Genetic Structure and Cryptic Diversity of *Onychodactylus japonicus* (Amphibia, Caudata, Hynobiidae) in Northeastern Honshu, Japan, as Revealed by Allozymic Analysis

Author(s): Natsuhiko Yoshikawa , Masafumi Matsui and Kanto Nishikawa
Source: Zoological Science, 29(4):229-237. 2012.
Published By: Zoological Society of Japan
DOI: http://dx.doi.org/10.2108/zsj.29.229
URL: http://www.bioone.org/doi/full/10.2108/zsj.29.229

ZOOLOGICAL SCIENCE **29**: 229–237 (2012)

Genetic Structure and Cryptic Diversity of *Onychodactylus japonicus* (Amphibia, Caudata, Hynobiidae) in Northeastern Honshu, Japan, as Revealed by Allozymic Analysis

Natsuhiko Yoshikawa, Masafumi Matsui*, and Kanto Nishikawa

Graduate School of Human and Environmental Studies, Kyoto University, Yoshida Nihonmatsu-cho Sakyo-ku, Kyoto 606-8501, Japan

We conducted a comprehensive allozymic analysis of 393 specimens of the Japanese clawed salamander, *Onychodactylus japonicus*, from 33 populations of northeastern Honshu, Japan. As a result, these populations exhibited extensive geographic genetic differentiation, and four major genetic groups (N-Tohoku, S-Tohoku, Tsukuba, and SW-Honshu groups) were consistently recognized. Of these, the Tsukuba group was geographically isolated from all the others, whereas the N- and S-Tohoku groups, and the S-Tohoku and SW-Honshu groups, respectively, were nearly parapatric, without distinct geographic barriers. The magnitude of genetic distances between the four groups, except for between the N- and S-Tohoku groups, was as large as that normally found among different hynobiid species. A structure analysis detected no admixture of the N- and S-Tohoku groups, whereas few hybrids were found between the S-Tohoku and SW-Honshu around their contact zone. However, genetic exchange between these parapatric groups appeared to be infrequent, suggesting the presence of some isolation mechanisms between them. Within each group, only the S-Tohoku group exhibited an extensive level of population genetic structure that roughly distinguishes the eastern, central, and northwestern subgroups, indicating the complexity of the phylogeographic traits of this group. These results strongly suggest that populations of *O. japonicus* from northeastern Japan encompass several cryptic species.

Key words: *Onychodactylus japonicus*, cryptic species, allozyme, genetic structure, reproductive isolation, northeastern Japan

INTRODUCTION

Molecular phylogenetic approaches contributed greatly to the inference of detailed phylogeography within species and the identification of cryptic species in morphologically conserved taxa. Especially, cytoplasmic mitochondrial DNA (mtDNA), which is highly variable and easy to analyze, has been extensively utilized in many studies. However, mtDNA does not always reflect exact species boundaries and phylogenies, because of the presence of occasional hybridization and introgression (e.g., Weisrock et al., 2006; Liu et al., 2010). Many recent works have therefore incorporated nuclear genetic markers to allow for more robust conclusions, especially for systematic purposes. Among such nuclear genetic markers, allozymes have long been used in many systematic studies of amphibians (e.g., Matsui, 1987; Good and Wake, 1992), and are still extensively utilized (e.g., Tominaga et al., 2005; Kuchta, 2007; Nishikawa et al., 2007; Fu and Zeng, 2008; Matsui et al., 2008; Arntzen and Wielstra, 2010).

Onychodactylus japonicus is a montane salamander widely occurring in Honshu and Shikoku Islands of mainland Japan. Recently, a molecular phylogenetic study based on mtDNA by Yoshikawa et al. (2008) clearly demonstrated that this species comprises four major clades (Clades I, II, II, and IV) and further subclades (two each within II and IV, and three in III). Of these, Clades III and IV occur sympatrically in western and southern parts of Honshu. Allozymic studies by Yoshikawa et al. (2010a, b) clearly demonstrated that sympatric Clade III and IV are reproductively isolated and are candidates of cryptic species. These previous findings on the western clades of *O. japonicus* suggest presence of further diversity and more candidates of cryptic species in this wide-ranging species, when the remaining eastern clades are studied.

In northeastern Japan, four genetic groups are known: N-Tohoku (corresponding to Clade I of mtDNA), S-Tohoku (Subclade II-A), SW-Honshu (Clade III), and Tsukuba (Subclade II-B) groups (Yoshikawa et al., 2008, 2010a). Of these, the former three groups are parapatric, whereas the Tsukuba group is completely isolated from all the other groups and is restricted to the Tsukuba Mountains. The magnitudes of allozymic genetic distances between these four groups are large, and are equal to or greater than the interspecific estimates of hynobiid salamanders (Yoshikawa et al., 2010a). From this, it can be hypothesized that each of the four major genetic groups recognized in the previous studies represents different cryptic species, as is the case in the western clade of *O. japonicus*. However, in evaluating

* Corresponding author. Tel. : +81-75-753-6846;
Fax : +81-75-753-6846;
E-mail: fumi@zoo.zool.kyoto-u.ac.jp
doi:10.2108/zsj.29.229

this hypothesis, geographic sampling of previous studies was not always sufficient to detect the presence of reproductive isolation between the groups. We therefore perform a comprehensive allozymic analysis focusing on genetic groups from northeastern Honshu (N-Tohoku, S-Tohoku, SW-Honshu, and Tsukuba groups) of *O. japonicus*, to obtain fundamental information for determining taxonomic status of each group and to understand their population genetic structures.

MATERIALS AND METHODS

A total of 393 specimens of *O. japonicus* (including metamorphs and larvae) were collected from 33 localities in Tohoku and Kanto Districts in the northeastern part of Honshu, Japan (Fig. 1, Table 1). These populations cover entire ranges of the N-Tohoku, S-Tohoku, and Tsukuba groups, and of eastern part of SW-Honshu group, which is also found in northeastern Honshu (Yoshikawa et al., 2008, 2010a). As we could not obtain fresh tissues of the topotypic population (Hakone-machi, Kanagawa Prefecture), a geographically and genetically close population from Izu-shi (Pop. 32; Yoshikawa et al., 2008), was used in this study.

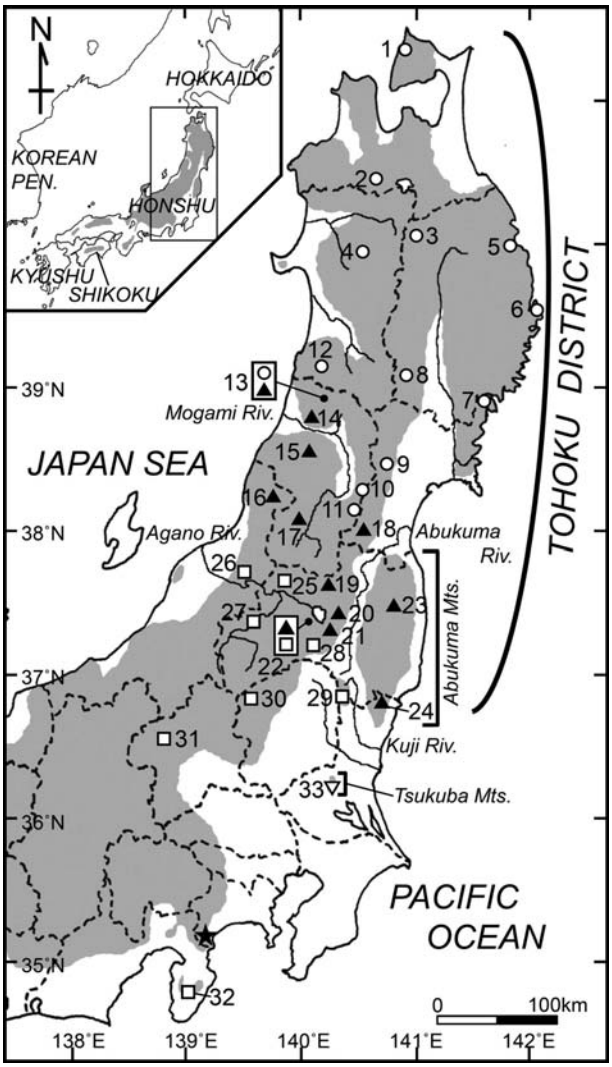

Fig. 1. A map of northeastern Honshu of Japan, showing distributional range of *Onychodactylus japonicus* (shaded) and sampling locations. Four groups recognized in this study, N-Tohoku, S-Tohoku, SW-Honshu, and Tsukuba groups, are indicated by open circles, closed triangles, open squares, and an open inverse triangle, respectively. The star indicates the type locality of *O. japonicus* (Hakone-machi, Kanagawa Prefecture). For population number, refer to Table 1.

Table 1. Population numbers, sampling localities, sample sizes, and assigned groups of *Onychodactylus japonicus* examined.

Pops. no.	Locality	Latitude (°N)	Longitude (°E)	n	Group
1	Sai-mura, Aomori Pref.	41.437	140.870	10	N-Tohoku
2	Hirakawa-shi, Aomori Pref.	40.473	140.608	10	N-Tohoku
3	Hachimantai-shi, Iwate Pref.	40.107	140.909	5	N-Tohoku
4	Kita-akita-shi, Akita Pref.	40.048	140.607	5	N-Tohoku
5	Noda-mura, Iwate Pref.	40.063	141.775	6	N-Tohoku
6	Miyako-shi, Iwate Pref.	39.572	142.014	6	N-Tohoku
7	Kesennuma-shi, Miyagi Pref.	38.978	141.513	8	N-Tohoku
8	Oshu-shi, Iwate Pref.	39.106	140.891	16	N-Tohoku
9	Taiwa-cho, Miyagi Pref.	38.463	140.678	6	N-Tohoku
10	Yamagata-shi, Yamagata Pref.	38.332	140.504	8	N-Tohoku
11	Kaminoyama-shi, Yamagata Pref.	38.119	140.395	10	N-Tohoku
12	Yurihonjo-shi, Akita Pref.	39.144	140.104	16	N-Tohoku
13	Sakata-shi, Yamagata Pref.	38.967	140.131	4	N-Tohoku, S-Tohoku
14	Sakata-shi, Yamagata Pref.	38.843	140.029	9	S-Tohoku
15	Nishikawa-machi, Yamagata Pref.	38.501	139.998	14	S-Tohoku
16	Asahi-mura, Niigata Pref.	38.245	139.636	11	S-Tohoku
17	Nagai-shi, Yamagata Pref.	38.106	139.948	13	S-Tohoku
18	Shichikashuku-machi, Miyagi Pref.	38.049	140.483	10	S-Tohoku
19	Kitashiobara-mura, Fukushima Pref.	37.724	140.068	16	S-Tohoku
20	Koriyama-shi, Fukushima Pref.	37.430	140.179	21	S-Tohoku
21	Koriyama-shi, Fukushima Pref.	37.358	140.117	11	S-Tohoku
22	Aizuwakamatsu-shi, Fukushima Pref.	37.433	139.973	13	S-Tohoku, SW-Honshu
23	Nihonmatsu-shi, Fukushima Pref.	37.543	140.661	10	S-Tohoku
24	Kitaibaraki-shi, Ibaraki Pref.	36.883	140.630	16	S-Tohoku
25	Kitakata-shi, Fukushima Pref.	37.701	139.718	15	SW-Honshu
26	Aga-machi, Niigata Pref.	37.739	139.450	24	SW-Honshu
27	Kaneyama-machi, Fukushima Pref.	37.360	139.474	13	SW-Honshu
28	Shirakawa-shi, Fukushima Pref.	37.239	140.137	10	SW-Honshu
29	Daigo-machi, Ibaraki Pref.	36.914	140.265	10	SW-Honshu
30	Nikko-shi, Tochigi Pref.	36.967	139.614	21	SW-Honshu
31	Naganohara-machi, Gunma Pref.	36.555	138.566	12	SW-Honshu
32	Izu-shi, Shizuoka Pref.	34.840	138.920	12	SW-Honshu
33	Sakuragawa-shi, Ibaraki Pref.	36.234	140.098	22	Tsukuba

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

Enzyme	E.C.number	Locus	Buffer system[a]
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
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).

Table 3. Allele frequencies and genetic variability at 11 polymorphic loci among 33 populations of *O. japonicus*. For population number, refer to Table 1.

	N-Tohoku													S-Tohoku				
Locus	1	2	3	4	5	6	7	8	9	10	11	12	13A	13B	14	15	16	17
	n = 10	n = 10	n = 5	n = 5	n = 6	n = 6	n = 8	n = 16	n = 6	n = 8	n = 10	n = 16	n = 3	n = 1	n = 9	n = 14	n = 11	n = 12
ATA-2	b0.200 e0.800	e	e	d0.100 e0.900	d0.083 e0.917	e	e0.938 g0.063	e	e	b0.063 e0.938	b0.050 e0.950	e	e	e	e	c0.036 e0.964	b0.091 e0.818 g0.091	e0.875 g0.125
GPI	c0.200 d0.800	c0.350 d0.650	c0.600 d0.850	c0.500 d0.500	c0.500 d0.500	c0.250 d0.750	c0.125 d0.875	c0.344 d0.656	c0.500 d0.500	c0.375 d0.625	b0.050 c0.400 d0.550	d	c0.167 d0.833	d	d	d	d	c0.042 d0.958
IDH-1	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
IDH-2	c	c	c	c	c	c	c	c	c	c	c	a0.033 c0.967	c	c	c	b0.107 c0.893	b0.045 c0.955	b0.042 c0.916 d0.042
LDH-1	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
MDH-1	c	b0.100 c0.900	c	c	c0.917 g0.083	c0.917 h0.083	c0.875 g0.125	c0.969 d0.031	c	c	c	c	c	c0.500 f0.500	a0.111 c0.500 d0.167 f0.222	a0.179 c0.750 d0.036 f0.036	a0.227 c0.273 d0.455 g0.045	a0.042 c0.667 d0.291
MDH-2	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b0.964 c0.036	a0.045 b0.818 c0.136	b0.958 c0.042
PGDH	c	c	c	c	c	c	c	b0.281 c0.719	c	b0.125 c0.875	b0.200 c0.800	b0.100 c0.900	c	c	b0.056 c0.944	b0.036 c0.893 d0.071	a0.045 c0.955	b0.042 c0.792 d0.166
PGM-1	b	b	b	b	b	b	b	b	b	b	b	a0.067 b0.933	b	b	b	b	b	b
PGM-3	c0.600 d0.400	a0.050 b0.350 c0.500 e0.100	a0.100 b0.300 c0.600	a0.100 b0.200 c0.400 d0.100 e0.200	b0.083 c0.583 d0.333	b0.083 c0.417 d0.333 e0.167	c0.375 d0.500 e0.125	b0.031 c0.500 d0.375 e0.063 f0.031	b0.083 c0.667 d0.250	b0.125 c0.500 d0.375	b0.200 c0.700 d0.050 e0.050	c0.567 d0.367 e0.066	c0.667 d0.333	c	b0.111 c0.833 d0.056	a0.036 b0.071 c0.822 d0.071	c0.955 d0.045	b0.083 c0.875 d0.042
SOD	b	b	b	b	b0.917 c0.083	b	b	b	b	b	a0.050 b0.950	b	b	a0.500 b0.500	a0.889 b0.111	a0.643 b0.357	a0.500 b0.500	a0.792 b0.208
A	1.27	1.45	1.27	1.55	1.55	1.45	1.45	1.64	1.27	1.45	1.73	1.45	1.18	1.18	1.64	2.09	2.00	2.09
(S.E.)	0.14	0.28	0.19	0.37	0.21	0.28	0.21	0.36	0.19	0.21	0.30	0.21	0.12	0.12	0.31	0.34	0.30	0.30
P	27.27	27.27	18.18	27.27	45.45	27.27	36.36	36.36	18.18	36.36	45.45	36.36	18.18	18.18	36.36	63.64	63.64	72.73
Ho	0.127	0.136	0.109	0.109	0.121	0.121	0.125	0.114	0.091	0.136	0.118	0.091	0.091	0.182	0.121	0.149	0.157	0.159
(S.E.)	0.078	0.081	0.078	0.073	0.051	0.071	0.069	0.061	0.065	0.068	0.054	0.055	0.065	0.122	0.064	0.058	0.060	0.040
He	0.102	0.114	0.093	0.129	0.136	0.110	0.104	0.138	0.090	0.127	0.137	0.083	0.066	0.091	0.114	0.155	0.186	0.173
(S.E.)	0.054	0.066	0.062	0.077	0.061	0.067	0.056	0.069	0.060	0.064	0.061	0.049	0.045	0.061	0.062	0.052	0.069	0.048

	S-Tohoku							SW-Honshu									Tsukuba
Locus	18	19	20	21	22A	23	24	22B	25	26	27	28	29	30	31	32	33
	n = 10	n = 16	n = 21	n = 11	n = 11	n = 10	n = 16	n = 1	n = 14	n = 24	n = 13	n = 10	n = 10	n = 21	n = 12	n = 12	n = 22
ATA-2	e	a0.310 e0.969	e	e	e	e	b0.156 e0.844	e	a0.179 e0.821	a0.021 e0.979	a0.038 e0.962	a0.100 e0.900	e	e	e	e	e0.500 f0.500
GPI	c0.150 d0.850	a0.031 c0.031 d0.938	c0.048 d0.929 e0.023	d0.955 e0.045	c0.045 d0.955	d	a0.031 d0.875 f0.094	d	d	c0.167 d0.833	c0.077 d0.923	a0.050 c0.200 d0.700 e0.050	c0.600 d0.400	c0.048 d0.952	c0.042 d0.958	d	c0.977 d0.023
IDH-1	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
IDH-2	c	c	c	c0.909 d0.091	b0.045 c0.955	a0.250 c0.750	c	c	c	b0.021 c0.875 d0.104	c0.962 d0.038	c	c0.350 d0.650	c0.738 d0.262	c	c	c
LDH-1	b	b	a0.071 b0.929	b	b	b	b	b	b	b	a0.038 b0.962	a0.050 b0.950	b	a0.024 b0.976	b	b	b
MDH-1	c0.200 d0.750 g0.050	c0.219 d0.781	c0.214 d0.786	c0.409 d0.591	c0.318 d0.682	a0.100 d0.900	d	c	c0.536 g0.464	a0.021 c0.875 g0.104	c0.923 g0.077	c0.850 d0.050 g0.100	c0.950 g0.050	c0.976 g0.024	c	c	e
MDH-2	b	b	b	b	b	b	a0.031 b0.969	b	b	b	b	b	b	b	b	a0.042 b0.958	b
PGDH	c	a0.125 c0.719 d0.156	a0.048 c0.833 d0.095 e0.024	a0.045 c0.773 d0.045 e0.136	c0.955 e0.045	c	a0.156 c0.844	e	c0.143 e0.500 f0.357	a0.042 c0.104 e0.688 f0.167	c0.231 e0.615 f0.154	c0.150 e0.600 f0.250	c0.650 e0.350	c0.238 e0.476 f0.286	c0.333 e0.542 f0.125	c0.458 e0.125 f0.417	c
PGM-1	b	a0.031 b0.969	b	a0.045 b0.955	b	b	b	b	b	b	b	a0.050 b0.950	b	b	a0.083 b0.917	b	b
PGM-3	c0.950 d0.050	c0.969 d0.031	b0.095 c0.833 d0.071	b0.136 c0.864	b0.091 c0.864 d0.045	b	b0.875 c0.125	a	a0.107 b0.643 c0.214 e0.036	a0.063 b0.875 c0.063	0.192 b0.731 c0.038 e0.038	a0.200 b0.600 c0.200	a0.100 b0.800 d0.100	a0.190 b0.738 c0.071	a0.292 b0.708	b	c0.910 e0.045 g0.045
SOD	a0.850 b0.150	a0.844 b0.156	a0.643 b0.357	a0.818 b0.182	a0.773 b0.227	a	a0.813 b0.187	a	a0.964 b0.036	a	a0.923 b0.077	a	a	a	a0.833 b0.167	a0.958 b0.042	b
A	1.45	1.82	1.91	1.82	1.64	1.18	1.64	1.00	1.73	2.00	2.00	2.09	1.55	1.73	1.55	1.36	1.36
(S.E.)	0.21	0.23	0.31	0.26	0.20	0.12	0.20	0.00	0.30	0.33	0.27	0.31	0.21	0.24	0.21	0.20	0.20
P	36.36	63.64	54.55	63.64	54.55	18.18	54.55	0.00	45.45	54.55	72.73	63.64	45.45	54.55	45.45	27.27	27.27
Ho	0.109	0.125	0.121	0.157	0.116	0.045	0.108	0.000	0.169	0.106	0.147	0.200	0.191	0.104	0.144	0.076	0.029
(S.E.)	0.053	0.047	0.040	0.049	0.046	0.031	0.039	0.000	0.074	0.043	0.051	0.069	0.083	0.052	0.063	0.060	0.018
He	0.091	0.123	0.150	0.158	0.117	0.050	0.121	0.000	0.181	0.134	0.147	0.201	0.166	0.149	0.137	0.069	0.065
(S.E.)	0.043	0.047	0.051	0.051	0.047	0.036	0.039	0.000	0.075	0.049	0.054	0.068	0.065	0.068	0.061	0.054	0.046

A total of 11 loci encoding eight enzymes were analyzed (Table 2). The experimental conditions, techniques, and interpretations of zymograms were essentially similar to those described by Yoshikawa et al. (2010a). For each population, genetic variabilities were assessed by calculating the mean number of alleles per locus (A), the proportion of polymorphic loci (P), and the mean observed (Ho) and expected (He) heterozygosities. Variable loci were checked using chi-square goodness-of-fit tests to determine whether they were in Hardy-Weinberg (HW) equilibrium. To evaluate the spatial pattern of heterogeneity, *F*-statistics (Wright, 1965) were employed. All these statistics were calculated by using GENALEX 6.14 (Peakall and Smouse, 2006). Populations of small sample size (less than three) were omitted from calculations of statistics and genetic distances (see below) because the estimates based on too small samples may not be accurate.

To infer overall genetic differentiation, we calculated Nei's unbiased genetic distance (Nei's D: Nei, 1978) between populations, and then constructed an UPGMA dendrogram using PHYLIP ver. 3.5C (Felsenstein, 1993). Confidences of tree topologies were tested by 2000 non-parametric bootstrap pseudo-replicates (Felsenstein, 1985). We also conducted principal coordinates analysis (PCO) on a pairwise matrix of Nei's D using GENALEX 6.14 (Peakall and Smouse, 2006) to understand the genetic relationships among populations. This type of analysis can detect clinal and complicated relationships that might be overlooked in clustering procedures (Felsenstein, 1982). Correlations between genetic and geographic distances were also tested by Mantel test (Mantel, 1967) for pairs of major genetic groups (N-Tohoku vs. S-Tohoku groups, S-Tohoku vs. SW-Honshu groups, and Tsukuba vs. the other groups).

Prior to the calculations of genetic variability and genetic distances, some individuals were omitted because they belonged to different genetic groups considered to be candidate cryptic species, or their hybrids (see RESULTS).

Allozyme data were also analyzed by structure v2.3.3 (Pritchard et al., 2000) using the admixture model to estimate population genetic structure and individual ancestries. We conducted an analysis with 10 iterations for each population size (*k*) of 1 to 10, and with MCMC running for 500,000 generations and initial burn-in of 50,000 generations. The ΔK values described by Evanno et al. (2005) were then calculated to identify the most reasonable *k*.

RESULTS

All 11 loci scored except IDH-1 were polymorphic and 74 alleles were detected (Table 3). The most variable locus was MDH-1 with eight alleles, followed by PGM-3 with seven alleles, and GPI and PGDH each with six alleles.

As stated above, we omitted one individual each from Pops. 13, 17 and 25, and two from Pop. 22 (shown in Fig. 6) from estimation of genetic variability of populations and pairwise genetic distances, as the result of structure analysis indicated the heterogeneous nature of each population (see below).

Among 33 populations examined after omitting above individuals, the mean number of allele per locus (A) varied from 1.18–2.09, the proportion of polymorphic loci (P) from 18.18–72.73, and the mean observed (Ho) and expected (He) heterozygosities ranged 0.029–0.200 and 0.050–0.201, respectively (Table 3). The mean for F_{st} (Wright, 1965) was 0.32 (range = 0.05–0.74), and the value was high for SOD (0.74), MDH-1 (0.58), PGM-3 (0.41), and PGDH (0.39) loci. In 10 populations (Pops. 1, 8, 15, 16, 20, 26, 28, 29, 30, and 33), several loci showed significant deviations from HW expectations in heterozygote frequency. Most of these were heterozygote-deficient.

In the UPGMA dendrogram (Fig. 2), four major genetic groups were recognized, as reported by Yoshikawa et al. (2010a). Designation of genetic groups was made based on the allocations of populations used in the previous study (N-Tohoku group: Pops. 1, 9, and 10; S-Tohoku group: Pops. 16 and 18; SW-Honshu group: Pops. 28, 29, 31, and 32; Tsukuba group: Pop. 33). Although bootstrap support for N-Tohoku group was high (85%), supports for the other groups were low (< 50%).

Nei's D varied between populations, with the mean of all pairs being 0.137 (Table 4). Among four groups, mean Nei's D ranged from 0.152–0.510, and the smallest and largest values were observed between the N-Tohoku and S-Tohoku, and between the SW-Honshu and Tsukuba groups, respectively. As shown in Table 4, Nei's Ds obtained between the genetic groups were: N-Tohoku vs. S-Tohoku = 0.047–0.327 (mean = 0.152), N-Tohoku vs. SW-Honshu = 0.151–0.277 (0.215), N-Tohoku vs. Tsukuba = 0.153–0.260 (0.191), S-Tohoku vs. SW-Honshu = 0.105–0.283 (0.161), S-Tohoku vs. Tsukuba = 0.236–0.500 (0.317), and SW-Honshu vs. Tsukuba = 0.460–0.552 (0.510).

The results of PCO are plotted in Fig. 3, where the first and second axis, respectively, explained 54.2% and 24.3% of the total variation. In the scattergram, four major groups found in the UPGMA dendrogram were clearly recognized. Distribution of plots did not correspond to that of sampling locations. Populations of N-Tohoku and SW-Honshu groups were densely plotted, whereas populations of S-Tohoku group were scatteredly plotted, with Pops. 23 and 24 distant from the others on the first axis.

Straight geographic distances between populations ranged from 9 to 752 km (Table 4). Between N- and S-

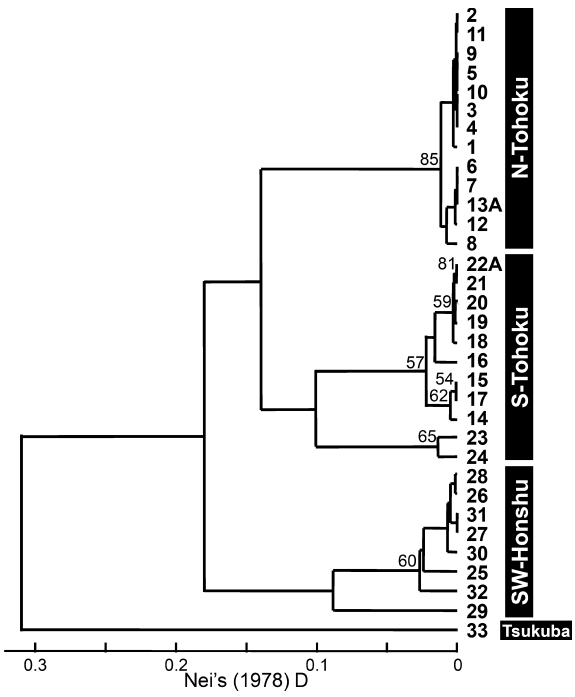

Fig. 2. An UPGMA phenogram based on Nei's (1978) unbiased genetic distance. Nodal values indicate the bootstrap support (> 50, 2000 replicates). For population number, refer to Fig. 1 and Table 1.

Table 4. Nei's unbiased genetic distance (below diagonal) and geographic distance (above diagonal) between populations of *O. japonicus*. Populations 13B and 22B were omitted due to small sample sizes. For population number, refer to Table 1.

Pops.	N-Tohoku													S-Tohoku										
	1	2	3	4	5	6	7	8	9	10	11	12	13A	14	15	16	17	18	19	20	21	22A	23	24
1	–	109	148	156	170	229	277	258	330	346	371	263	281	297	336	367	378	378	418	448	457	451	432	508
2	0.017	–	48	47	109	156	182	153	223	238	262	154	172	188	226	258	269	269	308	339	348	342	326	400
3	0.025	0.000	–	26	74	112	135	111	183	200	225	127	144	160	196	230	237	232	275	303	312	308	286	360
4	0.013	0.000	0.000	–	99	131	140	106	176	191	215	109	127	143	181	213	223	222	262	292	301	295	278	353
5	0.004	0.005	0.000	0.000	–	59	122	130	201	222	247	177	188	203	233	269	269	251	300	324	333	332	297	369
6	0.000	0.003	0.014	0.000	0.000	–	78	109	168	190	214	171	177	190	212	248	242	215	266	286	296	297	254	323
7	0.002	0.020	0.037	0.016	0.010	0.000	–	55	92	114	137	123	121	129	142	176	167	137	189	208	217	219	177	248
8	0.010	0.016	0.019	0.009	0.004	0.003	0.010	–	74	93	119	69	70	81	104	140	139	124	171	197	206	203	176	250
9	0.007	0.004	0.000	0.000	0.000	0.002	0.017	0.006	–	22	46	91	75	71	60	89	76	50	99	123	132	131	103	178
10	0.000	0.005	0.006	0.000	0.000	0.000	0.004	0.000	0.000	–	25	96	78	70	47	70	54	32	78	103	113	110	89	163
11	0.014	0.003	0.000	0.000	0.001	0.009	0.026	0.005	0.000	0.000	–	117	98	86	54	62	39	11	53	78	87	85	69	140
12	0.005	0.022	0.042	0.026	0.020	0.003	0.003	0.014	0.022	0.011	0.023	–	19	34	70	104	116	126	158	189	198	190	185	257
13A	0.000	0.007	0.017	0.007	0.000	0.000	0.000	0.003	0.000	0.000	0.006	0.000	–	16	54	86	97	107	138	170	179	171	166	238
14	0.116	0.117	0.141	0.138	0.121	0.114	0.118	0.129	0.126	0.126	0.113	0.103	0.099	–	39	71	82	97	124	156	165	156	155	225
15	0.062	0.068	0.088	0.084	0.071	0.063	0.067	0.076	0.074	0.071	0.062	0.052	0.047	0.007	–	36	43	65	85	118	126	117	121	189
16	0.085	0.095	0.120	0.117	0.097	0.090	0.090	0.108	0.102	0.103	0.094	0.077	0.073	0.025	0.023	–	27	72	67	100	106	95	117	175
17	0.092	0.095	0.115	0.113	0.098	0.093	0.098	0.102	0.102	0.100	0.085	0.081	0.077	0.003	0.001	0.018	–	47	44	77	84	75	89	150
18	0.157	0.154	0.171	0.174	0.149	0.153	0.159	0.168	0.157	0.165	0.150	0.148	0.139	0.023	0.049	0.020	0.025	–	52	73	83	82	59	132
19	0.171	0.173	0.196	0.197	0.173	0.170	0.174	0.178	0.178	0.181	0.163	0.154	0.152	0.028	0.052	0.024	0.023	0.003	–	33	41	33	57	108
20	0.128	0.125	0.149	0.148	0.129	0.122	0.126	0.135	0.135	0.135	0.124	0.113	0.111	0.031	0.046	0.013	0.025	0.004	0.003	–	9	18	46	76
21	0.134	0.129	0.153	0.154	0.137	0.130	0.136	0.141	0.141	0.141	0.124	0.117	0.115	0.010	0.025	0.018	0.004	0.004	0.002	0.004	–	16	52	71
22A	0.135	0.130	0.154	0.151	0.137	0.128	0.132	0.142	0.143	0.141	0.129	0.120	0.118	0.015	0.032	0.020	0.012	0.004	0.005	0.003	0.000	–	63	86
23	0.319	0.259	0.308	0.298	0.309	0.281	0.288	0.322	0.327	0.308	0.305	0.294	0.304	0.125	0.172	0.153	0.137	0.109	0.118	0.103	0.100	0.088	–	75
24	0.262	0.216	0.257	0.249	0.257	0.236	0.243	0.266	0.273	0.255	0.249	0.249	0.254	0.115	0.157	0.116	0.119	0.085	0.086	0.070	0.079	0.067	0.014	–
25	0.237	0.209	0.246	0.233	0.236	0.218	0.217	0.224	0.252	0.226	0.212	0.218	0.224	0.097	0.111	0.164	0.093	0.154	0.141	0.145	0.101	0.098	0.142	0.138
26	0.269	0.221	0.245	0.240	0.256	0.242	0.257	0.240	0.265	0.239	0.224	0.255	0.255	0.148	0.153	0.242	0.134	0.222	0.208	0.208	0.147	0.146	0.163	0.168
27	0.217	0.179	0.207	0.195	0.214	0.193	0.203	0.197	0.222	0.195	0.186	0.201	0.204	0.119	0.118	0.202	0.106	0.194	0.182	0.177	0.123	0.118	0.153	0.154
28	0.229	0.192	0.207	0.203	0.213	0.208	0.223	0.203	0.221	0.203	0.186	0.221	0.216	0.111	0.118	0.196	0.099	0.171	0.159	0.164	0.111	0.108	0.166	0.160
29	0.267	0.209	0.204	0.206	0.221	0.234	0.262	0.241	0.230	0.225	0.216	0.279	0.257	0.181	0.180	0.283	0.164	0.240	0.260	0.250	0.186	0.191	0.181	0.210
30	0.239	0.202	0.231	0.221	0.238	0.216	0.227	0.220	0.245	0.218	0.207	0.220	0.224	0.122	0.122	0.215	0.108	0.201	0.189	0.188	0.126	0.128	0.154	0.166
31	0.186	0.151	0.179	0.166	0.187	0.163	0.173	0.170	0.193	0.167	0.161	0.169	0.173	0.113	0.105	0.190	0.101	0.191	0.181	0.170	0.120	0.114	0.154	0.153
32	0.217	0.174	0.207	0.196	0.217	0.191	0.204	0.206	0.224	0.197	0.189	0.201	0.204	0.115	0.116	0.202	0.109	0.195	0.189	0.180	0.132	0.133	0.122	0.131
33	0.208	0.182	0.153	0.167	0.154	0.202	0.230	0.204	0.160	0.189	0.173	0.252	0.208	0.306	0.280	0.236	0.293	0.275	0.313	0.273	0.310	0.306	0.500	0.416

Pops.	SW-Honshu								Tsukuba
	25	26	27	28	29	30	31	32	33
1	427	428	468	470	506	509	576	752	581
2	318	320	359	361	397	399	468	643	472
3	287	292	330	325	360	367	442	611	435
4	273	275	315	315	350	353	425	597	425
5	319	328	362	344	375	393	477	633	450
6	289	302	332	305	333	357	447	593	407
7	212	227	255	227	256	279	370	515	330
8	188	198	232	218	251	264	347	506	327
9	121	135	164	144	177	192	279	433	253
10	99	113	142	125	160	171	257	413	235
11	76	93	118	100	135	146	233	387	210
12	164	166	106	212	249	246	315	490	323
13A	146	149	188	193	230	227	298	471	303
14	131	133	172	179	216	212	282	156	289
15	92	96	134	140	177	173	246	417	250
16	63	62	102	121	158	145	212	387	227
17	50	60	93	99	136	130	208	374	208
18	78	97	119	94	128	143	233	383	204
19	31	54	68	55	93	93	182	337	165
20	49	72	65	23	60	72	168	310	133
21	52	73	60	15	52	63	160	301	125
22A	37	57	47	29	64	61	154	303	133
23	86	110	110	55	79	113	211	339	153
24	123	143	119	58	33	91	182	273	85
25	–	24	44	65	100	82	159	325	165
26	0.025	–	41	85	117	88	150	326	176
27	0.018	0.001	–	66	89	48	156	285	138
28	0.014	0.002	0.000	–	37	58	157	289	111
29	0.134	0.072	0.082	0.082	–	57	151	260	76
30	0.030	0.007	0.003	0.010	0.060	–	99	244	91
31	0.032	0.012	0.000	0.007	0.090	0.009	–	192	136
32	0.041	0.029	0.020	0.031	0.094	0.019	0.020	–	188
33	0.486	0.548	0.515	0.471	0.460	0.562	0.498	0.538	–

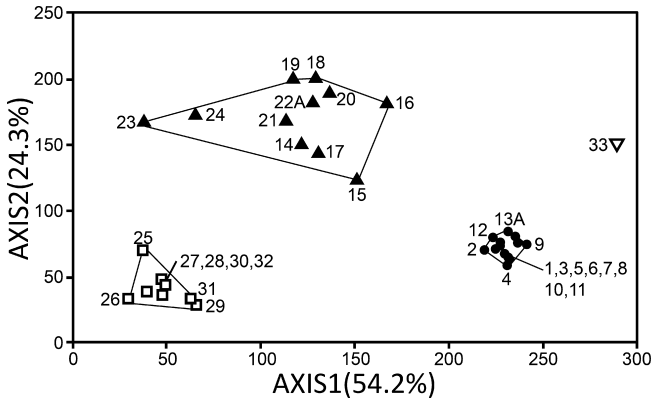

Fig. 3. Principal Co-ordinate Analysis (PCO) based on the Nei's (1978) unbiased genetic distance from 11 allozyme loci. The relative PCO values are plotted for the first and second axes. The N-Tohoku, S-Tohoku, SW-Honshu, and Tsukuba groups are indicated by closed circles, closed triangles, open squares, and an open inverse triangle, respectively. For population number, refer to Fig. 1 and Table 1.

Tohoku groups, geographic and genetic distances were significantly correlated ($r = 0.251$, $P < 0.05$: Fig. 4A). No correlation was found in the distances between populations of the S-Tohoku and SW-Honshu, with genetic distances larger than 0.100 in all pairs regardless of their geographic distance (Fig. 4B). Similarly, there was no correlation in the distances between the Tsukuba and the other groups, with

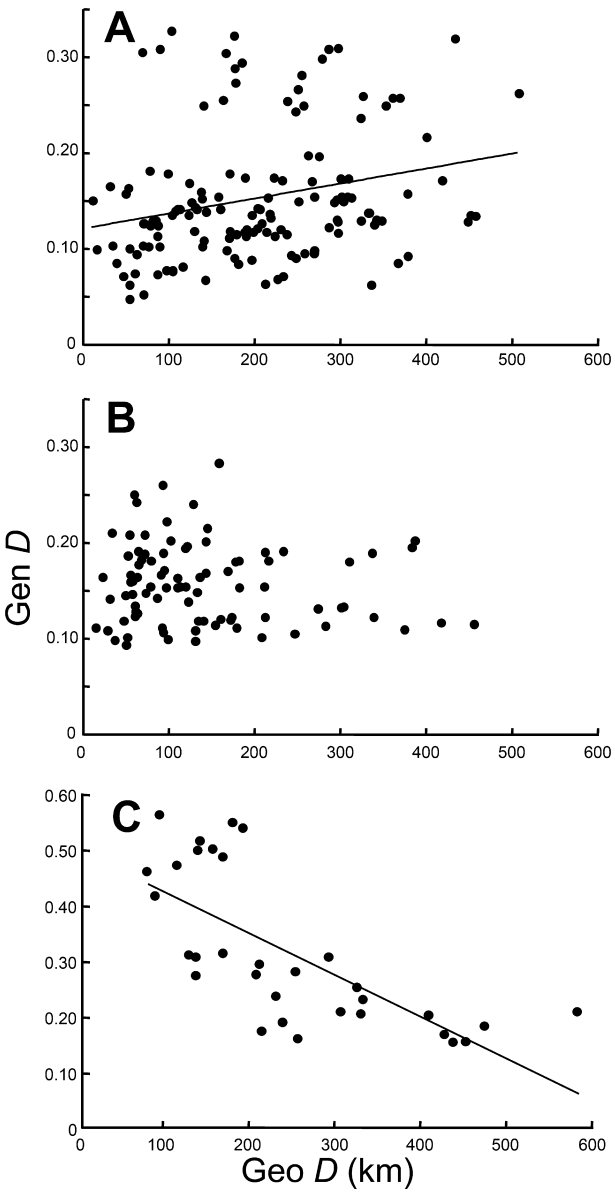

Fig. 4. Correlation between geographic distance (Geo *D*) and Nei's (1978) unbiased genetic distance (Gen *D*) pairs of populations between genetic groups recognized in *O. japonicus*. (A) Combination between populations of N-Tohoku and S-Tohoku groups: Gen*D* = 0.0002 Geo*D* + 0.1195, r^2 = 0.0629, P < 0.05. (B) Combination between populations of S-Tohoku and SW-Honshu groups: r^2 = 0.010, P = 0.056. (C) Combination between population of Tsukuba and all other populations: r^2 = 0.500, P < 0.05.

genetic distances larger than 0.150 in all pairs (Fig. 4C).

The N-Tohoku group was genetically uniform across its range (Nei's D = 0–0.043, mean 0.007), despite its relatively large distributional range (Pops. 1–13, Fig. 1). This group had no private allele, but was unique in SOD is nearly fixed by the allele [b] (Table 3). The mean F_{st} value for this group was very low (0.088).

Within S-Tohoku group, two populations from the Abukuma Mountains (Pops. 23 and 24) were differentiated from the others with Nei's D of 0.070–0.172 (mean 0.112). Except for these two populations, degree of genetic variation

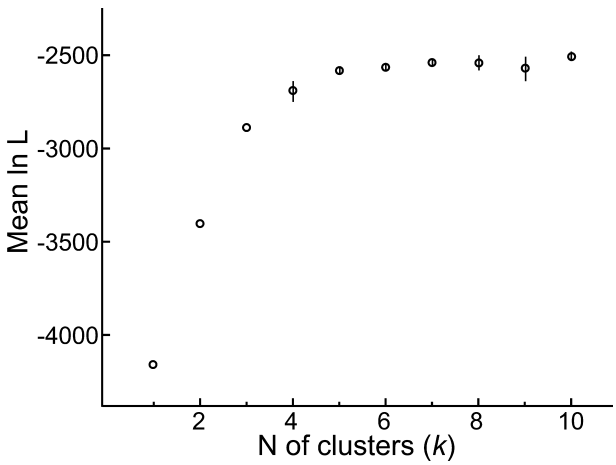

Fig. 5. Plots of *k* (1–10) versus mean ln likelihood values of 10 MCMC runs for the allozyme data under the admixture model. Vertical bars denote standard deviations.

between populations were relatively low (Nei's D = 0–0.052, mean 0.016), although they were scatteredly plotted in PCO scattergram. This group was characterized by high frequency of MDH-1[d], an allele that was rare in the other groups, although the frequency decreased in northeastern populations. Populations from the Abukuma Mountains (Pops. 23 and 24) were nearly fixed by PGM-3[b], which is rare in other S-Tohoku populations but dominant in SW-Honshu group (Table 3). The mean F_{st} value for this group was relatively high (0.145).

In SW-Honshu group, Pop. 29 was diverged from the others with Nei's D of 0.057–0.136 (mean 0.088), in contrast to moderate genetic variation between the remaining populations (0–0.042, mean 0.016). The population closest to the type locality of the species (Pop. 32) was included in this group. There was no specific allele in this group, but PGM-3[b], which is rare in other groups, was dominant (Table 3). The mean F_{st} value for this group was relatively high (0.135).

The Tsukuba group, found only in Tsukuba Mountains (Pop. 33), was geographically isolated from the other populations with the smallest distance of 76 km (between Pop. 29). In this group, unique alleles were found in MDH-1 (allele [e]) and ATA-2 (allele [f]), and the former was completely fixed (Table 3).

Structure was run for up to k = 10. The likelihood values reached plateau after k = 4 (Fig. 5: mean lnL = –2697.8), although the values continued to increase gradually as k increase. At k = 4, clusters obtained corresponded to four major genetic groups recognized in dendrogram and PCO (Fig. 6). At k = 5 and 6, the S-Tohoku group was further divided into two and three clusters, respectively (Fig. 6). The division of clusters in the S-Tohoku group corresponded roughly to northwestern, central, and eastern parts of its range, but were highly admixed. Population structure broke down when k was greater than 6. Therefore we interpreted that *O. japonicus* in the study area could be separated into four major genetic components.

We also estimated ΔK values (Evanno et al., 2005) to detect true number of clusters, and it was highest at k = 3 (data not shown), indicating the true number is three. How-

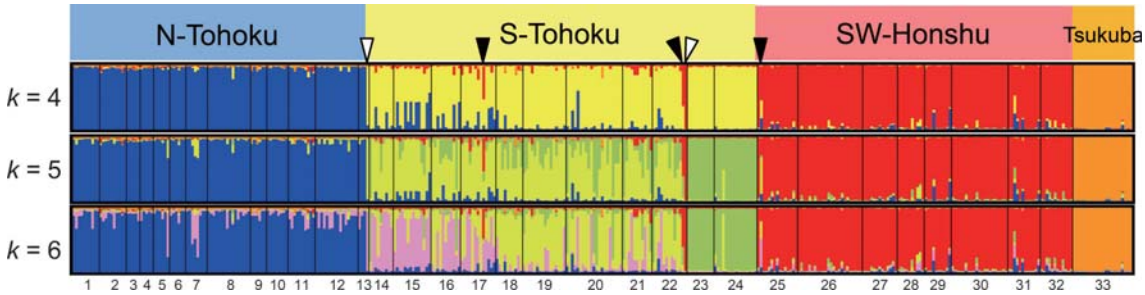

Fig. 6. Barplots of 393 individuals of *O. japonicus* from 33 populations in northeastern Honshu by structure (k = 4–6). Open arrows indicate individuals of pure ancestry in sympatric locality. Closed arrows indicate hybrids.

ever, we rejected this result, as the N-Tohoku and Tsukuba groups were assigned to the same cluster. These two groups were geographically separate (Fig. 1) and genetically divergent and distinctive (Figs. 2, 3; Table 4) as mentioned above. Therefore we considered that it is unreasonable to treat these groups as a single genetic component, despite the results of ΔK estimation.

According to the result of structure at k = 4–6 (Fig. 6), the Tsukuba group was robust and clearly distinguishable. The N- and S-Tohoku groups, with almost parapatric distribution, were clearly separated between Pops. 11 and 18 at the eastern contact zone despite close geographic distance between them (11 km). At the western contact zone, both of these genetic types were found in Pop. 13 (three and one individuals of N-Tohoku and S-Tohoku groups, respectively) without admixture. Although slight admixture between them was estimated in neighboring populations (Pops. 12–17), these populations were assigned to the S-Tohoku group when k = 5 or 6.

The S-Tohoku and SW-Honshu groups were also found nearly parapatric with few exceptions. In Pop. 22, each one individual was estimated to be a pure SW-Honshu and a hybrid between S-Tohoku and SW-Honshu, while all the remaining individuals were judged to be in the S-Tohoku group. Moreover, each one individual estimated to be hybrid between two genetic types was also found in Pops. 17 and 25.

DISCUSSION

Candidates of cryptic species in *O. japonicus*

In the UPGMA dendrogram, PCO, and structure analyses, we recognized four major genetic groups (N-Tohoku, S-Tohoku, SW-Honshu, and Tsukuba) that are parapatric or allopatric with each other, in 33 populations of *O. japonicus* from northeastern Honshu. This result is consistent with those reported in previous studies based on mtDNA and allozymes (Yoshikawa et al., 2008, 2010a). Among these four groups, Tsukuba group was genetically most divergent from the others.

The Tsukuba group occurs only on Mt. Tsukuba (Pop. 33) and several adjacent mountains that are located in the center of the Kanto plain. This group is geographically isolated from the others and even the nearest population is 76 km distant (Pop. 29: SW-Honshu group). Genetic distances between Tsukuba and other groups did not increase with increment of geographic distances, and were very large even between geographically closest populations, such as Pops. 24 and 29 (Nei's D of 0.416 and 0.460, respectively). This result indicates that the large genetic differentiation of the Tsukuba group is not due to the isolation by distance. In addition, occurrence of unique alleles in ATA-2 and MDH-1 also supports genetic distinctiveness, which may have been caused by long-term population isolation and/or bottleneck, or founder effect (Yoshikawa et al., 2010a). The magnitude of Nei's Ds between Tsukuba and other genetic groups are equal or greater than those observed between candidates of cryptic species in *O. japonicus* from western Japan (SW-Honshu vs. Shikoku = 0.160–0.334: Yoshikawa et al. [2010a]; SW-Honshu vs. Kinki = 0.190–0.365: Yoshikawa et al. [2010b]). These results strongly suggest that allopatric Tsukuba group is genetically distinct from any other genetic group, and is a candidate cryptic species.

The S-Tohoku and SW-Honshu groups are nearly parapatric, and their genetic distances were lower than that of the candidate cryptic species mentioned above. However, genetic distances did not correlate with geographic distances, and were greater than 0.100 in all pairs. This type of relationship is commonly found between parapatric species with restricted gene exchange (Good and Wake, 1992; Tilly and Mahoney, 1996). Moreover, structure analysis clearly demonstrated presence of only little admixture between the two groups and their sympatry in Pops. 22 and 25. These data indicate the presence of restricted gene flow between these parapatric groups through hybrids, as well as of occasional hybridization between them.

Between the N- and S-Tohoku groups, the level of genetic differentiation was even lower than that found between the S-Tohoku and SW-Honshu groups. Genetic distances between these groups positively correlated with geographic distances, but the regression line did not pass through the origin. This relationship suggests restricted gene flow between the N- and S-Tohoku groups (Good and Wake, 1992). Structure analysis revealed lack of admixture between them at the eastern part of the contact zone (between Pops. 11 and 18) despite their geographic proximity. Similarly, it seems that genetic exchange between N-and S-Tohoku groups is rare even in the western contact zone, as all individuals in Pops. 14 and 15 were assigned to the S-Tohoku group and separated from N-Tohoku group when k = 4 (clearer when k = 5 or 6: see below), and pure N- and S-Tohoku groups were found to be sympatric in Pop. 13. These data indicate that these two parapatric groups are effectively reproductively isolated.

In this way, *O. japonicus* from northeastern Honshu are

split into four groups, i.e., N-Tohoku, S-Tohoku, Tsukuba, and SW-Honshu groups, which are genetically distinctive and most probably reproductively isolated from each other. Contact zones between these genetic groups are located around Yamagata and Miyagi Prefectures of middle Tohoku District (between N- and S-Tohoku groups: Fig. 1), and Niigata Prefecture of Hokuriku District to Ibaraki Prefecture of northern Kanto District through Fukushima Prefecture of southern Tohoku District (between S-Tohoku and SW-Honshu groups: Fig. 1), but such genetic borders do not correspond to potential geographic barriers such as Mogami (between N- and S-Tohoku groups) and Agano (between S-Tohoku and SW-Honshu groups) river basins. Thus, genetic borders are maintained through some biological interactions between the genetic groups, irrespective of geographic barriers. Although further morphological and ecological studies are necessary to reach concrete taxonomic conclusions, the four genetic groups are surely candidates of cryptic species. Thus, the species currently called *O. japonicus* is most probably not a single species, but a complex of several species, and the name *O. japonicus* should be applied only to the SW-Honshu group, which is considered to include the topotypic population. All the other genetic groups require new names.

Patterns of genetic differentiation within groups

Observed pattern of population genetic structures within each genetic group, except the Tsukuba group, which consists of only one population, differs from each other. Extremely low genetic differentiation in N-Tohoku group could usually be interpreted as a result of recent rapid range expansion (Hutchison and Templeton, 1999). However, such interpretation is inconsistent with the result of mtDNA (cytb) analysis by Yoshikawa et al. (2008), where considerable levels of genetic divergences were found among populations (maximum p-distance = 4.91%). This type of incongruence is commonly interpreted as a result of male-mediated gene flow (e.g., Roberts et al., 2004; Fu and Zeng, 2008), which is caused by the differences in dispersion abilities between sexes, and modes of inheritance between maternal mtDNA and biparental nuclear loci (Avise, 2000), although no behavioral data are available for *O. japonicus*. On the other hand, a pattern of isolation-by-distance, which would be expected to be found in the species of limited dispersion ability, is not detected. Therefore, genetic variations in the ancestral N-Tohoku populations might have been so low as to generate inter-population differentiation. In addition, such a genetic uniformity across distributional range is not found in other genetic groups. Therefore, some ecological and/or geohistorical factors specific to the N-Tohoku group, such as activated volcanisms in northeastern Honshu around 350–100 MYA (Yoshida et al., 1999), might have affected the formation of the genetic structure of this group.

Within the SW-Honshu group, populations are moderately differentiated except for one (Pop. 29), and largely concordant with the result of Yoshikawa et al. (2010a). The diversification of Pop. 29 resulted from its unique allele compositions of GPI[c] and IDH-2[d], which are relatively rare in adjacent populations. This may be caused by a founder effect or result of random genetic drift in an isolated population.

Extensive level of population genetic structure observed in S-Tohoku group may reflect its complex phylogeographic traits. Differentiation of eastern populations (Abukuma Mountains) is contrasting to the results of mtDNA analysis (Yoshikawa et al., 2008). Combined with the previous results, populations in the Abukuma Mountains are estimated to have undergone some demographic fluctuation events (bottleneck or founder effects) to form current unique allelic compositions. The Abukuma Mountains are relatively low and not steep, and isolated from the other mountain systems by the Abukuma and Kuji river basins (Fig. 1). These topological features can result in limited distributional range and small population size that increase genetic differentiation. Separation of northwestern part of S-Tohoku group in structure analysis is interesting, because populations from this area possess relatively high frequencies of MDH-1[c] and SOD[b] that are dominant in neighboring N-Tohoku group. This suggests past genetic exchange between the two groups, although they seem to be currently strongly isolated reproductively as mentioned above. This situation may be a remnant of history of secondary contact and following reinforcement between the two groups. In any case, because three geographic genetic clusters recognized in S-Tohoku group are highly admixed and difficult to separate from each other, it is reasonable that they share a single gene pool, even though genetic differentiation between them is not small.

This study uncovered extensive genetic diversity and complex phylogeography of *O. japonicus* in northeastern Honshu, which would actually be a complex of several cryptic species. This area, especially Tohoku District, has long been regarded as poor in amphibian diversity, with only one nearly endemic salamander, *Hynobius lichenatus*. However, this study suggests presence of high genetic diversity and endemism in northeastern Japan, and provides new insights for biodiversity of this region.

ACKNOWEDGMENTS

We thank G. Aoki, T. Hayashi, S. Ikeda, M. Kanemaru, S. Kanemaru, M. Kato, Y. Misawa, I. Niibe, N. Sato, T. Sugahara, T. Sugihara, S. Tanabe, and K. Tsurumi for help in collecting specimens and N. Kuraishi, T. Shimada, and A. Tominaga for assisting with experiments. We are also grateful to two anonymous reviewers for valuable comments on earlier version of the manuscript. This research was partly supported by grants from the Ministry of Education, Science, and Culture, Japan (Nos. 63540599, 01304001, and 11640697) and the Ministry of Environment to MM, and the Showa Seitoku Memorial Foundation to KN.

REFERENCES

Arntzen JW, Wielstra B (2010) Where to draw the line? A nuclear genetic perspective on proposed range boundaries of the crested newts *Triturus karelinii* and *T. arntzeni*. Amphibia-Reptilia 31: 311–322

Avise JC (2000) Phylogeography. Harvard University Press, Cambridge, Massachusetts

Boyer SH, Fainer DC, Watson EJ (1963) Lactate dehydrogenase variation from human blood: evidence for molecular subunit. Science 141: 642–643

Clayton JW, Tretiak DN (1972) Amine-citrate buffers for pH control in starch gel electrophoresis. J Fish Res Board Canada 29: 1169–1172

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of

clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14: 2611–2620

Felsenstein J (1982) How can we infer geography and history from gene frequencies? J Theor Biol 96: 9–20

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics. University of Washington, Seattle, Washington, USA

Fu J, Zeng X (2008) How many species are in the genus *Batrachuperus*? A phylogeographical analysis of the stream salamanders (family Hynobiidae) from southwestern China. Mol Ecol 17: 1469–1488

Good DA, Wake DB (1992) Geographic variation and speciation in the torrent salamanders of the genus *Rhyacotriton* (Caudata: Rhyacotritonidae). Univ Calif Publ Zool: 1–91

Hutchison DW, Templeton AR (1999) Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. Evolution 53: 1898–1914

Kuchta SR (2007) Contact zones and species limits: Hybridization between lineages of the California newt, *Taricha torosa*, in the southern Sierra Nevada. Herpetologica 63: 332–350

Liu K, Wang F, Chen W, Tu L, Min M, Bi K, et al. (2010) Rampant historical mitochondrial genome introgression between two species of green pond frogs, *Pelophylax nigromaculatus* and *P. plancyi*. BMC Evol Biol 10: 201

Mantel N (1967) The detection of decrease clustering and a generalized regression approach. Cancer Res 27: 209–220

Matsui M (1987) Isozyme variation in salamanders of the *nebulosus-lichenatus* complex of the genus Hynobius from eastern Honshu, Japan, with a description of a new species. Jpn J Herpetol 12: 50–64

Matsui M, Yoshikawa N, Tominaga A, Sato T, Takenaka S, Tanabe S, et al. (2008) Phylogenetic relationships of two *Salamandrella* species as revealed by mitochondrial DNA and allozyme variation (Amphibia: Caudata: Hynobiidae). Mol Phylogenet Evol 48: 84–93

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583–590

Nishikawa K, Matsui M, Tanabe S, Sato S (2007) Morphological and allozymic variation in *Hynobius boulengeri* and *H. stejnegeri* (Amphibia: Urodela: Hynobiidae). Zool Sci 24: 752–766

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6: 288–295

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945–959

Roberts MA, Schwartz TS, Karl SA (2004) Global population genetic structure and male-mediated gene flow in the green sea turtle (*Chelonia mydas*): analysis of microsatellite loci. Genetics 166: 1857–1870

Shaw CR, Prasad R (1970) Starch gel electrophoresis of enzyme – a compilation of recipes. Biochem Genet 4: 297–330

Tilly SG, Mahoney MJ (1996) Patterns of genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Amphibia: Plethodontidae). Herpetol Monogr 10: 1–42

Tominaga A, Matsui M, Nishikawa K, Tanabe S, Sato S (2005) Genetic differentiations of *Hynobius naevius* (Amphibia, Hynobiidae) as revealed by allozyme analysis. Biochem Syst Ecol 33: 921–937

Weisrock DW, Shaffer HB, Storz BL, Storz, Voss SR (2006) Multiple nuclear gene sequences identify phylogenetic species boundaries in the rapidly radiating clade of Mexican ambystomatid salamanders. Mol Ecol 15: 2489–2503

Wright S (1965) The interpretation of population structure by *F*-statistics with special regard to systems of mating. Evolution 19: 395–420

Yoshida T, Nagahashi Y, Oguchi T, Ohira H, Aizawa K, Sato H, et al. (1999) Geohistory of the active periods of volcanoes and the formation of Late Neogene calderas in northeastern Honshu Arc. Chikyu Monthly/Extra 27: 123–129 (in Japanese)

Yoshikawa N, Matsui M, Nishikawa K, Kim JB, Kryukov A (2008) Phylogenetic relationships and biogeography of the Japanese clawed salamander, *Onychodactylus japonicus* (Amphibia: Caudata: Hynobiidae), and its congener inferred from the mitochondrial cytochrome b gene. Mol Phylogenet Evol 49: 249–259

Yoshikawa N, Matsui M, Nishikawa K, Misawa Y, Tanabe S (2010a) Allozymic variation of the Japanese clawed salamander, *Onychodactylus japonicus* (Amphibia: Caudata: Hynobiidae), with special reference to the presence of two sympatric genetic types. Zool Sci 27: 33–40

Yoshikawa N, Matsui M, Nishikawa K (2010b) Allozymic variation and phylogeography of two genetic types of *Onychodactylus japonicus* (Amphibia: Caudata: Hynobiidae) sympatric in the Kinki District, Japan. Zool Sci 27: 344–355

(Received October 17, 2011 / Accepted November 29, 2011)