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Genetic Uniformity of Japanese Giant Salamander (Amphibia, Caudata) from Kiso River, Central Japan

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Abstract: The Japanese giant salamander, *Andrias japonicus*, is known to exhibit very low genetic diversity, but the number of individuals surveyed in a population is limited by now. We investigated partial sequences (673 bp) of mitochondrial cytochrome b gene in 180 specimens of a population from the Inuyama Head Waters on the Kiso River, Central Japan, over nine years so as to clarify the degree of genetic diversity. The result again confirmed a tendency of lack of diversity; only one individual differed from the remaining 179 with an uncorrected p-distance of only 1.5%. The individual had the sequence identical with that reported for an individual from San'in District of Western Japan, far from the Kiso River, and is thought to have been introduced artificially. The healthy condition of the salamander population in spite of highly reduced genetic diversities might be due to possible decrease of inbreeding depression, resulting from the past purging effect of ancestral inbreeding wherein deleterious recessive alleles were eliminated from the gene pool.

Key words: *Andrias japonicus*; Genetic uniformity; Giant salamander; Mitochondrial DNA; Reduced genetic diversities

INTRODUCTION

The Japanese giant salamander, *Andrias japonicus*, is one of the largest extant am-

phibians in the world and is endemic to the three main islands of Japan (Matsui, 2017). It has been designated as a protected species by the Japanese government since 1952 and has also been listed in Appendix I of CITES since 1975. Thus, collecting of this species is completely prohibited throughout its range of distribution, and this has prevented research that

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requires fresh tissue, such as population genetic studies like allozymic analyses. However, through the development of DNA analyses, which use very small amount of samples, we can now conduct genetic studies on this species.

The Inuyama Head Works, where the present salamander survey was made, is set on the Kiso River about 57 km upstream from the river mouth at Ise Bay, between Inuyama City, Aichi Prefecture, and Kakamigahara City, Gifu Prefecture (35°23'17"N, 136°56'05"E, 33 m asl; Fig. 1). It is an intake structure of agricultural water and directly managed by Ministry of Agriculture, Forestry and Fisheries of Japan. There is a fishway on the middle of the dam, but salamanders seem unable to climb up this, and many individuals remain in two pools (called A and B) formed just downstream of the headworks (Komada et al., 1996, 2002, 2006, 2012a, b).

Cryptobranchid members are known to have extraordinarily low levels of genetic variation (Merkle et al., 1977; Shaffer and Breden, 1989), and a population of *A. japonicus* from the Aoyama River of the Kizugawa River system in Mie Prefecture has been reported to show no variations in 27 allozymic loci studied (Matsui and Hayashi, 1992). In the subsequent study on 27 populations across the range of the species using 3664 bp sequences of the mitochondrial genes NADH1, NADH3, cytochrome b gene (cyt b) and CR, partial NADH6 and intervening genes, *A. japonicus* was divided into central and western clades, with low genetic differentiation (Matsui et al., 2008), in strong contrast to other Japanese amphibians that exhibit great intraspecific differentiation (e.g., small salamanders: Matsui et al., 2007; newts: Tominaga et al., 2013; frogs: Eto and Matsui, 2014).

Of the two clades of *A. japonicus* recognized, the central clade comprised eastern populations from Gifu and Aichi prefectures, while the western clade comprised all the remaining populations from western Japan (from Shiga and Mie prefectures westwards). In that study (Matsui et al.,

2008), two individuals from the Inuyama Head Works were studied, and proved to have sequences identical with other six individuals from three other populations of the eastern clade (from Ontani River, Gujo City, Gifu Prefecture; Jagabora River, Seto City, Aichi Prefecture; Komatsu City, Ishikawa Prefecture). However, no study has been done to examine genetic variations within the population from the Inuyama Head Works. Therefore, we conducted a molecular analysis of the population to uncover patterns of genetic differentiation using cyt b gene, which has been extensively used in molecular phylogenetics and phylogeography (Matsui et al., 2007).

MATERIALS AND METHODS

We made field surveys around pools A and B of the headworks 32 times in nine years between 2005 and 2013, and accumulated small pieces of skin tissue (up to ca. 10×2 mm) from 180 individuals, each marked by PIT (passive integrated transponder) tags (Table 1), with authorization from the Agency for Cultural Affairs (Bunka-cho). Total DNA was extracted from ethanol-preserved tissue using standard phenol–chloroform extraction procedures (Hillis et al., 1996) and DNeasy Blood & Tissue Kit (QIAGEN). Fragments containing 673 bp of cyt b sequence were amplified by PCR. The PCR primers were L14239 (5'-TMWCAKWCWTGATGAAATTWTGGCTC-3') and H14935 (5'-GGAAATATCATTCTGGTTGAAT-3') of Matsui et al. (2008). The reaction conditions were initial denaturation at 94C for 4 min; 35 cycles of 94C (30 s), 49–50C (40 s), and 72C (50 s); and a final extension at 72C for 7 min. The amplified PCR products were purified by polyethylene glycol (PEG) precipitation, and then cycle sequencing reactions were done using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed on ABI 3100 and ABI 3130 automatic sequencers. Obtained sequences were aligned by the Clustal option

TABLE 1. Year and the numbers of survey and individuals sampled.

Year	N of Survey	N of individuals
2005	5	38
2006	3	9
2007	4	8
2008	5	22
2009	5	25
2010	4	35
2012	4	35
2013	2	8
Total	32	180

in the BioEdit software (Hall, 1999). We calculated nucleotide and haplotype diversities for total ($n=180$) and common ($n=179$, see result) individuals in DnaSP 5 (Rozas et al., 2003). The uncorrected p-distance (Nei and Kumar, 2000) for pairwise combinations of haplotypes was calculated using MEGA, version 4 (Tamura et al., 2007).

RESULTS

We successfully sequenced 673 bp of *cyt b* gene for all individuals. All the 180 individuals, except for one, had identical sequences that are part (from 391th to 1063th sequence positions) of AB445781 already reported for a specimen from the same locality. The exceptional individual (PIT number of 00012676F6) was captured on 21 July 2007 at pool A, and had the total length of 1230 mm and body mass of 15 kg. It differed from the common type by 10 of 673 bp (Table 2), with an uncorrected p-distance of only 1.5%. The

TABLE 2. Polymorphic sites found in *cyt b* gene. Site 1 corresponds to 391th sequence position of AB445781 for common and AB445772 for exceptional type.

Haplotype	Polymorphic site									
	5	7	78	164	168	339	437	470	515	650
Common	A	T	G	C	T	G	A	T	T	T
Exceptional	G	C	A	T	C	A	G	C	C	C

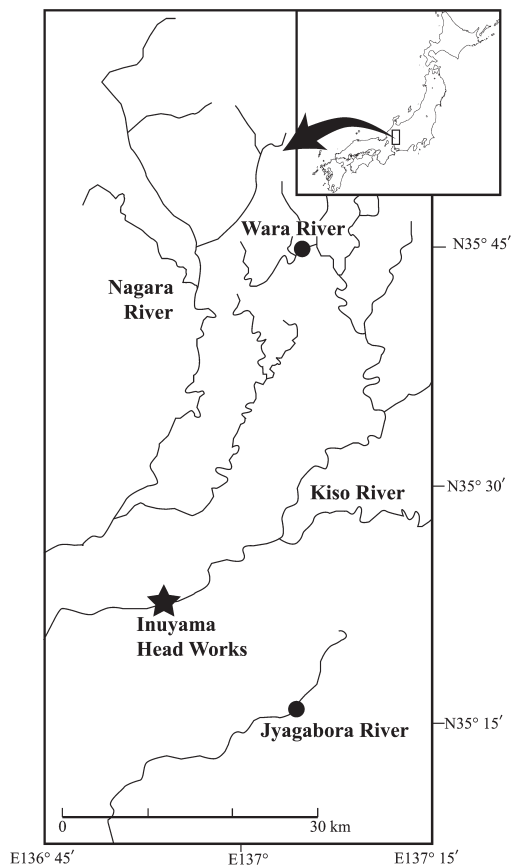


FIG. 1. A map of Tokai Region showing the position of Inuyama Head Works (filled star) and relevant river systems shown in the text.

sequence was identical with corresponding part of AB445772 reported for an individual from Hino Town, Tottori Prefecture. The haplotype diversity ($h \pm SD$) was 0.011 ± 0.011 for total and 0.000 ± 0.000 for common type individuals, and the nucleotide diversity ($\pi \pm SD$) was 0.00017 ± 0.00016 and

0.00000±0.00000, respectively.

DISCUSSION

In Gifu Prefecture, the distribution center of *A. japonicus* lies in a mountainous area and records have been made in the upper Hida River (Wara River), the upper Nagara River, and the Shirakawa River (a branch of the Hida River), whereas in Aichi Prefecture, the species is restricted to the Jagabora River, adjacent to Gifu (Fig. 1). Thus, the occurrence of a large number of salamanders at the Inuyama Head Works on the lower reach of the Kiso River, between Inuyama City and Kakamigahara City, is exceptional. The origin of this population of salamanders is unknown, but there are several hypotheses: (1) they were washed downstream by floodwater and prevented from returning upstream by the dam; (2) they widely inhabit the river, but aggregate just below the headworks because prey items, mainly fishes, are abundant there; (3) they were artificially moved to the headworks for unknown reasons. Occurrence of breeding in this region was long unknown, but eggs and hatched larvae were found recently (Komada et al., 2012a). Thus, the population around the Inuyama Head Works is not a temporal one and requires more intensive investigations, including genetic ones, to develop effective conservation measures.

Matsui et al. (2007) reported in various groups of animals, such as fishes, salamanders, snakes, and birds, that the cyt b gene was much more variable than CR (D-loop) gene in the percentages of variable and phylogenetically informative characters. We thus used cyt b to assess degree of genetic diversity in this study. The results, however, indicated that *A. japonicus* is very uniform in genetic traits and that the variation is almost absent even among individuals captured over nine years.

The exceptional individual we found in this study had the sequence identical with that found in the population from Hino Town,

Tottori Prefecture, within the western clade, and might have been introduced from that region artificially. Such kind of artificial translocation of giant salamanders is not uncommon in Japan (Matsui, 2017), and is one of the major threats for conserving their genetic structure.

Reduction and ultimate loss of genetic diversity are considered crucial genetic factors that tend to produce inbreeding depression, reduced adaptation and fitness, and decreased long-term species survival (e.g., Desalle and Amato, 2004; Frankham, 2005). The genetic uniformity is thought to have resulted from the effect of recent demographic and environmental pressures, or older historical influence like Pleistocene climatic fluctuations (e.g., Chen et al., 2012).

In *A. japonicus*, Matsui et al. (2008) found reduced genetic diversity and attributed that to its life history traits (polygyny, delayed sexual maturity, and notable longevity) and its large size. Also, Matsui et al. (2008) suspected extremely low genetic diversity in *A. japonicus* to be a result of past population bottlenecks. Probably, ancestors of *A. japonicus* would have experienced severe Pleistocene climate oscillations (Sibrava, 1986; Machida, 1999), and separation and extinction of many populations would have occurred during cool periods.

In contrast to the observed reduced genetic diversity in mtDNA, *A. japonicus* might actually have yet uncovered genetic diversification. For example, we may recover loci that display polymorphism in sequences containing microsatellite repeats (Yoshikawa et al., 2011, 2012), although Chen et al. (2012) found extreme genetic uniformity among populations of *Ranodon sibiricus* in not only mtDNA but also microsatellites. Similarly, amplified fragment length polymorphism (AFLP) analyses would show genetic diversities, although the results may be difficult to interpret (Matsui and Tominaga, 2007). Alternatively, reduced genetic diversity as revealed by previous studies may indicate that it has little to do with species survival in giant

salamanders, as most of ideas relating reduction of genetic diversity to decreased species survival came from studies of middle- to large-sized mammals and birds (e.g., Hoelzel et al., 2002; Fernando et al., 2006; Nims et al., 2008; Johnson et al., 2009; Schultz et al., 2009; Rodriguez et al., 2011) and not amphibians. This might be due to the past purging effect associated with ancestral inbreeding, in which deleterious recessive alleles would have been eliminated from the gene pool, resulting in decrease of inbreeding depression (Swindell and Bouzat, 2006).

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