**	0.243**	0.242**	0.207*	-	0.772**	0.888**	0.998**	0.994**	0.886**	0.842**	0.865**
17	0.704**	0.689**	0.698**	0.746**	0.823**	0.506**	0.423**	0.422**	0.513**	0.487**	0.333**	0.234**	0.243**	0.203**	0.200**	0.217**	-	0.263*	0.963**	0.934**	0.868**	0.847**	0.872**
18	0.920**	0.883**	0.892**	0.920**	0.959**	0.767**	0.662**	0.679**	0.730**	0.656**	0.572**	0.499**	0.512**	0.487**	0.446**	0.139	0.439**	-	1.000**	0.964**	0.937**	0.874**	0.896**
19	0.795**	0.775**	0.786**	0.808**	0.874**	0.544**	0.266**	0.257**	0.482**	0.526**	0.391**	0.288**	0.301**	0.160**	0.291**	0.374**	0.339**	0.609**	-	0.632**	0.917**	0.576**	0.507**
20	0.730**	0.717**	0.724**	0.744**	0.798**	0.542**	0.225**	0.273**	0.613**	0.596**	0.458**	0.438**	0.444**	0.417**	0.449**	0.481**	0.456**	0.663**	0.394**	-	0.780**	0.164**	0.193**
21	0.649	0.631	0.646	0.712	0.855*	0.499*	0.373*	0.393*	0.499*	0.455**	0.254*	0.190*	0.181*	0.125	0.208*	0.218	0.155	0.601	0.342*	0.294	-	0.507**	0.619**
22	0.503**	0.492**	0.504**	0.556**	0.652**	0.269**	0.272**	0.295**	0.305**	0.304**	0.130**	0.139**	0.143**	0.159**	0.234**	0.257**	0.264**	0.463**	0.286**	0.239**	0.082	-	0.203**
23	0.566**	0.560**	0.573**	0.616**	0.690**	0.305**	0.200**	0.218**	0.371**	0.348**	0.154**	0.164**	0.178**	0.173**	0.221**	0.237**	0.236**	0.453**	0.237**	0.220**	0.130	0.036	-
24	0.619**	0.631**	0.640**	0.645**	0.752**	0.519**	0.325**	0.340**	0.457**	0.506**	0.390**	0.321**	0.327**	0.272**	0.327**	0.334**	0.348**	0.518**	0.285**	0.189**	0.195*	0.201**	0.196**
25	0.578	0.582*	0.592*	0.622*	0.768**	0.526**	0.400**	0.422**	0.455**	0.496**	0.341**	0.288**	0.282**	0.250**	0.325**	0.304*	0.305**	0.562**	0.379**	0.227**	0.071	0.113*	0.152**
26	0.555	0.575**	0.587*	0.594*	0.745**	0.558**	0.406**	0.431**	0.477**	0.517**	0.386**	0.353**	0.356**	0.332**	0.398**	0.396*	0.415**	0.631**	0.414**	0.225**	0.197	0.157**	0.208**
27	0.567	0.589**	0.600**	0.641*	0.770**	0.469**	0.332**	0.358**	0.456**	0.403**	0.249**	0.219**	0.233**	0.223**	0.249**	0.257	0.252**	0.528**	0.351**	0.178**	0.038	0.068	0.100*
28	0.593**	0.596**	0.605**	0.620**	0.719**	0.463**	0.249**	0.245**	0.469**	0.452**	0.326**	0.252**	0.280**	0.231**	0.217**	0.268**	0.281**	0.456**	0.254**	0.267**	0.131	0.226**	0.181**
29	0.564**	0.548**	0.554**	0.593**	0.708**	0.430**	0.332**	0.341**	0.407**	0.415**	0.261**	0.201**	0.198**	0.148**	0.229**	0.239**	0.205**	0.430**	0.284**	0.244**	0.037	0.107**	0.140**
30	0.732	0.685	0.695	0.747	0.881**	0.703*	0.642*	0.669*	0.685*	0.667**	0.523*	0.485*	0.448*	0.455*	0.532*	0.562	0.437*	0.859*	0.634*	0.487*	0.242	0.337*	0.354*
31	0.598**	0.577**	0.579**	0.622**	0.687**	0.449**	0.380**	0.380**	0.477**	0.452**	0.327**	0.271**	0.251**	0.218**	0.276**	0.300**	0.216**	0.437**	0.331**	0.347**	0.060	0.237**	0.220**
32	0.683**	0.684**	0.693**	0.727**	0.807**	0.549**	0.567**	0.577**	0.527**	0.563**	0.430**	0.413**	0.347**	0.414**	0.450**	0.371**	0.383**	0.613**	0.542**	0.572**	0.428*	0.379**	0.312**
33	0.744	0.721**	0.731**	0.782**	0.868**	0.655**	0.631**	0.643**	0.625**	0.636**	0.480**	0.444**	0.386**	0.407**	0.493**	0.499**	0.362**	0.762**	0.585**	0.601**	0.423	0.379**	0.318**
34	0.638	0.621**	0.629**	0.676**	0.795**	0.590**	0.541**	0.551**	0.549**	0.586**	0.425**	0.377**	0.328**	0.323**	0.414**	0.396**	0.299**	0.657**	0.478**	0.485**	0.254	0.304**	0.254**
35	0.528**	0.537**	0.547**	0.579**	0.694**	0.443**	0.393**	0.397**	0.381**	0.451**	0.315**	0.271**	0.220**	0.219**	0.301**	0.272**	0.258**	0.488**	0.310**	0.406**	0.244*	0.243**	0.189**
36	0.493	0.478*	0.487*	0.555**	0.705**	0.424**	0.409**	0.421**	0.430**	0.449**	0.275**	0.249**	0.190**	0.225**	0.294**	0.273**	0.219**	0.554**	0.389**	0.380**	0.095	0.170**	0.158**
37	0.650**	0.646**	0.656**	0.688**	0.784**	0.607**	0.559**	0.564**	0.544**	0.593**	0.460**	0.417**	0.385**	0.382**	0.446**	0.409**	0.371**	0.623**	0.493**	0.549**	0.403*	0.375**	0.307**
38	0.518**	0.512**	0.529**	0.576**	0.678**	0.377**	0.315**	0.313**	0.345**	0.360**	0.220**	0.173**	0.131**	0.109**	0.196**	0.194**	0.154**	0.390**	0.222**	0.347**	0.122	0.166**	0.128**
39	0.506**	0.507**	0.519**	0.564**	0.668**	0.407**	0.304**	0.300**	0.405**	0.416**	0.262**	0.222**	0.212**	0.187**	0.233**	0.231**	0.215**	0.429**	0.265**	0.361**	0.176	0.185**	0.141**
40	0.542**	0.543**	0.551**	0.588**	0.699**	0.484**	0.373**	0.376**	0.470**	0.489**	0.348**	0.295**	0.273**	0.258**	0.292**	0.274**	0.239**	0.476**	0.347**	0.326**	0.147	0.214**	0.166**
41	0.469**	0.450**	0.455**	0.494**	0.605**	0.514**	0.416**	0.430**	0.505**	0.518**	0.427**	0.388**	0.380**	0.367**	0.393**	0.375**	0.385**	0.505**	0.415**	0.268**	0.214*	0.231**	0.281**
42	0.766**	0.760**	0.767**	0.782**	0.864**	0.749**	0.653**	0.677**	0.728**	0.699**	0.629**	0.596**	0.596**	0.596**	0.619**	0.657**	0.617**	0.805**	0.672**	0.464**	0.523*	0.415**	0.474**
43	0.759	0.714*	0.727*	0.797*	0.886**	0.516**	0.473**	0.486**	0.520**	0.434**	0.234**	0.185**	0.182**	0.125**	0.257**	0.360*	0.199*	0.692**	0.391**	0.484**	0.106	0.176**	0.203**
44	0.797	0.756*	0.771**	0.824*	0.899**	0.544**	0.272**	0.272**	0.573**	0.492**	0.339**	0.287**	0.308**	0.247**	0.278**	0.365*	0.306**	0.699**	0.247**	0.390**	0.293	0.261**	0.217**
45	0.831**	0.786**	0.798**	0.849**	0.905**	0.664**	0.560**	0.569**	0.617**	0.506**	0.416**	0.379**	0.421**	0.393**	0.422**	0.517**	0.468**	0.749**	0.512**	0.591**	0.487*	0.375**	0.369**
46	0.792	0.752*	0.766*	0.821*	0.899**	0.513**	0.292**	0.292**	0.572**	0.506**	0.323**	0.255**	0.246**	0.167**	0.241**	0.338*	0.204**	0.683**	0.229**	0.379**	0.145	0.224**	0.194**

Table 4-5. (continued)

	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
1	0.977**	0.929**	0.952**	0.948**	0.912**	0.945**	0.963**	0.949**	0.972**	0.938**	0.950**	0.978**	0.977**	0.967**	0.975**	0.953**	0.965**	0.992**	0.989**	0.978**	0.989**	0.989**	0.982**
2	0.990**	0.955**	0.975**	0.972**	0.926**	0.958**	0.994**	0.951**	0.984**	0.959**	0.970**	0.990**	0.997**	0.979**	0.985**	0.965**	0.977**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
3	0.990**	0.955**	0.975**	0.972**	0.926**	0.958**	0.994**	0.951**	0.984**	0.959**	0.970**	0.990**	0.997**	0.979**	0.985**	0.965**	0.977**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
4	0.991**	0.956**	0.976**	0.972*	0.928**	0.960**	0.994**	0.953**	0.984**	0.960**	0.971**	0.990**	0.997**	0.980**	0.985**	0.966**	0.978**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
5	0.976**	0.950**	0.964**	0.962**	0.932**	0.954**	0.973**	0.952**	0.974**	0.955**	0.962**	0.978**	0.978**	0.970**	0.975**	0.961**	0.969**	0.988**	0.985**	0.977**	0.977**	0.986**	0.981**
6	0.928**	0.884**	0.947**	0.931**	0.748**	0.848**	0.989**	0.865**	0.958**	0.910**	0.931**	0.972**	0.993**	0.947**	0.963**	0.917**	0.942**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
7	0.928**	0.884**	0.947**	0.931**	0.748**	0.848**	0.989**	0.865**	0.958**	0.910**	0.931**	0.972**	0.993**	0.947**	0.963**	0.917**	0.942**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
8	0.928**	0.884**	0.947**	0.931**	0.748**	0.848**	0.989**	0.865**	0.958**	0.910**	0.931**	0.972**	0.993**	0.947**	0.963**	0.917**	0.942**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
9	0.964**	0.834**	0.899**	0.866**	0.765**	0.852**	0.958**	0.802**	0.920**	0.851**	0.877**	0.947**	0.968**	0.906**	0.914**	0.865**	0.897**	0.987**	0.986**	0.984**	0.984**	0.984**	0.978**
10	0.839**	0.625**	0.691**	0.507**	0.638**	0.693**	0.643**	0.609**	0.651**	0.587**	0.583**	0.699**	0.669**	0.663**	0.698**	0.632**	0.628**	0.758**	0.783**	0.825**	0.825**	0.794**	0.757**
11	0.803**	0.543**	0.614**	0.464**	0.574**	0.637**	0.563**	0.589**	0.630**	0.544**	0.546**	0.679**	0.627**	0.642**	0.718**	0.605**	0.609**	0.755**	0.761**	0.772**	0.772**	0.757**	0.692**
12	0.876**	0.641**	0.723**	0.646**	0.632**	0.712**	0.773**	0.703**	0.778**	0.695**	0.712**	0.821**	0.806**	0.780**	0.833**	0.736**	0.758**	0.885**	0.884**	0.870**	0.870**	0.859**	0.816**
13	0.868**	0.592**	0.688**	0.467**	0.592**	0.677**	0.671**	0.580**	0.666**	0.564**	0.570**	0.734**	0.711**	0.677**	0.764**	0.622**	0.631**	0.822**	0.839**	0.863**	0.863**	0.843**	0.797**
14	0.898**	0.647**	0.744**	0.512**	0.625**	0.716**	0.728**	0.581**	0.700**	0.588**	0.598**	0.769**	0.763**	0.704**	0.791**	0.642**	0.657**	0.861**	0.877**	0.902**	0.902**	0.889**	0.855**
15	0.788**	0.542**	0.610**	0.531**	0.565**	0.636**	0.631**	0.671**	0.682**	0.605**	0.611**	0.702**	0.671**	0.688**	0.726**	0.660**	0.669**	0.792**	0.788**	0.763**	0.763**	0.674**	0.598**
16	0.948**	0.766**	0.852**	0.808**	0.711**	0.813**	0.924**	0.783**	0.895**	0.790**	0.827**	0.920**	0.946**	0.879**	0.915**	0.823**	0.867**	0.982**	0.979**	0.972**	0.972**	0.965**	0.946**
17	0.941**	0.784**	0.857**	0.812**	0.727**	0.816**	0.917**	0.723**	0.867**	0.811**	0.836**	0.907**	0.930**	0.875**	0.908**	0.832**	0.863**	0.967**	0.965**	0.959**	0.959**	0.933**	0.911**
18	0.971**	0.828**	0.906**	0.867**	0.748**	0.845**	0.980**	0.783**	0.924**	0.848**	0.878**	0.949**	0.987**	0.908**	0.937**	0.861**	0.898**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
19	0.737**	0.841**	0.932**	0.909**	0.640**	0.766**	0.986**	0.834**	0.946**	0.887**	0.912**	0.964**	0.991**	0.933**	0.953**	0.896**	0.926**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
20	-0.028	0.755**	0.876**	0.843**	0.521**	0.640**	0.926*	0.802**	0.903**	0.835**	0.859**	0.926**	0.939**	0.892**	0.921**	0.854**	0.882**	0.970**	0.967**	0.232	0.232	0.971**	0.961**
21	0.794**	0.341*	0.660**	0.546**	0.251*	0.199*	0.750**	0.657**	0.773**	0.583**	0.633**	0.840**	0.838**	0.761**	0.840**	0.666**	0.727**	0.944**	0.937**	0.812**	0.812**	0.947**	0.910**
22	0.128*	0.574**	0.739**	0.680**	0.372**	0.446**	0.774**	0.733**	0.792**	0.716**	0.731**	0.827**	0.808**	0.792**	0.839**	0.754**	0.774**	0.885**	0.880**	0.046	0.046	0.891**	0.857**
23	0.194**	0.664**	0.791**	0.751**	0.481**	0.567**	0.818**	0.780**	0.835**	0.767**	0.783**	0.859**	0.846**	0.831**	0.867**	0.799**	0.820**	0.910**	0.903**	0.201*	0.201*	0.913**	0.885**
24	-	0.761**	0.883**	0.850**	0.520**	0.642**	0.935**	0.803**	0.908**	0.840**	0.864**	0.932**	0.948**	0.897**	0.925**	0.857**	0.887**	0.975**	0.973**	0.137	0.137	0.976**	0.968**
25	0.056	-	0.219	0.330**	0.360**	0.424**	0.601**	0.617**	0.666**	0.539**	0.554**	0.732**	0.681**	0.675**	0.761**	0.619**	0.640**	0.833**	0.828**	0.722**	0.722**	0.835**	0.792**
26	0.073	0.004	-	0.444**	0.551**	0.666**	0.748**	0.666**	0.763**	0.632**	0.660**	0.821**	0.808**	0.759**	0.829**	0.696**	0.731**	0.911**	0.906**	0.882**	0.882**	0.921**	0.882**
27	0.058	0.007	0.079	-	0.471**	0.577**	0.474**	0.418**	0.483**	0.341**	0.324**	0.606**	0.556**	0.517**	0.672**	0.443**	0.424**	0.755**	0.801**	0.840**	0.840**	0.892**	0.840**
28	0.179**	0.207**	0.245**	0.172**	-	0.317**	0.573**	0.645**	0.636**	0.561**	0.563**	0.681**	0.625**	0.651**	0.717**	0.620**	0.625**	0.751**	0.748**	0.453**	0.453**	0.769**	0.721**
29	0.154**	0.054	0.160**	0.054	0.128**	-	0.709**	0.677**	0.730**	0.648**	0.659**	0.777**	0.749**	0.737**	0.797**	0.695**	0.712**	0.845**	0.845**	0.606**	0.606**	0.868**	0.828**
30	0.364*	0.295	0.395	0.286	0.249*	0.153	-	0.074	0.130	0.094	0.093	0.306*	0.273	0.148	0.553**	0.187*	0.113	0.676*	0.917**	0.971**	0.971**	0.983**	0.971**
31	0.264**	0.230**	0.346**	0.179**	0.161**	0.068**	0.069	-	0.287**	0.296**	0.266**	0.341**	0.277**	0.317**	0.479**	0.345**	0.280**	0357**	0.542**	0.787**	0.787**	0.812**	0.787**
32	0.407**	0.376**	0.462**	0.381**	0.394**	0.380**	0.504*	0.353**	-	0.143**	0.089*	0.175**	0.011	0.116**	0.510**	0.211**	0.051	0.084	0.729**	0.916**	0.916**	0.937**	0.916**
33	0.438**	0.368**	0.473**	0.402**	0.425**	0.346**	0.514	0.296**	0.176*	-	-0.042	0.231**	0.120*	0.093*	0.438**	0.019	0.040	0.292**	0.587**	0.816**	0.816**	0.870**	0.816**
34	0.325**	0.238**	0.348**	0.284**	0.289**	0.206**	0.253	0.161**	0.195**	0.046	-	0.211**	0.092	0.091*	0.456**	0.107*	0.019	0.256**	0.641**	0.853**	0.853**	0.898**	0.853**
35	0.235**	0.215**	0.275**	0.240**	0.231**	0.223**	0.328*	0.224**	0.120**	0.103	0.065	-	0.079	0.186**	0.559**	0.288**	0.183**	0.377**	0.823**	0.949**	0.949**	0.962**	0.949**
36	0.249**	0.151*	0.239*	0.165*	0.158**	0.103**	0.145	0.087**	0.188**	0.149*	0.042	0.066	-	0.078	0.557**	0.192*	0.049	0.126	0.940**	0.984**	0.984**	0.989**	0.984**
37	0.377**	0.329**	0.405**	0.378**	0.383**	0.351**	0.471*	0.341**	0.129**	0.065	0.079	0.040	0.171**	-	0.457**	0.181**	0.061*	0.205**	0.684**	0.898**	0.898**	0.923**	0.898**
38	0.213**	0.171**	0.250**	0.172**	0.177**	0.128**	0.279	0.133**	0.191**	0.125	0.079	0.026	0.044	0.122**	-	0.481**	0.475**	0.704**	0.822**	0.933**	0.933**	0.948**	0.933**
39	0.246**	0.199**	0.244**	0.217**	0.194**	0.188**	0.354*	0.224**	0.235**	0.184**	0.149*	0.055	0.083**	0.139**	0.026	-	0.065	0.338**	0.614**	0.844**	0.844**	0.881**	0.844**
40	0.155**	0.093	0.178**	0.137*	0.178**	0.138**	0.237	0.162**	0.174**	0.120*	0.055	0.059**	0.039	0.112**	0.063**	0.077	-	0.145**	0.664**	0.886**	0.886**	0.914**	0.886**
41	0.149**	0.115**	0.117**	0.137**	0.271**	0.197**	0.275**	0.286**	0.418**	0.405**	0.303**	0.259**	0.201**	0.372**	0.246**	0.252**	0.176**	-	1.000**	1.000**	1.000**	1.000**	1.000**
42	0.242**	0.350**	0.334**	0.280**	0.451**	0.397**	0.618*	0.438**	0.653**	0.719**	0.616**	0.513**	0.508**	0.643**	0.479**	0.507**	0.412**	0.181**	-	1.000**	1.000**	1.000**	1.000**
43	0.370**	0.311	0.417*	0.242*	0.302**	0.156**	0.528	0.181**	0.482**	0.468**	0.369**	0.300**	0.223*	0.457**	0.155**	0.230**	0.279**	0.364**	0.673**	-	0.000	1.000**	1.000**
44	0.322**	0.366*	0.416*	0.290	0.236**	0.268**	0.628	0.298**	0.515**	0.570**	0.459**	0.314**	0.307*	0.494**	0.219**	0.251**	0.312**	0.358**	0.676**	0.282	-	1.000**	1.000**
45	0.460**	0.495**	0.534**	0.433**	0.387**	0.391**	0.728*	0.410**	0.601**	0.673**	0.589**	0.436**	0.435**	0.592**	0.344**	0.388**	0.439**	0.437**	0.734**	0.408**	0.253**	-	1.000**
46	0.320**	0.330*	0.412*	0.258	0.235**	0.194**	0.556	0.207**	0.495**	0.505**	0.391**	0.288**	0.255**	0.465**	0.163**	0.214**	0.270**	0.353**	0.668**	0.142	0.101	0.447**	-

Table 4-6. Frequencies of alleles (indicated as base pairs) in five microsatellite loci of *Hynobius tokyoensis* . N = number of individuals examined.

Locus (motif)	Size (bp)	Population																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
HN002 (AACTC)n	234	0	0	0	0	0	0	6	4	0	0	0	0	0	0	0	0	0	0	0	24	2	16	10	24
	236	4	5	4	0	0	40	34	36	40	68	40	40	40	40	40	20	40	40	40	12	6	24	30	16
	241	8	15	16	20	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	0	0	0
HN004 (ACTC)n	154	0	0	0	0	0	0	0	0	4	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
	158	0	0	0	0	0	0	0	0	1	0	3	3	6	3	3	3	0	0	0	2	0	3	12	10
	162	12	20	20	20	40	40	40	40	35	68	36	37	34	37	37	17	22	40	39	38	10	37	28	30
	166	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0	0	0	0	0	0	0
HN019 (AAT)n	421	3	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15
	424	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0
	430	0	5	4	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	12	14	0
	433	0	8	8	0	1	0	0	0	2	14	5	1	0	0	0	0	0	0	0	0	0	0	0	0
	436	9	0	0	0	0	0	0	0	4	54	16	0	11	2	0	2	0	0	0	0	0	0	0	0
	439	0	0	0	3	35	28	33	31	0	0	10	0	0	0	0	0	0	0	14	31	0	10	18	6
	442	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	15	11	38	0	0	2	0	0	0
	445	0	7	8	0	0	12	0	0	0	0	9	8	16	15	6	2	22	2	3	6	6	13	8	0
	448	0	0	0	9	0	0	0	0	34	0	0	6	9	17	0	0	0	0	19	3	0	3	0	15
	451	0	0	0	3	0	0	7	9	0	0	0	0	0	0	3	0	0	0	0	0	2	0	0	4
	454	0	0	0	0	0	0	0	0	0	0	0	16	2	0	22	1	7	0	0	0	0	2	0	0
	457	0	0	0	0	0	0	0	0	0	0	0	0	2	6	2	0	0	0	0	0	0	0	0	0
	460	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	463	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HN020 (AAT)n	176	0	0	0	0	0	0	0	0	9	29	21	15	6	9	7	1	5	0	0	1	2	17	15	2
	179	12	20	20	20	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	182	0	0	0	0	0	7	36	38	4	14	7	18	15	26	32	16	35	40	40	39	8	14	22	38
	185	0	0	0	0	0	33	4	2	27	25	12	7	19	5	1	3	0	0	0	0	0	9	3	0
HN021 (AAT)n	374	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	377	0	0	0	0	0	40	40	40	40	68	40	40	40	40	40	20	40	40	40	40	10	34	38	38
	380	12	20	20	20	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	2	2
	383	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N		6	10	10	10	20	20	20	20	20	34	20	20	20	20	20	10	20	20	20	20	5	20	20	20

Table 4-6. (continued)

Locus (motif)	Size (bp)	Population																						Total
		25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	
HN002 (AACTC)n	234	14	16	12	0	11	0	0	0	0	0	0	0	0	0	0	12	42	36	0	0	0	0	229
	236	6	4	8	22	17	1	60	39	24	16	35	12	38	45	38	24	6	0	20	20	39	20	1153
	241	0	0	0	18	12	9	52	1	0	8	5	8	2	5	2	4	12	4	0	0	1	0	248
HN004 (ACTC)n	154	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
	158	6	4	3	8	6	3	33	39	22	19	27	10	38	22	17	26	14	0	0	0	0	0	346
	162	14	16	16	32	34	7	79	1	2	5	13	9	2	28	23	14	46	40	20	20	40	20	1258
	166	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	20
HN019 (AAT)n	421	0	0	5	6	1	2	31	11	0	0	6	1	3	3	1	7	17	35	0	0	0	0	151
	424	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9
	430	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	44
	433	0	0	1	0	0	0	0	1	1	0	2	3	0	2	0	0	17	0	6	10	37	4	123
	436	0	2	3	0	0	0	2	0	0	0	3	0	0	6	1	0	0	2	0	0	0	0	117
	439	0	3	2	8	0	0	0	0	0	0	3	0	1	2	8	1	0	0	0	7	0	6	260
	442	3	0	2	0	5	0	2	14	0	1	1	0	7	2	3	2	4	0	0	0	0	0	119
	445	6	0	5	0	21	8	77	11	19	17	11	11	15	22	12	16	16	3	14	3	0	10	440
	448	6	8	0	1	7	0	0	1	3	6	14	0	14	11	5	3	6	0	0	0	0	0	200
	451	5	7	0	12	6	0	0	0	0	0	0	4	0	2	10	11	0	0	0	0	3	0	88
	454	0	0	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	56
	457	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	19
	460	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3
	463	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
HN020 (AAT)n	176	5	5	6	3	11	0	20	1	3	3	2	2	0	8	8	4	0	0	6	0	8	0	234
	179	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	119
	182	15	15	14	37	29	10	92	23	20	21	31	13	33	38	31	36	54	40	14	20	32	20	1087
	185	0	0	0	0	0	0		16	1	0	7	5	0	4	1	0	6	0	0	0	0	0	190
HN021 (AAT)n	374	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	377	18	14	20	40	40	10	112	34	18	19	26	15	24	37	22	30	30	31	20	20	40	20	1368
	380	2	4	0	0	0	0	0	5	6	5	14	5	16	13	18	10	30	9	0	0	0	0	259
	383	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
N		10	10	10	20	20	5	56	20	12	12	20	10	20	25	20	20	30	20	10	10	20	10	Total N = 815

Table 4-7. Number of individuals sampled (N), number of alleles (N_A), and observed (H_O) and expected (H_E) heterozygosities of five microsatellite loci in 46 populations of *Hynobius tokyoensis* (no asterisks P>0.05; * P<0.05; ** P<0.01). For the localities of 46 populations, see Fig. 4-1.

Population		HN002	HN004	HN019	HN020	HN021
1	N_A	2	1	2	1	1
(N = 6)	H_O	0.000	0.000	0.500	0.000	0.000
	H_E	0.444	0.000	0.375	0.000	0.000
2	N_A	2	1	3	1	1
(N = 10)	H_O	0.500	0.000	0.800	0.000	0.000
	H_E	0.375	0.000	0.655	0.000	0.000
3	N_A	2	1	3	1	1
(N = 10)	H_O	0.400	0.000	1.000	0.000	0.000
	H_E	0.320	0.000	0.640	0.000	0.000
4	N_A	1	1	5	1	1
(N = 10)	H_O	0.000	0.000	0.600	0.000	0.000
	H_E	0.000	0.000	0.710	0.000	0.000
5	N_A	1	1	3	1	1
(N = 20)	H_O	0.000	0.000	0.150	0.000	0.000
	H_E	0.000	0.000	0.224	0.000	0.000
6	N_A	1	1	2	2	1
(N = 20)	H_O	0.000	0.000	0.350	0.350	0.000
	H_E	0.000	0.000	0.439	0.289	0.000
7	N_A	2	1	2	2	1
(N = 20)	H_O	0.000	0.000	0.350	0.200	0.000
	H_E	0.255	0.000	0.289	0.180	0.000
8	N_A	2	1	2	2	1
(N = 20)	H_O	0.000	0.000	0.450	0.100	0.000
	H_E	0.180	0.000	0.349	0.095	0.000
9	N_A	1	3	3	3	1
(N = 20)	H_O	0.000	0.150	0.300	0.550	0.000
	H_E	0.000	0.224	0.265	0.484	0.000
10	N_A	1	1	2	3	1
(N = 34)	H_O	0.000	0.000	0.294	0.735	0.000
	H_E	0.000	0.000	0.327	0.641	0.000
11	N_A	1	3	4	3	1
(N = 20)	H_O	0.000	0.200	0.450	0.700	0.000
	H_E	0.000	0.184	0.711	0.604	0.000
12	N_A	1	2	5	3	1
(N = 20)	H_O	0.000	0.150	0.800	0.850	0.000
	H_E	0.000	0.139	0.726	0.626	0.000
13	N_A	1	2	5	3	1
(N = 20)	H_O	0.000	0.300	0.800	0.450	0.000
	H_E	0.000	0.255	0.709	0.611	0.000
14	N_A	1	2	4	3	1
(N = 20)	H_O	0.000	0.150	0.450	0.450	0.000
	H_E	0.000	0.139	0.654	0.511	0.000
15	N_A	1	2	5	3	1
(N = 20)	H_O	0.000	0.150	0.900	0.400	0.000
	H_E	0.000	0.139	0.636	0.329	0.000
16	N_A	1	2	4	3	1
(N = 10)	H_O	0.000	0.100	0.500	0.400	0.000
	H_E	0.000	0.255	0.415	0.335	0.000

Table 4-7. (continued)

Population		HN002	HN004	HN019	HN020	HN021
17	N_A	1	2	3	2	1
(N = 20)	H_O	0.000	0.100	0.850	0.150	0.000
	H_E	0.000	0.495	0.591	0.219	0.000
18	N_A	1	1	2	1	1
(N = 20)	H_O	0.000	0.000	0.100	0.000	0.000
	H_E	0.000	0.000	0.095	0.000	0.000
19	N_A	1	2	4	1	1
(N = 20)	H_O	0.000	0.050	0.700	0.000	0.000
	H_E	0.000	0.049	0.636	0.000	0.000
20	N_A	3	2	3	2	1
(N = 20)	H_O	0.200	0.100	0.300	0.050	0.000
	H_E	0.540	0.095	0.371	0.049	0.000
21	N_A	3	1	3	2	1
(N = 5)	H_O	0.000	0.000	0.400	0.400	0.000
	H_E	0.560	0.000	0.560	0.320	0.000
22	N_A	2	2	5	3	2
(N = 20)	H_O	0.000*	0.150	0.700	0.850	0.000
	H_E	0.480	0.139	0.734	0.646	0.255
23	N_A	2	2	3	3	2
(N = 20)	H_O	0.000*	0.400	0.700	0.800	0.000
	H_E	0.375	0.420	0.635	0.551	0.095
24	N_A	2	2	4	2	2
(N = 20)	H_O	0.000*	0.500	0.850	0.100	0.000
	H_E	0.480	0.375	0.686	0.095	0.095
25	N_A	2	2	4	2	2
(N = 10)	H_O	0.000	0.400	0.700	0.300	0.000
	H_E	0.420	0.420	0.735	0.375	0.180
26	N_A	2	2	4	2	3
(N = 10)	H_O	0.000	0.400	1.000	0.500	0.200
	H_E	0.320	0.320	0.685	0.375	0.460
27	N_A	2	3	7	2	1
(N = 10)	H_O	0.000	0.200	0.700	0.400	0.000
	H_E	0.480	0.335	0.820	0.420	0.000
28	N_A	2	2	6	2	1
(N = 20)	H_O	0.500	0.400	0.900	0.150	0.000
	H_E	0.495	0.320	0.786	0.139	0.000
29	N_A	3	2	5	2	1
(N = 20)	H_O	0.250*	0.300	0.700	0.550	0.000
	H_E	0.654	0.255	0.655	0.399	0.000
30	N_A	2	2	2	1	1
(N = 5)	H_O	0.200*	0.600	0.400	0.000	0.000
	H_E	0.180	0.420	0.320	0.000	0.000
31	N_A	2	2	4	2	1
(N = 56)	H_O	0.214	0.375	0.286	0.357	0.000
	H_E	0.497	0.416	0.450	0.293	0.000
32	N_A	2	2	6	3	3
(N = 20)	H_O	0.050	0.050	0.850	0.550	0.300
	H_E	0.049	0.049	0.723	0.509	0.261

Table 4-7. (continued)

Population		HN002	HN004	HN019	HN020	HN021
33	N_A	1	2	4	3	2
(N = 12)	H_O	0.000	0.167	0.417	0.167	0.500
	H_E	0.000	0.153	0.354	0.288	0.375
34	N_A	2	2	3	2	2
(N = 12)	H_O	0.167	0.417	0.583	0.250	0.083
	H_E	0.444	0.330	0.434	0.219	0.330
35	N_A	2	2	7	3	2
(N = 20)	H_O	0.150	0.550	0.800	0.450	0.600
	H_E	0.219	0.439	0.765	0.366	0.455
36	N_A	2	3	5	3	2
(N = 10)	H_O	0.200	0.700	0.600	0.600	0.300
	H_E	0.480	0.545	0.630	0.505	0.375
37	N_A	2	2	5	2	2
(N = 20)	H_O	0.100	0.100	0.700	0.350	0.300
	H_E	0.095	0.095	0.700	0.289	0.480
38	N_A	2	2	8	3	2
(N = 25)	H_O	0.200	0.880	0.640	0.480	0.440
	H_E	0.180	0.493	0.734	0.390	0.385
39	N_A	2	2	7	3	2
(N = 20)	H_O	0.100	0.850	0.950	0.450	0.700
	H_E	0.095	0.489	0.785	0.359	0.495
40	N_A	3	2	6	2	2
(N = 20)	H_O	0.000*	0.500	0.750	0.200	0.400
	H_E	0.540	0.455	0.725	0.180	0.375
41	N_A	3	2	5	2	2
(N = 30)	H_O	0.000*	0.400	0.733	0.200	0.667
	H_E	0.460	0.358	0.754	0.180	0.500
42	N_A	2	1	3	1	2
(N = 20)	H_O	0.200	0.000	0.250	0.000	0.450
	H_E	0.180	0.000	0.226	0.000	0.349
43	N_A	1	1	2	2	1
(N = 10)	H_O	0.000	0.000	0.600	0.600	0.000
	H_E	0.000	0.000	0.420	0.420	0.000
44	N_A	1	1	3	1	1
(N = 10)	H_O	0.000	0.000	0.700	0.000	0.000
	H_E	0.000	0.000	0.605	0.000	0.000
45	N_A	2	1	2	2	1
(N = 20)	H_O	0.050	0.000	0.150	0.400	0.000
	H_E	0.049	0.000	0.139	0.320	0.000
46	N_A	1	1	3	1	1
(N = 10)	H_O	0.000	0.000	0.500	0.000	0.000
	H_E	0.000	0.000	0.620	0.000	0.000

Table 4-8. Analysis of molecular variance (AMOVA) of five microsatellite loci in 46 populations of *Hynobius tokyoensis* in 12 regions.

Source of variation	Sum of squares	Variance components	Percentage variation (%)	P-value
Among regions	638.04	0.39	30.4	<0.001
Among populations within regions	231.25	0.18	14.1	<0.001
Among individuals within populations	588.10	0.05	3.7	<0.001
Within individuals	546.00	0.67	51.8	<0.001
Total	2003.39	1.29	100	

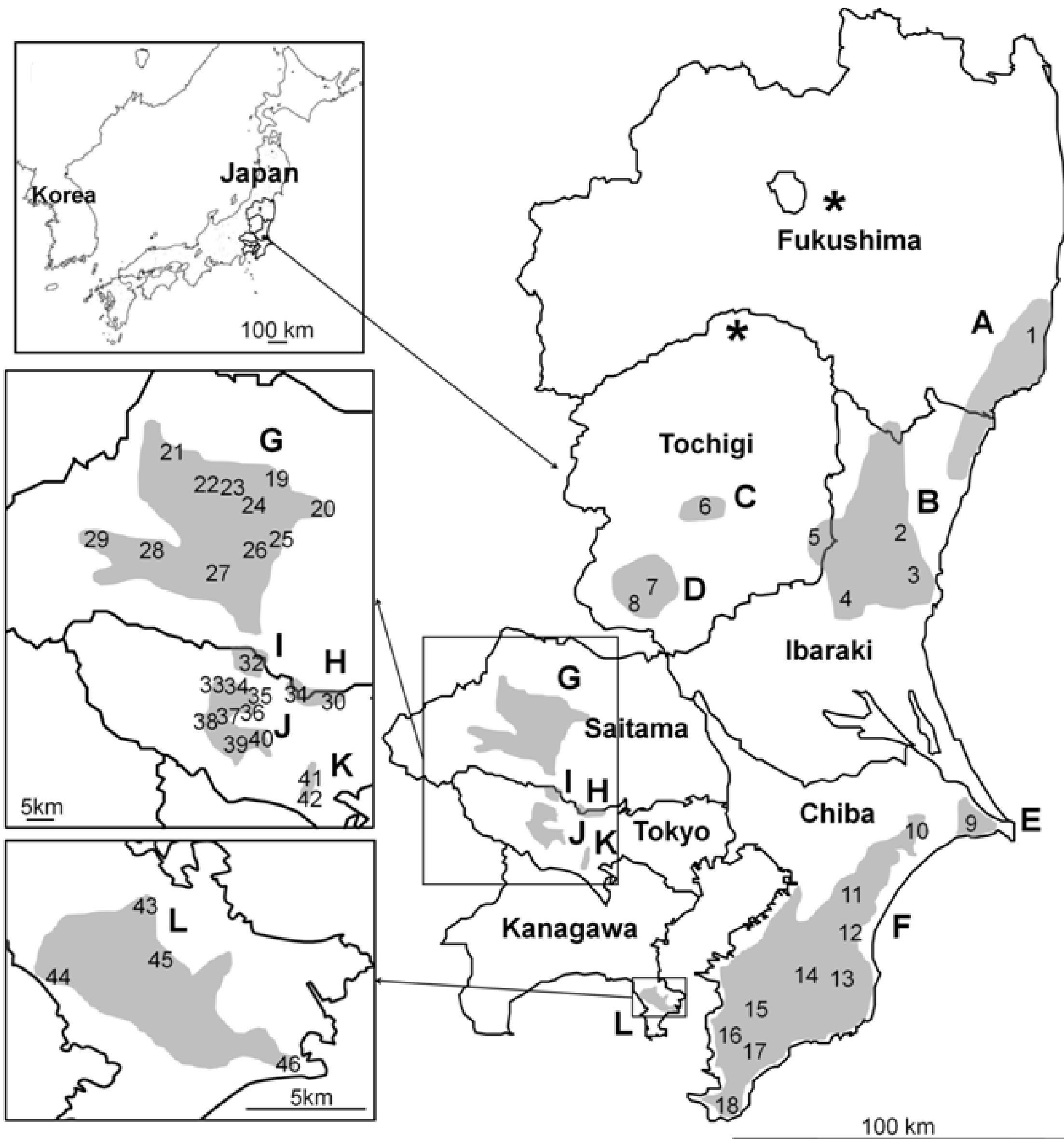

Fig. 4-1. A total of 46 populations (1-46) of *Hynobius tokyoensis* in central Japan. Darkened areas show 12 separated regions (A-L) of all detailed distribution records (see references in the text). Fukushima, Ibaraki, Tochigi, Chiba, Saitama, Tokyo, and Kanagawa indicate prefectures with prefectural boundaries. For locality of 46 populations, see Table 4-1. Asterisks show the collecting sites of two individuals of the outgroup species *H. lichenatus* in Fukushima and Tochigi Prefectures.

Fig. 4-2. Phylogenetic trees of *Hynoubius tokyoensis* based on 72 haplotypes of 650-bp mitochondrial cyt b sequences by the neighbor-joining (a) and maximum likelihood (b) methods. T1 to T72 show haplotypes and numerals in parentheses are population numbers 1-46 (see Fig. 4-1 and Table 4-1). The scale bar indicates the genetic distance (substitutions per site) of each tree. Numerals next to nodes indicate bootstrap values with 1,000 replications. Asterisks indicate the connecting points of the outgroup *H. lichenatus* as shown in the inserted small trees in which L1 and L2 are the haplotypes of outgroup obtained from Fukushima and Tochigi Prefectures, respectively (see Fig. 4-1).

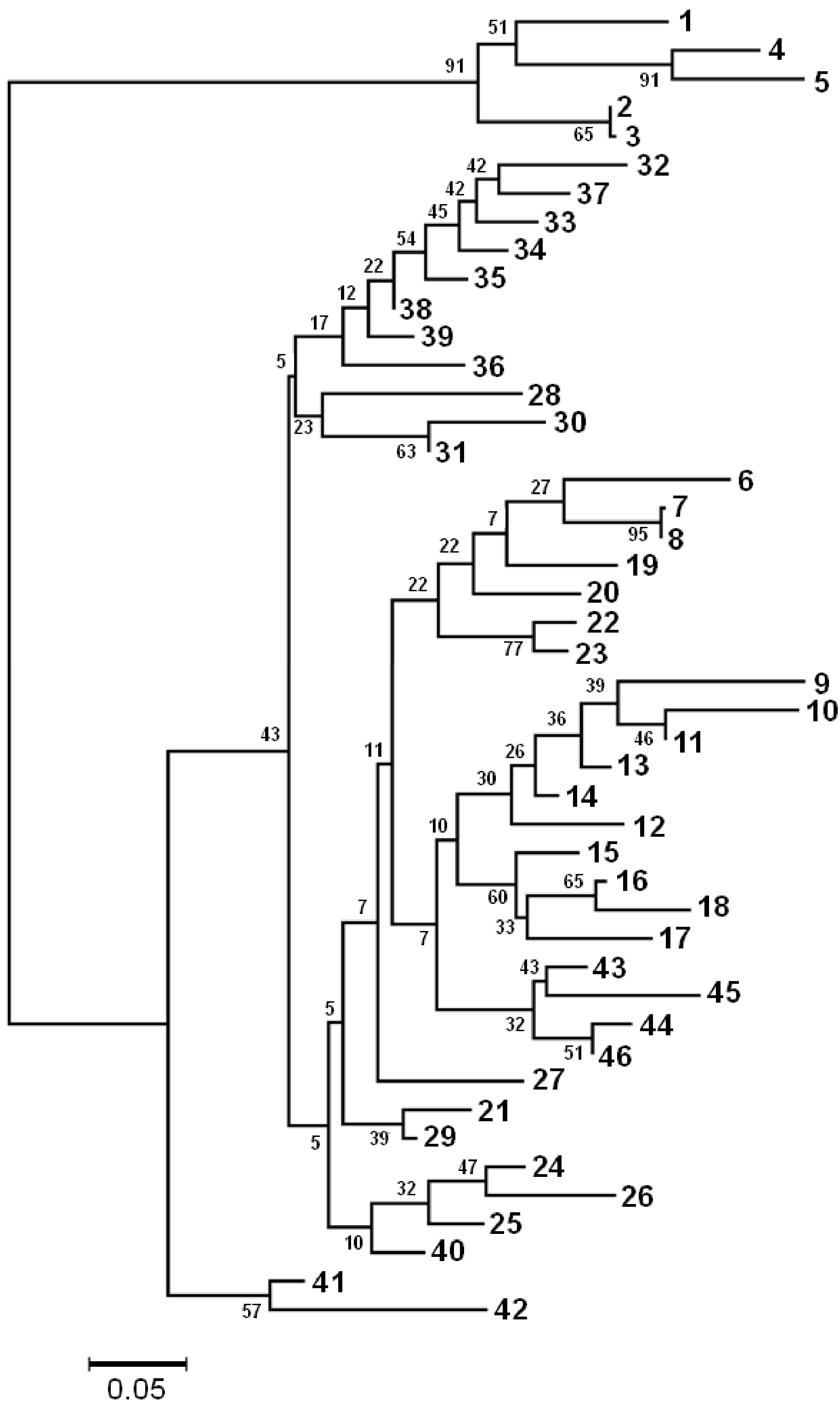

Fig. 4-3. Phylognetic tree of 46 populations of *Hynobius tokyoensis* based on the allele frequencies of five microsatellite loci by the neighbor-joining method. Numerals next to nodes indicate bootstrap values with 1,000 replications.

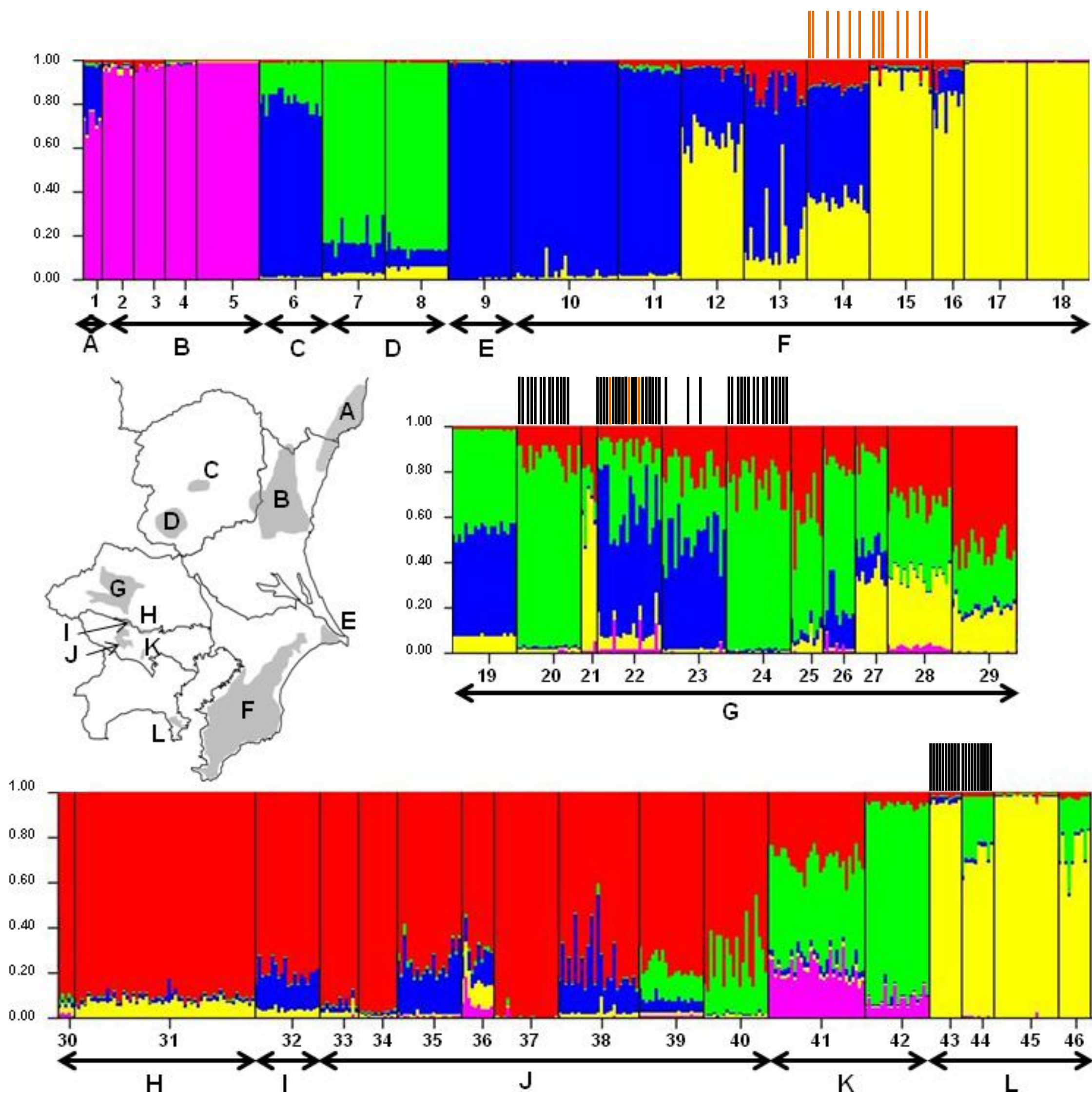

Fig. 4-4. Genetic clusters obtained from the STRUCTURE analysis (K = 5) for all 815 individuals of *Hynobius tokyoensis* from 46 populations. Individuals are represented with vertical lines with the estimated proportion of five genetic clusters. For the arrangement of 12 regions A-L, black and orange bars on the several bar plots indicate the individuals with same mitochondrial haplotype, T13 and T45, respectively. See the inserted map and also Fig. 4-1.

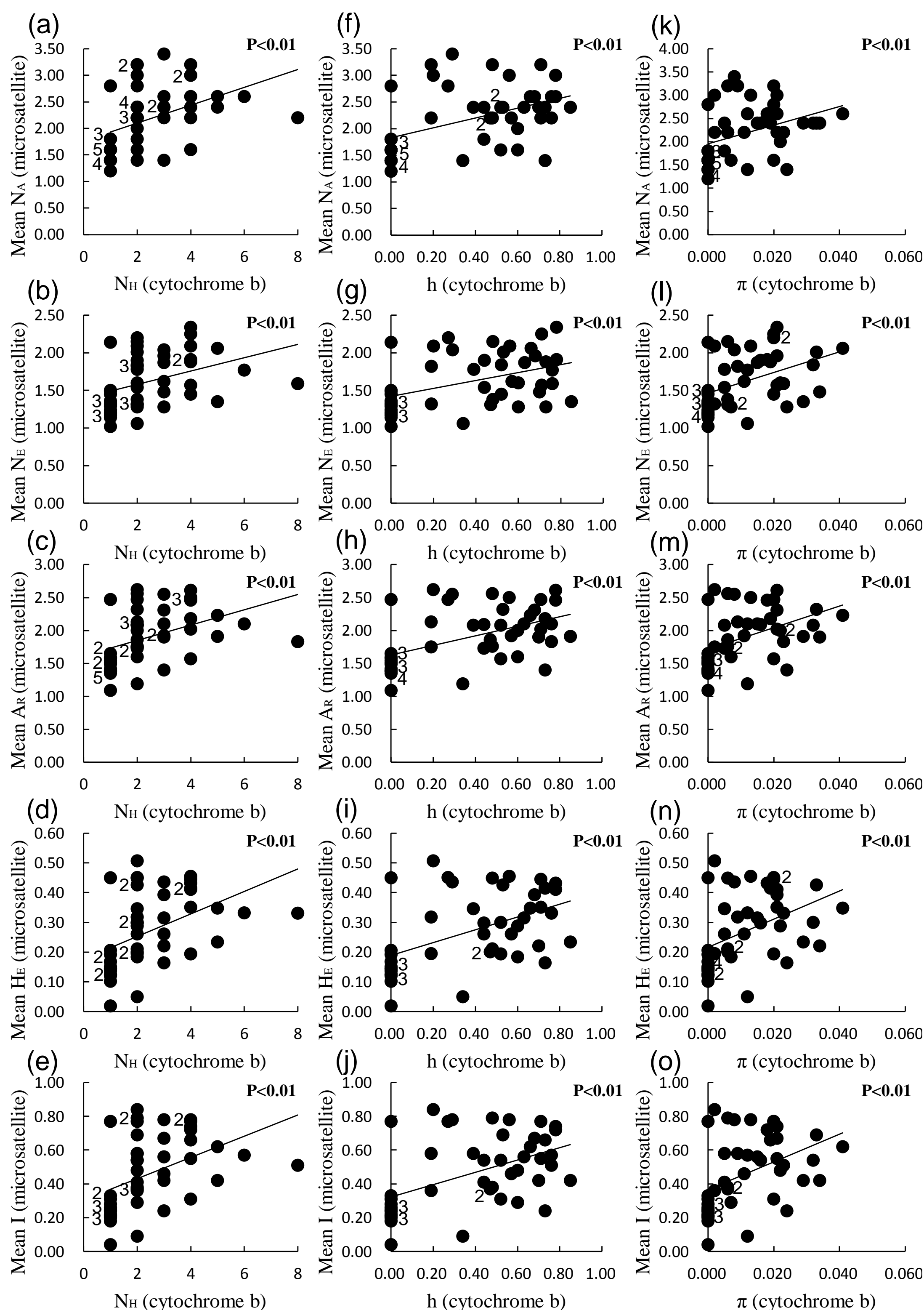

Fig. 4-5. Correlation analyses of the genetic diversities between mitochondrial and microsatellite DNAs. (a) N_H vs. mean N_A, (b) N_H vs. mean N_E, (c) N_H vs. mean A_R, (d) N_H vs. mean H_E, (e) N_H vs. mean I, (f) h vs. mean N_A, (g) h vs. mean N_E, (h) h vs. mean A_R, (i) h vs. mean H_E, (j) h vs. mean I, (k) π vs. mean N_A, (l) π vs. mean N_E, (m) π vs. mean A_R, (n) π vs. mean H_E, and (o) π vs. mean I. Numerals indicate the number of plots with the same value. For statistic results, see the text.

5. Population genetic structure and genetic diversity of Oita salamander

5-1. Introduction

Effects of habitat fragmentation are now unavoidable for wild animals living in human-inhabited areas. Fragmentations are caused by agriculture, forestry, and other human activities (e.g., urban development), and they can produce some geographic barriers to dispersal of animals (Kolozsvary and Swihart 1999). If their dispersal ability is very low, human-induced habitat fragmentation produces a lot of isolated small populations. Even in well-conserved forest area, construction of roads can severely divide populations of such animals with very low mobility (Trombulak and Frissell 2000). Small populations are very susceptible to stochastic events and often results in extinction (Frankham et al. 2002). Such populations also tend to have less genetic diversity than larger ones due to the loss of alleles through genetic drift and the increased chance of inbreeding (Frankham et al. 2002). Therefore, once the species distribution ranges are heavily fragmented and gene flow is quite limited by their rare dispersal events, we must manage such populations carefully to avoid extinction, understanding of the species ecology, demography, and genetics (Frankham et al. 2002). Recent development of molecular techniques enables us to assess a fine scale population genetic structure in the wild. Such population genetic data are available for monitoring genetic diversity (Schwartz et al. 2007), identifying management units to conserve (Palsbøll et al. 2007), and detecting genetic pollution and hybridization (Simison et al. 2013).

The Oita salamander *Hynobius dunni*, originally described from Oita Prefecture, of Kyushu by Tago (1931), is distributed in eastern Kyushu (Oita, Kumamoto, and Miyazaki Prefectures) and a southwestern part of Shikoku (Kochi Prefecture). This salamander is a lowland lentic breeder and endemic to above-mentioned southwestern Japan (Sato and Horie 2000). The distribution area is inhabited by human and subject to effects of anthropogenic activities (Sato 1979). This salamander has been listed as a class B2 endangered species in the IUCN Red List of Threatened Species (International Union for Conservation of Nature and Natural Resources 2014). Kochi population has extremely few individuals, but well conserved by the zoo and landowners (Watabe et al. 2005). Kochi population has now relatively stable number of individuals with support from many naturalists. In eastern Kyushu, two populations are protected by local governments but others are unprotected, and recent extinction of 13 small populations has been reported (Nagano and Kurafuchi 2011). Therefore, urgent conservation plans

are needed in Kyushu. All previous studies of *H. dunni* dealt with reproductive periods, egg-laying behavior, and/or fecundity (Mashiba 1969; Sato 1979; Sueyoshi 2001; Sueyoshi and Kushima 2004; Nagano and Kurafuchi 2011). Only one study revealed local genetic variation of this species based on mitochondrial D-loop sequences (Michigoshi 2000). According to it, northern Kyushu (Oita) populations differ genetically from other southern Kyushu (Miyazaki) populations and western Shikoku (Kochi) population. However, this pioneer study is based on only 222-bp D-loop sequences of a total of 63 individuals. Therefore, to confirm this result and to detect more important genetic factor for future conservation of the threatened *H. dunni* in Kyushu, we analyzed a total of 242 individuals using both mitochondrial and microsatellite DNAs. The aim of this study is to answer: (1) Are *H. dunni* populations genetically structured across its entire distribution range in eastern Kyushu? (2) Do mitochondrial and nuclear genetic markers show concordant results? (3) Which populations have low genetic diversity?

5-2. Materials and methods

5-2-1. Sampling

From February to April in 2012 and from March to May in 2013, 242 salamanders were sampled from 12 populations in Kyushu (Fig. 5-1); 20 from Usa (Pop. 1), 20 from Ajimu (Pop. 2), 20 from Yokoo (Pop. 3), 22 from Dannoharu (Pop. 4), 20 from Souda (Pop. 5), 20 from Saeki (Pop. 6), 20 from Kiyokawa (Pop. 7), 20 from Takeda (Pop. 8), 20 from Yoshida (Pop. 9), 20 from Takamori (Pop. 10), 20 from Furujyo (Pop. 11), and 20 from Tano (Pop. 12). Populations used in this study mean the individuals found in a single wetland including small ponds, marshes, and streams. At present, the distribution rage of this species was greatly separated into two regions A and B (Sato, 1998; Iwasaki, 1999; Sueyoshi, 2002; Sueyoshi and Iwakiri, 2009). Populations 1-10 are in the region A and populations 11 and 12 are in the region B (Fig. 5-1).

A single tailbud embryo was removed from each paired egg sac and preserved in 99.5% ethanol. To avoid sampling of individuals born to the identical female, we did never take DNA-samples from larvae, juveniles, and adults. The single tailbud embryo of the clouded salamander *Hynobius nebulosus* (Temminck and Schlegel 1838) was sampled at the northern part of Usa in April 2013, and used for the outgroup, because Kyushu populations of *H. nebulosus* is closely related to *H. dunni* (Zheng et al. 2012). When we collected samples for DNA extraction in each population, we also searched

paired egg sacs as much as possible and counted their numbers. In salamanders, estimation of rough female population size is usually possible because one female deposits one pair of egg sacs in each breeding season.

5-2-2. Mitochondrial DNA analysis

Total genomic DNA was extracted from the embryos preserved in 99.5% ethanol, using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). For the 242 samples from 12 populations, a total of 610-bp fragment of the mitochondrial DNA (41-bp tRNA-Glu + 569-bp cytochrome b (cyt b)), was amplified using Ex *Taq*® (TaKaRa, Tokyo, Japan) with primers L14010 (5′-TAHGGWGAHGGATTWGAWGCMACWGC-3′) and H14778 (5′-AARTAYGGGTGRAADGRRAYTTTRTCT-3′) (Matsui et al. 2007). The PCR reaction mix (total volume 10 μl) contained 1.0 μl 10× Ex Taq Buffer, 0.8 μl 25 mM dNTP mix, 0.5 μl each of the forward and reverse primers (10 pM), 0.05 μl *Taq* polymerase, 6.15 μl distilled deionized water, and 1.0 μl template DNA. Using a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA), the PCR protocol is as follows: an initial 10-min denaturing step at 95°C, 30 cycles of 60 s at 95°C, 60 s at 53°C, and 120 s at 72°C, with a final 10-min extension at 72°C. The PCR products were purified with Illustra™ ExoStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) and sequenced using BigDye® Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Direct sequencing data were aligned using MEGA5 (Tamura et al. 2011). Using the aligned sequences, phylogenetic analyses were performed with the neighbor-joining method (NJ) based on p-distance and maximum likelihood estimation (ML) based on Tamura 3-parameter model using MEGA5 (Tamura et al. 2011). The best-fit nucleotide substitution model was estimated based on the Bayesian Information Criterion (BIC: Schwarz, 1978) using MEGA5 (Tamura et al. 2011). A haplotype network was constructed by median-joining method using the NETWORK software package ver. 4.6.1.1 (Bandelt et al. 1999). The genetic variations among and within regions or among and within populations were subjected to analysis of molecular variance (AMOVA) using Arlequin ver. 3.5 (Excoffier and Lischer 2010). The haplotype diversity h and nucleotide diversity π were calculated using Arlequin ver. 3.5 (Excoffier and Lischer 2010). Significance of the pairwise Φ_{ST} was determined using Arlequin ver. 3.5 (Excoffier and Lischer 2010).

5-2-3. Microsatellite DNA analysis

We amplified seven microsatellite loci (HN002, HN004, HN019, HN020, HN023, HN043, and HN058) using the primer sets developed for *H. nebulosus* by Yoshikawa et al. (2013) for five individuals of each population, but only the following three loci were polymorphic and were amplified for all individuals: NH002 with the repeat motif $(AACTC)_n$ using the primers 5′-ATTTCATCTGGCCAACCCG-3′ and 5′-TCATTCCTCCAAGGCAGGG-3′; HN004 with $(ACTC)_n$ using 5′-GCCGTACCGATGTTGATAGC-3′ and 5′-TCCTGGCCACTCTATTGCC-3′; and HN020 with $(AAT)_n$ using 5′-AGACATGACGTGTGGAGGC-3′ and 5′-GGAAAGCCACACTGACTGC-3′. The PCR was performed on a T100™ thermal cycler (Bio-Rad) using KOD FX Neo DNA polymerase (Toyobo, Tokyo, Japan) with a thermal profile consisting of 94°C for 120 s, followed by 35 cycles at 94°C for 15 s, 59°C for 30 s, and 68°C for 60 s. The reaction mix (total volume 10 μl) contained 4.8 μl of 2× KOD FX Neo Buffer, 2.0 μl 2 mM dNTPs, 0.5 μl fluorescent (6-FAM or HEX) forward primer (10 pM), 0.5 μl reverse primer (10 pM), 0.1 μl Taq polymerase, 1.1 μl distilled deionized water, and 1.0 μl template DNA. The PCR products were then diluted (1:10) and mixed with GeneScan™ LIZ® 500 Size Standard (Applied Biosystems) and Hi-Di Formamide (Applied Biosystems), combining 0.2 μl Liz, 8.8 μl Hi-Di, and 1 μl diluted product. Fragment analysis data were collected using an ABI 3130xl Genetic Analyzer (Applied Biosystems). Allele lengths were scored using Peak Scanner ver. 1.0 (Applied Biosystems).

The effective number of alleles (N_E), observed (H_O) heterozygosity, expected (H_E) heterozygosity, and information index (I) in each population were calculated using GenAlEx 6.5 (Peakall and Smouse 2012), and the allelic richness (A_R) was calculated using FSTAT ver. 2.9.3.2 (Goudet 1995). Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) in H_O and H_E were estimated using Genepop'007 (Rousset 2008). Significance of the pairwise F_{ST} was determined using FSTAT ver. 2.9.3.2 (Goudet 1995). The genetic differentiation among regions, among populations within regions, among individuals within populations, and within individuals was subjected to analysis of molecular variance (AMOVA) using Arlequin ver. 3.5 (Excoffier and Lischer 2010). Current genetic structure of this species was assessed using the program STRUCTURE ver. 2.3.3 (Pritchard et al. 2000) with LOCPRIOR model for accurate inferences (Hubisz et al. 2009). We used the correlated (Falush et al. 2003) and independent (Pritchard et al. 2000) allele frequency models. The former model assumes that the populations all diverged from a common ancestral

population at the same time, but the latter does not assume it. Ten runs were set with a burn-in length of 50,000 and a Markov chain Monte Carlo (MCMC) run of 50,000 for each K (1 to 7). ΔK was estimated to decide the true K number (Evanno et al. 2005).

5-3. Results

5-3-1. Mitochondrial DNA analysis

The number of egg sacs did not differ among populations; the number of paired egg sacs found in each population habitat ranged from 20 to 52 (Table 5-1). Of the 610-bp sequences of 242 individual *H. dunni*, nucleotide substitutions occurred at 19 positions (Table 5-2), producing 15 haplotypes, D1-D15 (GenBank accession numbers LC003294 – LC003308) (Table 5-3). The phylogenetic trees, using the corresponding sequence of the outgroup *H. nebulosus* (GenBank accession number LC003293), were nearly same between NJ and ML estimates (Fig. 5-2). The haplotypes were separated into two groups, the region A consisting of populations 1-10 and the region B of populations 11 and 12. Analysis of the haplotype network also showed these two regional groups (Fig. 5-3). The most variance (83.4%) was explained by among regions (AMOVA; sum of squares 251.8, variance components 3.63, $P<0.001$), and only 10.3% and 6.3% were explained by among populations within region (sum of squares 93.7, variance components 0.45, $P<0.001$) and within populations (sum of squares 63.0, variance components 0.27, $P<0.001$), respectively. Pairwise Φ_{ST} values between populations differed significantly in most population combinations after the Bonferroni corrections (above diagonal in Table 5-4).

5-3-2. Microsatellite DNA analysis

Microsatellite analysis detected three, nine, and seven alleles in HN002, HN004, and HN020 loci, respectively (Table 5-5). The observed and expected heterozygosities ranged from 0.000 to 0.800 and 0.000 to 0.613, respectively (Table 5-1). No significant LD was detected in any combinations of loci for the 12 populations and deviations from HWE were identified in HN020 locus of five populations after Bonferroni corrections (Table 5-1). The most variance (71.1%) was explained by within individuals (AMOVA; sum of squares 122.0, variance components 0.50, $P<0.001$), and 19.1% was by among regions (sum of squares 60.3, variance components 0.14, $P<0.001$). Variance among populations within regions and variance among individuals within populations

explained only 3.2% (sum of squares 9.1, variance components 0.02, $P<0.001$) and 6.5% (sum of squares 137.2, variance components 0.05, $P<0.05$) of variation, respectively. Pairwise multilocus F_{ST} between populations revealed that the frequency distributions of alleles at the three loci were different among populations after the Bonferroni corrections (below diagonal in Table 5-4). The significant pairwise multilocus F_{ST} were observed in the most combinations including populations 1, 2, 6, 11, and 12, although the F_{ST} values between populations 11 and 12 did not differ from zero (Table 5-4).

In the correlated allele frequency model of STRUCTURE, ΔK calculated for $K = 2$ to 7 was highest at $K = 2$ ($\Delta K = 18.7$, SD = 4.6), followed by $K = 4$ (5.2, 8.5) and $K = 3$ (1.4, 13.2). In the independent allele frequency model of STRUCTURE, ΔK calculated for $K = 2$ to 7 was also highest at $K = 2$ ($\Delta K = 742.9$, SD = 2.0), followed by $K = 4$ (8.6, 4.9) and $K = 3$ (5.0, 5.0). Genetic structures obtained by these two models were similar: when $K = 2$, the populations 1, 2, 11, and 12 tended to differ from the other populations (3-10); when $K = 3$, the populations 1 and 2, and the populations 11 and 12 tended to differ from the other populations (3-10); and when $K = 4$, the populations 1 and 2, the population 6, and the populations 11 and 12 tended to differ from the other populations (3-5, 7-10) (Fig. 5-4).

5-3-3. Genetic diversity

The number of mitochondrial haplotypes (N_H), haplotype diversities (h), and nucleotide diversities (π) within populations ranged from 1 to 5, from 0.00 to 0.75, and from 0.000 to 0.114, respectively (Table 5-6). The mean number of microsatellite alleles (N_A), mean effective number of alleles (N_E), mean allelic richness (A_R), mean expected heterozygosities (H_E), and mean information indices (I) varied from 1.00 to 3.67, from 1.00 to 2.24, from 1.00 to 3.67, from 0.00 to 0.55, and from 0.00 to 0.92, respectively (Table 5-6). In the mitochondrial analysis, populations 2, 5, 10, and 11 had only one haplotype and populations 1, 6, 7, and 12 had only two (Table 5-6). In the microsatellite analysis, populations 11 and 12 had much lower diversity than the other populations, and several diversity indices were also lower in populations 1, 2, 3, 8, and 10 (Table 5-6). Thus, the southern group (populations 11 and 12) restricted to the narrow area had low genetic diversity in both mitochondrial and microsatellite DNAs. In the northern group, the populations 1, 2, 3, and 10 had lower genetic diversity both in mitochondrial and microsatellite analyses (Table 5-6).

5-4. Discussion

Michigoshi (2000) reported that genetic differentiation occurs between the regions A and B based on his analysis of the mitochondrial D-loop. Our results based on mitochondrial cyt b sequences also supported this differentiation. The similar genetic differentiation was obtained from our microsatellite DNA analysis, that is populations 11 and 12 differed from the other populations. However, in the microsatellite analysis, the populations 1 and 2, and the population 6, had some unique genetic characteristics by pairwise multilocus F_{ST} and STRUCTURE analyses. This may be caused by the fixation of the single common allele in the locus HN004 in the populations 1, 2, and 11 (also in 12) and by the private allele in the same locus in the population 6. At present, the distribution area of *H. dunni* is separated into northern and southern parts (Fig. 5-1), but the reason why so separated has been unknown.

Another population (only one population) of *H. dunni* is known from Kochi of western Shikoku (Sato 1998). Michigoshi (2000) reported that this Kochi population is closely related to populations of the region B (populations 11 and 12 in our study) in his mitochondrial D-loop sequences. However, he analyzed only one individual of Kochi population, so that in the further analyses including microsatellite DNA will be needed.

Populations 11 and 12 had low genetic diversity; particularly population 11 had only one mitochondrial haplotype and the single allele per microsatellite locus. Michigoshi (2000) already pointed it out in his mitochondrial analysis. This fact is an important implication for future management of *H. dunni*. There is no habitat of *H. dunni* and any other species of lentic *Hynobius* between the regions A and B (Sato 1998). Such completely isolated populations have significant deleterious effects on loss of genetic diversity, inbreeding, and extinction risk (Eldridge et al. 1999). Inbreeding is unavoidable in small populations and reduces reproductive fitness (Ralls and Ballou 1983). According to Halverson et al. (2006), inbreeding negatively affected the survival of amphibians. Therefore, in preservation of *H dunni*, the southern populations must be treated prior to the northern populations. An effective management in the recovery of small, inbred populations is to introduce individuals from the other populations with closer relationship (Frankham et al. 2002). In the case of *H. dunni*, artificial transportation from the northern region causes genetic pollution because genetic differentiation between the both regions is clear, and so that it must be avoided. Artificial transportation may be allowed between populations 11 and 12 in the region B. However, it is usually difficult to decide artificial introduction only when these two populations were completely genetically identical, or after one of them completely went

extinct.

Even in the northern region, forests are strongly fragmented by human use and the wetlands inhabited by *H. dunni* are distributed patchily (e.g., Sato and Horie 2000). Habitat fragmentation leads to reductions in population size, and to reduced migration or gene flow among populations, so causing decrease in genetic diversity (Frankham et al. 2002). In fact, the number of paired egg sacs deposited in each population was small, ranged from 20 to 52, and our results show evidence of decreasing of the genetic diversity in some populations of this region. Particularly populations 1, 2, 3, and 10 had a lower genetic diversity than the others. The low genetic diversities in these populations can be explained by population demographic history, because these populations are located in the peripheral region where the populations are more frequently isolated and receive fewer immigrants than those in central region (Channel 2004). Therefore, these populations must be monitored to prevent from further habitat loss and population decline caused by inbreeding depression. In the future, the requirement of the investigation of other microsatellite loci or genetic variation relevant to adaptive traits should be considered before reaching some implication for the management because few loci are used in this study. However, the allelic variation of each locus is sufficiently examined due to the enough number of individuals per population, and our genetic data will provide valuable information on genetic management and will serve as reference for comparative studies of *Hynobius* species.

Table 5-1. Number of individuals sampled (N_1), number of paired egg sacs observed (N_2), number of alleles (N_A), and observed (H_O) and expected (H_E) heterozygosities of three microsatellite loci in 12 populations of *Hynobius dunni* (no asterisks $P>0.05$; * $P<0.05$; ** $P<0.01$). For the localities of 12 populations, see Fig. 5-1.

Population		HN002	HN004	HN020
1	N_A	3	1	2
(N_1 = 20)	H_O	0.250	0.000	0.250
(N_2 = 33)	H_E	0.301	0.000	0.349
2	N_A	3	1	3
(N_1 = 20)	H_O	0.750	0.000	0.550
(N_2 = 21)	H_E	0.524	0.000	0.629
3	N_A	3	2	2
(N_1 = 20)	H_O	0.500	0.650	0.000*
(N_2 = 31)	H_E	0.511	0.439	0.255
4	N_A	2	4	3
(N_1 = 22)	H_O	0.182	0.682	0.091**
(N_2 = 35)	H_E	0.351	0.600	0.361
5	N_A	3	4	3
(N_1 = 20)	H_O	0.350	0.700	0.050**
(N_2 = 22)	H_E	0.576	0.606	0.451
6	N_A	2	5	4
(N_1 = 20)	H_O	0.250	0.700	0.150**
(N_2 = 30)	H_E	0.289	0.613	0.524
7	N_A	3	3	4
(N_1 = 20)	H_O	0.400	0.500	0.350*
(N_2 = 37)	H_E	0.395	0.545	0.439
8	N_A	3	3	2
(N_1 = 20)	H_O	0.400	0.600	0.350
(N_2 = 26)	H_E	0.339	0.434	0.289
9	N_A	3	3	3
(N_1 = 20)	H_O	0.500	0.600	0.350
(N_2 = 52)	H_E	0.579	0.486	0.296
10	N_A	3	2	2
(N_1 = 20)	H_O	0.800	0.600	0.100
(N_2 = 20)	H_E	0.559	0.420	0.320
11	N_A	1	1	1
(N_1 = 20)	H_O	0.000	0.000	0.000
(N_2 = 24)	H_E	0.000	0.000	0.000
12	N_A	1	2	1
(N_1 = 20)	H_O	0.000	0.450	0.000
(N_2 = 33)	H_E	0.000	0.349	0.000

Table 5-2. Variable nucleotide positions of the mtDNA cytochrome b region of *Hynobius dunni* .

Haplotype	Nucleotide Position																		
	122	149	161	176	182	250	294	311	429	434	449	470	473	510	511	512	536	542	545
D1	T	C	C	A	T	T	G	C	G	C	A	G	T	A	T	G	T	C	A
D2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G
D3	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
D4	-	T	A	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-
D5	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D6	-	-	A	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
D7	-	-	A	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-
D8	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D9	-	-	A	-	-	-	-	-	-	-	-	A	-	G	-	-	-	-	-
D10	-	-	A	-	-	-	-	-	-	-	-	A	-	G	G	-	-	-	-
D11	-	-	A	-	-	-	-	A	-	-	-	A	-	G	-	-	-	-	-
D12	-	-	A	-	C	-	-	A	-	-	-	A	-	G	G	-	-	-	-
D13	-	-	A	-	C	-	-	A	-	-	-	A	-	G	-	-	-	-	-
D14	-	-	-	G	-	-	A	A	-	-	G	-	C	-	-	A	C	T	-
D15	-	-	-	G	-	-	-	A	-	-	G	-	C	-	-	A	C	T	-

Table 5-3. Frequencies of cytochrome b haplotypes (D1-D15) in 12 populations of *Hynobius dunni* . For the localities of 12 populations, see Fig. 5-1.

Population	Haplotype															Total
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	
1	11	9														20
2	20															20
3				1			2	17								20
4				2		2	9	9								22
5								20								20
6					13			7								20
7								10	10							20
8			5					11	4							20
9								3		2	6	1	8			20
10								20								20
11															20	20
12														3	17	20
Total	31	9	5	3	13	2	11	97	14	2	6	1	8	3	37	242

Table 5-4. Pairwise genetic distances (above diagonal Φ_{ST}, based on mtDNA and below diagonal F_{ST}, based on microsatellite DNA) among 12 populations of *Hynobius dunni* (no asterisks P>0.05; * P<0.05; ** P<0.01). For the localities of 12 populations, see Fig. 5-1.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1	-	0.421**	0.733**	0.648**	0.825**	0.777**	0.699**	0.637**	0.783**	0.825**	0.976**	0.965**
2	0.148**	-	0.836**	0.685**	1.000**	0.860**	0.744**	0.676**	0.805**	1.000**	1.000**	0.987**
3	0.327**	0.341**	-	0.080	0.020	0.493**	0.410**	0.142**	0.696**	0.020	0.984**	0.975**
4	0.203**	0.273**	0.013	-	0.214**	0.435**	0.400**	0.220**	0.670**	0.214**	0.961**	0.953**
5	0.247**	0.222**	0.008	0.018	-	0.632**	0.474**	0.161**	0.728**	0.000	1.000**	0.989**
6	0.373**	0.391**	0.067**	0.068**	0.075**	-	0.552**	0.412**	0.729**	0.632**	0.983**	0.974**
7	0.228**	0.265**	0.024	0.005	0.041**	0.059**	-	0.159*	0.424**	0.474**	0.964**	0.957**
8	0.378**	0.392**	0.017	0.048**	0.066**	0.056**	0.015	-	0.566**	0.161**	0.958**	0.950**
9	0.311**	0.301**	-0.002	0.040**	0.022	0.076**	0.008	0.014	-	0.728**	0.945**	0.940**
10	0.355**	0.343**	0.007	0.060**	0.031	0.081**	0.039	0.035*	0.001	-	1.000**	0.989**
11	0.161*	0.425**	0.473**	0.327**	0.406**	0.484**	0.340**	0.508**	0.442**	0.489**	-	0.105
12	0.179**	0.386**	0.362**	0.232**	0.316**	0.369**	0.246**	0.382**	0.342**	0.385**	0.211	-

Table 5-5. Frequencies of alleles (indicated as base pairs) in three microsatellite loci of *Hynobius dunni* . N = number of individuals examined. For the localities of 12 populations, see Fig. 5-1.

Locus (motif)	Size (bp)	Population												Total
		1	2	3	4	5	6	7	8	9	10	11	12	
HN002 (AACTC)n	236	2	1	9	10	7	7	8	5	9	7	0	0	65
	241	33	20	26	34	23	33	30	32	23	24	40	40	358
	246	5	19	5	0	10	0	2	3	8	9	0	0	61
HN004 (ACTC)n	158	0	0	0	0	0	0	0	1	0	0	0	0	1
	162	40	40	13	19	13	4	18	11	14	12	40	31	255
	166	0	0	0	0	0	0	2	0	0	0	0	0	2
	170	0	0	0	0	0	0	0	0	0	0	0	9	9
	174	0	0	0	2	4	1	0	0	0	0	0	0	7
	178	0	0	0	0	2	7	0	0	1	0	0	0	10
	182	0	0	27	20	21	23	20	28	25	28	0	0	192
	186	0	0	0	3	0	0	0	0	0	0	0	0	3
	190	0	0	0	0	0	5	0	0	0	0	0	0	5
HN020 (AAT)n	173	9	13	6	9	12	0	0	0	0	0	0	0	49
	176	0	8	0	0	0	0	7	7	6	0	0	0	28
	179	0	0	0	0	0	6	2	0	0	0	0	0	8
	182	31	19	34	34	27	26	29	33	33	32	40	40	378
	185	0	0	0	0	0	7	0	0	0	0	0	0	7
	188	0	0	0	1	1	0	2	0	1	8	0	0	13
	191	0	0	0	0	0	1	0	0	0	0	0	0	1
N		20	20	20	22	20	20	20	20	20	20	20	20	Total N = 242

Table 5-6. Number of haplotypes (N_H), haplotype diversities (h), and nucleotide diversities (π) in 569-bp cytochrome b sequences, and the mean number of alleles (N_A), mean effective number of alleles (N_E), mean allelic richness (A_R), mean expected heterozygosities (H_E), and mean information indices (I) in three microsatellite loci of *Hynobius dunni*. The lower six values are indicated with bold. N = number of individuals examined. SD = standard deviation. For the localities of 12 populations, see Fig. 5-1.

Population	N	Cytochrome b					Microsatellite									
		N_H	h	SD	π	SD	Mean N_A	SD	Mean N_E	SD	Mean A_R	SD	Mean H_E	SD	Mean I	SD
1	20	**2**	0.52	0.04	0.033	0.030	**2.00**	0.82	**1.32**	0.23	**2.00**	0.82	**0.28**	0.19	**0.37**	0.26
2	20	**1**	**0.00**	0.00	**0.000**	0.000	**2.33**	0.94	1.93	0.70	**2.33**	0.94	**0.38**	0.34	**0.61**	0.44
3	20	3	**0.28**	0.12	**0.026**	0.026	**2.33**	0.47	**1.72**	0.29	**2.33**	0.47	**0.40**	0.13	**0.64**	0.19
4	22	4	0.68	0.06	0.068	0.049	3.00	0.82	1.87	0.45	2.97	0.82	0.44	0.14	0.73	0.22
5	20	**1**	**0.00**	0.00	**0.000**	0.000	3.33	0.47	2.24	0.30	3.33	0.47	0.55	0.08	0.92	0.15
6	20	**2**	0.48	0.07	0.029	0.028	3.67	1.25	2.03	0.48	3.67	1.25	0.48	0.17	0.88	0.31
7	20	**2**	0.53	0.04	0.078	0.055	3.33	0.47	1.88	0.23	3.33	0.47	0.46	0.08	0.79	0.08
8	20	3	0.63	0.08	0.073	0.052	2.67	0.47	**1.56**	0.15	2.67	0.47	**0.35**	0.07	**0.60**	0.10
9	20	5	0.75	0.06	0.114	0.073	3.00	0.00	1.91	0.39	3.00	0.00	0.45	0.14	0.76	0.18
10	20	**1**	**0.00**	0.00	**0.000**	0.000	**2.33**	0.47	**1.82**	0.33	**2.33**	0.47	0.43	0.12	0.69	0.19
11	20	**1**	**0.00**	0.00	**0.000**	0.000	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**0.00**	0.00	**0.00**	0.00
12	20	**2**	**0.27**	0.11	**0.016**	0.020	**1.33**	0.47	**1.18**	0.25	**1.33**	0.47	**0.12**	0.20	**0.18**	0.25

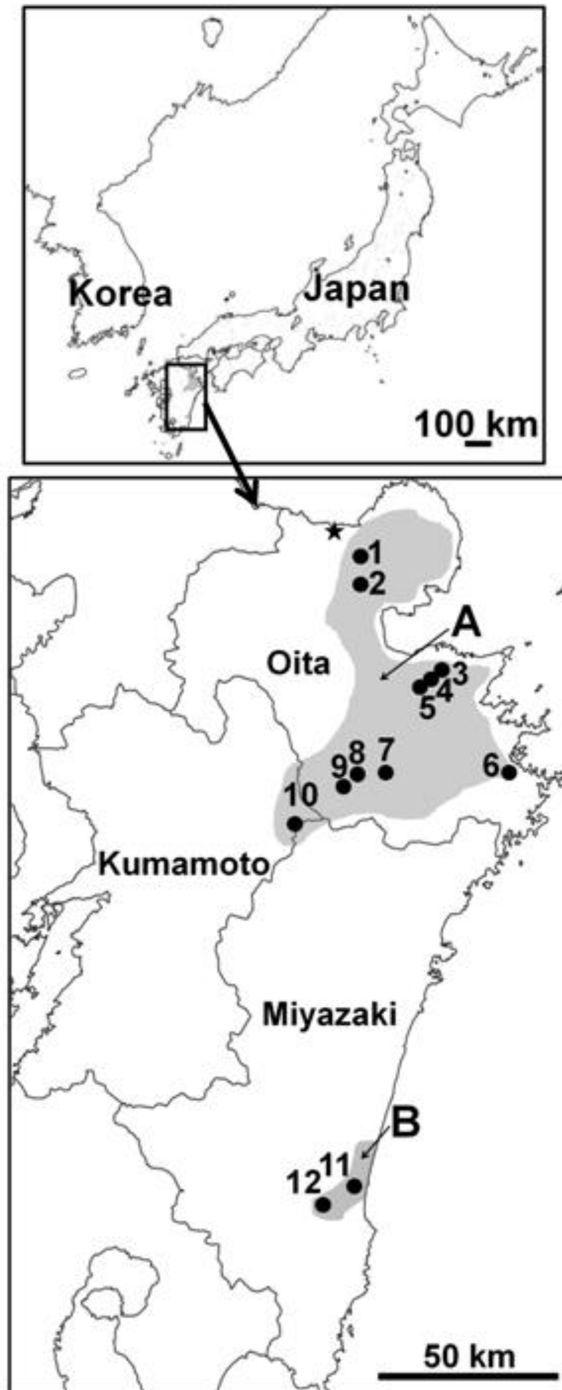

Fig. 5-1. Twelve populations of *Hynobius dunni* collected in Kyushu, southwestern Japan. Dotted areas show their known distribution areas which across to Oita, Kumamoto, and Miyazaki Prefectures (A, northern region; B, southern region). 1, Usa; 2, Ajimu; 3, Yokoo; 4, Dannoharu; 5, Souda; 6, Saeki; 7, Kiyokawa; 8, Takeda; 9, Yoshida; 10, Takamori; 11, Furujyo; and 12, Tano. The place indicated with a star is the collecting site of the outgroup *Hynobius nebulosus*.

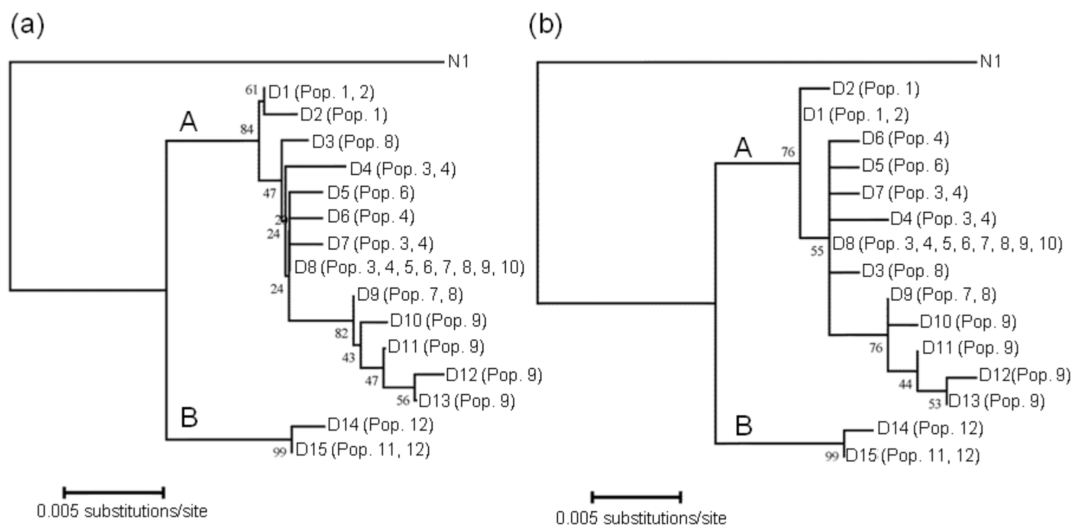

Fig. 5-2. NJ (a) and ML (b) phylogenetic trees based on 41-bp tRNA-Glu and 569-bp cytochrome b (cyt b) sequences. D1-D15, haplotypes of *Hynobius dunni*; N1, *H. nebulosus* (outgroup); Pop. 1-12, populations; A and B, regions (see Fig. 5-1). Numerals indicated near branches are bootstrap probabilities with 1,000 replications.

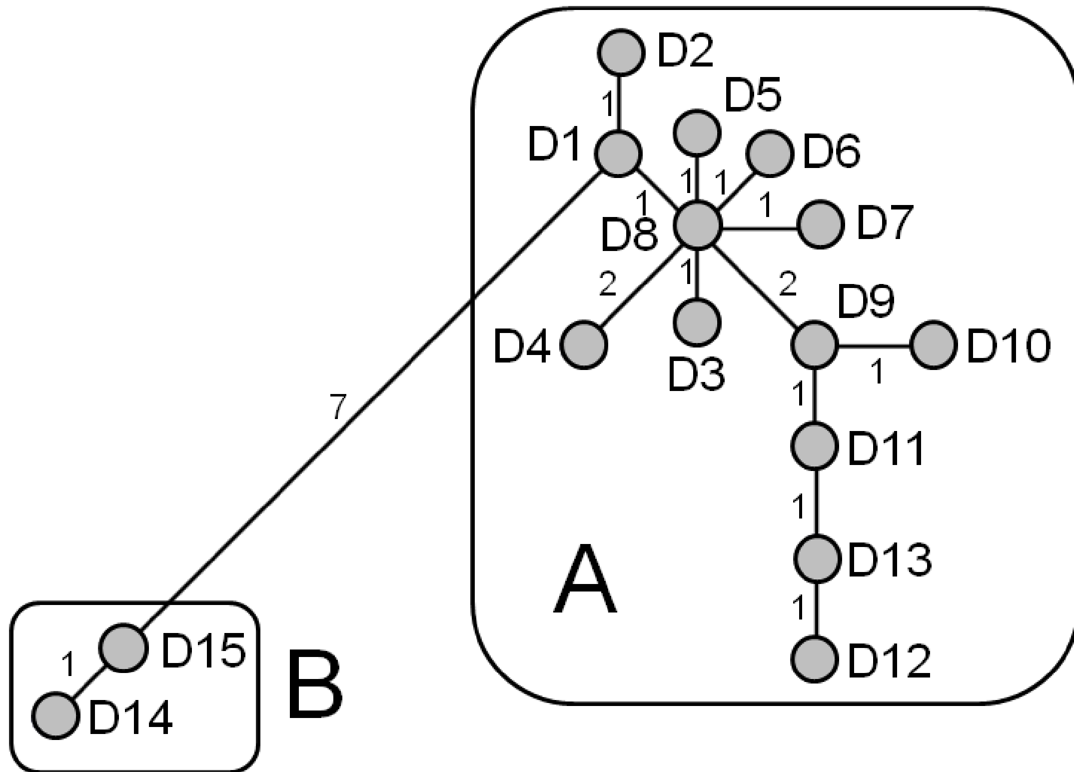

Fig. 5-3. Fifteen haplotypes network (D1-D15) based on 569-bp cytochrome b sequences by the median-joining method. A and B indicate the northern and southern regions in *Hynobius dunni* distribution range (see Fig. 5-1). Numbers of mutational steps are exhibited near the lines connecting haplotypes.

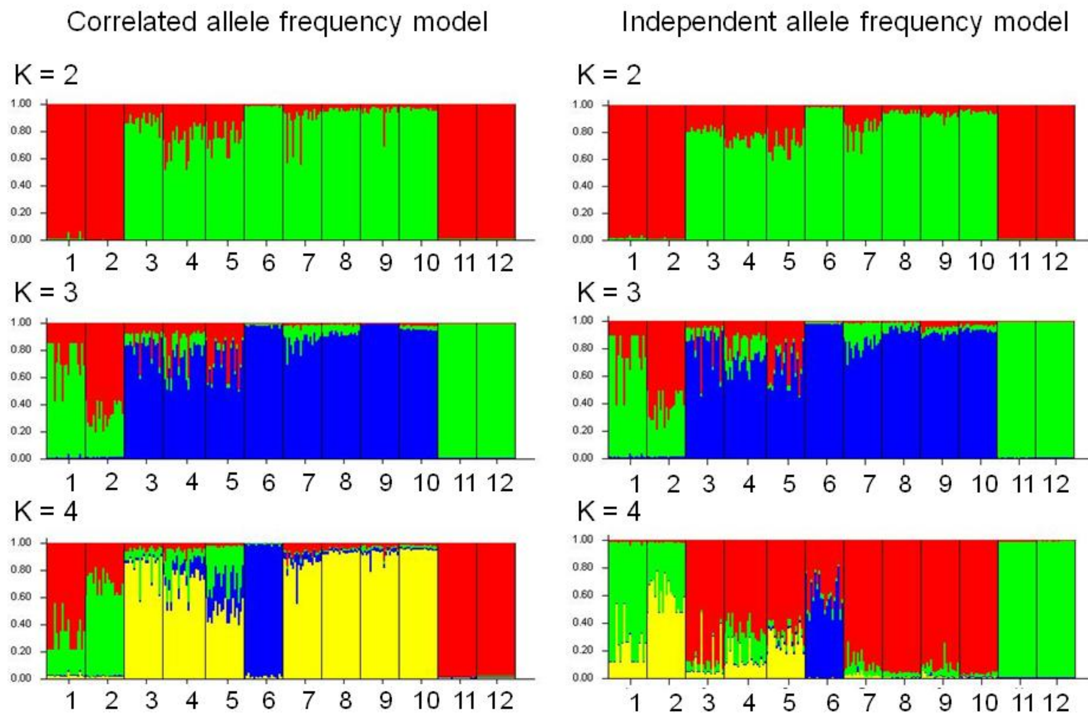

Fig. 5-4. Genetic clusters obtained from the STRUCTURE analysis using the correlated allele frequency model and the independent allele frequency model in K = 2, 3, and 4 for all 242 *Hynobius dunni* from 12 populations. Individuals are represented with vertical lines with the estimated proportion of each genetic cluster which is represented by a distinct color. Populations 1-10 are in the northern region A and Populations 11 and 12 are in the southern region B (see Fig. 5-1).

6. General discussion

6-1. Genetic monitoring

Many studies on fine-scale spatial genetic monitoring using molecular markers have been conducted on salamander species around the world (Table 6-1). Results of the spatial genetic monitoring on the two species, *H. tokyoensis* and *H. dunni*, showed the remarkable genetic differentiation among populations despite they have relatively narrower distribution range. Usually, salamanders have relatively large genetic differentiation among populations, because they have low dispersal ability (Velo-Antón et al. 2013). In the present results, genetic differentiations among breeding site were also large and artificial transportation may lead to serious genetic disturbance. There are striking genetic clines between populations of *H. tokyoensis* despite the narrower distribution ranges: across about 120 km range of Boso Peninsula and about 110 km range of Tochigi to Tokyo. In the case of *H. tokyoensis*, migration among populations may occurr in the Boso Peninsula and central Kanto District (Tochigi Prefecture to Tokyo) during recent years. This phenomenon can be confirmed on the low dispersal species (e.g., Sotka et al. 2004), but observed case of genetic cline may be rare in salamander species (Table 6-1). According to Devitt et al. (2013), genetic cline was also confirmed on Large-blotched salamander (*Ensatina eschscholtzii klauberi*) across about 50 km range of Palomar Mountain to Cuyamaca Rancho State Park in California. Present results provide a good example of genetic cline on salamander species in narrow areas.

The distribution patterns and population genetic structures of salamander species often depend on geographical histories (e.g., formations of mountains or rivers) or distance effect (e.g. geographic or stream distances), because they have low dispersal ability (Table 6-1). In *H. tokyoensis*, populations of the Boso Peninsula and the Miura Peninsula have a similar population genetic structure. This result may be suggest the geographical connectivity between these peninsulas in the past. In Japan, molecular research conducted by Honda et al. (2012) using the individuals of Anderson’s crocodile newt (*Echinotriton andersoni*) also showed evidence of geographical connectivity between the Amami Islands and the Okinawa Islands in the past.

The total threat experienced by a population is the cumulative effects of deterministic factors, demographic, environmental and genetic stochasticity, and occasional catastrophe (Frankham et al. 2002). The temporal genetic monitoring is essential because they have a long life-span. According to Kusano et al. (2014), a single

population's age structure in the western Tama area is 4 or 5 to 21. Adults after breeding scatter widely in the forested areas around breeding pond, so destruction of the aquatic environment is not a deadly factor for adult individuals. In this case, the monitoring may be conducted at relatively longer time interval (e.g., decennial monitoring). On the other hand, deforestation in the habitat of salamanders may lead to extinction of populations because it has negative impact on the number of adult individuals. In this case, prompt and temporal genetic monitoring is needed (e.g., every few years). Identifying the most important factors determining extinction risk can help identify possible remedial action for threatened populations or species (Frankham et al. 2002). Population viability analysis (PVA) is defined as the process of evaluating the combined effects (e.g., age structure, population size, catastrophic event, environmental change, population fluctuation, inbreeding rate, and so on) of threats faced by populations on their risks of extinction and their chances of recovery within defined time frames (Frankham et al. 2002). The analysis has already tried on *H. tokyoensis* without genetic factors (Kusano et al. 2014). However, the analysis was only performed on one population in western Tama area, and the population has the lower extinction risk, because it has larger population size than the other threatened populations (e.g., populations of Miura Peninsula) (Kusano et al. 2013). In addition, western Tama populations may have had the higher genetic diversity based on microsatellite DNA analysis. For the future prospects, estimations of the extinction risk using PVA with genetic factors are essential on several threatened populations of this species.

6-2. Management unit

According to Moritz (1995), species require management as separate units. Monophyly of *H. tokyoensis* was strongly supported by mitochondrial DNA analysis. Thus, this species probably has no taxonomic and phylogenetic problems. On the other hand, monophyly of Kyushu populations of *H. dunni* was strongly supported, but the Kochi population of *H. dunni* had distantly related sequences. It can be inferred from the results, Kochi population should not be merged into the Kyushu populations as same species or management unit at this time. After this, phylogentic analysis using high variable nuclear markers is needed because introgression may have occurred with other species of western Japan group (e.g., *H. nebulosus*).

Not just species, populations within species may be on the path to speciation (Frankham et al. 2002). If they show large adaptive differentiation to different habitats (ecological niches) or large genetic differentiation, they may justify management as

separate units for conservation (Moriz 1995). Two salamander species, *H. tokyoensis* and *H. dunni*, had relatively large genetic differentiation each breeding site and these populations are expected to conserve separately. However, closely related populations based on STRUCTURE analysis may justify management as single unit.

Crandall et al. (2000) point out the major two problems on management unit. First, management unit is unlikely to be detected within species with high gene flow, even though populations may have adaptive differences and warrant separate management. Second, in taxa with low gene flow, populations that have differentiated by genetic drift may be designated as separate management unit, even though they may not be adaptively distinct and in this case they may benefit from gene flow (Crandall et al. 2000). Salamanders correspond to the latter type and gene flows may bring a positive effect to the populations. However, they have low dispersal ability and cannot migrate themselves among populations frequently, so human intervention is essential to improve their management on gene flow in the wild. However, managing gene flow involves many issues and we must address the issues: (1) Which individuals to translocate? (2) How many? (3) How often? (4) From where to where? (5) When should translocations begin? (6) When should they be stopped? (Frankham et al. 2002). Answering these questions, management populations should be genetically monitored using computer simulation as well as field surveys because there are so many variables to optimize (Frankham et al. 2002). The goal of computer simulation is to decide a realistic conservation plan: it is necessary to maintain as genetically viable populations with acceptable costs and fit within other management constraints (Frankham et al. 2002). These analyses will also be necessary in the future conservation of this species.

6-3. Genetic diversity

Peripheral populations exhibit low genetic diversity and greater genetic differentiation as a consequence of smaller effective population size and greater geographical isolation relative to geographically central populations (Eckert et al. 2008), and also completely isolated population fragments, lacking gene flow, are the most severe form of fragmentation (Westemeier et al. 1998). The genetic diversity of the two species, *H. tokyoensis* and *H. dunni*, also tended to be low in the peripheral region and completely isolated populations of their distribution range. The edge effect is confirmed in several salamander species (Table 6-1). Loss of genetic diversity severely diminishes the capacity of populations to respond to novel pathogens pressure, and following sequential assaults by different pathogens, populations with high genetic diversity are

more likely to persist than populations with low genetic diversity (Lively et al. 1990). Furthermore, in small populations, the role of chance predominates and the effects of selection are typically reduced, and these populations become inbred at a faster rate than large populations (Frankham et al. 2002). Thus, peripheral and small isolated populations should be preferentially protected, because they have usually low genetic diversities.

There are two major considerations for future conservation of the two species. First, it is necessary to verify the relationships between population size and genetic diversity. Generally, small populations have lower genetic diversity than large populations (Frankham 1996), but the large populations have not always maintained the high genetic diversity. In this study, population 19 of *H. tokyoensis* has lower genetic diversity despite it has relatively larger population size (221 egg sacs: at least there are 221 female individuals within the population). Second, it is also necessary to verify the relationships between genetic diversity and inbreeding depression. Loss of genetic diversity induces an increase in malformation or mortality rate during the developmental stage (Frankham et al. 2002). In this study, mortality rate of two populations, population 10 (higher genetic diversity) and 19 (lower genetic diversity), were calculated. Consequently, population 19 had higher mortality rate (43.0%) than population 10 (2.1%) despite population 19 had larger population size (145 vs. 221). However, this phenomenon may be transient, so continued monitoring is needed. After this, these surveys should be also conducted on other populations with low genetic diversities.

An effective management strategy in the recovery of small inbred populations with low genetic diversity is to introduce individuals from other populations to improve their reproductive fitness and resurrect their genetic diversity (Frankham et al. 2002). There is extensive experimental evidence and this approach can be successful (Westemeier et al. 1998). However, the option of crossing a threatened population to a related population requires careful consideration because there may be a serious risk of outbreeding depression (Frankham et al. 2002). Thus, such crosses must be evaluated based on experimental outcomes prior to implementation of conservation activities. From a genetic perspective, the worst situation is where a threatened population exists as a single inbred population with no related populations (Frankham et al. 2002). The populations with lower genetic diversity have compromised ability to cope with changes in its physical or biotic environment (Frankham et al. 2002). Management plans of these fragile populations should be instituted to (1) increase their population size, (2) establish populations in several location to minimize the risk of catastrophes, (3) maximize their

reproductive rate by improving their environment (e.g., removing predators and competitors), and (4) insulate them from environmental change (Frankham et al. 2002). In *H. tokyoensis*, populations 41 and 42 have the unique population genetic structure based on the STRUCTURE analysis. At this time, management plans of these populations should be instituted to insulate them from environmental change. In fact, there have been attempts this effort by the local residents (Kusano et al. 2014).

Many threatened species have fragmented habitats. The management options for fragmented populations to maximize genetic diversity and minimize inbreeding and extinction risk are to (1) increase the habitat area, (2) increase the suitability of available habitat (increase density), (3) artificially increase the migration rate by translocation, (4) re-establish populations in suitable habitat where they have gone extinct, and (5) create habitat corridors (Frankham et al. 2002). A plan of (2) should be conducted at this time, because (1) and (5) is unrealistic plan. To perform the plan of (3) and (4), further genetic and ecological monitoring is needed.

6-4. Genetic pollution

Results of present study suggest the possibility of genetic pollution on *H. tokyoensis* based on mitochondrial DNA analysis: between Saitama and Miura Peninsula of Kanagawa and between Saitama and Boso Peninsula of Chiba. Genetic pollutions may be rare in the case of salamander species among populations based on bibliographic survey (Table 6-1), although some cases of introgression between two species are reported (e.g., Weisrock et al. 2005). These populations have well-mixed population genetic structures based on microsatellite DNA analysis. Options for addressing the problem of genetic pollution include eliminating the introduced individuals, or translocating ‘pure’ individuals into isolated regions (Frankham et al. 2002). In the case of *H. tokyoensis*, however, success for these options is hard to achieve. Thus, we should make effort for non-proliferation of these populations at this time. In addition, crossing of genetically differentiated population may lead to outbreeding depression in the F1 and subsequent generations (Osborne and Norman 1991; Sunnucks and Tait 2001). To detect the impact of outbreeding depression, the decades of continuous survey using genetic markers such as microsatellite DNA is essential, because salamanders have a long life history.

Recently, some caution is expressed about mixing populations, but an effective management strategy in the recovery of small inbred populations with low genetic diversity is to introduce individuals from other non-inbred populations (Shields 1983;

Frankham et al. 2002). On introduction to inbred populations from other non-inbred populations, salamanders have several advantages that mammals or birds do not have. It is relatively easy to collect the egg sacs, and rearing of larvae is also easy until metamorphosis. Thus, individuals of larvae or juveniles can readily be introduced to wild populations. Generally, individuals used for introduction should maximize the chances of recovering a self-sustaining wild population, so individuals with low inbreeding coefficients and high genetic diversity are ideal (Frankham et al. 2002). However, the prompt introduction into inbred populations from non-inbred populations may lead to the severe genetic pollution. Furthermore, the option of crossing a population to a related population requires very careful consideration, because there may be a serious risk of outbreeding depression (Frankham et al. 2002). To select individuals or populations to be newly introduced, breeding experiments about survival rate and reproductive success of pure and hybrid individuals using variable markers (e.g. microsatellite makers used in this study) are essential.

For the future conservation of urban neighborhood-dwelling salamanders, assessment of these four matters (i.e., genetic monitoring, management unit, genetic diversity, and genetic pollution) is indispensable. Population dynamics of threatened populations of *H. tokyoensis* and *H. dunni* should be temporally investigated using both ecological and genetic monitoring methods in their future conservation.

Table 6-1. Summary of the population genetic study of salamander species.

No.	Species	Family	Study site	mtDNA					nDNA					Microsatellite				Factors							Comments	Reference
				No. of Population	n	site	bp	N_H	No. of Population	n	site	bp	N_H	No. of Population	n	loci	F_{ST}	Geographical History	Distance Effect	Resistance Effect	Bottleneck Effect	Edge Effect	Genetic Cline	Genetic Pollution		
1	*Hynobius tokyoensis*	Hynobiidae	eastern Honshu, Japan	46	815	Cyt b	650	72						46	815	5	0.423	+			m, f	+	+	+	urban neighborhood-dwelling	This study
2	*Hynobius tokyoensis*	Hynobiidae	eastern Honshu, Japan	22	34	D-loop	204	8																	urban neighborhood-dwelling	Yoshizawa et al. 2005
3	*Hynobius tokyoensis*	Hynobiidae	eastern Honshu, Japan	37	64	D-loop	316	20										+							urban neighborhood-dwelling	Hayashi and Kusano 2006
4	*Hynobius tokyoensis*	Hynobiidae	eastern Honshu, Japan	14	31	Cyt b, D-loop	1956	32										+							urban neighborhood-dwelling	Matsui et al. 2007
5	*Hynobius dunni*	Hynobiidae	eastern Kyushu, Japan	12	242	Cyt b	610	12						12	242	3						+			urban neighborhood-dwelling	This study
6	*Hynobius lichenatus*	Hynobiidae	northeastern Honshu, Japan	75	215	Cyt b	1141	126										+								Aoki et al. 2013
7	*Hynobius nebulosus*	Hynobiidae	Kitakyusyu, Japan	16	199	D-loop	474	32											+						urban neighborhood-dwelling	Yamane and Nishida 2010
8	*Hynobius retardatus*	Hynobiidae	Hokkaido, Japan	28	105	D-loop	490	20										+	+							Azuma et al. 2013
9	*Hynobius yatsui*	Hynobiidae	Kyushu, Japan	24	181	Cyt b	637	49										+								Sakamoto et al. 2009
10	*Pachyhynobius shangchengensis*	Hynobiidae	Dabieshan Mountains, China	7	93	Cyt b	942	51											+							Zhao et al. 2013
11	*Pachyhynobius shangchengensis*	Hynobiidae	Dabieshan Mountains, China	4	175	D-loop	768	28																		Pan et al. 2014
12	*Ranodon sibiricus*	Hynobiidae	Tibet, China	6	123	Cyt b, D-loop, 12S	1323	3						5	123	2		+							fragmented into isolated refugia	Chen et al. 2012
13	*Salamandrella keyserlingii*	Hynobiidae	across Russia	12	26	Complete	16356	26																	current dispersion from refugia	Malyarchuk et al. 2013
14	*Salamandrella schrenckii*	Hynobiidae	eastern Russia	35	167	Cyt b	825	62																		Malyarchuk et al. 2009
15	*Andrias davidianus*	Cryptobranchidae	central China	9	20	ATPase 6, cyt b	678	17																		Murphy et al. 2000
16	*Andrias davidianus*	Cryptobranchidae	central China	4	28	D-loop	771	27																		Tao et al. 2005
17	*Andrias davidianus*	Cryptobranchidae	central China											3	28	11										Meng et al. 2012
18	*Andrias japonicus*	Cryptobranchidae	western Japan	27	46	Cyt b, D-loop, ND1, ND6	3664	13																		Matsui et al. 2008
19	*Cryptobranchus alleganiensis alleganiensis*	Cryptobranchidae	southeastern Missouri, USA											77	1203	12			+						isolation by stream distance	Unger et al. 2013
20	*Cryptobranchus alleganiensis alleganiensis*	Cryptobranchidae	southeastern Missouri, USA											4	138	7										Crowhurst et al. 2011
21	*Cryptobranchus alleganiensis bishopi*	Cryptobranchidae	southeastern Missouri, USA											4	138	7										Crowhurst et al. 2011
22	*Pseudobranchus axanthus*	Sirenidae	central to southern Frorida, USA	12	108	Cyt b	786	60										+								Liu et al. 2006
23	*Pseudobranchus striatus*	Sirenidae	South Carolina to Frorida, USA	27	197	Cyt b	786	106										+								Liu et al. 2006
24	*Ambystoma californiense*	Ambystomatidae	central California, USA											16	514	13										Wang et al. 2009
25	*Ambystoma jeffersonianum*	Ambystomatidae	Halton Hills, Ontario, Canada											2	168	9										Ramsden 2008
26	*Ambystoma laterale*	Ambystomatidae	Iwoa to Minnesota, USA											3	100	5										Eastman et al. 2007
27	*Ambystoma leorae*	Ambystomatidae	southern Mexico											6	96	9	0.021		-		m, f					Sunny et al. 2014
28	*Ambystoma macrodactylum columbianum*	Ambystomatidae	northeastern Montana, USA											21	549	7									directional dispersal	Giordano et al. 2007
29	*Ambystoma macrodactylum sigillatum*	Ambystomatidae	Sierra Nevada, California, USA											54	1142	18	0.270									Savage et al. 2010
30	*Ambystoma maculatum*	Ambystomatidae	Tompkins county, New York, USA											29	592	11	0.073		+							Zamudio and Wieczorek 2007
31	*Ambystoma maculatum*	Ambystomatidae	northeastern Ohio, USA											17	407	8			-							Purrenhage et al. 2009
32	*Ambystoma mavoritium melanostictum*	Ambystomatidae	Yellowstone National Park, Wyoming, USA											10	199	8	0.240		+							Spear et al. 2005
33	*Ambystoma mavoritium melanostictum*	Ambystomatidae	Yellowstone National Park, Wyoming, USA											10	199	8					m, f					Spear et al. 2006
34	*Ambystoma mavoritium sttebinsi*	Ambystomatidae	southern Arizona, USA											16	276	10			-		m, f					Storfer et al. 2014
35	*Ambystoma opacum*	Ambystomatidae	southwestern Ohio, USA											2	37	6	0.020									Bartoszek and Greenwald 2009
36	*Ambystoma tigrinum*	Ambystomatidae	Long Island, New York, USA											17	439	12										Titus et al. 2014
37	*Dicamptodon atterimus*	Dicamptodontidae	Idaho and Montana, USA											24	361	14	0.390	+	+						isolation by stream distance	Mullen et al. 2010
38	*Dicamptodon tenebrosus*	Dicamptodontidae	British Columbia to California, USA											8	78	5										Abbott and Curtis 2010
39	*Dicamptodon tenebrosus*	Dicamptodontidae	British Columbia to Washington, USA											39	979	9				+	m, f	+				Dudaniec et al. 2012
40	*Rhyacotriton variegatus*	Rhyacotritonidae	Oregon to California, USA	72	189	Cyt b	850	59											+							Miller et al. 2006
41	*Rhyacotriton variegatus*	Rhyacotritonidae	eastern Oregon and northern California, USA											19	367	10			+		m, f				independent of stream network	Emel and Storfer 2014
42	*Proteus anguinus*	Proteidae	Slovenia, Croatia, and Bosnia and Herzegovina	26	84	Cyt b, D-loop, Spacer	1098	20																		Gorički and Trontelj 2006
43	*Aneides flavipunctatus*	Plethodontidae	southern Oregon to northern California, USA	4	58	Cyt b, ND4, 12S	1734	27	4	98	POMC	481	4												current dispersion from refugia	Reilly et al. 2013
44	*Batrachoseps wrighti*	Plethodontidae	northern Oregon, USA	22	22	Cyt b	774	18																		Miller et al. 2005
45	*Desmognathus fuscus*	Plethodontidae	New York City, USA											5	110-136	5					m, f				urban neighborhood-dwelling	Munshi-South et al. 2013
46	*Eurycea nana*	Plethodontidae	San Marcos Springs, Texas, USA	3	103	ND4	817	2	3	69	RAG-1	721	3													Lucas et al. 2009
47	*Ensatina eschscholtzii klauberi*	Plethodontidae	California to Baja California, USA and Mexico	14	68	ND4	800	27						14	68	10		+	+	+			+		current dispersion from refugia	Devitt et al. 2013
48	*Phaeognathus hubrichti*	Plethodontidae	southern Alabama, USA											21	105	10	0.150		-		m, f					Apodaca et al. 2012
49	*Plethodon cinereus*	Plethodontidae	Giles, Virginia, USA											8	221	7	0.019		+		-				urban neighborhood-dwelling	Cabe et al. 2007
50	*Plethodon cinereus*	Plethodontidae	Shenandoah National Park, Virginia, USA	3	6	Cyt b	404	6																-		Carpenter et al. 2001
51	*Plethodon cinereus*	Plethodontidae	Mount Royal, Quebec, Canada											8	384	5									urban neighborhood-dwelling	Noël et al. 2007
52	*Plethodon larselli*	Plethodontidae	eastern Washington and northern Oregon, USA	15	44	Cyt b	850	13																		Wagner et al. 2005
53	*Plethodon shenandoah*	Plethodontidae	Shenandoah National Park, Virginia, USA	2	3	Cyt b	404	3																-		Carpenter et al. 2001
54	*Pseudoeurycea leprosa*	Plethodontidae	southern Mexico											23	351	9				+						Velo-Antón et al. 2013
55	*Calotriton arnoldi*	Salamandridae	southern France and northern Spain	7	105	Cyt b	374	4	7	75	RAG-1	530	3													Valbuena-Ureña et al. 2013
56	*Calotriton asper*	Salamandridae	southern France and northern Spain	15	315	Cyt b	374	7	4	33	RAG-1	530	2													Valbuena-Ureña et al. 2013
57	*Cynops ensicauda*	Salamandridae	Amami and Okinawa Islands, Japan	45	274	Cyt b	1141	99										+								Tominaga et al. 2010
58	*Cynops ensicauda*	Salamandridae	Amami and Okinawa Islands, Japan	11	18	Cyt b, ND6	1407	16	11	18	RAG-1	1208	8													Tominaga et al. 2013
59	*Cynops pyrrhogaster*	Salamandridae	Honshu, Shikoku, and Kyushu, Japan	155	224	Cyt b, ND6	1407	141	155	224	RAG-1	1208	25					+								Tominaga et al. 2013
60	*Echinotriton andersoni*	Salamandridae	Amami and Okinawa Islands, Japan	29	93	Cyt b	1141	38										+								Honda et al. 2012
61	*Euproctus platycephalus*	Salamandridae	Sardinia Island, Italy	4	74	D-loop, 12S	915	22										+								Lecis and Norris 2004
62	*Ichthyosaura alpestris*	Salamandridae	eastern Germany and westen Poland	13	27	ND2, ND4	1957	7						13	354	6	0.078	+	+						current dispersion from refugia	Pabijan and Babik 2006
63	*Notophthalmus viridescens louisianensis*	Salamandridae	eastern Iwoa, USA	14	282	Cytb, D-loop	1054	9						14	282	3			+							Whitmore et al. 2013
64	*Paramesotriton caudopunctatus*	Salamandridae	Guizhou and Chongqing, China	5	42	D-loop	690	13																		Chen et al. 2011
65	*Salamandra infraimmaculata*	Salamandridae	northern Israel											20	475	15			+							Blank et al. 2013
66	*Salamandra salamandra*	Salamandridae	North Rhine Weatphalia, Germany	19	438	D-loop	758	3																		Steinfartz et al. 2007
67	*Salamandrina perspicillata*	Salamandridae	central Italy	8	144	Cyt b	630	5						7	162	7										Hauswaldt et al. 2008
68	*Salamandrina perspicillata*	Salamandridae	northern to central Italy	8	36	Cyt b, 12S, 16S	1455	7										+								Mattoccia et al. 2005
69	*Salamandrina terdigitata*	Salamandridae	southern Italy	2	42	Cyt b	630	5						2	42	7										Hauswaldt et al. 2008
70	*Salamandrina terdigitata*	Salamandridae	southern Italy	3	9	Cyt b, 12S, 16S	1455	2										+								Mattoccia et al. 2005
71	*Tylototriton kweichowensis*	Salamandridae	Guizhou and Yunnan, China	14	58	D-loop	808	28																		Zhang et al. 2013
72	*Tylototriton shanjing*	Salamandridae	south western China	19	146	Cyt b, D-loop, 12S	2373	58										+							current dispersion from refugia	Yu et al. 2013

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9. Appendices

9-1. Publications

Main paper

Sugawara H, Nagano M, Sueyoshi T, Hayashi F (2015) Local genetic differentiation and diversity of Oita salamander (*Hynobius dunni*) in Kyushu revealed by mitochondrial and microsatellite DNA analyses. Current Herpetology (in press)

Sub paper

Sugawara H, Takahashi H, Hayashi F (2015) Microsatellite analysis of the population genetic structure of *Anolis carolinensis* introduced to the Ogasawara Islands. Zoological Science 32: 47–52.

9-2. Title and summary in Japanese

学位論文要旨（博士（理学））

日本産止水性 *Hynobius* 属サンショウウオ類の集団遺伝構造と遺伝的多様性：希少種の保全に向けて（英文）

菅原　弘貴（首都大学東京　理工学研究科　生命科学専攻）

低地の止水で繁殖するサンショウウオ類は，人為的な環境変化により個体群の衰退が著しく，保護が急務とされている．野生生物を保護する際，生態学的な知見 (生活史，個体群密度，個体数変動，生残率) に加えて，集団遺伝学的な知見が欠かせない．特に，集団遺伝学的モニタリング (genetic monitoring)，管理単位 (management unit) の推定，遺伝的多様性 (genetic diversity) の評価，そして遺伝子汚染 (genetic pollution) の有無は，適切な保全計画を立てる上で必須である. 本研究では, 関東に生息するトウキョウサンショウウオ (*Hynobius tokyoensis*) および九州東部に生息するオオイタサンショウウオ (*H. dunni*) に関して，将来的な保全に向けて，これら 4 項目を含む集団遺伝学的解析を行った．

まず，トウキョウサンショウウオ 46 集団 815 個体について，ミトコンドリア DNA の cytochrome b (650-bp) および核 DNA のマイクロサテライト 5 遺伝子座に基づく集団遺伝学的解析を行った．トウキョウサンショウウオは *Hynobius* 属の中で分子系統学的によくまとまった集団を形成し，福島南部から房総および三浦半島南部まで断続的に生息する．本種は，地理的に比較的狭い範囲に分布しているにもかかわらず，集団ごとの遺伝的差異は顕著であった．特に，北部と南部では遺伝的差異が大きく，銚子から房総半島にかけてと，栃木から東京にかけて，それぞれ異なる遺伝的な傾斜が認められた．一方，三浦半島と房総半島南部の集団は遺伝的に類似しており，両半島が陸続きであった時代に移動

分散が起こった可能性を支持している．遺伝的多様性に関して，ミトコンドリア DNA と核 DNA に基づく多様度を比較した結果，両者の間には正の相関が認められ，分布の周縁域でとくに遺伝的多様度が低い集団が見られた．また，埼玉集団と三浦半島集団および埼玉集団と房総半島集団の間にはそれぞれ遺伝子汚染が生じている可能性が示唆された．

次に，オオイタサンショウウオ 12 集団 242 個体において，ミトコンドリア DNA の cytochrome b (569-bp) および核 DNA のマイクロサテライト 3 遺伝子座に基づく集団遺伝学的解析を行った．オオイタサンショウウオについては，形態分類と分子系統解析の結果が一致しなかったため，九州東部の集団のみを解析対象とした．本種もトウキョウサンショウウオと同様に，大分県から宮崎県という比較的狭い分布域にも関わらず，大きな分集団化が認められた．また，主に分布域の周縁部で遺伝子多様度の低い集団が見られた．

これら都市近郊に分布する低移動性サンショウウオ類に関しては，繁殖場所ごとに遺伝的な分化が生じている可能性が示唆された．そのため，保全管理単位の決定を行う際には，比較的狭い範囲の管理単位とする必要がある．また，分布域の周縁部では，両種ともに遺伝子多様性が低い傾向があった．やむを得ずこれらの集団に対して遺伝的多様性の回復を目的とした導入を行う際には，遺伝子汚染に十分注意する必要がある．

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Local Genetic Differentiation and Diversity of the Oita Salamander (*Hynobius dunni*) in Kyushu Revealed by Mitochondrial and Microsatellite DNA Analyses

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Abstract: The Oita salamander *Hynobius dunni* Tago, 1931, endemic to eastern Kyushu and western Shikoku of southwestern Japan, is a lowland lentic breeder and has declined throughout its distribution range. To contribute to the future conservation of this salamander, current population genetic structures and genetic diversities were examined for 12 populations of eastern Kyushu, using a mitochondrial cytochrome b gene and three microsatellite loci. Populations were found to be genetically separated into northern and southern groups, and microsatellite analysis showed some genetic differences even in the northern regions. The southern group was restricted to a narrow area and had low genetic diversity in both mitochondrial and microsatellite DNAs. In the northern group, the mitochondrial and microsatellite DNA diversities were also low in some peripheral populations. For the accurate genetic management of this species, we need to pay more detailed attention to such genetic differentiation and diversity.

Key words: Genetic differentiation; Genetic diversity; Microsatellite DNA; Mitochondrial DNA; Oita salamander

Introduction

The effects of habitat fragmentation are now unavoidable for wild animals living in inhabited areas. Fragmentation is caused by agriculture, forestry, and other human activities (e.g., urban development), and they can produce geographic barriers to the dispersal of animals (Kolozsvary and Swihart, 1999). If their dispersal ability is very low, human-induced habitat fragmentation produces many small, isolated populations. Even in well-conserved forest areas, construction of roads can severely divide populations of animals with very low mobility (Trombulak and Frissell, 2000). Small populations are very susceptible

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to stochastic events, which often result in extinction (Frankham et al., 2002). Such populations also tend to have less genetic diversity than larger ones due to the loss of alleles through genetic drift and the increased chance of inbreeding (Frankham et al., 2002). Therefore, once the species distribution range becomes heavily fragmented and gene flow is limited by rare dispersal events, we must manage such populations carefully to avoid extinction and to understand the species ecology, demography, and genetics (Frankham et al., 2002). Recent development of molecular techniques enables us to assess, on a fine scale, population genetic structure in the wild. Such population genetic data are available for monitoring genetic diversity (Schwartz et al., 2007), identifying management units to conserve (Palsbøll et al., 2007), and detecting genetic pollution and hybridization (Simison et al., 2013).

The Oita salamander *Hynobius dunni*, originally described from Oita Prefecture of Kyushu by Tago (1931), is distributed in eastern Kyushu (Oita, Kumamoto, and Miyazaki Prefectures) and the southwestern part of Shikoku (Kochi Prefecture). This salamander is a lowland lentic breeder and is endemic to the above mentioned southwestern Japan (Sato and Horie, 2000). The distribution area is inhabited by humans and subject to the effects of anthropogenic activities (Sato, 1979). This salamander has been listed as a class B2 endangered species in the IUCN Red List of Threatened Species (International Union for Conservation of Nature, 2014). The Kochi population has extremely few individuals, but is well conserved by zoos and landowners (Watabe et al., 2005). The Kochi population has now a relatively stable number of individuals thanks to support from many naturalists. In eastern Kyushu, two populations are protected by local governments but others are unprotected, and the recent extinction of 13 small populations has been reported (Nagano and Kurafuchi, 2011). Thus urgent conservation plans are needed in Kyushu. All previous studies of *H. dunni* dealt with reproductive periods, egg-laying behavior, and/or fecundity (Mashiba, 1969; Sato, 1979; Sueyoshi, 2001; Sueyoshi and Kushima, 2004; Nagano and Kurafuchi, 2011). Only one study revealed local genetic variation of this species based on mitochondrial D-loop sequences (Michigoshi, 2000). According to this study, northern Kyushu (Oita) populations differ genetically from other southern Kyushu (Miyazaki) populations and the western Shikoku (Kochi) population. However, this pioneer study was based on only 222 bp D-loop sequences of a total of 63 individuals. Therefore, to confirm this result and to detect more important genetic factors for future conservation of the threatened *H. dunni* in Kyushu, we analyzed a total of 242 individuals using both mitochondrial and microsatellite DNAs. The aim of this study is to answer: (1) Are *H. dunni* populations genetically structured across their entire distribution range in eastern Kyushu? (2) Do mitochondrial and nuclear genetic markers show concordant results? (3) Which populations have low genetic diversity?

Materials and Methods

Sampling

From February to April in 2012 and from March to May in 2013, 242 salamanders were sampled from 12 populations in Kyushu (Fig. 1); 20 from Usa (Pop. 1), 20 from Ajimu (Pop. 2), 20 from Yokoo (Pop. 3), 22 from Dannoharu (Pop. 4), 20 from Soda (Pop. 5), 20 from Saeki (Pop. 6), 20 from Kiyokawa (Pop. 7), 20 from Takeda (Pop. 8), 20 from Yoshida (Pop. 9), 20 from Takamori (Pop. 10), 20 from Furujyo (Pop. 11), and 20 from Tano (Pop. 12). Individuals representing each of the populations were found in a single wetland including small ponds, marshes, and streams. At present, the distribution range of this species is greatly separated into two regions A and B (Sato, 1998; Iwasaki, 1999; Sueyoshi, 2002; Sueyoshi and Iwakiri, 2009). Populations 1–10 were in region A and populations 11 and 12 were in region B (Fig. 1).

A single tailbud embryo was removed from

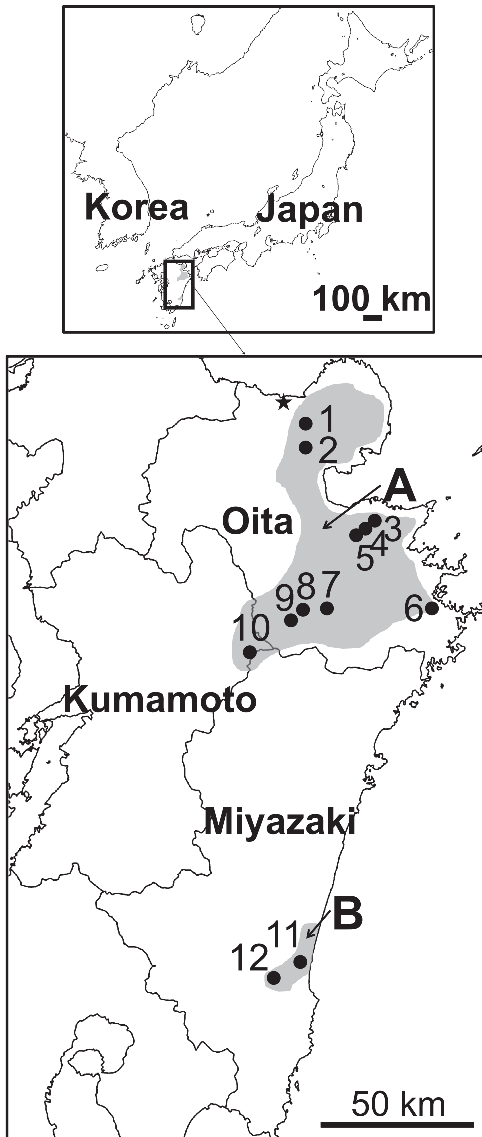

Fig. 1. Twelve populations of *Hynobius dunni* collected in Kyushu, southwestern Japan. Dotted areas show their known distribution areas across to Oita, Kumamoto, and Miyazaki Prefectures (A, northern region; B, southern region). 1, Usa; 2, Ajimu; 3, Yokoo; 4, Dannoharu; 5, Soda; 6, Saeki; 7, Kiyokawa; 8, Takeda; 9, Yoshida; 10, Takamori; 11, Furujo; and 12, Tano. The place indicated with a star is the collecting site of the outgroup *Hynobius nebulosus*.

each paired egg sac and preserved in 99.5% ethanol. To avoid sampling of individuals born to an identical female, we never took DNA-samples from larvae, juveniles, or adults. A single tailbud embryo of the clouded salamander *Hynobius nebulosus* (Temminck and Schlegel, 1838) was sampled in the northern part of Usa in April 2013, and used for the outgroup, because Kyushu populations of *H. nebulosus* are closely related to *H. dunni* (Zheng et al., 2012). When we collected samples for DNA extraction in each population, we also searched for pairs of egg sacs as much as possible and counted their numbers. In salamanders, rough estimation of female population size is usually possible because one female deposits a single pair of egg sacs in each breeding season.

Mitochondrial DNA analysis

Total genomic DNA was extracted from the embryos preserved in 99.5% ethanol, using a DNeasy Blood and Tissue Kit (Qiagen). For 242 samples from 12 populations, 610 bp fragments of the mitochondrial DNA (41 bp $tRNA^{Glu}$+569 bp cytochrome b (cyt b)) were amplified using Ex Taq (TaKaRa) with primers L14010 (5'-TAHGGWGAHGGATTWGA WGCMACWGC-3') and H14778 (5'-AARTA YGGGTGRAADGRRAYTTTRTCT-3') (Matsui et al., 2007). The PCR reaction mix (total volume 10 μl) contained 1.0 μl 10× Ex Taq Buffer, 0.8 μl 25 mM dNTP mix, 0.5 μl each of the forward and reverse primers (10 pM), 0.05 μl Taq polymerase, in 6.15 μl distilled deionized water, and 1.0 μl template DNA. Using a T100™ thermal cycler (Bio-Rad), the PCR protocol was as follows: an initial 10 min denaturing step at 95C, 30 cycles of 60 s at 95C, 60 s at 53C, and 120 s at 72C, with a final 10 min extension at 72C. The PCR products were purified with Illustra™ ExoStar™ 1-Step (GE Healthcare) and sequenced using BigDye Terminator ver. 3.1 (Applied Biosystems) on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Direct sequencing data were aligned using MEGA5 (Tamura et al., 2011). Using the

aligned sequences, phylogenetic analyses were performed with the neighbor-joining method (NJ) based on p-distance and maximum likelihood estimation (ML) based on a Tamura 3-parameter model using MEGA5 (Tamura et al., 2011). The best-fit nucleotide substitution model was estimated based on the Bayesian Information Criterion (BIC: Schwarz, 1978) using MEGA5 (Tamura et al., 2011). A haplotype network was constructed by median-joining method using the NETWORK software package ver. 4.6.1.1 (Bandelt et al., 1999). The genetic variations among and within regions or among and within populations were subjected to analysis of molecular variance (AMOVA) using Arlequin ver. 3.5 (Excoffier and Lischer, 2010). The haplotype diversity h and nucleotide diversity π were calculated using Arlequin ver. 3.5 (Excoffier and Lischer, 2010). Significance of the pairwise Φ_{ST} was determined using Arlequin ver. 3.5 (Excoffier and Lischer, 2010).

Microsatellite DNA analysis

We amplified seven microsatellite loci (HN002, HN004, HN019, HN020, HN023, HN043, and HN058) using the primer sets developed for *H. nebulosus* by Yoshikawa et al. (2013) for five individuals of each population, but only the following three loci were polymorphic and were amplified for all individuals. The PCR was performed on a T100™ thermal cycler (Bio-Rad) using KOD FX Neo DNA polymerase (Toyobo) with a thermal profile consisting of 94C for 120 s, followed by 35 cycles at 94C for 15 s, 59C for 30 s, and 68C for 60 s. The reaction mix (total volume 10 μl) contained 4.8 μl of 2× KOD FX Neo Buffer, 2.0 μl 2 mM dNTPs, 0.5 μl fluorescent (6-FAM or HEX) forward primer (10 pM), 0.5 μl reverse primer (10 pM), 0.1 μl Taq polymerase, 1.1 μl distilled deionized water, and 1.0 μl template DNA. The PCR products were then diluted (1:10) and mixed with GeneScan™ LIZ® 500 Size Standard (Applied Biosystems) and Hi-Di Formamide (Applied Biosystems), combining 0.2 μl Liz, 8.8 μl Hi-Di, and 1 μl diluted product. Fragment analysis data were collected using an ABI 3130xl Genetic Analyzer (Applied Biosystems). Allele lengths were scored using Peak Scanner ver. 1.0 (Applied Biosystems).

The effective number of alleles (N_E), observed (H_O) heterozygosity, expected (H_E) heterozygosity, and information index (I) in each population were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012), and the allelic richness (A_R) was calculated using FSTAT ver. 2.9.3.2 (Goudet, 1995). Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) in H_O and H_E were estimated using Genepop '007 (Rousset, 2008). Significance of the pairwise F_{ST} was determined using FSTAT ver. 2.9.3.2 (Goudet, 1995). The genetic differentiation among regions, among populations within regions, among individuals within populations, and within individuals was subjected to analysis of molecular variance (AMOVA) using Arlequin ver. 3.5 (Excoffier and Lischer, 2010). The current genetic structure of this species was assessed using the program STRUCTURE ver. 2.3.3 (Pritchard et al., 2000) with LOCPRIOR model for accurate inferences (Hubisz et al., 2009). We used correlated (Falush et al., 2003) and independent (Pritchard et al., 2000) allele frequency models. The former model assumes that the populations all diverged from a common ancestral population at the same time, but the latter does not assume it. Ten runs were set with a burn-in length of 50,000 and a Markov chain Monte Carlo (MCMC) run of 50,000 for each K (1 to 7). ΔK was estimated to decide the true K number (Evanno et al., 2005).

Results

Mitochondrial DNA analysis

The number of egg sacs did not differ greatly among populations; the number of paired egg sacs found in each population habitat ranged from 20 to 52 (Table 1). Of the 610 bp sequences of 242 individual *H. dunni*, nucleotide substitutions occurred at 19 positions, producing 15 haplotypes, D1–D15 (GenBank

TABLE 1. Number of individuals sampled (N1), number of paired egg sacs observed (N2), number of alleles (N_A), and observed (H_O) and expected (H_E) heterozygosities of three microsatellite loci in 12 populations of Hynobius dunni (no asterisks P>0.05; * P<0.05; ** P<0.01). For the localities of 12 populations, see Fig. 1.

Population		HN002	HN004	HN020
1	N_A	3	1	2
(N_1=20)	H_O	0.250	0.000	0.250
(N_2=33)	H_E	0.301	0.000	0.349
2	N_A	3	1	3
(N_1=20)	H_O	0.750	0.000	0.550
(N_2=21)	H_E	0.524	0.000	0.629
3	N_A	3	2	2
(N_1=20)	H_O	0.500	0.650	0.000*
(N_2=31)	H_E	0.511	0.439	0.255
4	N_A	2	4	3
(N_1=22)	H_O	0.182	0.682	0.091**
(N_2=35)	H_E	0.351	0.600	0.361
5	N_A	3	4	3
(N_1=20)	H_O	0.350	0.700	0.050**
(N_2=22)	H_E	0.576	0.606	0.451
6	N_A	2	5	4
(N_1=20)	H_O	0.250	0.700	0.150**
(N_2=30)	H_E	0.289	0.613	0.524
7	N_A	3	3	4
(N_1=20)	H_O	0.400	0.500	0.350*
(N_2=37)	H_E	0.395	0.545	0.439
8	N_A	3	3	2
(N_1=20)	H_O	0.400	0.600	0.350
(N_2=26)	H_E	0.339	0.434	0.289
9	N_A	3	3	3
(N_1=20)	H_O	0.500	0.600	0.350
(N_2=52)	H_E	0.579	0.486	0.296
10	N_A	3	2	2
(N_1=20)	H_O	0.800	0.600	0.100
(N_2=20)	H_E	0.559	0.420	0.320
11	N_A	1	1	1
(N_1=20)	H_O	0.000	0.000	0.000
(N_2=24)	H_E	0.000	0.000	0.000
12	N_A	1	2	1
(N_1=20)	H_O	0.000	0.450	0.000
(N_2=33)	H_E	0.000	0.349	0.000

accession numbers LC003294–LC003308). The phylogenetic trees, using the corresponding sequence of the outgroup *H. nebulosus* (GenBank accession number LC003293), were nearly the same between NJ and ML estimates (Fig. 2). The haplotypes were separated into two groups, region A consisting of populations 1–10 and region B populations 11 and 12. Analysis of the haplotype network also showed these two regional groups (Fig. 3). The greatest variance (88.8%) was seen among regions (AMOVA; sum of squares 321.2, variance components 1.55, P<0.001), and only 9.3% and 13.6% were seen among populations within regions (sum of squares 24.3, variance components 0.19, P<0.001) and within populations (sum of squares 63.0, variance components 0.27, P<0.001). Pairwise Φ_{ST} values between populations differed significantly in most population combinations after Bonferroni corrections (above diagonal in Table 2).

Microsatellite DNA analysis

Microsatellite analysis detected three, nine, and seven alleles in HN002, HN004, and HN020 loci, respectively. The observed and expected heterozygosities ranged from 0.000 to 0.800 and 0.000 to 0.613, respectively (Table 1). No significant LD was detected in any combinations of loci for the 12 populations and deviations from HWE were identified in HN020 locus of five populations after Bonferroni corrections (Table 1). The greatest variance (71.1%) was seen within individuals (AMOVA; sum of squares 122.0, variance components 0.50, P<0.001), and 19.1% was seen among regions (sum of squares 60.3, variance components 0.14, P<0.001). Variance among populations within regions and variance among individuals within populations explained only 3.2% (sum of squares 9.1, variance components 0.02, P<0.001) and 6.5% (sum of squares 137.2, variance components 0.05, P<0.05) of variation. Pairwise multilocus F_{ST} between populations revealed that the frequency distributions of alleles at the three loci were different among popula-

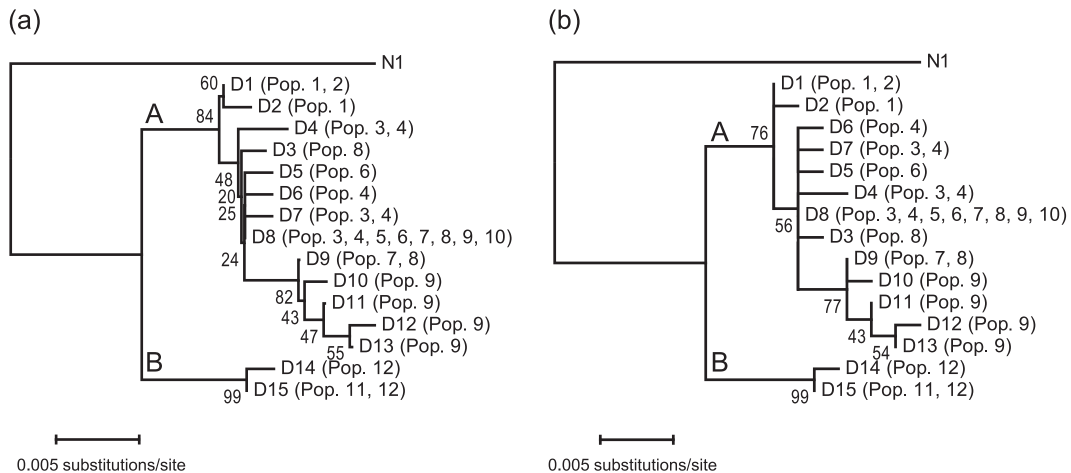

FIG. 2. NJ (a) and ML (b) phylogenetic trees based on 41 bp tRNA^{Glu}+569 bp cytochrome b (cyt b) sequences. D1–D15, haplotypes of *Hynoubius dunni*; N1, *H. nebulosus* (outgroup); Pop. 1–12, populations; A and B, regions (see Fig. 1). Numerals near branches are bootstrap probabilities with 1,000 replications.

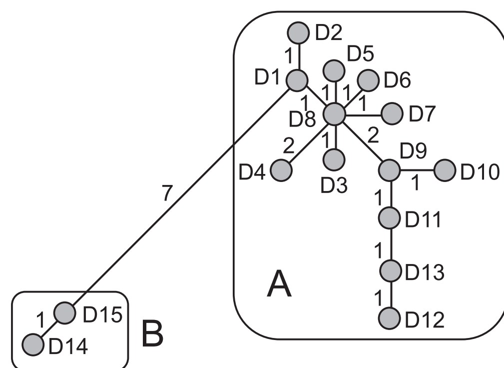

FIG. 3. Fifteen haplotypes network (D1–D15) based on 569 bp cytochrome b sequences by the median-joining method. A and B indicate the northern and southern regions in *Hynobius dunni* distribution range (see Fig. 1). Numbers of mutational steps are shown exhibited near the lines connecting haplotypes.

tions after the Bonferroni corrections (lower diagonal in Table 2). The significant pairwise multilocus F_{ST} were observed in the most combinations including populations 1, 2, 6, 11, and 12, although the F_{ST} value between populations 11 and 12 did not differ from zero (Table 2).

In the correlated allele frequency model of STRUCTURE, ΔK calculated for K=2 to 7 was highest at K=2 (ΔK=18.7, SD=4.6), followed by K=4 (5.2, 8.5) and K=3 (1.4, 13.2). In the independent allele frequency model of STRUCTURE, ΔK calculated for K=2 to 7 was also highest at K=2 (ΔK=742.9, SD=2.0), followed by K=4 (8.6, 4.9) and K=3 (5.0, 5.0). Genetic structures obtained by these two models were similar: when K=2, the populations 1, 2, 11, and 12 tended to differ from the other populations (3–10); when K=3, the populations 1 and 2, and the populations 11 and 12 tended to differ from the other populations (3–10); and when K=4, the populations 1 and 2, the population 6, and the populations 11 and 12 tended to differ from the other populations (3–5, 7–10) (Fig. 4).

Genetic diversity

The number of mitochondrial haplotypes (N_H), haplotype diversities (h), and nucleotide diversities (π) within populations ranged from 1 to 5, from 0.00 to 0.75, and from 0.000 to 0.114, respectively (Table 3). The mean number of microsatellite alleles (N_A), mean effective number of alleles (N_E), mean allelic richness (A_R), mean expected heterozygosities (H_E), and mean information indices (I) varied from 1.00 to 3.67, from 1.00 to 2.24, from 1.00 to 3.67, from 0.00 to 0.55, and from 0.00 to 0.92 (Table 3). In the mitochondrial analysis,

TABLE 2. Pairwise genetic distances (upper diagonal Φ_{ST}, based on mtDNA and lower diagonal F_{ST}, based on microsatellite DNA) among 12 populations of *Hynobius dunni* (no asterisks P>0.05; * P<0.05; ** P<0.01). For the localities of 12 populations, see Fig. 1.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1	—	0.421**	0.733**	0.648**	0.825**	0.777**	0.699**	0.637**	0.783**	0.825**	0.976**	0.965**
2	0.148**	—	0.836**	0.685**	1.000**	0.860**	0.744**	0.676**	0.805**	1.000**	1.000**	0.987**
3	0.327**	0.341**	—	0.080	0.020	0.493**	0.410**	0.142**	0.696**	0.020	0.984**	0.975**
4	0.203**	0.273**	0.013	—	0.214**	0.435**	0.400**	0.220**	0.670**	0.214**	0.961**	0.953**
5	0.247**	0.222**	0.008	0.018	—	0.632**	0.474**	0.161**	0.728**	0.000	1.000**	0.989**
6	0.373**	0.391**	v0.067**	0.068**	0.075**	—	0.552**	0.412**	0.729**	0.632**	0.983**	0.974**
7	0.228**	0.265**	0.024	0.005	0.041**	0.059**	—	0.159*	0.424**	0.474**	0.964**	0.957**
8	0.378**	0.392**	0.017	0.048**	0.066**	0.056**	0.015	—	0.566**	0.161**	0.958**	0.950**
9	0.311**	0.301**	–0.002	0.040**	0.022	0.076**	0.008	0.014	—	0.728**	0.945**	0.940**
10	0.355**	0.343**	0.007	0.060**	0.031	0.081**	0.039	0.035*	0.001	—	1.000**	0.989**
11	0.161*	0.425**	0.473**	0.327**	0.406**	0.484**	0.340**	0.508**	0.442**	0.489**	—	0.105
12	0.179**	0.386**	0.362**	0.232**	0.316**	0.369**	0.246**	0.382**	0.342**	0.385**	0.211	—

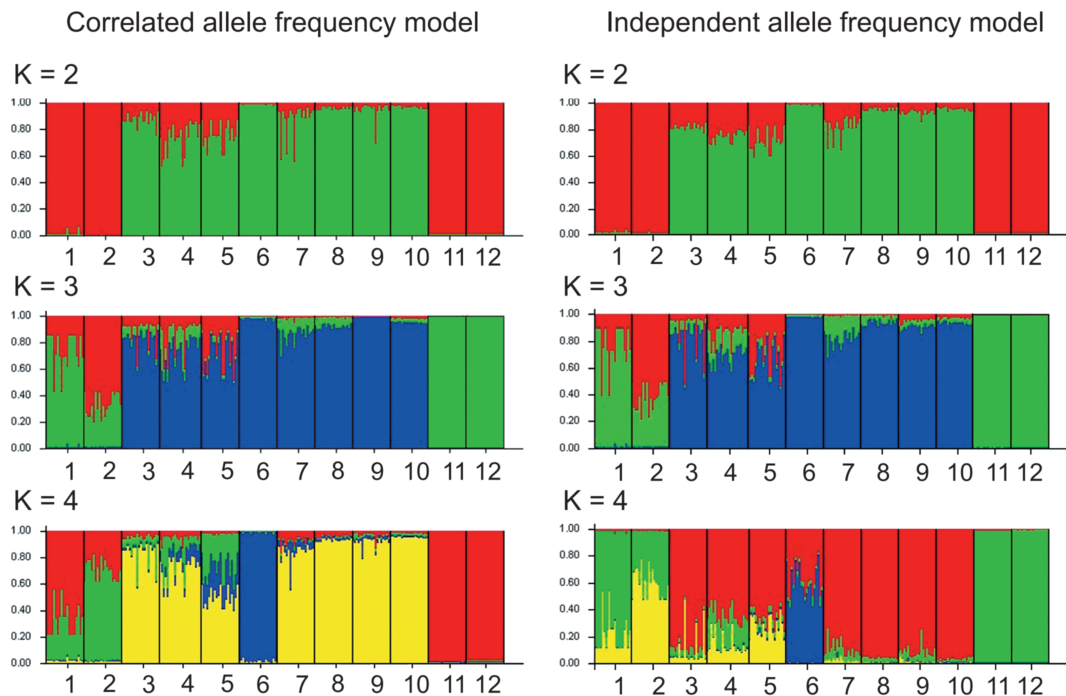

FIG. 4. Genetic clusters obtained from the STRUCTURE analysis using a correlated allele frequency model and an independent allele frequency model in K=2, 3, and 4 for all 242 *Hynobius dunni* from 12 populations. Individuals are represented with vertical lines with the estimated proportion of each genetic cluster which is represented by a distinct color. Populations 1–10 are in the northern region A and Populations 11 and 12 are in the southern region B (see Fig. 1).

populations 2, 5, 10, and 11 had only one haplotype and populations 1, 6, 7, and 12 had only two (Table 3). In the microsatellite analysis, populations 11 and 12 had much lower diversity than the other populations, and several diversity indices were also lower in populations 1, 2, 3, 8, and 10 (Table 3). Thus, the southern group (populations 11 and 12)

Table 3. Number of haplotypes (N_H), haplotype diversities (h), and nucleotide diversities (π) in 569 bp cytochrome b sequences, and the mean number of alleles (N_A), mean effective number of alleles (N_E), mean allelic richness (A_R), mean expected heterozygosities (H_E), and mean information indices (I) in three microsatellite loci of *Hynobius dunni*. The lower six values are indicated with bold type face. N=number of individuals examined. SD=standard deviation. For the localities of 12 populations, see Fig. 1.

Population	N	Cytochrome b					Microsatellite									
		N_H	h	SD	π	SD	Mean N_A	SD	Mean N_E	SD	Mean A_R	SD	Mean H_E	SD	Mean I	SD
1	20	**2**	0.52	0.04	0.033	0.030	**2.00**	0.82	**1.32**	0.23	**2.00**	0.82	**0.28**	0.19	**0.37**	0.26
2	20	**1**	**0.00**	0.00	**0.000**	0.000	**2.33**	0.94	1.93	0.70	**2.33**	0.94	**0.38**	0.34	**0.61**	0.44
3	20	3	**0.28**	0.12	**0.026**	0.026	**2.33**	0.47	**1.72**	0.29	**2.33**	0.47	**0.40**	0.13	**0.64**	0.19
4	22	4	0.68	0.06	0.068	0.049	3.00	0.82	1.87	0.45	2.97	0.82	0.44	0.14	0.73	0.22
5	20	**1**	**0.00**	0.00	**0.000**	0.000	3.33	0.47	2.24	0.30	3.33	0.47	0.55	0.08	0.92	0.15
6	20	**2**	0.48	0.07	0.029	0.028	3.67	1.25	2.03	0.48	3.67	1.25	0.48	0.17	0.88	0.31
7	20	**2**	0.53	0.04	0.078	0.055	3.33	0.47	1.88	0.23	3.33	0.47	0.46	0.08	0.79	0.08
8	20	3	0.63	0.08	0.073	0.052	2.67	0.47	**1.56**	0.15	2.67	0.47	**0.35**	0.07	**0.60**	0.10
9	20	5	0.75	0.06	0.114	0.073	3.00	0.00	1.91	0.39	3.00	0.00	0.45	0.14	0.76	0.18
10	20	**1**	**0.00**	0.00	**0.000**	0.000	**2.33**	0.47	**1.82**	0.33	**2.33**	0.47	0.43	0.12	0.69	0.19
11	20	**1**	**0.00**	0.00	**0.000**	0.000	**1.00**	0.00	**1.00**	0.00	**1.00**	0.00	**0.00**	0.00	**0.00**	0.00
12	20	**2**	**0.27**	0.11	**0.016**	0.020	**1.33**	0.47	**1.18**	0.25	**1.33**	0.47	**0.12**	0.20	**0.18**	0.25

restricted to the narrow area had low genetic diversity in both mitochondrial and microsatellite DNAs. In the northern group, the populations 1, 2, 3, and 10 had lower genetic diversity both in mitochondrial and microsatellite analyses (Table 3).

Discussion

Michigoshi (2000) reported that genetic differentiation occurs between the regions A and B based on his analysis of the mitochondrial D-loop. Our results based on mitochondrial cyt b sequences also supported this differentiation. Similar genetic differentiation was obtained from our microsatellite DNA analysis, that is, populations 11 and 12 differed from the other populations. However, in the microsatellite analysis, populations 1 and 2 and population 6 had some unique genetic characteristics by pairwise multilocus F_{ST} and STRUCTURE analyses. This may be caused by the fixation of the single common allele in the locus HN004 in populations 1, 2, and 11 (also in 12) and by the private allele in the same locus in population 6. At present, the distribution of *H. dunni* is separated into northern and southern parts (Fig. 1), but the reason why they are separated in this way is still unknown.

Another population (only one population) of *H. dunni* is known from Kochi of western Shikoku (Sato, 1998). Michigoshi (2000) reported that this Kochi population is closely related to populations of the region B (populations 11 and 12 in our study) in his mitochondrial D-loop sequences. However, he analyzed only one individual of the Kochi population, so that in the future analyses it will be necessary to include microsatellite DNA.

Populations 11 and 12 had low genetic diversity; particularly population 11 had only one mitochondrial haplotype and a single allele per microsatellite locus. Michigoshi (2000) already pointed it out in his mitochondrial analysis. This fact is an important implication for future management of *H. dunni*. Neither *H. dunni* nor any other species of lentic *Hynobius* occurs between regions A and B (Sato, 1998). Such completely isolated populations have significant deleterious effects such as loss of genetic diversity, inbreeding, and extinction risk (Eldridge et al., 1999). Inbreeding is unavoidable in small populations and reduces reproductive fitness (Ralls and Ballou, 1983). According to Halverson et al. (2006), inbreeding negatively affects the survival of amphibians. Therefore, in the conservation of

H dunni, the southern populations must be treated prior to the northern populations. An effective management in the recovery of small, inbred populations is to introduce individuals from the other populations with closer relationship (Frankham et al., 2002). In the case of *H. dunni*, artificial transportation from the northern region causes genetic pollution because genetic differentiation between both regions is clear, and so pollution must be avoided. Artificial transportation may be allowed between populations 11 and 12 in region B. However, it is usually difficult to decide on artificial introduction when only these two populations are completely genetically identical, or after one of them is completely extinct.

Even in the northern region, forests are strongly fragmented by human use and the wetlands inhabited by *H. dunni* are distributed patchily (e.g., Sato and Horie, 2000). Habitat fragmentation leads to reductions in population size, and to reduced migration or gene flow among populations, thus causing decrease in genetic diversity (Frankham et al., 2002). In fact, the number of paired egg sacs deposited in each population was small, ranged from 20 to 52, and our results show evidence of decreasing genetic diversity in some populations of this region. Particularly populations 1, 2, 3, and 10 had a lower genetic diversity than the others. The low genetic diversities in these populations can be explained by population demographic history, because these populations are located in the peripheral region where the populations are more frequently isolated and receive fewer immigrants than those in central region (Channel, 2004). Therefore, these populations must be monitored to prevent further habitat loss and population decline caused by inbreeding depression. In the future, the requirements of the investigation of other microsatellite loci or genetic variation relevant to adaptive traits should be considered before reaching some implication for management because few loci were used in this study. However, the allelic variation of each locus was sufficiently examined because a sufficient number of individuals per population were studied, and our genetic data will provide valuable information on genetic management and will serve as reference for comparative studies of *Hynobius* species.

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Microsatellite Analysis of the Population Genetic Structure of *Anolis carolinensis* Introduced to the Ogasawara Islands

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DNA analysis can reveal the origins and dispersal patterns of invasive species. The green anole *Anolis carolinensis* is one such alien animal, which has been dispersed widely by humans from its native North America to many Pacific Ocean islands. In the Ogasawara (Bonin) Islands, this anole was recorded from Chichi-jima at the end of the 1960s, and then from Haha-jima in the early 1980s. These two islands are inhabited. In 2013, it was also found on the uninhabited Ani-jima, close to Chichi-jima. Humans are thought to have introduced the anole to Haha-jima, while the mode of introduction to Ani-jima is unknown. To clarify its dispersal patterns within and among these three islands, we assessed the fine-scale population genetic structure using five microsatellite loci. The results show a homogeneous genetic structure within islands, but different genetic structures among islands, suggesting that limited gene flow occurs between islands. The recently established Ani-jima population may have originated from several individuals simultaneously, or by repeated immigration from Chichi-jima. We must consider frequent incursions among these islands to control these invasive lizard populations and prevent their negative impact on native biodiversity.

Key words: alien species, genetic diversity, green anole, invasive species, microsatellite DNA

INTRODUCTION

True oceanic islands have an unbalanced fauna that often lacks competitors, predators, and parasites for nonindigenous invasive animal species due to their discrete geographical nature and long-term isolation from continental invasion (Case and Bolger, 1991). Moreover, on inhabited oceanic islands, the disturbance of the original environment creates new opportunities for nonindigenous invasive species (Borges et al., 2006). For these reasons, alien species often become established readily on oceanic islands once they are introduced, and the frequent, wide-ranging introduction of alien species has become a major problem in terms of protecting the native biodiversity of oceanic islands. Typically, limited information is available on the route of introduction, frequency of overseas dispersal, and offspring dispersal abilities of alien species; however, such information is essential for preventing their unintentional introduction and/or controlling their populations. Fine-scale genetic analyses offer important insights into the gene flow patterns of populations (e.g., Bossart and Pashley Prowell, 1998). The estimated population genetic structure lies between two extremes: a well-mixed population genetic structure determined by high dispersal abilities and great differentiation among populations because of low dispersal.

The green anole *Anolis carolinensis* is a lizard native to the southeastern United States, but has been introduced to other areas, particularly the islands of the Pacific Ocean (Lever, 2003; Glor et al., 2005). The Ogasawara (Bonin) Islands comprise more than 30 islands in the Pacific Ocean, approximately 1000 km from the Japanese mainland. This archipelago has been isolated from the continent since its formation. Many organisms have undergone unique evolutionary processes there (Shimizu, 2003) and the Ogasawara was declared a natural World Heritage Site in June 2011 (Shibagaki, 2012). Green anoles were first found in the northern part of Chichi-jima in the late 1960s and progressively expanded their range on this island (Matsumoto et al., 1980; Hasegawa, 1986; Hasegawa et al., 1988). Subsequently, the anole was introduced from Chichi-jima to central Haha-jima in the early 1980s and has become widespread on this island (Miyashita, 1991; Suzuki and Nagoshi, 1999). In 2013, *A. carolinensis* was also found on Ani-jima (Okochi, 2013; Shimizu, 2013). Humans are thought to have introduced the anole to Haha-jima (Miyashita, 1991), while the mode of introduction to Ani-jima is unknown (Okochi, 2013; Shimizu, 2013). In the Ogasawara Islands, the green anole has had very negative effects on native organisms, particularly insects, due to its strong predation pressure (Abe et al., 2008; Karube, 2009; Okochi, 2009; Kawakami and Okochi, 2010). This lizard was placed on the list of Japan's Worst Invasive Alien Species (Japan Wildlife Research Center, 2008) and attempts to control the lizard populations have been started (Toda et al., 2010), although little information exists on its dispersal distance or frequency.

In its native United States, *A. carolinensis* is used as a model organism for evolutionary patterns and mechanisms, so the molecular phylogeny and population genetics of this species are well studied (Glor et al., 2005; Wordley et al., 2011; Campbell-Staton et al., 2012; Tollis et al., 2012; Tollis and Boissinot, 2014). A comparison of the mitochondrial

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16S ribosomal RNA and ND2 sequences of the native population and the introduced populations on Chichi-jima and Haha-jima (before it was found on Ani-jima) suggested that the source of the introduction was the coastal region between Louisiana and Jacksonville, Florida, in the southeastern United States (Hayashi et al., 2009). In this study, using microsatellite markers developed in the United States, we examined the current population genetic structure of *A. carolinensis* to understand its dispersal pattern and genetic properties on Chichi-jima, Haha-jima, and Ani-jima. We also determined a partial mitochondrial DNA sequence of the Ani-jima population to compare it with sequences for the Chichi-jima and Haha-jima populations.

MATERIALS AND METHODS

Sampling and DNA extraction

From March to October 2013, 182 lizards (59 from Ani-jima, 71 from Chichi-jima, and 52 from Haha-jima) were sampled from the three islands in cooperation with the local liaison committee on green anole emergency measures on Ani-jima. The sampling sites comprised four close to each other on Ani-jima, three oriented from north to south on Chichi-jima, and two oriented from north and south on Haha-jima (Fig. 1). The tail tips of lizards were removed and preserved in 99.5% ethanol. Total genomic DNA was extracted from these tail tissues using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany).

Mitochondrial DNA analysis

For the 59 samples from Ani-jima, a 429-bp fragment of the mitochondrial 16S ribosomal RNA gene was amplified using Ex *Taq*® (TaKaRa, Tokyo, Japan) with primers L2606 (5′-CTGACCGT-GCAAAGGTAGCGTAATCACT-3′) and H3056 (5′-CTCCGGTCT-GAACTCAGATCACGTAGG-3′) (Hedges et al., 1993). The PCR reaction mix (total volume 10 μl) contained 1.0 μl 10× Ex *Taq* Buffer, 0.8 μl 25 mM dNTP mix, 0.5 μl each of the forward and reverse primers (10 pM), 0.05 μl *Taq* polymerase, 6.15 μl distilled deionized water, and 1.0 μl template DNA. The PCR protocol followed Hayashi et al. (2009) using a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA): an initial 5-min denaturing step at 93°C, 30 cycles of 30 s at 93°C, 60 s at 50°C, and 85 s at 72°C, with a final 5-min extension at 72°C. The PCR products were purified with Illustra™ ExoStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) and sequenced using BigDye® Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). After direct sequencing, the sequences were aligned using MEGA 5 (Tamura et al., 2011).

Microsatellite DNA analysis

We amplified five microsatellite loci using the primer sets developed for *A. carolinensis* by Wordley et al. (2011): the Acar1 locus with the repeat motif $(AC)_n$ using the primers 5′-CCAAAAACCA-AAAAGGCTGA-3′ and 5′-TGGACACACATACACCCACA-3′; Acar4 with $(AC)_n$ using 5′-ACAGGGTACTGTGGACAGGG-3′ and 5′-AGG-AGCGTGGAGCTACAAAA-3′; Acar9 with $(AAGG)_n$ using 5′-AAAG-GCAATGGCAGAGAAAA-3′ and 5′-TAATGGGAAAGGAGGCAGTG-3′; Acar11 with $(AG)_n$ using 5′-AGTTTCCCAAGAAAACCCGT-3′ and 5′-GGGTTGCTCGTTCTGGACTA-3′; and Acar14 with $(AGAT)_n$ using 5′-TATGTTGGGAGAAAGACGGG-3′ and 5′-CCTGAGC-TACGTGACATGGA-3′. PCR was performed on a T100™ thermal cycler (Bio-Rad) using KOD FX Neo DNA polymerase (Toyobo, Tokyo, Japan) with a thermal profile consisting of 94°C for 120 s, followed by 35 cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 60 s. The reaction mix (total volume 10 μl) contained 4.8 μl of 2× KOD FX Neo Buffer, 2.0 μl 2 mM dNTPs, 0.5 μl fluorescent (6-FAM) forward primer (10 pM), 0.5 μl reverse primer (10 pM), 0.1 μl *Taq* polymerase, 1.1 μl distilled deionized water, and 1.0 μl template DNA. The PCR products were then diluted (1:10) and mixed with GeneScan™ LIZ® 500 Size Standard (Applied Biosystems) and Hi-Di Formamide (Applied Biosystems), combining 0.2 μl Liz, 8.8 μl Hi-Di, and 1 μl diluted product. Fragment analysis data were collected using an ABI 3130xl Genetic Analyzer (Applied Biosystems). Allele lengths were scored using Peak Scanner ver. 1.0 (Applied Biosystems).

The observed (H_O) and expected (H_E) heterozygosity in each population were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012). Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were estimated using Genepop'007 (Rousset, 2008). The significance of the inbreeding coefficients was

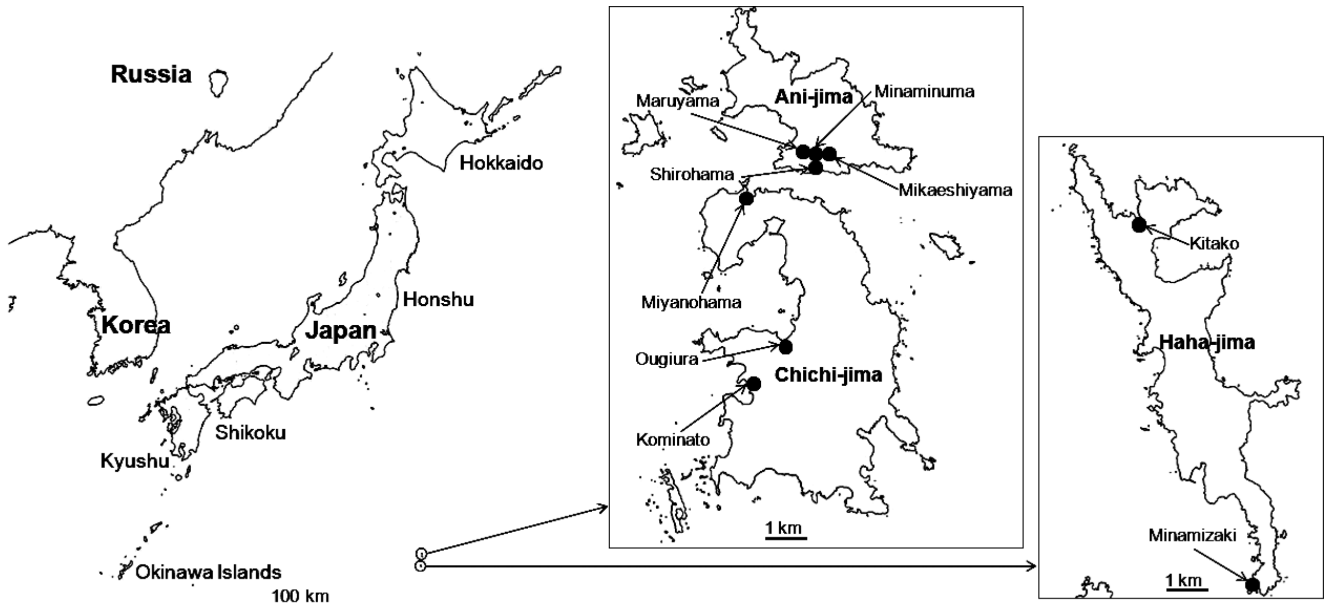

Fig. 1. Sites where the introduced *Anolis carolinensis* was sampled on the three Ogasawara islands. The distance between Chichi-jima and Haha-jima is about 45 km. At present, only Chichi-jima and Haha-jima are inhabited.

Table 1. Allele frequencies for five microsatellite loci of *Anolis carolinensis* introduced to three Ogasawara islands. N = number of lizards sampled. For the collection sites on the islands, see Fig. 1.

Locus (motif)	Size (bp)	Ani-jima					Chichi-jima				Haha-jima			Comments
		Maruyama	Mikaeshiyama	Minaminuma	Shirohama	All	Miyanohama	Ougiura	Kominato	All	Kitako	Minamizaki	All	
Acar1	111	3	0	12	9	24	13	13	4	30	6	8	14	
$(AC)_n$	113	9	2	26	57	94	37	35	40	112	48	42	90	
Acar4	120	0	0	0	0	0	0	0	0	0	1	0	1	Haha-jima only
$(AC)_n$	122	0	0	0	0	0	0	0	0	0	0	0	0	Absent
	124	0	0	0	0	0	0	0	0	0	0	0	0	Absent
	126	0	0	0	0	0	0	0	0	0	0	0	0	Absent
	128	0	0	0	0	0	0	0	0	0	0	0	0	Absent
	130	0	0	0	0	0	0	0	0	0	0	0	0	Absent
	132	0	0	2	16	18	12	4	10	26	10	6	16	
	134	0	0	0	0	0	0	0	0	0	0	0	0	Absent
	136	3	1	11	5	20	6	12	6	24	7	5	12	
	138	9	1	25	45	80	32	32	28	92	36	39	75	
Acar9	148	1	0	1	7	9	1	2	0	3	0	0	0	Ani- and Chichi-jima only
$(AAGG)_n$	152	0	0	0	0	0	0	0	0	0	0	0	0	Absent
	156	0	0	5	5	10	0	3	0	3	0	0	0	Ani- and Chichi-jima only
	160	4	1	13	25	43	25	21	24	70	36	37	73	
	164	4	1	6	6	17	9	8	8	25	7	9	16	
	168	3	0	13	23	39	15	14	12	41	11	4	15	
Acar11	183	0	0	0	1	1	0	3	0	3	0	2	2	
$(AG)_n$	185	2	0	0	5	7	3	9	6	18	3	11	14	
	187	0	0	8	6	14	0	3	2	5	1	1	2	
	189	4	0	16	29	49	15	11	16	42	11	14	25	
	191	0	0	0	0	0	1	0	1	2	5	7	12	Chichi- and Haha-jima only
	193	1	0	2	0	3	2	1	1	4	1	1	2	
	195	2	0	6	3	11	13	12	12	37	20	9	29	
	197	2	1	2	1	6	8	4	0	12	0	0	0	Ani- and Chichi-jima only
	199	0	0	1	4	5	1	1	1	3	1	0	1	
	201	1	1	3	17	22	7	4	5	16	12	5	17	
Acar14	183	1	0	0	2	3	1	5	1	7	2	2	4	
$(AGAT)_n$	187	4	2	14	23	43	4	7	7	18	14	12	26	
	191	1	0	8	8	17	3	3	3	9	9	7	16	
	195	1	0	6	16	23	12	17	14	43	17	18	35	
	199	1	0	7	15	23	8	10	11	29	7	4	11	
	203	4	0	3	2	9	22	6	8	36	5	7	12	
N		6	1	19	33	59	25	24	22	71	27	25	52	Total N = 182

determined using FSTAT ver. 2.9.3.2 (Goudet, 1995). Tests of significant genetic differentiation among populations were conducted using *F*-statistics (Weir and Cockerham, 1984) with each parameter tested against zero by a bootstrapping method using FSTAT ver. 2.9.3.2. The genetic variation among and within populations was subjected to analysis of molecular variance (AMOVA) using Arlequin ver. 3.5 (Excoffier and Lischer, 2010). Current genetic structure was assessed using the program STRUCTURE ver. 2.3.3 (Pritchard et al., 2000). As sampling location information could be used to provide accurate inferences, a LOCPRIOR model was performed (Hubisz et al., 2009). Ten runs were set with a burn-in length of 100,000 and a Markov chain Monte Carlo (MCMC) run of 200,000 for each K (1 to 8). ΔK was calculated to examine the true K number (Evanno et al., 2005).

RESULTS

Only one mitochondrial haplotype was detected in the Ani-jima population, which was the same as the most frequent haplotype (GenBank accession number AB473617) on Chichi-jima and Haha-jima (Hayashi et al., 2009). Another haplotype (AB473618) known on Chichi-jima was not found on Ani-jima. This haplotype is also unrecorded on Haha-jima (Hayashi et al., 2009).

The microsatellite analysis detected two, four, five, 10, and six alleles in Aca1, Aca4, Aca9, Aca11, and Aca14, respectively (Table 1). Of these 27 alleles, 25 (92.6%) were detected on Ani-jima, 26 (96.3%) were detected on Chichi-jima, and 24 (88.9%) were detected on Haha-jima (Table 1). Most alleles were common to all three islands, although some rare alleles were restricted to individual populations (Table 1). The four sampling sites on Ani-jima were within a small area (Fig. 1) and the sample size was insufficient at two sites (Table 1); therefore, all individuals were combined for the subsequent genetic analyses for Ani-jima. The observed and expected heterozygosities ranged from 0.182 to 0.983 and 0.165 to 0.827, respectively, and no significant LD or deviation from HWE was identified after the Bonferroni corrections (Table 2). AMOVA revealed that most of the variance (92.5%) was explained by within-individual variation (but insignificant statistically) and the variance explained by differences among populations (5.4%) was only significant (Table 3). The mean F_{ST} calculated for all loci was 0.029 (99% confidence interval 0.008–0.039). Pairwise multilocus F_{ST} between populations revealed that the frequency distributions of alleles at the five loci were similar within-island populations but differed among the three islands, although no significant differences were observed between

Table 2. Number of individuals (N), number of alleles (N_A), and observed (H_O) and expected (H_E) heterozygosities of five microsatellite loci for *Anolis carolinensis* introduced to the three Ogasawara islands.

Island	Site		Acar1	Acar4	Acar9	Acar11	Acar14
Ani-jima	All	N_A	2	3	5	9	6
	(N = 59)	H_O	0.373	0.61	0.932	0.983	0.797
		H_E	0.324	0.488	0.724	0.761	0.764
Chichi-jima	Miyanohama	N_A	2	3	4	8	6
	(N = 25)	H_O	0.52	0.64	0.68	0.8	0.8
		H_E	0.385	0.518	0.627	0.791	0.713
	Ougiura	N_A	2	3	5	9	6
	(N = 24)	H_O	0.542	0.667	0.708	0.917	0.958
		H_E	0.395	0.486	0.69	0.827	0.78
	Kominato	N_A	2	3	3	8	6
	(N = 22)	H_O	0.182	0.727	0.545	0.773	0.864
		H_E	0.165	0.525	0.595	0.758	0.773
	All	N_A	2	3	5	10	6
	(N = 71)	H_O	0.423	0.676	0.648	0.831	0.873
		H_E	0.333	0.518	0.642	0.806	0.78
Haha-jima	Kitako	N_A	2	4	3	8	6
	(N = 27)	H_O	0.222	0.593	0.37	0.704	0.704
		H_E	0.198	0.504	0.497	0.759	0.779
	Minamizaki	N_A	2	3	3	8	6
	(N = 25)	H_O	0.32	0.44	0.52	0.84	0.84
		H_E	0.269	0.367	0.414	0.809	0.766
	All	N_A	2	4	3	9	6
	(N = 52)	H_O	0.269	0.519	0.442	0.769	0.769
		H_E	0.233	0.443	0.463	0.805	0.775

Table 3. Analysis of molecular variance (AMOVA) of five microsatellite loci in the six *Anolis carolinensis* populations.

Source of variation	Sum of squares	Variance components	Percentage variation (%)	P-value
Among populations	462.96	1.21	5.4	< 0.001
Among individuals within populations	3802.09	0.47	2.1	0.305
Within individuals	3760	20.66	92.5	0.055
Total	8025.05	22.34		

Table 4. Pairwise F_{ST} estimates among six populations of *Anolis carolinensis* (no asterisks P > 0.05; * P < 0.05, ** P < 0.01).

	Ani-jima	Chichi-jima			Haha-jima	
		Miyanohama	Ougiura	Kominato	Kitako	Minamizaki
Ani-jima	–	0.042**	0.022*	0.022**	0.039**	0.052**
Miyanohama		–	0.016	0.012	0.034*	0.046**
Ougiura			–	0.008	0.023*	0.029**
Kominato				–	0.002	0.017
Kitako					–	0.007
Minamizaki						–

Kominato population and any other populations on Chichi-jima and Haha-jima, after the Bonferroni corrections (Table 4). For the genetic structure obtained with STRUCTURE, ΔK calculated for K = 2–7 was highest at K = 2, and the proportion of two genetic clusters of individuals seemed to be similar within island but different among islands (Fig. 2).

DISCUSSION

Divergent mitochondrial haplotypes of the 16S rRNA and ND2 genes are reported in the United States where *A. carolinensis* is native (Glor et al., 2005; Campbell-Staton et al., 2012; Tollis et al., 2012; Tollis and Boissinot, 2014). In comparison, only two haplotypes of the 1448-bp 16S/ND2 sequences were detected in Ogasawara, suggesting a strong bottleneck at the first introduction to Chichi-jima (Hayashi et al., 2009). Moreover, the fact that a single haplotype, the dominant type on Chichi-jima, was found on Haha-jima (Hayashi et al., 2009) and on Ani-jima (this study) suggests the restricted gene flow in secondary transfer from the source Chichi-jima population to the other two islands.

The microsatellite analysis in this study revealed that the genetic variation among individuals seems to be sufficient to compare fine-scale population genetic structures of *A. carolinensis* in Ogasawara. The obtained results showed no clear genetic differences within-island populations. These islands are all small and have low elevations: respectively, 7.9 km^2 and 254 m for Ani-jima, 23.8 km^2 and 326 m for Chichi-jima, and 20.8 km^2 and 462 m for Haha-jima. Although the natal dispersal and lifetime movement distances of individual *A. carolinensis* have not been reported, gene flow seems to occur rapidly over an island. However, analyses of pairwise multilocus F_{ST} and STRUCTURE revealed that the frequencies of alleles at the five microsatellite loci differed among the three islands. Between Chichi-jima and Haha-jima (45 km apart, both are inhabited), human-mediated transfer may occur through the release of individuals reared as pets, or through the escape of unintentional contaminants in cargo. In contrast, naturally occurring drift by oceanic currents, animals, and others may be more important than human-mediated transfer in the invasion from Chichi-jima to Ani-jima, because Ani-jima is uninhabited and the minimum distance from Chichi-jima is only 0.5 km. For example, Chiba (2014) suggests the possibility of dispersion by green anole individuals captured by the Ogasawara buzzard *Buteo buteo toyoshimai* and given as prey to egg-incubating females or nestlings, in cases in which the anoles manage to escape.

It has been suggested that invasion of Haha-jima is attributable to human-mediated transfer in the early 1980s (Miyashita, 1991). If the invasion to Haha-jima occurred

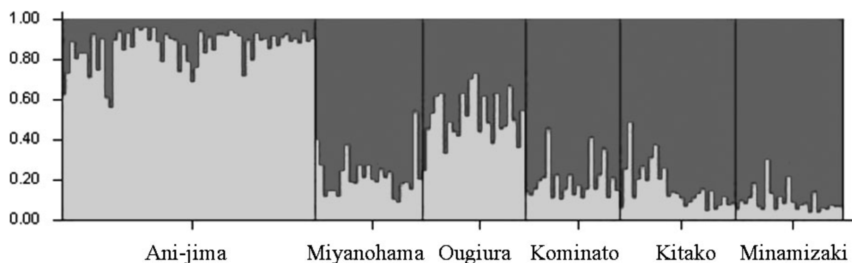

Fig. 2. Genetic clusters obtained from the STRUCTURE analysis (K = 2) for all 182 *Anolis carolinensis* from six populations on three islands (Ani-jima; Miyanohama, Ougiura, and Kominato on Chichi-jima; Kitako and Minamizaki on Haha-jima). Individuals are represented with vertical lines with the estimated proportion of two genetic clusters.

only once, several individuals may have been brought to this island, because the present genetic variation of microsatellite loci cannot be derived from only a single pregnant female or single mating pair. On the other hand, the mode of introduction to Ani-jima is unknown. Of 25 alleles confirmed, three alleles (148 and 156 of Acar9 and 197 of Acar11) were common to Ani-jima and Chichi-jima, but not found on Haha-jima, and no alleles were common to Ani-jima and Haha-jima, but not found on Chichi-jima (Table 1). This suggests that the Ani-jima population was derived from the Chichi-jima population. Immigration may have involved several individuals at the same time or repeated events, as the genetic diversity was rather high as in the case of Haha-jima. However, another factor may also have increased the genetic diversity of the newly established populations. Female *Anolis* are able to store sperm for several months after mating with several males and produce offspring with multiple paternities (Eales et al., 2008). On Chichi-jima, female *A. carolinensis* lay only one egg at a time, but continue to lay 0.95 eggs per week from April to October, or 13.7 eggs annually on average (Toda et al., 2013). This reproductive feature might contribute to greater gene flow than that expected from the immigration frequency alone.

The diversity and abundance of insects are greatly reduced on Chichi-jima and Haha-jima where *A. carolinensis* has become established, compared to islands without *A. carolinensis* (Abe et al., 2008; Karube, 2009). The condition of the fauna on Ani-jima is still good, and the Japanese and Tokyo governments started an *A. carolinensis* population control program immediately after finding it on the island (Okochi, 2013; Shimizu, 2013). To protect the fauna on this island from strong predation pressure, *A. carolinensis* is being excluded where possible, and a plan is in place to surround a protected area with a fence that this lizard cannot cross. The present results will provide valuable information in the genetic management to control and prevent further invasion of this alien lizard in the Ogasawara Islands.

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