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Allozymic Variation and Phylogeography of Two Genetic Types of *Onychodactylus japonicus* (Amphibia: Caudata: Hynobiidae) Sympatric in the Kinki District, Japan

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On the basis of allozyme and mtDNA sequence variation, we elucidated genetic relationships between two sympatric genetic types of *Onychodactylus japonicus* in Kinki and adjacent districts, and investigated their phylogeography. Allozymic analysis revealed the presence of two distinct genetic types (the SW-Honshu and Kinki groups) in this area, and their sympatric occurrence in three of 10 sampling sites. Fixed or nearly fixed allele differences in several loci strongly suggested reproductive isolation between the two types, although one hybrid specimen was found in a locality. Analyses of mtDNA using 194 specimens from 22 localities also demonstrated two genetic types. From phylogeographic and population genetic analyses, it was surmised that these two types diverged allopatrically, and secondarily contacted to become sympatric by the Pleistocene uplift of mountains. Our results indicate different specific status for these two types and separation of the Kinki group from *O. japonicus*, to which the SW-Honshu group belongs.

Key words: allozyme, mtDNA, allopatric speciation, cryptic species, phylogeography, *Onychodactylus japonicus*

INTRODUCTION

Molecular techniques are now widely utilized in the fields of systematics and biogeography, and are playing important roles in detecting genetic diversity and cryptic species in morphologically conservative taxa (e.g., Highton, 1999; Jockusch et al., 2001). These techniques are also powerful tools for inferring phylogeographic events that affected diversification within and between species (Avise, 2000). Many studies have recently been conducted to elucidate the genetic diversity and phylogeography of Japanese salamander species, and to detect cryptic species (e.g., Tominaga et al., 2003, 2005, 2006; Matsui et al., 2006, 2007, 2008; Nishikawa et al., 2001; Sakamoto et al., 2009; Yoshikawa et al., 2008).

Onychodactylus japonicus is a stream-breeding hynobiid salamander widely distributed in Honshu and Shikoku islands of Japan. On the basis of mitochondrial DNA (mtDNA), Yoshikawa et al. (2008) recently clarified the presence of four major clades (Clades I–IV), with further subclades in Clade II–IV, within this species. In western Japan, Clade III, comprising three parapatric subclades (III-A to -C), and Clade IV, composed of two allopatric subclades (IV-A and -B), are found. Of these, sympatric distributions are seen between Subclades III-B and IV-A in the Kinki to Hokuriku Districts of western Honshu, and between III-C and IV-B at several localities in the Chugoku Mountains (westernmost Honshu).

Yoshikawa et al. (2010) conducted an electrophoretic survey to elucidate genetic variation in *O. japonicus* based on biparental allozyme markers and demonstrated that this species is divided into six genetic groups that correspond to clades or subclades recognized in mtDNA analyses. Of these, the SW-Honshu group (corresponding to Clade III), Kinki group (Subclade IV-A), and Shikoku group (Subclade IV-B) are found in southwestern Japan, and reproductive isolation between the SW-Honshu and Shikoku groups in a sympatric locality has been clarified. However, reproductive isolation between the SW-Honshu and Kinki groups, which seemed likely from unique genetic features in the Kinki group, was not clear because sympatric localities were not included in the previous study (Yoshikawa et al., 2010). It was therefore necessary to clarify their genetic relationships for subsequent taxonomic reassessment of *O. japonicus*.

Moreover, the origin of the two genetic types and the process of formation of their sympatric distribution are interesting issues in understanding the biogeography of Japanese amphibians. The Kinki District is geologically separated into two regions, the Inner Zone (northern region in this study) and Outer Zone (southern region in this study = Kii Peninsula), bordered by the Median Tectonic Line (MTL). The Kii Peninsula is considered to be closely related floristically to Shikoku and southern Kyushu (e.g., Hotta, 1974). On the Kii Peninsula, montane salamanders with relatives in Shikoku and southern Kyushu (*Hynobius boulengeri* [sensu lato] and *Hynobius yatsui*) are distributed, and some geohistorical factors are considered to have influenced their distributions (Nishikawa et al., 2001). Yoshikawa et al. (2008)

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estimated the divergence time and historical biogeography of *O. japonicus* from analyses of mitochondrial phylogeny. Their hypothesis related to the SW-Honshu (Clade III) and Kinki (Subclade IV-A) groups is as follows: (1) Separation of Clade IV in the region corresponding to present Shikoku and the Kii Peninsula from Clade III in other parts of southwestern Honshu by the formation of the Second Setouchi Basin, which lay along ancient Osaka Bay, Lake Biwa, and Ise Bay. (2) Division of Subclades IV-A and IV-B (Shikoku group) by the formation of Kii Strait. (3) Range expansion and secondary contact of Clade III and Subclade IV-A subsequent to uplifting of the mountains that connect montane regions between the Kii Peninsula and other parts of Honshu. In this scenario, Yoshikawa et al. (2008) assumed contrasting events between the two lineages, southward expansion of Clade III and northward expansion of Subclade IV-A. If this scenario is correct, some similar signals of population expansion should be found between the southern populations of Clade III and northern populations of Subclade IV-A.

In this study, we conducted an electrophoretic survey to clarify the genetic relationship between the two genetic types in the Kinki District. We also performed phylogenetic, population genetic, and nested-clade phylogeographic analyses (NCPA) using mtDNA sequences to infer the local-scale phylogeographic history of the two types of *O. japonicus*. Hereafter, we call Clade III (and its component subclades) and Subclade IV-A recognized in mtDNA analyses as the SW-Honshu and Kinki groups, respectively.

MATERIAL AND METHODS

Sampling

For allozyme electrophoresis, we used 126 specimens of *O. japonicus* (including metamorphs and larvae) from 10 localities in Kinki and adjacent districts, and *Salamandrella keyserlingii* from one locality on Hokkaido as the outgroup (Fig. 1; Table 1).

For mtDNA analyses, we used a total of 194 specimens, including both metamorphs and larvae, from 22 localities (including all localities examined in the allozyme analysis) from Kinki and the adjacent regions (Fig. 1; Table 1). In the outgroup, we included previously published cyt b sequences from congeneric *O. fischeri* from Russia and South Korea (AB452956 and AB452959, respectively; Yoshikawa et al., 2008).

Allozyme electrophoresis

Experimental conditions, techniques, and interpretations of zymograms were basically same as described by Yoshikawa et al. (2010), and the buffers and enzymes used are shown in Table 2. For each sample of *O. japonicus*, genetic variability was assessed by calculating the mean number of alleles per locus (A), the proportion of polymorphic loci (P), and the mean heterozygosity by direct count (H). Variable loci were checked with chi-square goodness-of-fit tests to determine whether they were in Hardy-Weinberg equilibrium. The expected number of each genotype was calculated using Levene's (1949) formula for small sample sizes. To evaluate the spatial pattern of heterogeneity, *F*-statistics (Wright, 1965) were calculated. All these statistics were calculated by using the BIOSYS-1 computer program (Swofford and Selander, 1981). Genotypic similarities between samples were calculated for six loci that showed significant deviation from Hardy-Weinberg expectations (see RESULTS), based on the procedure described in Yoshikawa et al. (2010).

To infer overall genetic differentiation, Nei's unbiased genetic distance (Nei, 1978) was calculated between samples. We also

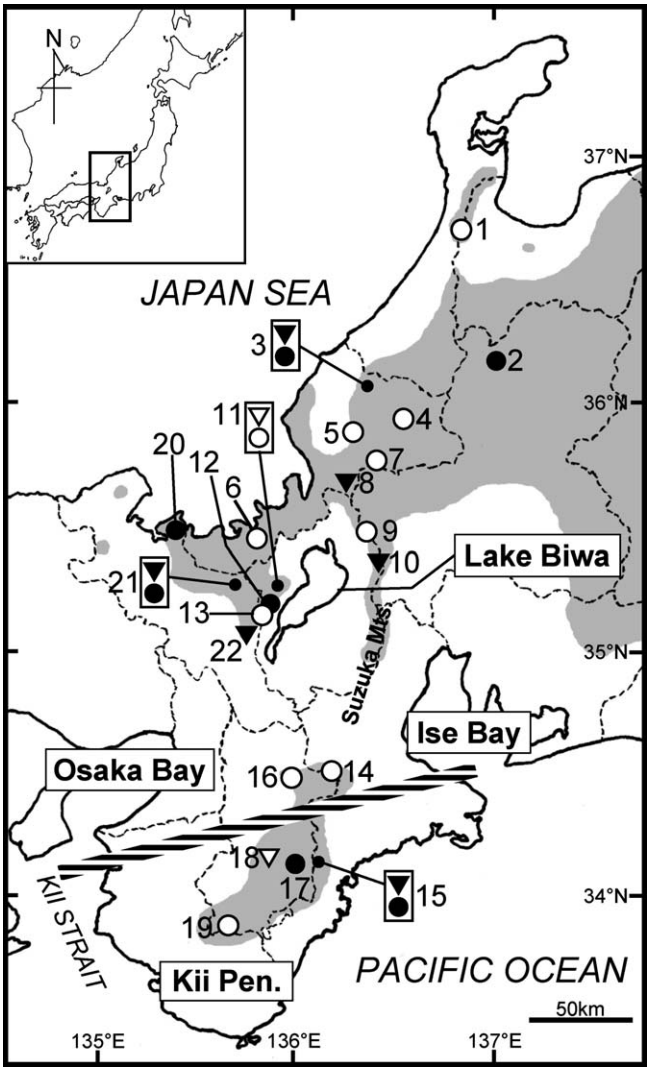

Fig. 1. Map of the Kinki and adjacent districts, Japan, showing the known distributional range (shaded) and sampling localities for *Onychodactylus japonicus* (circles, SW-Honshu group; inverse triangles, Kinki group). Open and closed symbols indicate sampling localities used for only mtDNA analysis and both mtDNA and allozymic analyses, respectively. Horizontal hatching, Median Tectonic Line (MTL). For sample numbers, refer to Table 1.

constructed a neighbor-joining (NJ; Saitou and Nei, 1987) tree using Cavalli-Sforza and Edwards' (1967) chord distance and a continuous maximum-likelihood (CONTML; Felsenstein, 1973) tree based on allelic frequencies. Confidence in tree topologies was tested by 1000 non-parametric bootstrap pseudo-replicates (Felsenstein, 1985). These analyses were performed with PHYLIP ver. 3.5C (Felsenstein, 1993).

To understand the genetic similarities among samples, we also conducted multidimensional scaling (MDS) using SAS (SAS, 1985). This analysis can detect clinal and complicated relationships that might be overlooked in clustering procedures (Felsenstein, 1982). Because the results of MDS are stable regardless of the genetic distance used (Lessa, 1990), we used only Nei's (1978) D in this study.

Mitochondrial DNA analyses

Total DNA was extracted from frozen or ethanol-preserved tissues by standard phenol-chloroform extraction (Hillis et al., 1996). The partial cyt b gene was amplified by PCR using the primer set

Table 1. Species, sample numbers, sampling localities, sample sizes, and haplotype (h) and nucleotide (π) diversities.

Sample no.	Locality	Latitude	Longitude	n		Haplotype (n)	$h \pm$ SD	$\pi \pm$ SD
				mtDNA	Allozyme			
Onychodactylus japonicus								
1	Hodatsushimizu-cho, Ishikawa Pref.	36°48'N	136°48'E	17	–	H1(14), H2(2), H3(1)	0.32 ± 0.14	0.0030 ± 0.0021
2	Shirakawa-mura, Gifu Pref.	36°15'N	136°57'E	13	10	H4(2), H5(6), H6(2), H7(1), H8(1), H9(1)	0.78 ± 0.11	0.0038 ± 0.0025
3A	Sakai-shi, Fukui Pref.	36°9'N	136°21'E	10	6	H4(8), H10(1), H11(1)	0.38 ± 0.18	0.0026 ± 0.0019
3B	Sakai-shi, Fukui Pref.	36°9'N	136°21'E	15	10	K1(7), K2(2), K3(1), K4(1), K5(1), K6(1), K7(1), K8(1)	0.79 ± 0.11	0.0030 ± 0.0021
4	Ono-shi, Fukui Pref.	36°1'N	136°37'E	3	–	H4(2), H5(1)	0.67 ± 0.31	0.0023 ± 0.0023
5	Echizen-shi, Fukui Pref.	35°52'N	136°16'E	5	–	H12(1), H13(4)	0.40 ± 0.24	0.0007 ± 0.0009
6	Wakasa-cho, Fukui Pref.	35°33'N	135°51'E	4	–	H12(2), H14(2)	0.67 ± 0.20	0.0011 ± 0.0013
7	Ikeda-cho, Fukui Pref.	35°47'N	136°25'E	1	–	H5(1)	–	–
8	Minami-Echizen-cho, Fukui Pref.	35°41'N	136°16'E	11	11	K9(2), K10(2), K11(2), K12(1), K13(1), K14(1), K15(1), K16(1)	0.95 ± 0.05	0.0041 ± 0.0027
9	Nagahama-shi, Shiga Pref.	35°30'N	136°20'E	2	–	H13(1), H15(1)	1.00 ± 0.50	0.0068 ± 0.0076
10	Maibara-shi, Shiga Pref.	35°31'N	136°23'E	2	2	K10(1), K11(1)	1.00 ± 0.50	0.0034 ± 0.0042
11A	Otsu-shi, Shiga Pref.	35°14'N	135°53'E	7	–	H12(5), H16(1), H17(1)	0.52 ± 0.21	0.0010 ± 0.0010
11B	Otsu-shi, Shiga Pref.	35°14'N	135°53'E	7	–	K11(1), K17(1), K18(2), K19(1), K20(1), K21(1)	0.95 ± 0.10	0.0033 ± 0.0024
12	Otsu-shi, Shiga Pref.	35°11'N	135°53'E	9	18	H18(2), H19(4), H20(2), H21(1)	0.78 ± 0.11	0.0060 ± 0.0038
13	Otsu-shi, Shiga Pref.	35°11'N	135°52'E	1	–	K21(1)	–	–
14	Tsu-shi, Mie Pref.	34°33'N	136°10'E	4	–	H18(4)	0	0
15A	Odai-cho, Mie Pref.	34°11'N	136°06'E	23	23	H22(7), H23(6), H24(2), H25(2), H26(2), H27(1), H28(1), H29(1), H30(1)	0.83 ± 0.06	0.0028 ± 0.0019
15B	Odai-cho, Mie Pref.	34°11'N	136°06'E	10	9	K22(5), K23(1), K24(2), K25(1), K26(1)	0.76 ± 0.13	0.0027 ± 0.0020
16	Nara-shi, Nara Pref.	34°34'N	135°57'E	1	–	H18(1)	–	–
17	Kamikitayama-mura, Nara Pref.	34°14'N	135°59'E	11	10	H22(1), H23(5), H31(3), H32(1), H33(1)	0.76 ± 0.11	0.0019 ± 0.0015
18	Tenkawa-mura, Nara Pref.	34°11'N	135°56'E	8	–	K22(1), K27(4), K28(1), K29(1), K30(1)	0.79 ± 0.15	0.0078 ± 0.0049
19	Totsukawa-mura, Nara Pref.	33°54'N	135°39'E	1	–	H34(1)	–	–
20	Maizuru-shi, Kyoto Pref.	35°32'N	135°22'E	6	6	H35(4), H36(1), H37(1)	0.60 ± 0.22	0.0017 ± 0.0015
21A	Nantan-shi, Kyoto Pref.	35°18'N	135°44'E	1	1	H38(1)	–	–
21B	Nantan-shi, Kyoto Pref.	35°18'N	135°44'E	10	9	K11(10)	0	0
22	Kyoto-shi, Kyoto Pref.	35°08'N	135°45'E	12	10	K11(1), K20(2), K31(6), K32(3)	0.71 ± 0.11	0.0036 ± 0.0024
Onychodactylus fischeri								
	Shkotovo, Primorsky, Russia (AB452956[a])	–	–	1	–	–	–	–
	Muju, Chunlabuk-do, South Korea (AB452959[a])	–	–	1	–	–	–	–
Salamandrella keyserlingii								
	Kushiro, Hokkaido, Japan	–	–	–	10	–	–	–

[a]Yoshikawa et al. (2008).

Table 2. Enzymes, loci, and buffer systems used in this study.

Enzyme	E.C.number	Locus	Buffer system[a]
Aconitate hydratase	4.2.1.3	ACOH-1	TC8
Aspartate transaminase	2.6.1.1	ATA-2	CAPM6, TC8
Glucose-6-phosphate isomerase	5.3.1.9	GPI	CAPM6
Isocitrate dehydrogenase	1.1.1.42	IDH-1	TC7
Isocitrate dehydrogenase	1.1.1.42	IDH-2	TC7
L-lactate dehydrogenase	1.1.1.27	LDH-1	CAPM6
Malate dehydrogenase	1.1.1.37	MDH-1	CAPM6, TC8
Malate dehydrogenase	1.1.1.37	MDH-2	CAPM6, TC8
Peptidase (leucyl-glycine)	3.4.11.-	PEPlg	TBE8.7
Peptidase (leucyl-glycyl-glycine)	3.4.11.-	PEPlgg	TBE8.7
Phosphogluconate dehydrogenase	1.1.1.44	PGDH	TC7
Phosphoglucomutase	5.4.2.2	PGM-1	TC7
Phosphoglucomutase	5.4.2.2	PGM-3	TC7
Superoxide dismutase	1.15.1.1	SOD	TBE8.7

[a] Buffer systems: CAPM6, Citrate aminopropylmorphorine, pH 6.0 (Clayton and Tretiak, 1972); TC7, Tris-citrate, pH 7.0 (Shaw and Prasad, 1970); TC8, Tris-citrate, pH 8.0 (Clayton and Tretiak, 1972); TBE8.7, Tris-borate-EDTA, pH 8.7 (Boyer, et al., 1963).

Onycho_Cytb_520_F (forward, 5'-GGTGGATTTTCAGTTGATAA-AGC-3') and salamander_Cytb_RN2 (reverse, 5'-YTYTCAATCTTK-GGYTTACAAGACC-3'). Experimental conditions and techniques were as described in Yoshikawa et al. (2008). Sequences obtained have been deposited in DDBJ under accession numbers AB526732–AB526801.

Partial cyt b gene sequences 586 bp long were obtained in both directions and were aligned by using Clustal X 1.8 (Thompson et al., 1997). The haplotype diversity (h) and nucleotide diversity (π) for each sample were computed with ARLEQUIN version 3.11 (Excoffier et al., 2005).

The optimum substitution model for each codon position (1st, 2nd, and 3rd positions) were selected by using Kakusan3 (Tanabe, 2007), based on the Akaike information criterion (AIC). We then constructed phylogenetic trees with the maximum likelihood (ML) and Bayesian (BI) methods using TREEFINDER ver. Oct. 2008 (Jobb, 2008) and MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001), respectively.

For the ML analysis, the TIM model with a gamma shape parameter (G), J2 + G, and TVM + G were selected as the optimal models for the 1st, 2nd, and 3rd codon positions, respectively. For the Bayesian analyses, HKY (Hasegawa et al., 1985) + G was selected as the best substitution model for the 1st position, and GTR (Tavaré, 1986) + G for the 2nd and 3rd positions. Two independent runs of four Markov chains were conducted for 6 million generations. We sampled one tree every 100 generations and calculated a consensus topology for 60,001 trees after discarding the first 30,000 trees (burn-in = 3,000,000).

Confidence in tree topology was tested by non-parametric bootstrap (bs) analysis (Felsenstein, 1985) with 1000 pseudo-replicates for the ML analysis. Branches with bootstrap values 70% or greater were regarded as sufficiently resolved (Huelsenbeck and Hillis, 1993). For the Bayesian analysis, posterior probabilities (bpp) were used as the indicator of node credibility, and values 95% or greater were considered to be significant (Leaché and Reeder, 2002).

To test the phylogeographical hypothesis for *O. japonicus* in Kinki and the adjacent regions, we conducted a nested clade phylogeographic analysis (NCPA; Templeton, 2004). Two major lineages in these regions (SW-Honshu and Kinki groups; see RESULTS) were separately analyzed by NCPA. First, statistical parsimony networks were constructed by using TCS version 1.21 (Clement et al., 2000), and ambiguous loops in the networks were resolved following the criteria described in Crandall and Templeton (1993) and Posada and Crandall (2001). Next, we nested both observed and inferred haplotypes by using standard nesting rules (Templeton et al., 1987; Templeton and Sing, 1993). We then calculated NCPA distance statistics based on geographical coordinates and their significances by comparison with a null distribution derived from 10,000 random permutations of clade against sampled populations by using GeoDis version 2.6 (Posada et al., 2000).

Table 3. Allele frequencies and genetic variation at 14 polymorphic loci among samples of *O. japonicus* (Samples 2–22) and *S. keyserlingii* (SK). For sample numbers, refer to Table 1.

	O. japonicus													SK
	2	3A	3B	8	10	12	15A	15B	17	20	21A	21B	22	–
Locus	n = 10	n = 6	n = 12	n = 11	n = 2	n = 16	n = 23	n = 9	n = 10	n = 6	n = 1	n = 9	n = 10	n = 10
ACOH-1	a0.900 b0.100	a	a0.542 b0.167 c0.292	a0.227 b0.636 c0.136	a0.250 b0.250 c0.500	a0.861 c0.139	a0.913 b0.087	a0.556 b0.444	a0.950 b0.050	a	a	b0.889 c0.111	b	d
ATA-2	a0.150 c0.800 d0.050	c	b	b	b	c	c0.978 d0.022	b	c	c	c	b0.944 c0.056	b0.500 c0.500	c
GPI	b	b	b	b	b	b	b	b	b	b	b	b	b	a
IDH-1	a	a	a0.917 b0.083	a	a	a	a	a	a	a	a	a	a	a
IDH-2	b	b0.583 c0.417	b	b	b	b	a0.043 b0.957	b	b	b	b	b	b	b
LDH-1	a	a0.917 c0.083	a	a	a	a	a	a	a0.950 c0.050	a	a	a	a	b
MDH-1	b	a0.083 b0.667 d0.250	a0.083 b0.917	b	b	b	b0.978 d0.022	b	b	b	b	b	b	c
MDH-2	a	a0.917 c0.083	a	a	a	a	a	a	a	a0.833 c0.167	a	a0.944 c0.056	a0.950 c0.050	b
PEPIg	b	b	b	b	b	b	b	b	b	b	b	b	b	a
PEPIgg	b	b	a	a	a	b	a0.022 b0.978	a	a0.050 b0.950	b	b	a	a	b
PGDH	c0.550 d0.300 e0.150	c0.667 d0.333	b0.333 c0.625 d0.042	c0.864 e0.045 f 0.091	c0.750 e0.250	b0.028 c0.278 d0.583 e0.083 f 0.028	c0.217 d0.783	c0.944 d0.056	c0.100 d0.900	c0.667 d0.250 f 0.083	c	c0.944 f 0.056	c	a
PGM-1	a	a	a	a0.955 c0.045	a	a	a	a	a	a	a	a	a0.900 c0.100	b
PGM-3	b0.050 c0.950	c	a0.125 b0.542 c0.208 e0.125	b0.182 c0.500 e0.318	b0.250 c0.500 e0.250	b0.194 c0.806	b0.043 c0.957	a0.500 c0.056 e0.444	b0.150 c0.850	c0.917 e0.083	c	b0.056 c0.889 e0.056	b0.200 c0.550 e0.250	d
SOD	a	a0.750 b0.250	b	a0.136 b0.864	a0.750 b0.250	a0.944 b0.056	a	a0.222 b0.778	a	a	a	a0.222 b0.778	a0.150 b0.850	a
A	1.43	1.50	1.64	1.57	1.43	1.50	1.50	1.36	1.36	1.29	1.00	1.50	1.43	1.00
(SE)	0.20	0.17	0.27	0.23	0.20	0.29	0.14	0.17	0.13	0.16	0	0.17	0.17	0
P	28.57	42.86	35.71	35.71	28.57	28.57	50.00	28.57	35.71	21.43	0	42.86	35.71	0
H	0.107	0.119	0.119	0.117	0.214	0.091	0.053	0.103	0.057	0.083	0	0.087	0.114	0
(SE)	0.062	0.044	0.056	0.051	0.101	0.050	0.021	0.055	0.025	0.052	0	0.035	0.051	0

Finally, the output from GeoDis was interpreted by employing the latest version of the inference key (Templeton, 2004; version dated December 18, 2008, http://darwin.uvigo.es/software/geodis.html).

Demographic parameters for each of the SW-Honshu and Kinki groups in the northern and southern regions were estimated from a mismatch distribution analysis of pairwise differences (Rogers and Harpending, 1992), and the fit of the observed data to a model of demographic expansion (Rogers and Harpending, 1992) was tested with 10,000 parametric bootstrap pseudo-replicates by using ARLEQUIN version 3.11 (Excoffier et al., 2005). This distribution is usually multimodal for sequences sampled from populations at demographic equilibrium, but typically unimodal in populations that recently expanded demographically (Slatkin and Hudson, 1991; Rogers and Harpending, 1992), or underwent a range expansion with high levels of migration between neighboring populations (Ray et al., 2003). The significance of Harpending's (1994) raggedness index (*H*rag) and the sum of squared deviation (SSD) of Rogers (1995), which measures the smoothness of the mismatch distribution, were also calculated in ARLEQUIN. In addition to the mismatch distribution analysis, Fu's tests of neutrality (Fu's *F*s; Fu, 1997) were performed. Significant negative values of Fu's statistics can be interpreted as a signature of demographic expansion. Fu's *F*s was calculated with ARLEQUIN; significance of values was assessed through 10,000 simulations, and we regarded *p* values of 0.05 or less as significant.

We estimated times of divergence between and within groups of *O. japonicus* in Kinki and the surrounding regions by using BEAST version 1.4.7 (Drummond and Rambaut, 2007). The analysis was run for 10 million generations under GTR + I + G model (partitioned by codon positions). The first one million generations were discarded as burn-in, and parameter values were sampled every 1000 generations. Parameter estimates and convergence were checked by using Tracer version 1.4 (Rambaut and Drummond, 2007). We calculated dates of divergence by setting the divergence time between the SW-Honshu and Kinki groups at 4.3 MYA (Yoshikawa et al., 2008) and by using the relaxed molecular clock method with an uncorrelated log-normal distribution of evolutionary rates among lineages.

RESULTS

Allozyme analysis

Within *O. japonicus*, 12 of 14 loci scored were polymorphic, and 36 alleles were detected (Table 3). The most variable loci were PGDH, with five alleles, followed by PGM-3 and ATA-2, each with four alleles. Compared with *O. japonicus*, *S. keyserlingii* had unique alleles for nine loci, though it was monomorphic for all loci.

For three of 10 samples (Samples 3, 15, and 21), significant deviation from Hardy-Weinberg expectations was found for several loci (ATA-2, PEPlgg, PGM-3, and SOD for Sample 3; ACOH-1, ATA-2, PEPlgg, PGDH, PGM-3, and SOD for Sample 15; ACOH-1, ATA-2, and PEPlgg for Sample 21; $p < 0.05$). For all samples, we estimated the genotypic similarity for six loci that significantly deviated from Hardy-Weinberg expectations by the method described in Yoshikawa et al. (2010). As a result, we found a bimodal distribution of genotypic similarity for three samples (Samples 3, 15, and 21) (Fig. 2A–C), but a unimodal distribution for all the remaining samples (Fig. 2D). We thus suspected that specimens from these three samples actually consisted of two distinct genetic types and divided them into two subsamples (designated with 'A' or 'B' after the sample numbers) according to the distributions of genotypic similarity. From the allocations of samples used in the previous study (Yoshikawa et al., 2010) in the clustering and MDS analyses (see below), we designated subsamples included in the SW-Honshu group as 'A', and those included in Kinki group as 'B'. Among these subsamples, only IDH-1 in Sample 3B showed significant deviation from Hardy-Weinberg expectations.

In Sample 15, one specimen was positioned between the two genetic types on the axis of genotypic similarity (Fig. 2B). This specimen was heterozygous at four of six loci used in the estimation of genotypic similarity (ab/bc/ab/cc/cc/ab for ACOH-1/ATA-2/PEPlgg/PGDH/PGM-3/SOD), possessing both alleles predominant in the SW-Honshu and Kinki groups. We then considered it as a hybrid of the two types and omitted it from later clustering and MDS analyses.

Within the ingroup, after splitting samples 3, 17, and 21 into two sub-samples (except for 21A, which was monomorphic and included only one individual), the mean number of allele per locus (A) ranged from 1.29–1.64, the proportion of polymorphic loci (P) from 21.43–50.0, and the mean heterozygosity by direct count (H) from 0.053–0.214. The highest A, P, and H values were observed in samples 8, 15A, and 10, respectively, whereas the lowest A, P, and H values were found in sample 20 (Table 3). F_{st} values (Wright, 1965) had a mean of 0.59 (range = 0.07–0.98), and

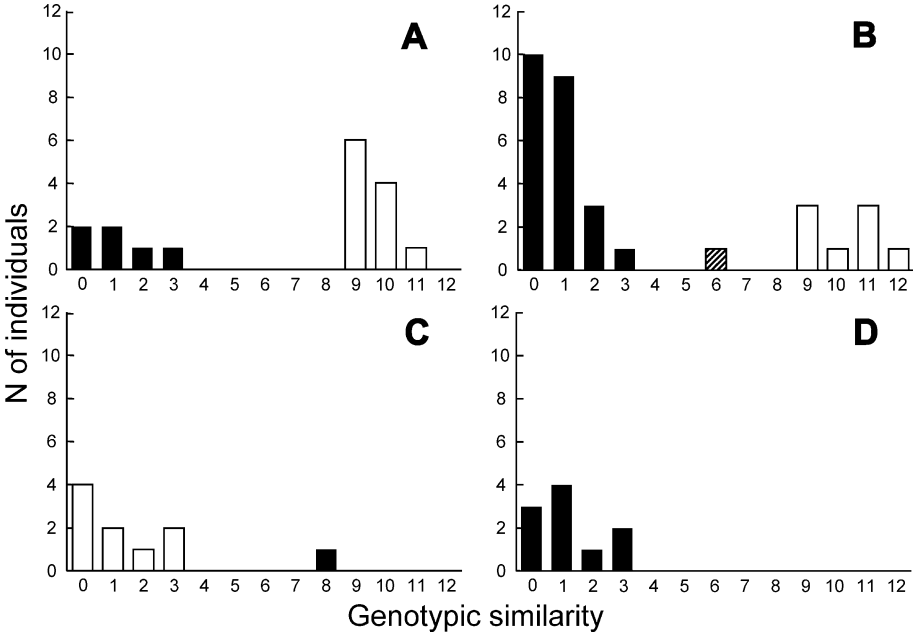

Fig. 2. Frequency distribution of specimens by genotypic similarity relative to the standard (most common) genotype at six loci for Samples 3 **(A)**, 15 **(B)**, 21 **(C)**, and 2 **(D)**. Filled, open, and hatched bars indicate specimens assigned to the SW-Honshu group and Kinki group, and as a hybrid, respectively.

large values were observed for the PEPIgg (0.98), ATA-2 (0.85), SOD (0.66), and ACOH-1 (0.54) loci.

The dendrograms constructed by the NJ and CONTML methods were nearly identical, and only the NJ tree is shown in Fig. 3. In this tree, samples of *O. japonicus* are clearly divided into two groups, one comprising Samples 2, 3A, 12, 15A, 17, 20, and 21A (bootstrap support = 60% for NJ and 79% for CONTML) and the other comprising Samples 3B, 8, 10, 15B, 21B, and 22 (bs = 99% and 85%). From the allocations of samples used in the previous study (SW-Honshu group, Samples 2 and 17; Kinki group, Sample 22; Yoshikawa et al., 2010), we regarded the former as the SW-Honshu group and the latter as the Kinki group. In applying MDS to our data, we repeated the estimation procedure for two-dimensional resolution until the converging values reached less than 0.01. Finally, a badness-of-fit standard value of 0.025 was achieved. In two-dimensional plots of the MDS analysis (Fig. 4), the two groups recognized in the clustering analysis were clearly separated in dimension 1. Compared with the SW-Honshu group, the Kinki group was more dispersed on the plot in MDS space.

The relative magnitudes of pairwise Nei's (1978) distances and the chord distances of Cavalli-Sforza and Edwards (1967) were similar (Table 4), and only the Nei's D values are described below. *Salamandrella keyserlingii* differed from samples/sub-samples of *O. japonicus* with large genetic distances (range = 0.993–1.907, mean = 1.350). Nei's D within the SW-Honshu and Kinki groups, and between the groups, ranged from 0–0.062 (mean = 0.021), 0.010–0.087 (0.040), and 0.190–0.365 (0.303), respectively. The greatest D was observed between Samples 3B and 15A, two genetic types from distant localities, while the smallest value was found between 15A and 17 from adjacent localities.

Between the SW-Honshu and Kinki groups, fixed (or nearly fixed) allele differences were found for the ATA-2 and PEPIgg loci, except for Sample 22, in which ATA-2 had both

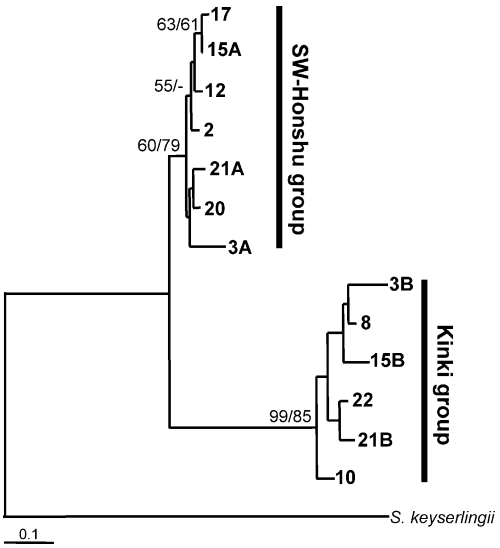

Fig. 3. Neighbor-joining (NJ) tree based on Cavalli-Sforza and Edwards' (1967) chord distances obtained from the allozyme analysis. Nodal values indicate bootstrap support (1000 replicates) for the NJ and CONTML analyses. For sample numbers, refer to Table 1.

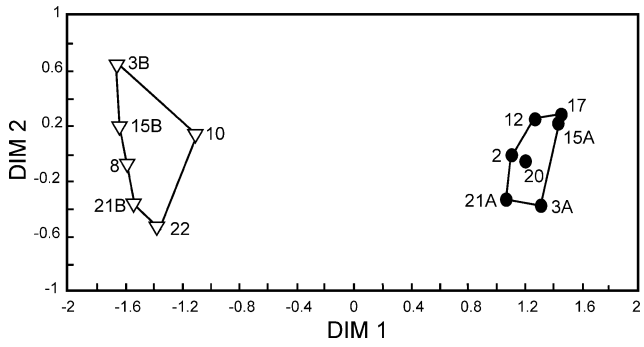

Fig. 4. Two-dimensional scattergram from multidimensional scaling (MDS) based on Nei's (1978) unbiased genetic distances obtained from the allozyme analysis. Closed circles and open inverse triangles indicate the SW-Honshu and Kinki groups, respectively. For sample numbers, refer to Table 1.

Table 4. Nei's (1978) unbiased genetic distances (above diagonal) and Cavalli-Sforza and Edwards' (1967) chord distances (below diagonal) among *O. japonicus* (Samples 2–22) and *S. keyserlingii* (SK). For population numbers, refer to Table 1.

Sample	2	3A	3B	8	10	12	15A	15B	17	20	21A	21B	22	SK
2	–	0.022	0.322	0.291	0.190	0.008	0.013	0.296	0.023	0.002	0.012	0.281	0.267	1.023
3A	0.116	–	0.342	0.323	0.252	0.030	0.033	0.326	0.045	0.016	0.024	0.314	0.289	1.084
3B	0.510	0.522	–	0.029	0.040	0.326	0.365	0.037	0.364	0.341	0.337	0.074	0.087	1.907
8	0.438	0.490	0.094	–	0.015	0.323	0.345	0.022	0.359	0.312	0.300	0.010	0.024	1.812
10	0.370	0.463	0.139	0.056	–	0.215	0.241	0.035	0.250	0.213	0.206	0.031	0.065	1.522
12	0.046	0.109	0.459	0.444	0.369	–	0.003	0.327	0.004	0.012	0.035	0.324	0.297	0.993
15A	0.038	0.102	0.516	0.469	0.412	0.040	–	0.349	0.000	0.018	0.046	0.336	0.316	1.017
15B	0.456	0.483	0.125	0.090	0.142	0.482	0.466	–	0.358	0.307	0.299	0.060	0.065	1.781
17	0.054	0.119	0.507	0.478	0.415	0.037	0.014	0.478	–	0.028	0.062	0.358	0.333	1.012
20	0.056	0.089	0.535	0.462	0.412	0.060	0.055	0.457	0.071	–	0.006	0.306	0.281	0.993
21A	0.057	0.106	0.529	0.457	0.395	0.087	0.085	0.461	0.108	0.040	–	0.289	0.266	1.030
21B	0.433	0.470	0.160	0.043	0.098	0.452	0.452	0.142	0.470	0.449	0.440	–	0.019	1.774
22	0.410	0.446	0.192	0.077	0.156	0.437	0.425	0.158	0.437	0.427	0.418	0.047	–	1.599
SK	1.175	1.209	1.554	1.501	1.437	1.165	1.167	1.488	1.165	1.161	1.161	1.457	1.407	–

alleles b and c. This was the case even in localities where the two types occurred sympatrically (Samples 3, 15, and 21) (Table 3). In addition, these two types also tended to have a heterogeneous allele composition at the SOD locus. Additional allelic differences at the local scale were detected for other loci between the two groups (Table 3). For ACOH-1, a nearly fixed allele difference was found among samples from west of Lake Biwa (Samples 12, 20, 21A, 21B, and 22), and for PGDH, the allele composition tended to differ among samples from the Kii Peninsula (Samples 15A, 15B, and 17). Further, for PGM-3, a difference in allele composition was nearly fixed in samples from the Kii Peninsula (Samples 15A, 15B, and 17).

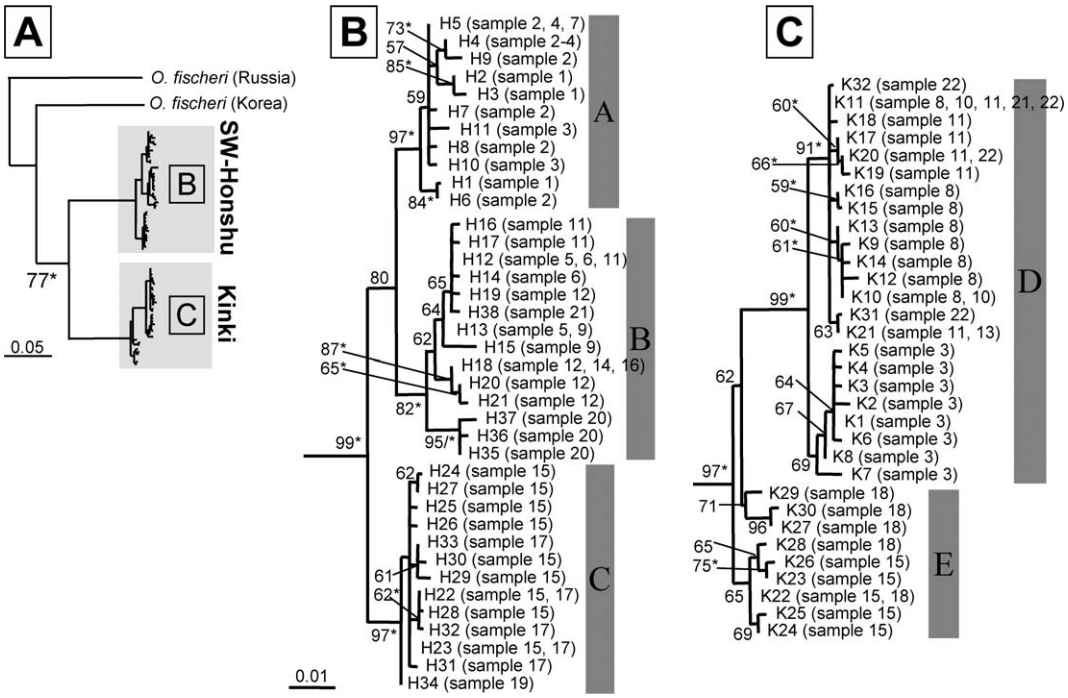

Fig. 5. Maximum likelihood tree based on 586 bp of the mitochondrial cyt b gene. **(A)** Total phylogenetic tree. **(B, C)** Detailed phylograms for the SW-Honshu **(B)**, and Kinki **(C)** groups. Nodal values indicate bootstrap support for the ML analysis. Nodes with asterisks indicate significant support (> 95%) by Bayesian inference. For sample numbers, refer to Table 1.

Sequence variation and phylogenetic relationships

We obtained 586-bp sequences of part of the mitochondrial cyt b gene from all 191 ingroup individuals analyzed; the sequences included 173 variable positions and 108 parsimony-informative positions. The haplotype and nucleotide diversities among samples/subsamples are shown in Table 1.

The ML and Bayesian analyses yielded essentially identical topologies (-lnL = 2149.133 and 2286.697, respectively), and only the ML tree is shown in Fig. 5. The samples of *O. japonicus* were divided into two well-supported monophyletic groups, the SW-Honshu (bs and bpp = 99% and 100%, respectively) and Kinki groups (97% and 100%), corresponding to Subclades III-B and IV-A in Yoshikawa et al. (2008), respectively. In all, 70 unique haplotypes were detected within the ingroup, of which 38 (H1–H38) were found in the SW-Honshu group and 32 (K1–K32) in

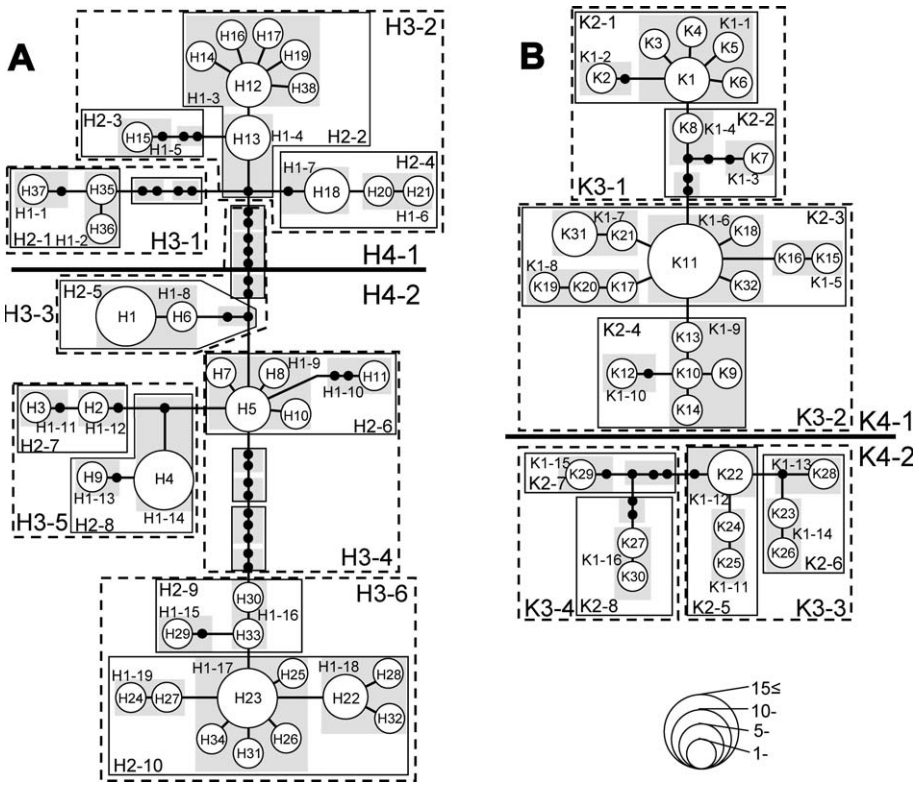

Fig. 6. Statistical parsimony networks and nested designs for cyt b haplotypes of the **(A)** SW-Honshu and **(B)** Kinki groups of *O. japonicus*. Shaded boxes, narrow lines, and dashed polygons indicate 1-step, 2-step, and 3-step clades, respectively, and thick lines separate 4-step clades. The size of each open circle is proportional to the haplotype frequency.

the Kinki group. These two groups were found sympatrically in four localities (Samples 3, 11, 15, and 21), and we divided these samples into two subsamples, designated as A (SW-Honshu group) and B (Kinki group; see above).

Within the SW-Honshu group, three haplotype groups, A (bs and bpp = 97% and 100%), B (82% and 96%), and C (97% and 100%) were recognized. These corresponded to nested clades H3-1 and -2, H3-3 to -5, and H3-6, respectively (see below) (Figs. 5, 6). Among these haplotype groups, divergence of C from A and B was supported in the ML analysis (bs = 80%), but not in Bayesian inference (bpp < 95%). Phylogenetic relationships within each haplotype group were largely unresolved. Nucleotide diversities for Haplotype Groups A, B, and C were 0.00566, 0.00697, and 0.00259, respectively.

The Kinki group consisted of two haplotype groups, D (99% and 100%) and E, though the latter group was a paraphyletic assemblage. These groups corresponded to nested clades K4-1 (K3-1 and -2) and K4-2 (K3-3 and -4), respectively. Haplotypes K9–K21, K31, and K32 formed a monophyletic group (91% and 100%), but relationships among the remaining haplotypes were mostly unresolved. Nucleotide diversities for Haplotype Groups D and E were 0.00662 and 0.00777, respectively.

Phylogeographic analysis

The statistical parsimony networks and nested designs for the SW-Honshu and Kinki groups are presented in Fig. 6. The TCS program linked all haplotypes of the SW-Honshu group, but did not link Haplotype Groups E and D of the Kinki group within the limit of 95% for statistical parsimony. On the basis of the three-step clades, six and four nested clades were found in the SW-Honshu (H3-1 to H3-6) and Kinki (K3-1 to K3-4) groups, respectively (Fig. 6).

In the SW-Honshu group, Haplotype Group B (Clades H3-1 and -2) occurred widely from north of the Median Tectonic Line (MTL) to the northern part of the Kinki and southern Hokuriku Districts (Samples 5, 6, 9, 11, 12, 14, 20, and 21) (Fig. 7). Haplotype Group A (Clades H3-3 to -5) was found in the Hokuriku and Chubu Districts (Samples 1, 2, 3, 4, and 7) (Fig. 7). Haplotype Group C (Clade H3-6) was restricted to the southern region (Kii Peninsula). Clade H4-1 was composed of Haplotype Group B, whereas H4-2 comprised Haplotype Groups A and C. This nesting pattern was different from the result from the phylogenetic analysis. In the Kinki group, Haplotype Group D (Clades K3-1 and -2) occurred in the northern region (Samples 3, 8, 10, 11, 13, 21, and 22), whereas haplotype group E (Clades K3-3 and -4) was restricted to two localities (Samples 15 and 18) in the southern region (Fig. 7).

In the nested contingency analysis, eight and six nested clades in the SW-Honshu and Kinki groups, respectively, showed significant distance measures (D_c, D_n, or I-T) and an association between clade and geographic location. Of these, three nested clades each in the SW-Honshu group (H1-3, H2-2, and H4-2) and Kinki group (K1-6, 1-7, and K3-2) had a conclusive outcome (Fig. 6; Table 5). Inferences on

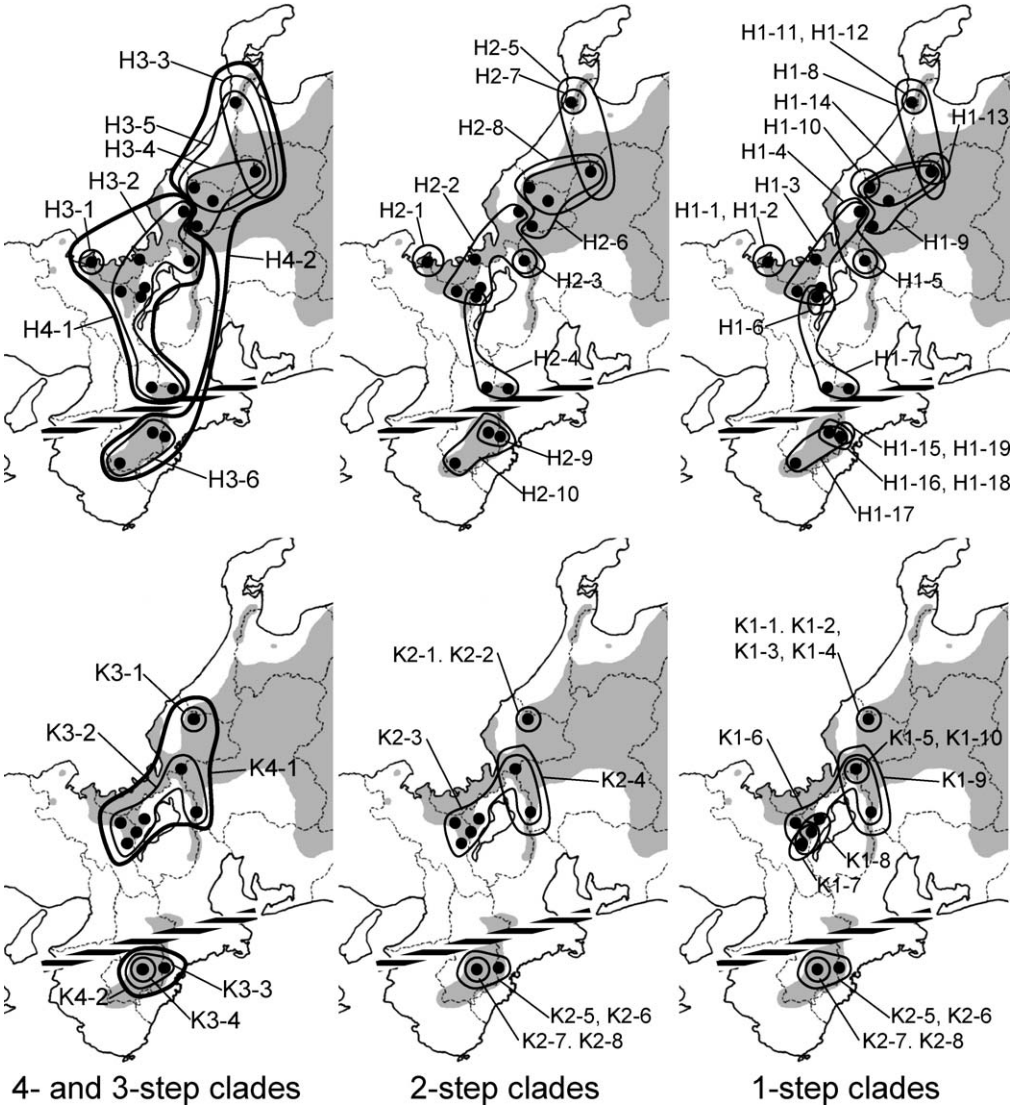

Fig. 7. Distribution of 1- to 4-step clades of the SW-Honshu (upper) and Kinki (lower) groups. Shaded areas, known distributional range of *O. japonicus*; horizontal hatching, MTL.

Table 5. Interpretation of the results of the nested clade phylogeographical analysis (Templeton, 2004) and the time frame of phylogeographic events. Only nested clades significantly associated with geographic locations are listed. Clades with significant χ^2 values but inconclusive outcomes are H1-8, H1-9, H3-5, the total cladogram for the SW-Honshu group, K4-1, K4-2, and the total cladogram for the Kinki group.

Clade	χ^2; *p* value	Inference chain	Demographic event inferred	Time in MY (95% CI)
H1-3	42.20; 0.006	1-2-3-5-6-7-YES	Restricted gene flow/dispersal but with some long distance dispersal	0.099 (0.034-0.179)
H2-2	17.44; 0.001	1-2-11-12-NO	Contiguous range expansion	0.163 (0.053-0.279)
H4-2	139.07; 0.000	1-2-11-12-13-YES-21-NO	Past larger range followed by extinction in intermediate areas or a past gradual range expansion followed by fragmentation	0.971 (0.565-1.419)
K1-6	22.17; 0.010	1-2-11-12-NO	Contiguous range expansion	0.082 (0.012-0.167)
K1-7	8.00; 0.030	1-19-NO	Allopatric fragmentation	0.049 (0.001-0.119)
K3-2	22.89; 0.001	1-2-3-4-NO	Restricted gene flow with isolation by distance	0.245 (0.113-0.396)

population structure and historical influences for clades within the SW-Honshu group are as follows (Fig. 7; Table 5):

1) For Clade H1-3, which consisted of six haplotypes (H12, H14, H16, H17, H19, and H38) and was distributed in the northern Kinki and southern Hokuriku Districts (Samples 5, 6, 11, 12, and 21), restricted gene flow/dispersal but with some long-distance dispersal was suggested.

2) Clade H2-2, which included Clade H1-3 (six haplotypes from five samples) and one haplotype (H13; Samples 5, 9), was estimated to have experienced contiguous range expansion.

3) Clade H4-2 (Haplotype Groups A and C) occurred in two disjunct areas, the Hokuriku District and the Kii Peninsula. A gradual range expansion in the past followed by fragmentation, or a larger range in the past followed by extinction in intermediate areas, was inferred for this clade.

The inferences for clades within the Kinki group are as follows (Fig. 7; Table 5):

4) K1-6, which consisted of three haplotypes (K11, K18, and K32) and was found in the northern Kinki and Hokuriku Districts (Samples 8, 10, 11, 21, and 22), was estimated to have experienced contiguous range expansion.

5) K1-7 comprised two haplotypes (K21 and K31) from west of Lake Biwa (Samples 11, 13, and 22), and was inferred to have experienced allopatric fragmentation.

6) For K3-2, which was monophyletic and composed of 15 haplotypes from the northern Kinki and Hokuriku Districts, restricted gene flow with isolation by distance was suggested.

Observed mismatch distributions for each genetic type in the northern and southern regions did not differ significantly from the model of sudden expansion (Table 6). However, although the mismatch distributions were unimodal for the southern region of the SW-Honshu group (Clade H3-6) and the northern region of the Kinki group (Clade K4-1), the distributions were bimodal in the northern region of the SW-Honshu group (Clades H3-1 to -5) and southern region of the Kinki group (Clade K4-2) (Fig. 8). As a result of Fu's neutrality test, significant negative values were detected in the SW-Honshu group from the southern region and the Kinki group from the northern region (Table 6). Haplotype diversities tended to be higher in the northern region for both genetic types, whereas nucleotide diversities were lower in the southern SW-Honshu and northern Kinki groups (Table 6).

Estimation of divergence times

When the divergence time of the SW-Honshu and Kinki groups was set at around 4.3 MYA, the divergence between haplotype groups C and A + B was estimated to have occurred 0.97 MYA (95% confidence interval = 0.57–1.42 MYA), and that between haplotype groups A and B to have occurred 0.76 MYA (0.42–1.11 MYA). Diversification within Haplotype Groups A, B, and C was dated to have started at around 0.36 MYA (0.17–0.59 MYA), 0.45 MYA (0.23–0.71

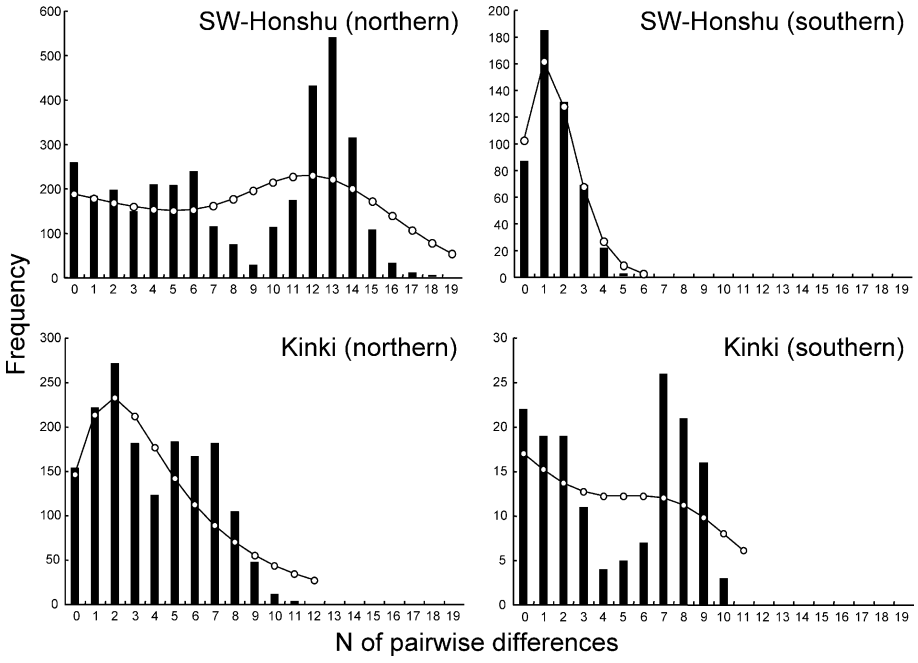

Fig. 8. Mismatch distributions of pairwise nucleotide differences among mtDNA haplotypes of the SW-Honshu and Kinki groups in the northern and southern regions. No distribution deviates significantly from the expectation of the population expansion model (open circles).

Table 6. Haplotype (h) and nucleotide (π) diversities, demographic parameters estimated by the mismatch distribution analysis under the population expansion model, and Fu's test of neutrality (Fu's *Fs*). τ, time parameter for a generation; θ_0, pre-expansion population size; θ_1, post-expansion population size; SSD, sum of squared deviations; H_{rag}, Harpending's raggedness index. NS, $P > 0.05$; **, $P < 0.05$,

			Population expansion model					
Group	$h \pm$ SD	$\pi \pm$ SD	τ	θ_0	θ_1	SSD	H_{rag}	Fu's *Fs*
(SW-Honshu)								
Northern	0.92 ± 0.01	0.0141 ± 0.0073	13.279	0.002	17.130	0.022^{NS} ($P = 0.11$)	0.019^{NS} ($P = 0.27$)	-2.020^{NS} ($P = 0.31$)
Southern	0.85 ± 0.05	0.0026 ± 0.0018	1.555	0.026	99999	0.004^{NS} ($P = 0.51$)	0.077^{NS} ($P = 0.28$)	-8.656^{**} ($P < 0.02$)
(Kinki)								
Northern	0.91 ± 0.03	0.0066 ± 0.0037	1.348	3.721	19.453	0.008^{NS} ($P = 0.54$)	0.012^{NS} ($P = 0.90$)	-9.808^{**} ($P < 0.02$)
Southern	0.86 ± 0.06	0.0078 ± 0.0045	8.783	0.002	7.996	0.024^{NS} ($P = 0.48$)	0.030^{NS} ($P = 0.83$)	-0.705^{NS} ($P = 0.36$)

MYA), and 0.24 MYA (0.09–0.40 MYA), respectively. Divergence within the Kinki group was estimated to have begun around 0.90 MYA (0.52–1.34). Diversification within each of Haplotype Groups D and E was calculated to have started around 0.43 MYA (0.22–0.67 MYA) and 0.50 MYA (0.24–0.83 MYA), respectively. Estimated time frames for inferred phylogeographic events are shown in Table 5.

DISCUSSION

Genetic isolation between the two genetic types

The allozyme analysis revealed the presence of two genetic types in *O. japonicus*, the SW-Honshu and Kinki groups, in Kinki and the adjacent districts, as previously reported by Yoshikawa et al. (2010). This is also consistent with the result of the mtDNA analysis (Yoshikawa et al., 2008). Moreover, discontinuous bimodal distributions of genotypic similarity in three localities indicated sympatric occurrence of these two types. Between the two types, fixed allelic differences were found for at least two loci, ATA-2 and PEPlgg, except for Sample 22. In addition, several other loci tended to differ between the two types. These results suggest that reproductive isolation between the types is maintained in their largely overlapping distributional range.

On the other hand, one hybrid individual was found in Sample 15. This was approximately 3.0% of total 33 specimens examined, a proportion lower than observed between the SW-Honshu and Shikoku groups (10.2%; Yoshikawa et al., 2010) in a locality where they were sympatric. This result suggests that reproductive isolation is more complete between tge SW-Honshu and Kinki groups than between the SW-Honshu and Shikoku groups.

The clustering and MDS analyses in this study also clearly separated the two genetic types, and no samples were found in intermediate positions between the types. However, Samples 10 and 22 of the Kinki group were placed relatively close to the SW-Honshu group in dimension 1 of MDS space. This may have resulted from similar allelic compositions at SOD (Sample 10) and ATA-2 (Sample 22), which possessed alleles predominant in the SW-Honshu group.

This study clearly demonstrated reproductive isolation between the two sympatric genetic types of *O. japonicus*. In addition, the genetic distance between them (mean D = 0.303) was larger than was reported for several salamander species, e.g., Nei's (1972) D between *Plethodon* species = 0.15 (Highton, 1989, 1990, 1999); Nei's (1978) D between *Hynobius* species = 0.11) (Kim et al., 2007). These results indicate that the two genetic types are different species, and that the Kinki group needs to be separated from *O. japonicus*, which should be retained for the SW-Honshu group (Yoshikawa et al., 2010).

Phylogeography of two genetic types of *O. japonicus*

The results of our phylogenetic analysis based on mtDNA showed the presence of two distinct genetic types in the Kinki District. This is concordant with the results of the allozyme analysis above and of the previous study (Yoshikawa et al., 2008).

Within the SW-Honshu group, three parapatric Haplotype Groups, A, B, and C, were recognized. In the phylogenetic tree, a basal split separated Haplotype Group C from A and B. Two haplotype groups, D and E, found in the Kinki group are separately distributed in the northern and southern regions, respectively. The relationships within each genetic group highlight major divisions between the northern (Haplotype Groups A, B, and D) and southern (Haplotype Groups C and E) regions in the Kinki District, and the estimated divergence times for these separations in the SW-Honshu and Kinki groups coincided. This may suggest that a common phylogeographical event was responsible for these major divisions.

From the NCPA, phylogeographic events were inferred for six nested clades. We based the inference of population phylogeography mainly on the results of the NCPA, although the nesting pattern was only slightly congruent with the results of the phylogenetic analysis. In inference 3, two alternative events were inferred (past gradual range expansion followed by fragmentation, or a past larger range followed by extinction in intermediate areas). For inference 3, we favor the former event, because a past larger range through the Hokuriku District to the Kii Peninsula was unlikely from the time frame of this event and the geological history of the area (see below). Based on the coalescent theory (Templeton, 1998), we interpret that this expansion occurred from Clade H3-3 or H3-4 (interior clades) to Clade H3-6 (tip clade), suggesting southward expansion of the SW-Honshu group.

Although no observed mismatch distributions significantly deviated from the population expansion model, contrasting patterns of frequency distribution between the SW-Honshu and Kinki groups in the northern and southern regions of the Kinki District suggest different population histories. In addition, Fu's neutrality test suggested demographic expansion in the SW-Honshu group from the south-

ern region and the Kinki group from the northern region. We believe that the NCPA, mismatch distributions, and Fu's *F*s indicate a northern origin and southward expansion of the SW-Honshu group, and in contrast, a southern origin and northward expansion of the Kinki group, and that they clearly support the phylogeographic hypothesis proposed by Yoshikawa et al. (2008). Below, we discuss the historical biogeography of the two genetic types of *O. japonicus* in the Kinki District, based on the NCPA, population genetic analyses, and divergence time estimates.

The ancestral form of *O. japonicus* was divided into a SW-Honshu group and ancestor of the Shikoku and Kinki groups around 4.3 MYA by the formation of the Second Setouchi Basin, which started about 5 MYA (Yoshida, 1992). Separation between the Shikoku and Kinki groups was estimated to have occurred around 2.5 MYA, due to formation of Kii Strait (Yoshikawa et al., 2008). After that, the Kinki and SW-Honshu groups would have been allopatrically distributed on the Kii Peninsula and in part of Honshu, respectively, separated by the freshwater basin that existed along ancestral Osaka Bay, Lake Biwa, and Ise Bay.

Uplift of the Suzuka Mountains, which connect mountains on the Kii Peninsula with the northern part of the Kinki District, started around 2 MYA and accelerated around 1 MYA (Okada, 1980). This geologic activity would have enabled the SW-Honshu group to disperse onto the Kii Peninsula (inference 3), and in contrast, the Kinki group to expand its range into the northern region. The estimated time frame for inference 3 and the divergence times between the northern and southern regions for both groups (approximately 0.9–1 MYA) are congruent with this assumption. By this expansion, the two genetic types would have contacted secondarily.

The Kinki group subsequently expanded its range into the northern Kinki and Hokuriku Districts. Because "restricted gene flow with isolation by distance" (inference 6) was suggested in southern Hokuriku to west of Lake Biwa around 0.25 MYA, the Kinki group may have reached this area by that time. Afterward, phylogeographic events at the level of 1-step clades occurred (inferences 1, 2, 4, 5) 0.16–0.05 MYA, from the middle to late Pleistocene. These events may have occurred in relation to the Pleistocene climatic oscillations rather than to geohistorical events, because formation of the basic landform of the Kinki and adjacent districts is considered to have been completed by the middle Pleistocene (Ota et al., 2004). It has been suggested that species adapted to a cool climate at higher elevations, like *O. japonicus* and several other salamanders, expand their range to lower elevations during glacial periods (Crespi et al., 2003; Yoshikawa et al., 2008; Sakamoto et al., 2009). Repeated climate changes in the Pleistocene may also have affected differentiation in this species.

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