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Phylogenetic Origins of a Newly Found Japanese Red-bellied Newt Population in Yokohama City and Other Populations in Kanagawa Prefecture

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Abstract: We uncovered the phylogenetic origins of the Japanese red-bellied newt (*Cynops pyrrhogaster*) population newly found in Yokohama city and 11 neighboring populations in Kanagawa Prefecture based on mitochondrial NADH6-tRNAGlu-cytochrome b DNA sequences. The Yokohama city population was found to be an alien population introduced from Western Japan. On the other hand, the other populations we investigated belong to either of two genetic lineages of the newt (CENTRAL and NORTHERN), and they are distributed parapatrically around the foot of the Tanzawa Mountains.

Key words: Alien population; *Cynops pyrrhogaster*; Tanzawa Mountains; Yokohama city

Introduction

The Japanese red-bellied newt, *Cynops pyrrhogaster*, is widely distributed in the Japanese archipelago and the adjacent islands (Sengoku et al., 1996). Previous studies showed that this species varies genetically by geography, and is composed of four distinct lineages (NORTHERN, CENTRAL, WESTERN, and SOUTHERN lineages), with one additional lineage (SOUTHERN IZU) (Tominaga et al., 2013, 2015). Despite its wide distribution across the country, this species is listed as endangered in Kanagawa Prefecture, located between Miura Peninsula and Izu Peninsula (Fig. 1), due to drastic habitat reduction, especially in Yokohama city, the prefectural capital city of Kanagawa Prefecture (Arai, 2006; Goto, 1994). More recently, a small population of the newt was found in the

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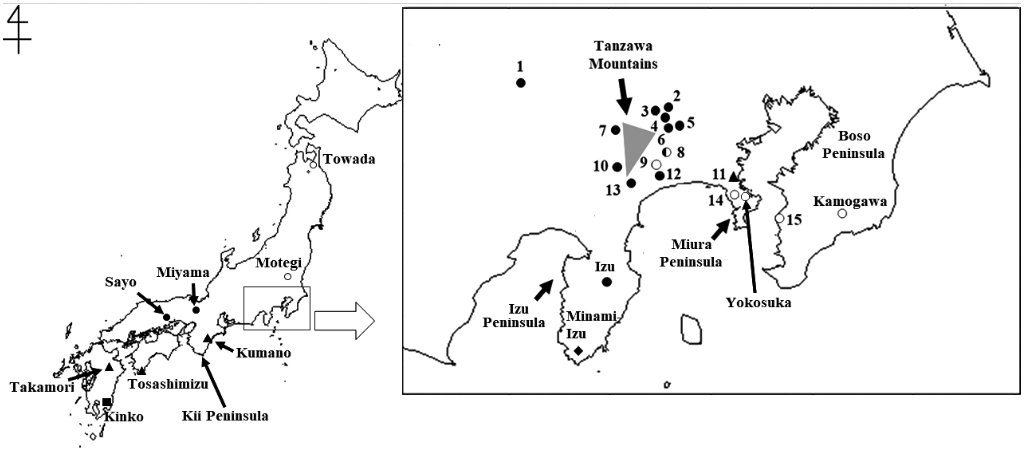

FIG. 1. Map of Japan and localities of 15 populations examined in this study. Black circle indicates CENTRAL lineage, whereas white circle indicates NORTHERN lineage. Black triangle indicates WESTERN lineage, whereas black square indicates SOUTHERN lineage. Black diamond indicates SOUTHERN IZU linage. Location of the Tanzawa Mountains is roughly shown. The numbers correspond to the population numbers in Table 1. Localities of 11 published sequences and the Kumano population of *Cynops pyrrhogaster* are also shown.

southern area of Yokohama city (Sano and Hirose, 2017). Considering the present status of the newt in Yokohama city, conservation activity needs to be initiated for protecting this population. On the other hand, an alien population of the newt, which was artificially introduced from another geographic area, has been reported around Kanagawa Prefecture (Hayashi and Matsui, 1990). Therefore, before starting a conservation program, it is important to confirm the geographic origin of the newly discovered Yokohama population. According to the previous study, the newts from the southern area of Kanagawa Prefecture (Miura Peninsula) were assigned to the NORTHERN lineage (Tominaga et al., 2013). Since the population in Yokohama city is located on the same peninsula, we expected the Yokohama population to be assigned to the NORTHERN lineage if it is a naturally occurring remnant population. In this study, we clarify the genetic lineages of the Yokohama population and other populations on the Kanagawa Prefecture by examining mitochondrial DNA partial sequences of cytochrome b.

MATERIALS AND METHODS

A total 40 specimens from 15 populations from Kanagawa, Yamanashi, and Chiba Prefectures were collected and used in this study (Table 1). Eleven of the 15 populations are located around the Tanzawa Mountains (P2–10, P12, and P13), two populations are in Miura Peninsula (P11 and P14), and the remaining population (P15) is located on the Boso Peninsula in Chiba Prefecture (Fig. 1). DNA was extracted from clipped toe or tail tissue of each newt using a DNeasy blood and tissue kit (QIAGEN), according to the manufacturer's instructions. The NADH6-tRNAGlu-cytochrome b partial gene sequence was amplified according to the method used in the previous study (Tominaga et al., 2013). We sequenced a total of 23 specimens, including at least one specimen per population, using an ABI 310 or 3100 genetic analyzer (Table 1). For the population in Yokohama city (P11), the gene sequences of all specimens were determined. For sequencing, we used an additional forward primer, L14841 (Kocher et al., 1989), and a reverse primer, SLD_cytb_H2

(Tominaga et al., 2010). The obtained sequences were deposited in the DDBJ under accession numbers LC661455–LC661462. The DNA sequences were aligned using MEGA 7 (Kumar et al., 2016), and a phylogenetic tree was constructed by the Maximum Likelihood (ML) method, using MEGA7 software, including an additional 11 published sequences of *Cynops pyrrhogaster* and three published sequences of two other salamandrid species (*C. ensicauda* AB754738 and AB754750, *Tritrus carnifex* HQ697272). Of the 11 additional sequences of *C. pyrrhogaster*, four sequences (AB754594, AB754613, AB754624, and AB754625) were assigned to the NORTHERN lineage, three sequences (AB754662, AB754651 and AB754664) were assigned to the CENTRAL lineage, and two sequences were assigned to the WESTERN lineage (AB754693 and AB754715); the remaining two sequences were assigned to the SOUTHERN lineage (AB754734) or the SOUTHERN IZU (LC016764) lineage of the previous studies (Tominaga et al., 2013, 2015). Localities for the 11 published sequences are Towada (AB754594), Motegi (AB754613), Yokosuka (AB754624), Kamogawa (AB754625), Miyama (AB754651), Izu (AB754662), Sayo (AB754664), Takamori (AB754693), Tosashimizu (AB754715), Kinko (AB754734), and Minamiizu (LC016764) (Tominaga et al., 2013, 2015). The optimum DNA substitution model was selected using the MEGA software, based on the Akaike information criterion (AIC). Bootstrap probabilities were also estimated with 1000 replications using the MEGA software. Furthermore, the mtDNA lineage of each specimen, except those of the Yokohama population, was determined by the PCR-RFLP method using a restriction enzyme (Alu I). For PCR-RFLP analysis, the PCR products from the sequencing analysis were used. Based on the DNA sequences described in a previous study (Tominaga et al., 2013), the number of restriction sites in the PCR product for the NORTHERN lineage is four (94th nucleotide, 112nd nucleotide, 423rd nucleotide and 1394th nucleotide), whereas that of the CENTRAL lineage is two (94th nucleotide and 1394th nucleotide). Therefore, the PCR products of the two lineages could be discriminated from each other by RFLP analysis using Alu I (Fig. 2).

RESULTS

The 408 bp sequences of cytochrome b gene were determined and compared. A total of eight haplotypes (haplotypes C1–C4, N1–N3, and YH) were detected from 23 sequences representing 15 populations. The sequence of the C4 haplotype of the CENTRAL lineage was identical to AB754662, whereas that of the N2 haplotype (NORTHERN lineage) was identical to AB754624. The T92+G model was found to be the optimum substitution model based on the AIC. Log likelihood of the ML tree was –1466.98. Based on the ML tree, five distinct lineages were identified. According to the classification described by Tominaga et al. (2013, 2015), these five lineages were identified as the NORTHERN lineage, SOUTHERN IZU lineage, CENTRAL lineage, WESTERN lineage, and SOUTHERN lineage (Fig. 3). All specimens of the Yokohama population showed an identical haplotype (YH), which was assigned to the WESTERN lineage. Of the eight populations from Kanagawa Prefecture, six (Minamiashigara, Yamakita, Aikawa, Hiratsuka, Sagamihara1–3, and Sagamihara4) belonged to the CENTRAL lineage, whereas the other two populations (Hayama and Hadano) were assigned to the NORTHERN lineage. The one remaining population (Isehara) included haplotypes of both of the latter lineages. PCR-RFLP analysis showed similar results (Table 1).

DISCUSSION

This study reveals that *Cynops pyrrhogaster* in Yokohama city belong to the WESTERN lineage, based on the mitochondrial cytochrome b sequence. On the other hand, those of the two adjacent populations belong to the NORTHERN lineage (P14: Hayama) or the

TABLE 1. List of specimens examined and their cytochrome b haplotypes. Numbers in parentheses indicate number of specimens having each haplotype.

Locality number	Locality name	Prefecture	No. of specimens	Cytochrome b haplotype	
				RFLP*[1]	DNA sequence*[2]
1	Koufu	Yamanashi	1	CENTRAL (1)	C1 (1)
2	Sagamihara1	Kanagawa	1	CENTRAL (1)	C4 (1)
3	Sagamihara2	Kanagawa	2	CENTRAL (2)	C2 (1)
4	Sagamihara3	Kanagawa	6	CENTRAL (6)	C4 (1)
5	Sagamihara4	Kanagawa	4	CENTRAL (4)	C2 (2)
6	Aikawa	Kanagawa	1	CENTRAL (1)	C4 (1)
7	Doushi	Yamanashi	1	CENTRAL (1)	C3 (1)
8	Isehara	Kanagawa	4	NORTHERN (1)/ CENTRAL (3)	N1 (1)/C4 (1)
9	Hadano	Kanagawa	6	NORTHERN (6)	N1 (2)
10	Yamakita	Kanagawa	1	CENTRAL (1)	C4 (1)
11	Yokohama	Kanagawa	6	Not examined	YH (6)
12	Hiratsuka	Kanagawa	2	CENTRAL (2)	C4 (1)
13	Minami-ashigara	Kanagawa	2	CENTRAL (2)	C4 (1)
14	Hayama	Kanagawa	2	NORTHERN (2)	N2 (1)
15	Kanaya	Chiba	1	NORTHERN (1)	N3 (1)
Total			40		

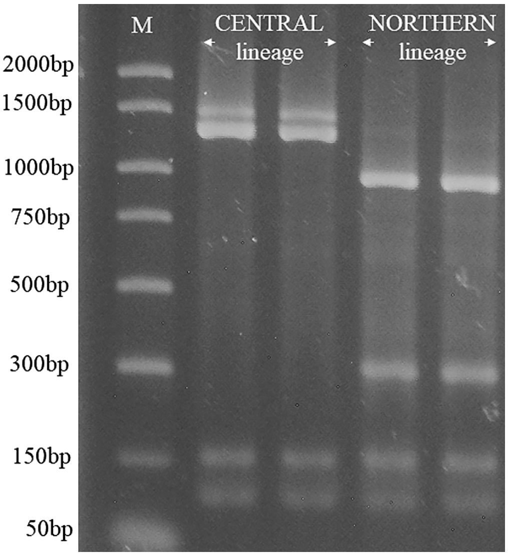

FIG. 2. Electrophoretic profile of NADH6-tRNAGlu-cytochrome b DNA fragment digested by Alu I in 3% agarose gel, showing difference between CENTRAL and NORTHERN lineages. M indicates DNA size marker.

CENTRAL lineage (P12: Hiratsuka) and not to the WESTERN lineage (Fig. 3). Also, a previous study showed that the closest population of the WESTERN lineage to Yokohama, geographically, is the Kumano population on the Kii Peninsula, far west of the Izu Peninsula (Tominaga et al., 2013: Fig. 1). Therefore, the recently discovered population of *C. pyrrhogaster* in Yokohama city may not be a native population, but rather an alien population artificially introduced from an area in western Japan. A similar case has recently been reported for *C. pyrrhogaster* on Hachijo-jima Island, Tokyo (Tominaga et al., 2016). Thus, artificial introduction of newts from western Japan to eastern Japan may be a repeating pattern. Although the mode of introduction for the Yokohama population is unclear, the pet trade in *C. pyrrhogaster* is a likely candidate for such an introduction (e.g. Drinkwater et al., 2021).

Thus far, the southern boundary of two lineages (CENTRAL and NORTHERN) on the

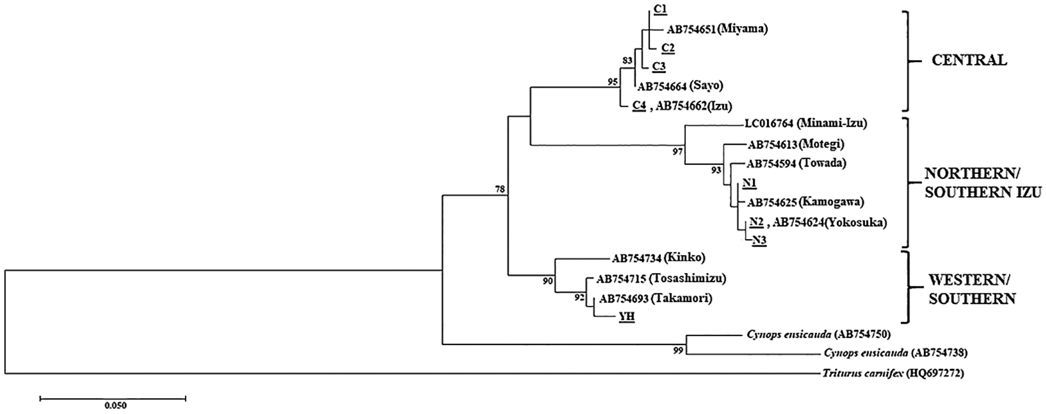

FIG. 3. Maximum likelihood tree based on 408 bp sequences of mitochondrial cytochrome b. Numeral at each node denotes the bootstrap probability (% of 1000 replicates).
Eight haplotypes (C1–4, N1–3 and YH) correspond with those in Table 1. Mitochondrial DNA lineages and localities of published sequences of *Cynops pyrrhogaster* are described in the Materials and Methods.

Pacific Ocean side has been predicted to be located between the Miura Peninsula and the Izu Peninsula (Tominaga et al., 2013). However, its exact location has not been identified. The present study shows that populations of *Cynops pyrrhogaster* around the northern and western parts of the Tanzawa Mountains belong to the CENTRAL lineage, whereas the newts around the southern foot of the Tanzawa Mountains belong to either the NORTHERN or the CENTRAL lineage. Furthermore, haplotypes of those two lineages were detected within just one population (P8) in latter area (Fig. 1). Thus, the two lineages may be distributed parapatrically around the southern foot of the Tanzawa Mountains. According to the previous study, a hybrid zone between the two lineages was identified in central Japan, and it has been maintained as a tension zone, meaning that the hybridized newts do not expand their distributions into either of the ancestral populations (Tominaga et al., 2021). To clarify whether such a hybrid zone similarly occurs around the foot of the Tanzawa Mountains, further study on nuclear genomes will be needed.

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