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Chapter 14

Genetic Diversity and Population Structure of the Hotoke Loach, *Lefua echigonia*, a Japanese Endangered Loach

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Additional information is available at the end of the chapter

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1. Introduction

In Japan, conservation and regeneration projects have been actively conducted for large-sized birds such as the Japanese crested ibis, *Nipponia Nippon*, the oriental white stork, *Ciconia boyciana* and the intermediate egret, *Ardea intermedia* (Photo 1) that inhabit rural areas [1, 2]. Many people are highly interested in these projects and a lot of information about growth and breeding for large-sized birds is broadcasted through television, radio and internet media. In such a situation, a conspicuous topic has been found in recent months, that is, 2 individuals of the Japanese crested ibis displayed beriberi symptom along with human being, because of overeating great favorite food that is the Dojo loach, *Misgurnus anguillicaudatus*. Their beriberi symptom appeared to be caused by eating the Dojo loach raw. The 2 individuals were diagnosed as follows; this beriberi symptom occurred as vitamin B1 in the individual bodies was destroyed by tiaminase enzyme contained in the Dojo loach. At present the two individuals may have completely recovered from the beriberi symptom through vitamin B1 supplementation by injection.

By the way, the presence of 10 or more loach species including the Dojo loach has been observed around paddy fields in rural areas, Japan. Most loach species appear to become food attractive for large-sized birds (Photo 1) [3] and one of the reasons is that the loach species cannot move as rapidly as swimming species such as the Japanese dace, *Tribolodon hakonensis* and the Ayu, *Plecoglossus altivelis altivelis*; hence large-sized birds are able to easily catch them. In addition, only the Dojo loach has been investigated, but nutrition contained in this loach was superior to other fish species; for instance, amount of calcium

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in the Dojo was 9 times that of the Japanese ell, *Anguilla japonica*, and also the Dojo had the most amount of vitamin B2 in all fish [4, 5]. Actually these precise nutrient components may somewhat differ among the loach species, but their nutrient components could have to be fundamentally similar.



Photo 1. 2 individuals of the intermediate egret, *Ardea intermedia* that are finding individuals of many loach species as their food in paddy field (unpublished photo)

However, some of the loach species have confronted a kind of serious concerns, especially a decrease in their population size. In Japan, we have conducted many land consolidation projects for rising rice production and easing agricultural works in rural area since 1960s. In land consolidation projects, concrete canals, drops, diversion weirs, etc. have been installed around paddy fields as agricultural infrastructures; therefore not only fish populations and their habitats but also all of ecosystem and biodiversity in rural area have been extremely damaged [6-9].

The Hotoke loach, *Lefua echigonia* endemic to Japan (the above in Photo 2) has been well known as a representative loach species has been adversely impacted on its habitat due to land consolidation projects. Since populations of this loach have rapidly declined in some rural areas, consequently the Hotoke loach has been designated as an endangered species on the Red List of Japan [10]. Ecology of the loach is briefed as follows; this species is widely distributed across the Honshu Island from the Tohoku region to the Kinki region. They usually inhabits earth canals and ditches around paddy fields into which ground water flows (the bottom in Photo 2) [11, 12]. The Hotoke loach often coexists with the Dojo loach in the habitat and geographic variations for this loach based on morphological characteristics is obscure [13, 14].

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Photo 2. An adult of the Hotoke loach, *Lefua echigonia* (the above) with approximately 60 mm in body length and typical earth ditch (the bottom) where the loach inhabits around paddy field (unpublished photo)

In recent years, because importance of ecosystem and biodiversity in rural areas has been deeply realized, various research activities have been carried out for conserving and recovering populations of the Hotoke loach. Distribution pattern and habitat characteristics of this loach were elucidated in some rural areas [15-18], manners of habitat utilization and migration routes for the species were investigated [11, 12, 19, 20] and techniques of artificial propagation were developed with human chorionic gonadotropin [21-23]. Further, molecular analyses of phylogeography of the Hotoke loach using DNA sequences of mitochondrial genes revealed that populations of the species were evolutionally separated into a total of 7 genetic clades in Japan [24-29].

Unfortunately, there is also another serious concern left in populations of the Hotoke loach. That is, as this loach has experienced, diminishment of population size may often cause to improve not only fragmentation among populations but also inbreeding among individuals. Such populations tend to have distinctly poor genetic diversity, occasionally threatened with extinction [30-33]. Usually, to evaluate genetic diversity including genetic population structure for such populations, polymorphism analysis has been performed using microsatellite loci in nuclear genome [33-35]. Only preliminary investigations, however, were implemented for populations the Hotoke loach [36-38], although microsatellite analyses have been carried out for populations of several endangered species.

Genetic properties of microsatellite loci are briefed as follows (Fig. 1). These loci are repeating sequences of 2 to 6 base pairs of DNA, for instance CACA…, CTCTCT… and CATCAT… Microsatellites that are typically neutral and co-dominant are used as molecular markers in genetics for kinship, population and other studies, because of often presenting high levels of inter- and intra-specific polymorphism [33-35]. Especially, CA nucleotide repeats appear to be very frequent in human and other genomes and present every few 10,000 to 100,000 base pairs. A repeat size in a locus is treated as an allele and a pair of repeat sizes which are inherited from both of parents is used as genotypes at a locus for a diploid organism. Heterozygous describes a genotype consisting of two different sizes (alleles), while homozygous does it consisting of two identical ones (Fig. 1).

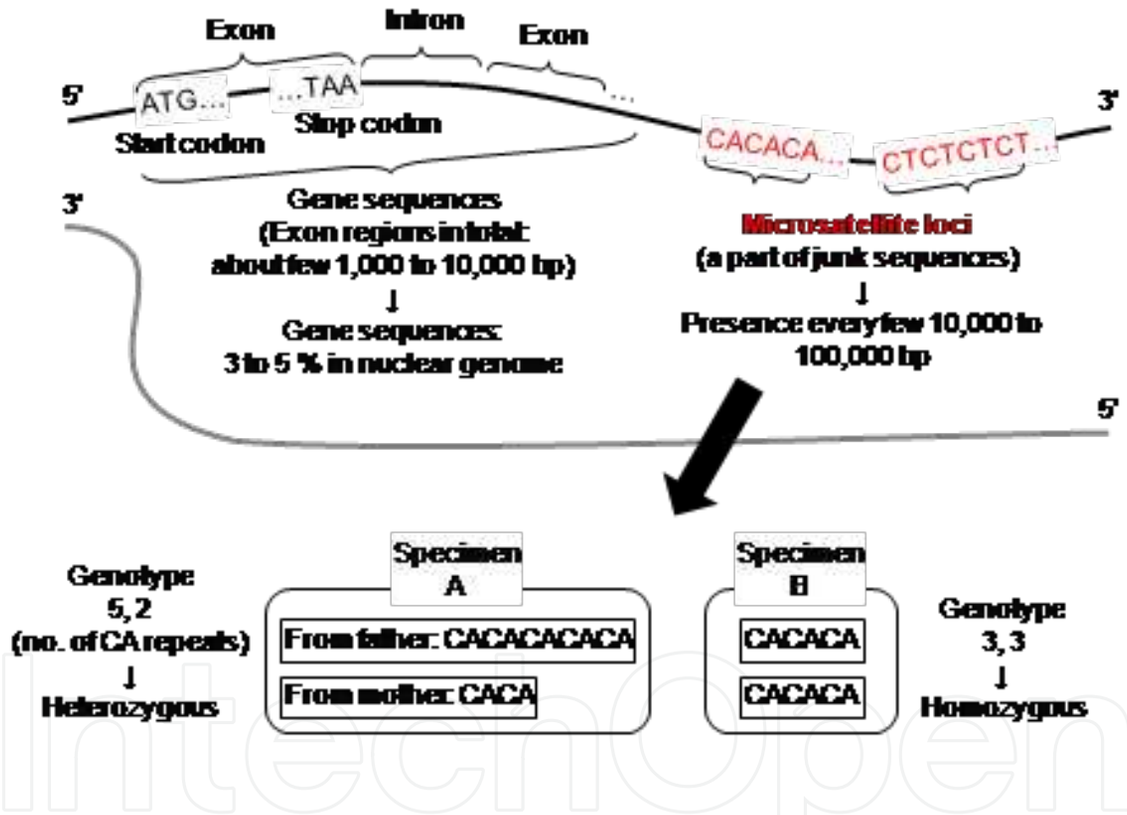


Figure 1. Scheme of microsatellite loci in nuclear genome DNA (unpublished figure)

In this chapter, to detect the existence of the above serious genetic issues, we carried out a series of analysis for genetic diversity and population structure in population of the Hotoke loach (Fig. 2). Novel microsatellite loci applied in this loach were developed and characterized in Section 2. Using these developed loci, genetic diversity and population structure were investigated for populations in the upper Kokai River along with adjacent rivers, the southeast part of Tochigi Prefecture as a case study in Section 3. Technical terms related to population and conservation genetics are often used in the sections; thus, details of meanings of these terms are able to be known by references cited in the end of this chapter.

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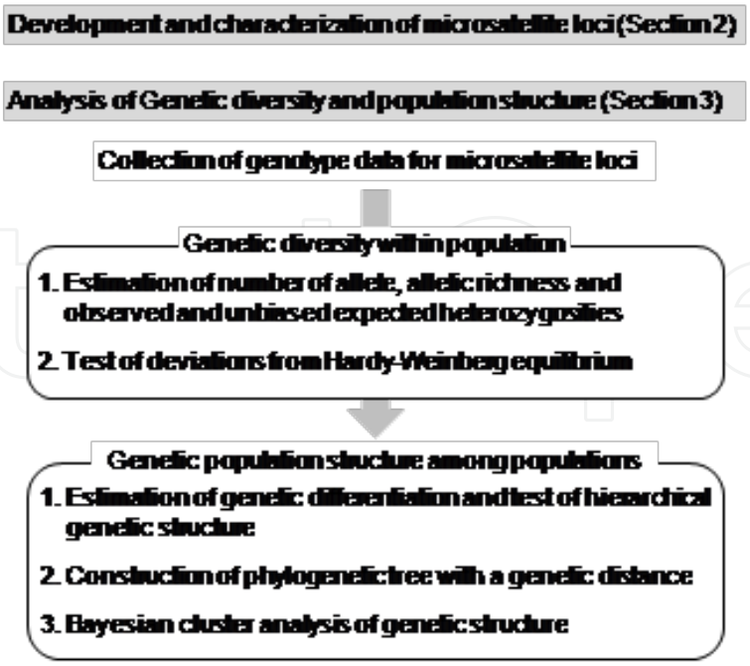


Figure 2. Analysis flow chart of microsatellite loci in this chapter (unpublished figure)

2. Development and characterization of microsatellite loci

2.1. Development of microsatellite loci

In Section 2, a total of 19 novel microsatellite loci for the Hotoke loach were isolated with an individual obtained in the Shitada R., Chiba Pref. and characterized using 32 individuals collected from the Koise R., Ibaraki Pref. The following development procedure [36] is partially improved based on the latest studies [39, 40].

A sample of this loach was collected from an agricultural canal in the Shitada R., Chiba Pref. in 2005 and preserved in 99% EtOH, and then stored at -30 °C. Genomic DNA was extracted from single caudal fin clip, approximately 5 mm × 5 mm, using a standard phenol-chloroform procedure [41]. Microsatellite enriched libraries were developed following the previous study [42] with some modifications. Briefly extracted DNA was digested with RsaI (New England Biolabs) and then ligated to SuperSNX linkers (SuperSNX24 Forward: 5′-GTT TAA GGC CTA GCT AGC AGA ATC-3′ and SuperSNX24+4P Reverse: 5′-phosphate-GAT TCT GCT AGC TAG GCC TTA AAC AAA A-3′). Linker-ligated DNA was enriched for microsatellites using streptavidin-coated magnetic beads (Dynal) treated with a blocking step [43] and using the pooled biotinylated probes $(CA)_{12}$ and $(CT)_{12}$.

Recovered DNA was amplified by the polymerase chain reaction (PCR) and PCR products were cloned using a TOPO-TA Cloning Kit (Invitrogen) following the manufacture's protocol. A total of 192 positive clones were sequenced on a 3130*xl* Genetic Analyzer (Applied Biosystems; ABI) using BigDye Terminator kit version 3.1 (ABI) and resultant sequences were

proofread for repeat regions using the software DNA BASER version 3.2 (Heracle BioSoft). Oligonucleotide primers (Table 1) were designed in flanking regions of the 19 targeted microsatellite loci using the software DNASIS PRO version 3.0 (Hitachi Software Engineering).

Locus	Primer sequence (5'-3')[a]	Repeat motif	Dye	GenBank accession no.
Lec01	F: M13-ATC CCT CCC TTC ACC GTC TG R: TCC GAA ACC AGC AGC ACC AC	$(CA)_{13}$	6-FAM	AB286032
Lec02	F: M13-TGT GCT GTA GGA TTG CTT GAG C R: ATG TCA GAG GCT GAT GG GAT AC	$(CA)_{30}AA(CA)_5$	VIC	AB286033
Lec03	F: M13-CGT CCA CCA GCC TTA CGA AC R: TGA CGC TCA GTA GTC GGA CC	$(CA)_{14}CG(CA)_3$	6-FAM	AB286034
Lec04	F: M13-GCA CTG CTG ATG ACA ATC ATT G R: GCT TTG GGT TAG AAC ATC AGT G	$(GA)_{29}$	6-FAM	AB286035
Lec05	F: M13-TGT CTG CTG TGA TGA TGA CAT C R: CTC ACA GCA CTA TTC ACT GAT G	$(GT)_{13}$	NED	AB286036
Lec06	F: M13-CCG TGT CTG TTT TGC TTT CTC R: CTC CCT TCA CAA AGT AAC TGG	$(CT)_{10}$	PET	AB286037
Lec07	F: M13-TGT GAA GAA ACC TGA ACA CGC R: ATT CTG TGT CCC TGA ACA CAC	$(CT)_7(GT)_{11}$	NED	AB286038
Lec08	F: M13-GAC GCA ACA ATC TCA GGG TC R: ACA GGA CCA AGT GGA CTC TC	$(GA)_5AA(GA)_8$ $AA(GA)_{17}$	6-FAM	AB286039
Lec09	F: M13-GGG GAT AGT GGA GAT GGG TG R: TTC ATC CCT CTT CCG CCC AC	$(GA)_{14}$	PET	AB286040
Lec10	F: M13-GGT TGG CAA TGC CAG CAA TG R: TGC TTT ACC AAG GTG ACG GC	$(GT)_7$	6-FAM	AB286041
Lec11	F: M13-CTG ACA CTG TGT GTG TAG CAG R: GGT TTC ACC TGG TCC ATA CAC	$(GT)_{11}$	NED	AB286042
Lec12	F: M13-GGC ACC AAA GGC AGA TTT TAC R: AGA GTG TGA GAT TAT GGC AGC	$(CT)_{14}CA(CT)_2$ $(CA)_6$	VIC	AB286043
Lec13	F: M13-GAC GCC ACG ACA AGA CGA AC R: TAT GTG TGG AGG GGG GTG AG	$(CT)_{21}$	NED	AB286044
Lec14	F: M13-ATT AGG AGC ATT ACC CAA CAG C R: CAA AGG AAG CAA AAA CAA GGG C	$(GT)_7$	NED	AB286045
Lec15	F: M13-GAG CAA GAG GTG TGT GCT TC R: TGC TGG TTC ACG CTC TAC AC	$(GT)_{11}$	PET	AB286046
Lec16	F: M13-CAC ACT AAC ACT TCT CCA GCG R: CAC AGT GAC CAA AGT CAC CAG	$(CA)_{10}$	6-FAM	AB286047
Lec17	F: M13-GTC CCC ATA AAA CAG GAA ACC C R: GAC TAT TGA GTG AGT GCC ACA C	$(GT)_7GCGTGG$ $(GT)_5$	VIC	AB286048
Lec18	F: M13-CGA CCA TCT TCT GGG GTT ACG R: CCT CGG ATG GGC TAA ATG ACC	$(GT)_9$	NED	AB439725
Lec19	F: M13-CTG TGT GTG GGT GTA TCT GAA C R: AAA GTG GCT CTT CTT CTG CTG G	$(GT)_6$	PET	AB439726

[a] Sequence of the M13 tails on forward primers: GCC AGT CAC GAC GTT GTA

Table 1. Characterization of 19 polymorphic microsatellite loci for 32 individuals of the Hotoke loach. Loci with gray color were used in analysis of genetic diversity and population structure in Section 3. (Modified from one of previous study [36])

2.2. Characterization of microsatellite loci

Each microsatellite locus was characterized for polymorphisms among 32 individuals obtained the Koise R., Ibaraki Pref. in 2006. DNA of the individuals was extracted using an automated DNA isolation system (GENE PREP STAR PI-80X, KURABO) following the manufacturer's instructions. PCR amplifications were performed on the 32 DNA extracts across all loci using 10 μl reaction volumes containing approximately 10 ng DNA template, 0.5 U Taq DNA polymerase (BIOTAQ, Bioline), 1×NH_4 buffer (BIOTAQ), 2.5 mM $MgCl_2$, 0.25 mM each dNTP, 0.03 μM M13-tailed forward primer, 0.25 μM reverse primer and 0.25 μM labeled M13 primer (5'-GCC AGT CAC GAC GTT GTA-3') [44]. The M13 primer was labeled at the 5' end with 6-FAM, VIC, NED or PET fluorescent dyes (ABI, Table 1).

Thermal profiles on iCycler and C1000 (both of Bio-Rad) of thermal cyclers were as follows. Initial denaturation at 94°C for 2 min was followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 15 s and extension at 72°C for 30 s. A single final extension at 72°C was done for 30 min. PCR products were resolved on a 3130*xl* Genetic Analyser with GeneScan 500 LIZ size standard (ABI). Electropherograms were analyzed with the software GENEMAPPER version 4.0 (ABI).

Measures of genetic diversity, tests for deviations from Hardy-Weinberg equilibrium (HWE) and estimates of linkage disequilibrium (LD) between loci were calculated using the software GENEPOP on the web version 4.0.10 [45]. The possible presence of null alleles was assessed with the software MICRO-CHECKER version 2.2.3 [46].

All the 19 loci were polymorphic (Table 1). The number of observed alleles per locus ranged from 2 to 9. The observed heterozygosity ranged from 0.125 to 0.844, while the expected heterozygosity varied from 0.148 to 0.876. No significant deviations from HWE or signs of LD were observed after sequential Bonferroni correction with the significant level at 0.05 [47] and there was no evidence of null alleles in any of the tested loci. Consequently, the high level of polymorphisms observed in these microsatellite loci may have to support future investigations to improve our knowledge of the genetic differentiation and genetic structure of populations of the Hotoke loach.

3. Analysis of genetic diversity and population structure

3.1. Study sites

In Section 3, genetic diversity and population structure of populations of the Hotoke loach in the upper Kokai R. including 4 adjacent rivers, the southeast part of Tochigi Pref. (Fig. 3) was detailed using the microsatellite loci developed in Section 2 (Table 1). As mentioned in Section 1, populations of the Hotoke loach have been often diminished and isolated by land consolidation projects in rural areas. Therefore it appears difficult to find populations distributed with a certain area. However, rich biota still continues to exist in the upper Kokai R. due to delay of land consolidation. This area sounds attractive for field scientists, and then their some activities were carried out to conserve and recover such a sound rural ecosystem

[48-52]. According to the results of these studies [48, 49], the populations of the Hotoke loach tended to be distributed in the upper zone of hill-bottom valleys in this area and also a negative correlation was observed between the population size and water temperature.

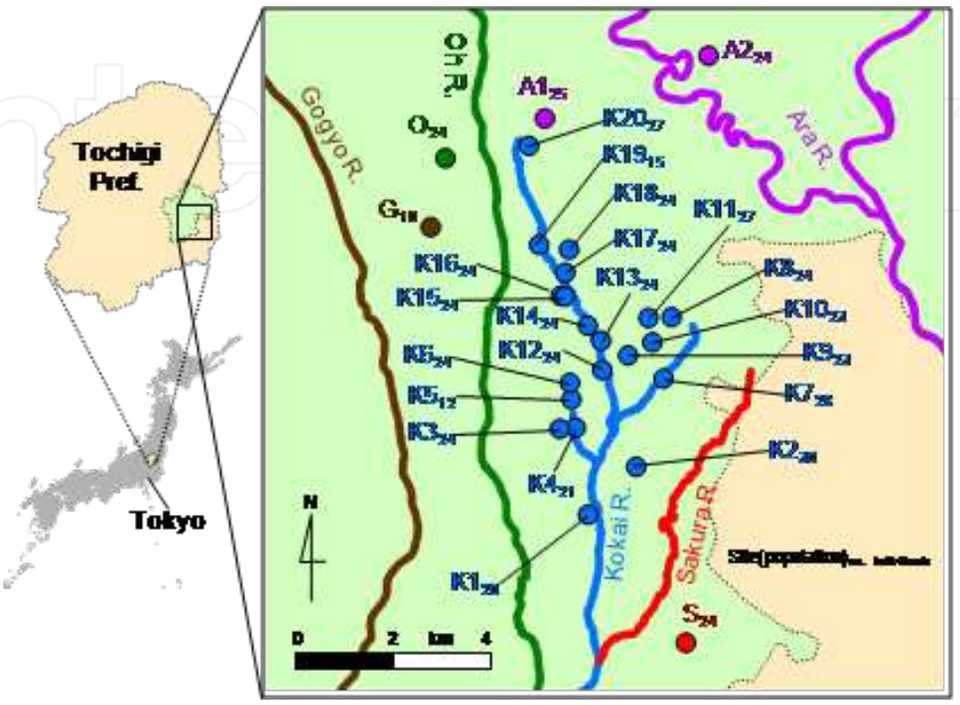


Figure 3. Collection sites for individuals of populations of the Hotoke loach in the upper of Kokai River (K1 to K20) along with adjacent the Oh, Sakura, Gogyo and Ara Rivers (O, S, G and A1, A2, respectively), the southeast part of Tochigi Prefecture (unpublished figure).

Considering such spatial distribution patterns in the previous studies [48, 49] and geographical conditions in this area, a total of 20 sites were established to collect individuals of the populations in the upper Kokai R. (K1 to K20 in Fig. 3). Additionally 5 collection sites of adjacent 4 rivers that are the Oh, Sakura, Gogyo and Ara (O, S, G and A1, A2, respectively in Fig. 3) were decided to compare with the populations of the Kokai R.

3.2. Sample collection

Sample collections in each site (Fig. 3) were performed using hand nets with reticulation at 2 mm, flame width at 30 to 40 cm in August 2007 to June 2008 (Photo 4). 10 to 24 individuals (a total of 573 individuals) of each population were collected in earth canals and ditches with water depth of 2 to 24 cm, water width of 15 to 110 cm, flow velocity of 5 to 25 cm/s and substrates consisting of silts, sands and gravels. There were no rain during the sample collections and a part of the caudal fin (3 mm × 3 mm) of each individual was removed and preserved in 99.5% EtOH at the sites, and then all individuals were immediately released alive. The preserved caudal fins were kept at -30 °C and the mean ± standard deviation in body length for all individuals was 46 ± 11 mm.

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Photo 3. Collection of individuals of the Hotoke loach in an earth ditch at the site K9 in the Kokai River (unpublished photo)

3.3. DNA chemical analysis

Total genomic DNA from the preserved caudal fins of each individual was extracted using an automated DNA isolation system following the manufacturer's instructions, and kept at 4 °C after being diluted to 10 ng/μl.

The microsatellite DNA analysis were performed using the following 11 loci that are *Lec01*, *Lec05*, *Lec06*, *Lec08*, *Lec12*, *Lec14*, *Lec15*, *Lec16*, *Lec17*, *Lec18* and *Lec19* with gray color in Table 1. These loci were confirmed to be appropriate for investigating the populations in the Kokai R. in the preliminary studies [37, 38]. In accordance with the procedure in Section 2, microsatellite amplification with PCR on iCycler and C1000 of thermal cyclers was conducted in 10 μl reaction volumes containing approximately 10 ng DNA templates. PCR products were electrophoresed on a 3130*xl* Genetic Analyzer with GeneScan 500 LIZ of size markers and the electrophoregrams were analyzed with the GENEMAPPER. Consequently genotype data composed of a pair of fragment sizes, which are inherited from both of parents and depends on length of repeat motif, was obtained for each individual in a PCR product of a locus. All genotype data were compiled in the software THE EXCEL MICROSATELLITE TOOLKIT [53].

3.4. DNA data analysis

3.4.1. Genetic diversity within population

The genetic diversity within the populations of the Hotoke loach in each collection site was evaluated with the genotype data of the 11 loci for all individuals. The number of allele (N_A) and allelic richness (A_r) [54], where bias caused by population size (the number of individuals) is removed from N_A, were estimated using the software GENALEX version 6.41 [55] and FSTAT version 2.9.3 [56], respectively. Differences of N_A and A_r among the populations were

tested by one-way analysis of variance (ANOVA) using the software EKUSERU-TOUKEI 2010 (Social Survey Research Information Co., Ltd.).

The observed and unbiased expected heterozygosities (H_O and H_E, respectively) [57] were calculated by GENALEX [55]. The software ARLEQUIN version 3.11 [58] was used to test deviations from Hardy-Weinberg equilibrium with Fisher's exact probability test, which was run through 100,000 iterations using the Markov chain Monte Carlo (MCMC). Significance values ($\alpha = 0.05$) of a multiple test were corrected following the sequential Bonferroni procedure [47]. Significant differences of H_O and H_E among the populations were detected by one-way ANOVA using EKUSERU-TOUKEI 2010.

3.4.2. Genetic population structure among populations

Genetic population structure among the populations in the Kokai R. including 4 adjacent rivers was elucidated with three analytical methods based on the assumption that mutation of alleles in each locus confirmed to an infinite allele model [59, 60].

First, genetic differentiation between the populations was evaluated with classical pairwise F_{ST} statistics [61] using ARLEQUIN [58]. Statistical significance ($\alpha = 0.05$) for values of F_{ST} was tested with applying 10,000 permutations, followed by sequential Bonferroni corrections [47] and these values were graded on four classifications for genetic differentiation in the previous study [62]. An analysis of molecular variance (AMOVA) [63] for F_{ST} was performed to estimate hierarchical genetic structure across the populations. In this AMOVA, the populations were divided into 2 to 6 groups according to geographical condition such as rivers and the distances among collection sites. And then variances among groups, among populations within groups, among individuals within populations and within all individuals were computed for 3 cases of genetic structure using GENALEX [55] with 10,000 permutations.

Second, a phylogenetic tree of a genetic distance D_A [64] between the populations was constructed with the neighbor-joining method [65] and the reliability of the obtained phylogenetic tree was evaluated using the aid of 1,000 bootstrap replicates [66]. The software POPULATIONS version 1.2.31 [67] was used to estimate D_A and to construct a phylogenetic tree and an appropriate shape of the phylogenetic tree was edited with the software MEGA version 5.05 [68].

Finally, Bayesian cluster analysis [69-73] that has been recently used as a popular method was implemented in the software STRUCTURE version 2.3.3 [70] to circumstantially investigate the occurrence of genetic structure among the populations without the prior identification of populations. Briefly, this analysis allows the inference of the number of genetically homogeneous clusters (K) that are implicitly genetic populations from individual genotypes at multiple loci and also assignment probability (Q) of individuals to each genetic cluster. The admixture model and correlated allele frequencies model were used along with LOCPRIOR model [74] and the software was run with 20 repetitions of 500,000 iterations of MCMC, following a burn-in of 500,000 iterations at K of 1 to 10.

The most likely number of genetic clusters was evaluated using the rate of change in the log probability between the values of successive K [75]. Distribution of the values of Q across

runs for each cluster were organized using the software STRUCTURE HARVESTER web version 0.6.92 [76] and then summarized using the software CLUMPP [77]. When individuals had the values of Q more than 0.7, they were assigned to be members of that particular cluster in this study. And also K usually appears to show the genetic structure at the uppermost hierarchical level [75]. Therefore, when a particular cluster was formed by some populations, additional analysis of each cluster was performed to investigate the detailed genetic structures after the first analysis.

3.5. Results and discussions

3.5.1. Genetic diversity within populations

All the 11 microsatellite loci were moderate to highly polymorphic, with the number of alleles (N_A) and observed and unbiased heterozygosities (H_O and H_E, respectively) per locus for all individuals ranging from 2 (*Lec19*) to 40 (*Lec06*) and from 0.147 (*Lec17* and *Lec19*) to 0.846 (*Lec05*) and from 0.155 (*Lec17*) and 0.915 (*Lec08*), respectively. Such a polymorphic level observed in these loci indicated to be beneficial to investigating genetic characteristics of populations in detail.

Means of N_A per locus in the populations varies from 4.5 (Population A2, hereafter Pop A2) to 8.0 (Pop K11). Allele richness (A_r) per locus was standardized by the minimum size of the population (10 individuals of Pop G) and its means per locus varied from 3.7 (Pop A2) to 5.6 (Pop K11) among populations (Fig. 4). The one-way analysis of variance (ANOVA) showed that significant differences of the means of both N_A and A_r were not confirmed among populations ($F_{24, 250} = 0.641$, $M_{SE} = 11.937$, $p > 0.05$ for N_A and $F_{24, 250} = 0.459$, $M_{SE} = 4.803$, $p > 0.05$ for A_r).

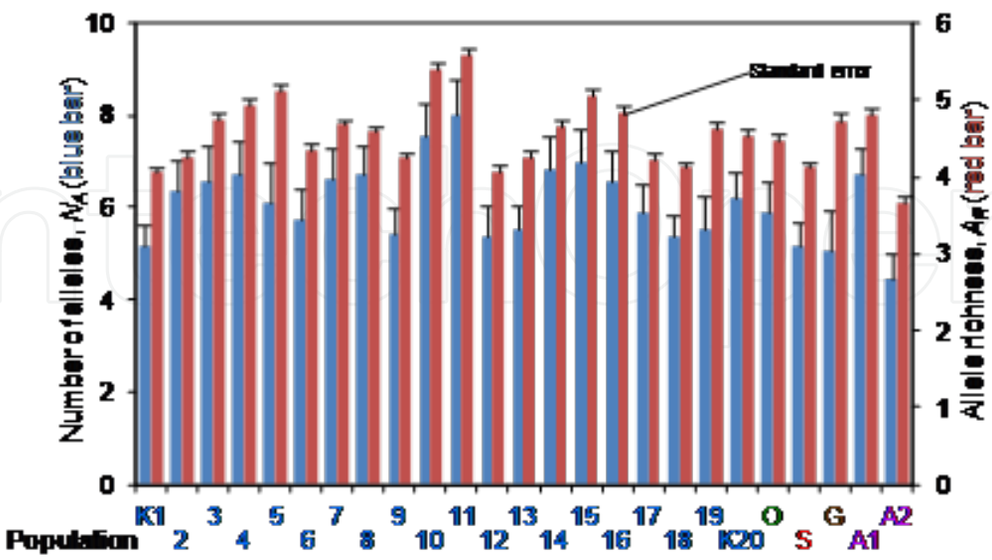


Figure 4. Means and standard errors of the number of alleles (N_A) and allelic richness (A_r) per locus in the populations (unpublished figure). A_r was standardized by the minimum size of the population (10 individuals of Population G) and there were no significant differences among the populations for both N_A and A_R ($p > 0.05$).

Means of the observed and unbiased expected heterozygosities (H_O and H_E, respectively) per locus across all population ranged from 0.418 (Pop A2) to 0.669 (Pop K11) and from 0.507 (Pop A2) to 0.674 (Pop K11), respectively (Fig. 5). Significant departures from the Hardy-Weinberg equilibrium (HWE) were not observed in all the populations. This result indicated that the populations could be applied to the following analyses of genetic population structure, because most analyses are often performed under the assumption that population conforms to HWE. The results of one-way ANOVA showed that there were no significant of the differences among the populations for both H_O and H_E ($F_{24, 250} = 0.377$, $M_{SE} = 0.090$, $p > 0.05$ for H_O and $F_{24, 250} = 0.207$, $M_{SE} = 0.079$, $p > 0.05$ for H_E).

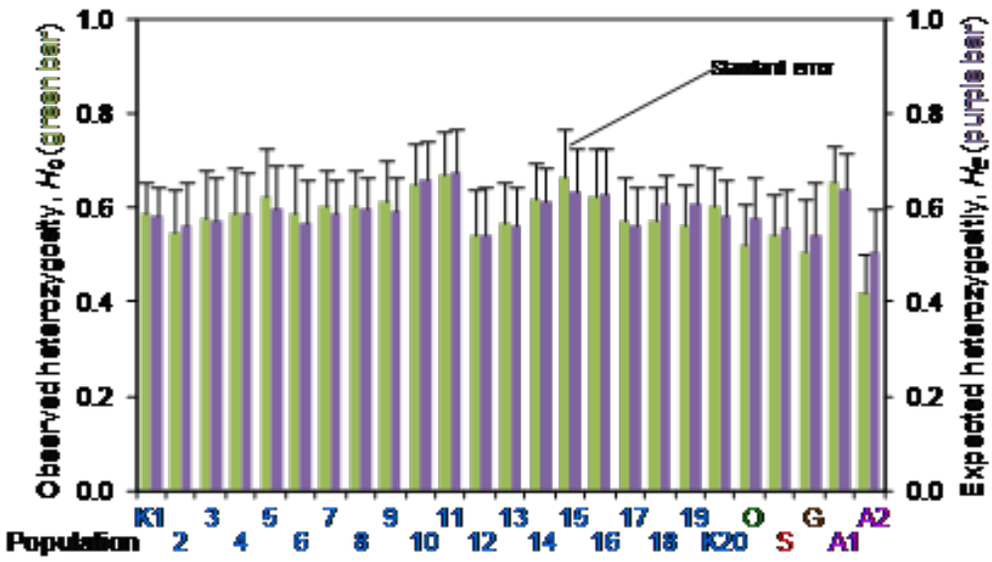


Figure 5. Means and standard errors of the observed and unbiased expected heterozygosities (H_O and H_E, respectively) per locus in the populations (unpublished figure). There were no significant differences among the populations for both H_O and H_E ($p > 0.05$).

Genetic diversity of the populations appeared not to degrade. Generally, when population size is small, inbreeding among individuals appears to progressively occur in a population [30-33] as mentioned in Section 1. It has been observed that such populations had low values of N_A, H_O and H_E [33]. For instance, means of N_A per locus for the Ethiopian wolf, *Canis simensis*, the Mauritius kestrel, *Falco punctatus* and the Northern hairy-nosed wombat, *Lasiorhinus krefftii* which are designated as worldwide endangered species, were only 2.4, 1.4 and 2.1, respectively. Means of H_E for the Ethiopian wolf, the Mauritius kestrel and the Northern hairy-nosed wombat were also 0.21, 0.10 and 0.32, respectively [78]. But then, values of representatively common freshwater fish species inhabiting agricultural canals and ditches in rural area, Japan such as the Dojo loach, the Field gudgeon, *Gnathopogon elongates elongatus* and the Amur goby (orange type), *Rhinogobius* sp. OR ranged from 3.3 to 17.7 (both of the Amur goby) for means of N_A per locus and from 0.463 (the Dojo loach) to 0.905 (the Field gudgeon) for means of H_E per locus [79-81].

Comparing with these values for the endangered and common species, the means of N_A and H_E per locus (4.5 to 8.0 and 0.507 to 0.674, respectively) observed in the populations indicated to be

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in relatively moderate level. Hence, a serious concern for genetic diversity could not occur in the populations at present. However, there are no confident that such a level of genetic diversity would be sustaining in the future. Monitoring genetic diversity may need including ordinary biological investigation such as an estimation of size and age composition of populations.

3.5.2. *Genetic population structure inferred from F_{ST}*

The lowest and highest values of F_{ST} were observed between Pops K15 & K16 and between Pops K18 & A2 (F_{ST} = 0.008 and 0.246, respectively, Fig. 6). The permutation test showed that all the F_{ST} were significantly different from zero (p > 0.05), except the lowest F_{ST} between Pops K15 & K16 after sequential Bonferroni corrections [47].

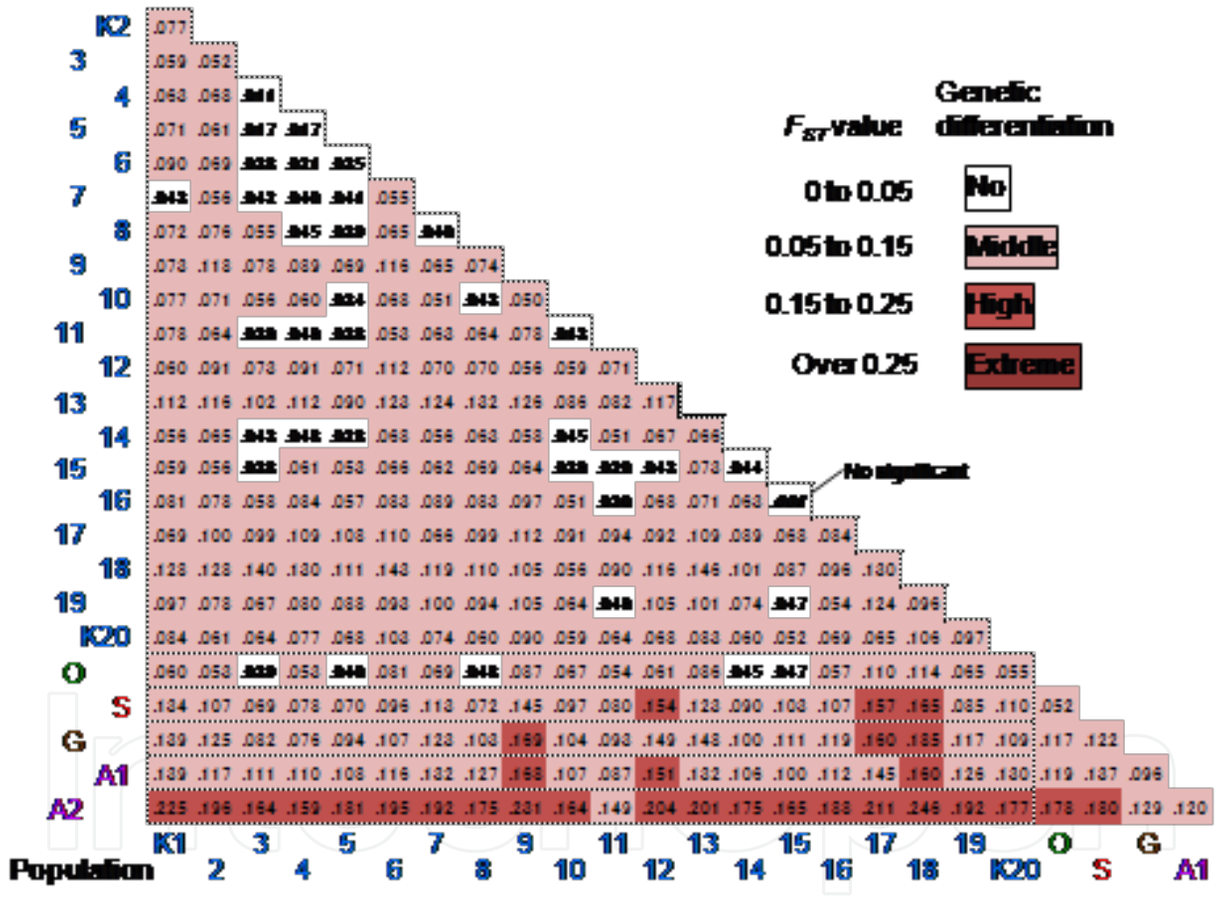


Figure 6. Values of pairwise F_{ST} between the populations and their grades of genetic differentiation composed of four classifications (unpublished figure). All the value of F_{ST} were significantly different from zero (p > 0.05), except between Populations K15 & K16 (F_{ST} = 0.008). Four classifications of genetic differentiation derive from the previous study [62].

Values of F_{ST} were graded on four classifications for genetic differentiation based on the previous study [62]. These classifications imply no, middle, high and extreme genetic differentiation when F_{ST} ranges from 0 to 0.05, from 0.05 to 0.15, from 0.15 to 0.25 and over 0.25. Applying this grade, 20.3% of the F_{ST} (32/190) between the populations within the Kokai R. (Pops K1 to K20)

were classified into no genetic differentiation and a part of such populations tended to be close located each other (Fig. 6). The remaining F_{ST} within the Kokai R. were classified into middle genetic differentiation. Between the populations in the Kokai R. and adjacent 4 rivers (Pops O to A2), their F_{ST} showed middle to high genetic differentiation, although the F_{ST} between the populations in the Kokai and Oh Rs were partially no differentiation.

The analysis of molecular variance (AMOVA) was implemented for the following Cases I to III, among which the number of groups and composition of the populations in groups differed. In Case I, the populations of the Kokai R. (K1 to K20) and 4 adjacent rivers (Pops O to A2) were divided into $Group_{CaseI}$ 1 and 2, respectively. In Case II, $Group_{CaseII}$ 1 was formed by the populations of the Kokai, Oh and Sakura Rs (Pops K1 to S) and $Group_{caseII}$ 2, 3 and 4 were formed by 3 remaining populations of 2 rivers (Pops G, A1 and A2). There were groups $Group_{CaseIII}$ 1 to 6 composed of the populations of the Kokai (Pops K1 to K20), Oh (Pop O), Sakura (Pop S), Gogyo (Pop G), one Ara (Pop A1) and another Ara (Pop A2) R. in Case III.

Significant genetic differentiations were observed at all hierarchical levels in all cases ($p < 0.01$, Table 2). The largest genetic variance in all variances was found at the level of within individuals in each case (from 82.5 % in Case II to 86.0 % in Case I). The genetic variances at the levels of among groups and among populations within groups accounted for 2.8% in Case I to 7.3 % in Case II and 6.9 % in Case II and III to 7.9 % in Case I, respectively (Table 2).

Case (no. groups)	Statistic	Hierarchy				
		Among groups (A)	Among pops within groups (B)	Among inds within pops (C)	Within inds (D)	Total (E)
I (2)	d.f.	1	23	548	573	1145
	MS	52.5	16.8	3.4	3.2	
	Var comp	0.102	0.291	0.124	3.184	3.702
	% of var	2.76	7.87	3.35	86.01	100.00
	F	0.028[a]	0.081[b]	0.106[c]	0.038[d]	0.140[e]
II (4)	d.f.	3	21	548	573	1145
	MS	34.6	15.9	3.4	3.2	
	Var comp	0.282	0.267	0.124	3.184	3.858
	% of var	7.32	6.93	3.22	82.53	100.00
	F	0.073	0.075	0.142	0.038	0.175
III (6)	d.f.	5	19	548	573	1145
	MS	28.5	15.6	3.4	3.2	
	Var comp	0.183	0.260	0.124	3.184	3.752
	% of var	4.88	6.94	3.31	84.86	100.00
	F	0.049	0.073	0.118	0.038	0.151

[a]$F_{A/E}$, [b]$F_{B/(B+C+D)}$, [c]$F_{(A+B)/E}$, [d]$F_{C/(C+E)}$, [e]$F_{(A+B+C)/E}$

Table 2. Results of analysis of molecular variance (AMOVA) for three Cases I, II and III (unpublished table). Compositions of the populations in groups for Case I to III are referred in the text. Genetic differentiations were significant at all hierarchical levels for each case ($p < 0.01$).

Genetic differentiation between the populations was significantly inferred from the analysis of F_{ST} and its relevant AMOVA. Geographical condition such as river and the distances among locations appeared to relate to degree of the genetic differentiation as illustrated in the previous studies [37, 38, 82-84]. However, only a part of genetic population structure could be indicated in this analysis, because the proportions of the genetic variances at the level of among groups were relatively low (2.8 to 7.3 % of among groups in Table 2). Investigating schematically and visually genetic structure may have to be implemented as further analysis as commented in the previous study [74].

3.5.3. Genetic population structure inferred from phylogentic tree

The calculated genetic distance D_A between the populations ranged from 0.073 (between Pops K15 & K16) to 0.99 (between Pops K6 & A2). In this phylogenetic tree of D_A using the neighbor-joining method [65] (Fig. 7), there was a few of highly significant divergences of population with the bootstrap probabilities over 90 % (e.g. 98 % between Pops K15 & K16, 93 % between Pops K10 & K11); while the probabilities left were less than 50 % on most divergences. But, the topology of the phylogenetic tree displayed that there were 4 distinct groups, $\text{Group}_{\text{Tree}}$ 1 to 4 despite weak condition of the statistical support. Both $\text{Group}_{\text{Tree}}$ 1 and 2 consisted of 3 populations of the Gogyo and Ara Rs (Pops G, A1 and A2) and of the Kokai, Oh and Sakura Rs (Pops K8, O and S), respectively. $\text{Group}_{\text{Tree}}$ 3 consisted of 7 populations collected in the lower part of the Kokai R. (Pops K1 to K7), while $\text{Group}_{\text{Tree}}$ 4 were formed by the 12 remaining populations coming from the middle and upper part of the Kokai R. (Pops K9 to K20).

The schematic genetic structure of the populations was showed by constructing the phylogenetic tree (Fig. 7). Including the results of the above F_{ST} analysis and AMOVA, the existence of 2 genetic populations that related to $\text{Group}_{\text{Tree}}$ 3 and 4 was indicated in the populations within the Kokai R., but these groups were statistically cryptic. It could be expected that characterization of admixture of gene flow and migrants among the populations was displayed by detailing structures of such cryptic genetic populations.

3.5.4. Genetic structure among populations inferred from Bayesian cluster analysis

The Bayesian clustering analysis supported the occurrence of two defined genetic clusters, Clusters A and B in the uppermost hierarchical level (Fig. 8). By accounting for the number of individuals with more than 70 % of assignment probability (Q) to each cluster, 98.6 6% of all individuals (507/514 individuals) in the populations from the Kokai, Oh and Sakura Rs (Pops K1 to S) were assigned to Cluster A. And also, 91.5 % of the remaining individuals (54/59 individuals) in the populations from the Gogyo and Ara Rs (Pops G to A2) were assigned to Cluster B (Fig. 8).

Further clustering analysis were performed to assign the populations in Clusters A and B to genetic clusters in the second hierarchical level. Applying the same procedure in the first analysis, the appropriate K were 2 in both analyses. According to the values of Q of the individuals, they were assigned to one of Clusters I, II or admixture of Cluster I & II in the analysis of Cluster A and Clusters III or IV in the analyses of Cluster B.

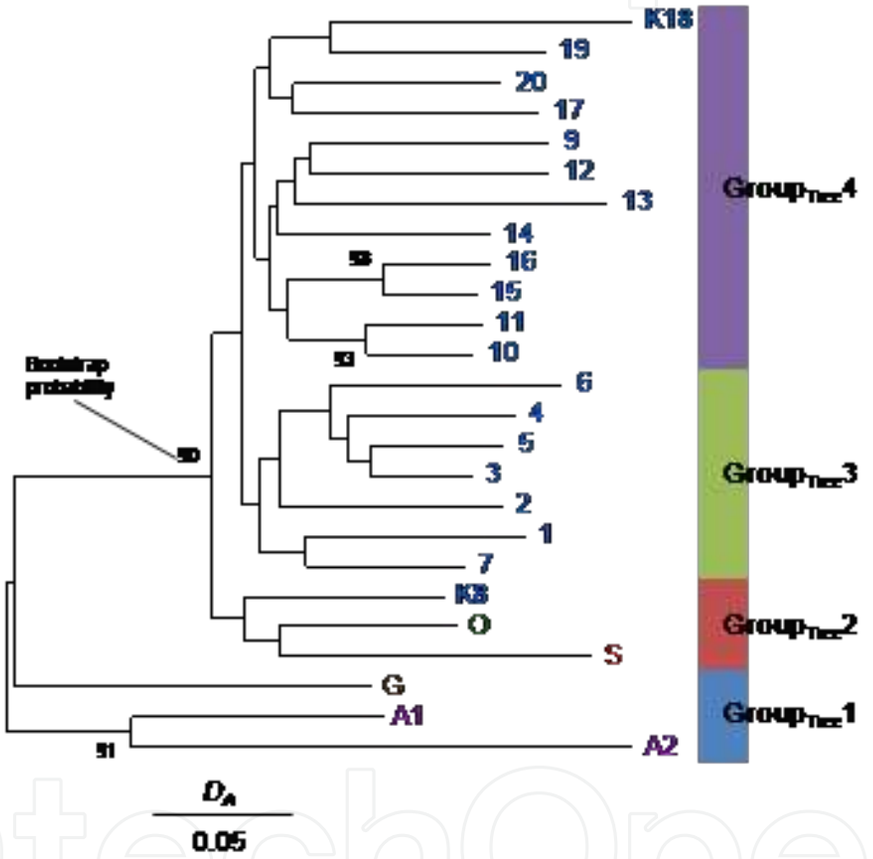


Figure 7. Phylogenetic tree of D_A for the populations with neighbour-joining method (unpublished figure)

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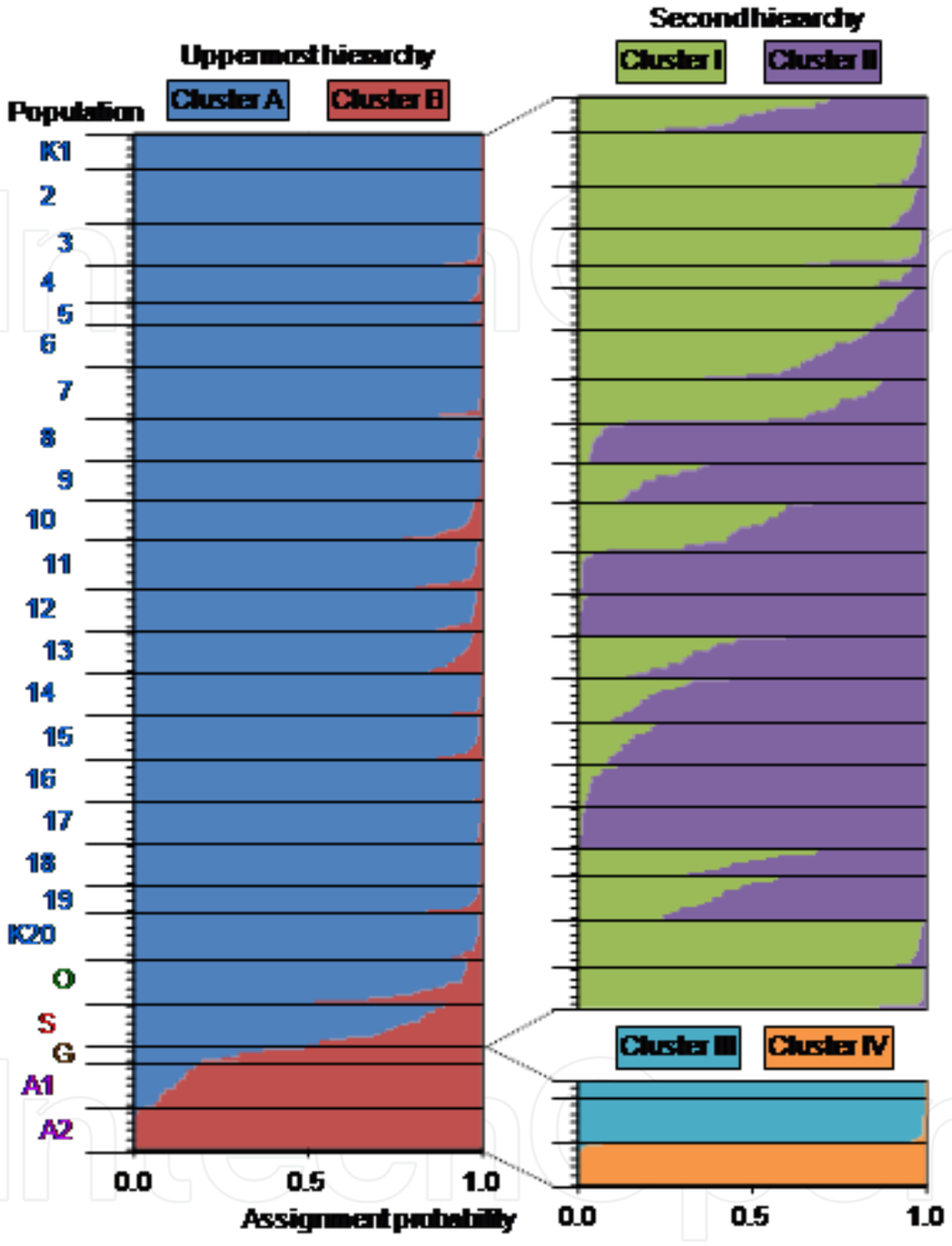


Figure 8. Structures of genetic clusters in the populations inferred by the Bayesian analysis (unpublished figure). Clusters A & B and I to IV imply the genetic populations at uppermost and second hierarchical levels, respectively. Each individual is represented by a horizontal line fragmented by assignment probabilities to the genetic clusters.

In the analysis of Cluster A, 77.0 to 98.6 % of individuals of Pops K2 to K6, K8, O and S (a total of 8 populations) in the Kokai, Oh and Sakura Rs were assigned to members of Cluster I (Fig. 8). Considering the geographical locations of the populations as performed in the previous studies [85-88], Cluster I mainly indicated to be the genetic population of the lower

part of the Kokai including the Oh and Sakura Rs (Fig. 9). 77.4 to 99.0 % of individuals of Pops K9, 10, 12, 13, 15 to 18 (a total of 8 populations) in the Kokai occupied members of Cluster II. Cluster II also implied to be the genetic population of the middle and upper parts of the Kokai R. The remaining individuals of Pops K1, K7, K11, K14, K19 and K20 (a total of 6 populations) in the Kokai R. were mainly classified into members of admixtures of Clusters I & II. In the analysis of Cluster B, almost all individuals (more than 99.1 %) of Pops G and A1 in the Gogyo and Ara Rs. and Pop A2 in the Ara R. were assigned to Clusters III and IV, respectively (Fig. 8). Cluster III and IV reflected the genetic populations of the Gogyo and one of Ara R. and another of the Ara R., respectively (Fig. 9).

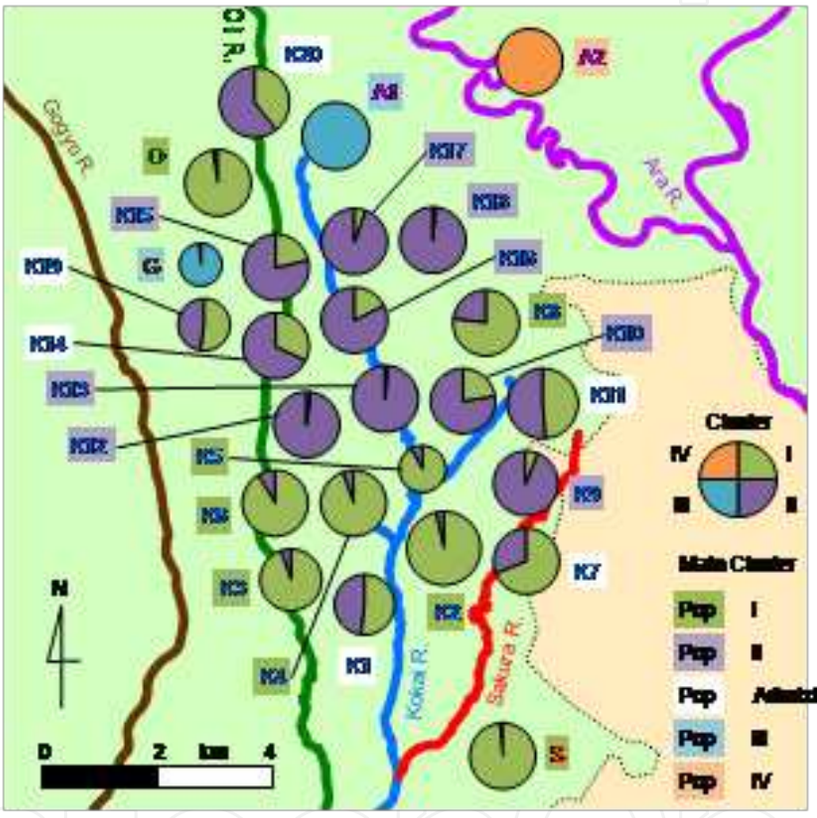


Figure 9. Spatial distribution and composition of genetic clusters in populations (unpublished figure). Size of circle reflects that of population.

Consequently the four genetic populations (Clusters I to IV) and a mixed genetic population (admixture of Cluster I & II) were confirmed in the populations using this clustering analysis. Clusters I, II and a pair of Clusters III & IV nearly coincided with a pair of $Group_{Tree}$ 2 & 3, $Group_{Tree}$ 4 and $Group_{Tree}$ 1 in the phylogenetic tree, respectively (Fig. 7). Moreover, the presence of the mixed genetic population, which could not be usually detected in a phylogenetic tree, was founded by the cluster analysis. As discussed in the previous studies [69-71, 73], this admixture of Cluster I & II may be established through gene flow caused by migrant; thus, events relative to individual movement and breeding could have occurred among some populations in the past.

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4. Conclusions

A series of the exhaustive genetic analysis in this chapter demonstrated that the populations of the Hotoke loach indicated to have moderate genetic diversity and to be supported with 4 genetic populations, of which distributions depended on the populations and the geographical locations. These 2 genetic characteristics showed that there could not be serious genetic concerns at present and the populations might be available as valuable biological resources such as bird food. To fulfill the effective utilization of this loach in near future, both biomedical and nutritional investigations for component contained in the body may also have to be practiced in the next research subjects along with proposing an optimal management plan for conserving the populations.

Further, the following 2 suggestions based on the results of this analysis should be realized in conduct of the next research. First, to sustain the present genetic features, habitats of the populations have to be maintained with monitoring the population size. As it is repeatedly described in the above, but reduction of the population size often appears to cause degradation of the genetic diversity and the lost genetic diversity could never be regained in the populations [30-33]. Avoiding such a decrease in the genetic diversity, habitat conservation might be important for a population management. It was also investigated that this species had relatively strict water temperature resistance compared with other common freshwater fish [48, 49]; hence the control of the water quality, especially water temperature could be one of essential factors for conserving habitats of the populations.

Second, spatial distribution and composition of the genetic populations should be taken account in the population management. In this area the genetic populations could be established by only geographical factors such as river and ground conditions (Fig. 9) and related to no human activities. The foregoing genetic populations often appear a kind of genetic heritages and it is recommended that their distributions do not have to be disturbed artificially [30-33, 35]. If perchance size diminishment of a specific population is observed and there is only individual translocation as a method to recover the population, selections of translated individuals and populations should be advisedly carried out based on the distribution of the genetic populations. Finally there still may be various and many biological resources left in the rural ecosystem in Japan. Genetic analyses performed in this chapter would have to contribute substantially to exploration and beneficial utilization of these resources.

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