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Discordance Between Mitochondrial DNA Genealogy and Nuclear DNA Genetic Structure in the Two Morphotypes of *Rana tagoi tagoi* (Amphibia: Anura: Ranidae) in the Kinki Region, Japan

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Two morphotypes, with a large and small body size, of a brown frog *Rana t. tagoi* occur sympatrically in the Kinki region, central Honshu of Japan. Previous mitochondrial (mt) DNA genealogical study recognized two main lineages (A and B) and several sublineages in *R. tagoi*, where the small type was placed in the group A-1b, and the large type in groups A-1a and B-2a. Using haplotype network and structure analysis of three nuclear genes, we examined the discrepancy between morphology and mitochondrial genealogy. The results showed that the small type is reproductively isolated from its co-occurring large type (A-1a or B-2a), and that unlimited gene flow occurred between parapatrically occurring two mtDNA lineages of large types (A-1a and B-2a). Discordant genetic relationships between mtDNA and nuclear DNA results may be caused by the past mitochondrial introgression, and possibly, the incomplete lineage sorting. These results also suggest a heterospecific relationship between the large (A-1a and B-2a) and small types (A-1b). The large type is identified as *Rana t. tagoi* as it is genetically very close to the topotypes of the nominal subspecies, while the small type remains unnamed.

Key words: *Rana tagoi*, genealogy, morphotype, mitochondrial DNA, nuclear DNA, introgression, speciation

INTRODUCTION

A brown frog *Rana tagoi* Okada, 1928 is widely distributed throughout main islands of Japan, except for Hokkaido, and includes three subspecies. However, recent phylogenetic study based on mitochondrial (mt) DNA analysis revealed that *R. tagoi* and its close relative *R. sakuraii* Matsui and Matsui, 1990 are highly divergent genetically with complex evolutionary histories, and include many cryptic taxa (Eto et al., 2012).

In the region of Kinki, central Honshu, two types (large and small types: Sugahara, 1990) of *R. t. tagoi* occur sympatrically and are different in morphology and breeding ecology (Sugahara and Matsui, 1992, 1993, 1994, 1995, 1996, and 1997). Interestingly, the large type is split into two major mitochondrial clades (A and B), one of which (Group A-1a) is more closely related to the small type (Group A-1b) in Clade A, while the other (Group B-2a) was nested in Clade B (Eto et al., 2012).

Mitochondrial (mt) DNA is very widely used in phylogenetic studies, given its high variability and many traits suitable for experiments and analyses (Avise, 2000). However, the results of some recent studies have revealed that phylogenies derived from mtDNA do not always agree with those obtained from other sources like morphology (e.g., Liu et al., 2010; Hamidy et al., 2011), as is the case in two morphotypes of *R. t. tagoi* described above. Thus, it is desirable to confirm the validity of phylogenetic relationships from mtDNA using other genetic markers.

In order to assess detailed genetic and taxonomic relationships of the two morphotypes with three mitochondrial lineages of *R. t. tagoi* in the Kinki region, we conducted phylogenetic and population genetic analyses using nuclear (n) DNA sequences. By doing this, we tried to infer the states of reproductive isolations among each of the mitochondrial lineages in question.

MATERIALS AND METHODS

For samples from the Kinki region, we distinguished the large and small types based on body size and other diagnostic characters as described by Sugahara and Matsui (1994). In fact, some samples with mtDNA and morphological traits of the large type showed body sizes intermediate between the two types, but these were treated as the large type.

We first ascertained mtDNA phylogeny of Eto et al. (2012) using 186 samples of *R. t. tagoi* from 41 localities in the Kinki region and nine samples from the type locality in the Chubu region (Fig. 1 and Table. 1). The data include 23 mtDNA sequences from GenBank (accession numbers AB639617, AB639621–AB639630, AB639633–AB639635, AB639706–AB639709, and AB639711–AB639715). We used *R. sauteri* and *R. tsushimensis* (AB685767 and AB639752) as outgroup taxa.

Based on the results of mtDNA analysis, we selected 126 samples from six locality groups of the Kinki region (see result) and nine

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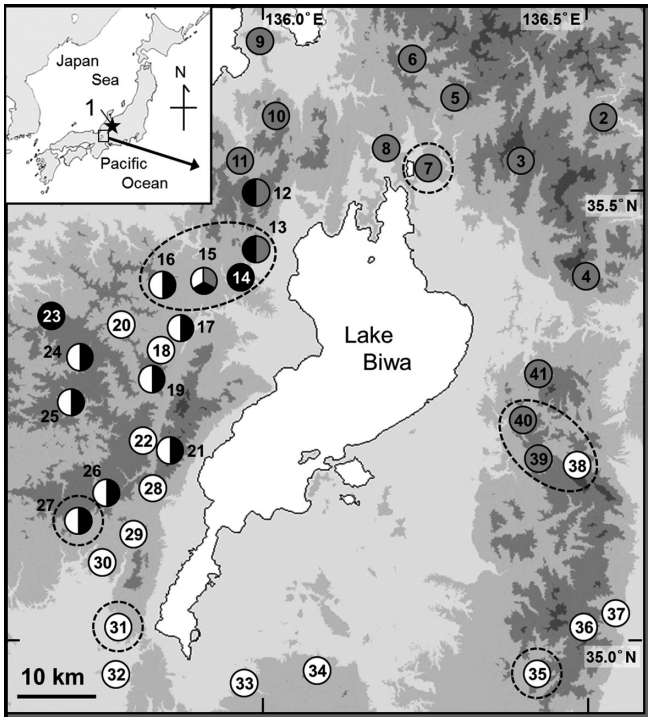

Fig. 1. Map of the Kinki region, Japan, showing sampling localities of *Rana t. tagoi*. Open, closed, and shaded circles indicate localities with mitochondrial genetic groups B-2a, A-1b, and A-2a, respectively, and the star shows the type locality in the Chubu region. Ranges encircled by dashed lines indicate localities used in nDNA analyses. Figures indicate localities shown in Table 1.

topotypic samples, and conducted genetic analyses using nDNA sequences. These localities were chosen to represent sites where (1) a single mitochondrial genetic group occurs, (2) two or three groups occur sympatrically, and/or (3) each mitochondrial group occurs parapatrically, exhibiting boundary areas. When samples belonging to different mitochondrial groups co-occurred in a locality, we treated them as different units in the analyses.

Following the experimental conditions and techniques described in Eto et al. (2012), we first analyzed approximately 600 bp of *ND1* (NADH dehydrogenase subunit 1) fragments of mtDNA. We constructed phylogenetic trees based on maximum likelihood (ML) and Bayesian inference (BI). The ML and BI analyses were respectively performed using TREEFINDER ver. Mar. 2011 (Jobb, 2011) and MrBayes v3.2.0 (Ronquist and Huelsenbeck, 2003). Methods for construction of trees also follow Eto et al. (2012). We then amplified partial sequences of three nuclear genes (*NCX1* [sodium-calcium exchanger 1], *POMC* [pro-opiomelanocortin], and *RAG1* [recombination activating gene 1]) by PCR using primer sets listed in Table 2. The experimental conditions and techniques were essentially same as those in mtDNA analysis. We used PHASE ver. 2.1 (Stephens et al., 2001) to separate and determine haplotypes of heterozygotic individuals. We considered haplotypes supported by BPP 0.95 or greater as significant; others were treated as missing data.

To estimate relationships between nDNA haplotypes, statistical parsimony networks for each gene were constructed by using TCS version 1.21 (Clement et al., 2000). We also performed population genetic analyses based on nDNA haplotypes. For each population, genetic variability was assessed by calculating the mean observed (Ho) and expected (He) heterozygosities, and all genes were checked with chi-square goodness-of-fit tests to determine whether or not they were deviated from Hardy-Weinberg (HW) equilibrium.

Table 1. Numbers and names of sampling localities, assigned mitochondrial genetic groups, and sample sizes of *Rana t. tagoi* examined.

No.	Locality	MtDNA group	n
Rana t. tagoi			
1	Takayama City, Gifu Pref.	A-1a	9
2	Ibigawa Town, Gifu Pref.	A-1a	1
3	Nagahama City, Shiga Pref.	A-1a	1
4	Maibara City, Shiga Pref.	A-1a	2
5	Nagahama City, Shiga Pref.	A-1a	2
6	Nagahama City, Shiga Pref.	A-1a	2
7	Nagahama City, Shiga Pref.	A-1a	11
8	Nagahama City, Shiga Pref.	A-1a	1
9	Mihama Town, Fukui Pref.	A-1a	1
10	Mihama Town, Fukui Pref.	A-1a	1
11	Mihama Town, Fukui Pref.	A-1a	1
12	Mihama Town, Fukui Pref.	A-1a	1
		A-1b	1
13	Takashima City, Shiga Pref.	A-1a	5
		A-1b	9
14	Takashima City, Shiga Pref.	A-1b	1
15	Takashima City, Shiga Pref.	A-1a	15
		A-1b	2
		B-2a	7
16	Takashima City, Shiga Pref.	A-1b	2
		B-2a	3
17	Takashima City, Shiga Pref.	A-1b	1
		B-2a	1
18	Takashima City, Shiga Pref.	B-2a	7
19	Takashima City, Shiga Pref.	A-1b	2
		B-2a	2
20	Takashima City, Shiga Pref.	B-2a	2
21	Otsu City, Shiga Pref.	A-1b	4
		B-2a	6
22	Otsu City, Shiga Pref.	B-2a	2
23	Oi Town, Fukui Pref.	A-1b	1
24	Nantan City, Kyoto Pref.	A-1b	2
		B-2a	6
25	Kyoto City, Kyoto Pref.	A-1b	1
		B-2a	1
26	Kyoto City, Kyoto Pref.	A-1b	1
		B-2a	3
27	Kyoto City, Kyoto Pref.	A-1b	16
		B-2a	14
28	Otsu City, Shiga Pref.	B-2a	3
29	Kyoto City, Kyoto Pref.	B-2a	2
30	Kyoto City, Kyoto Pref.	B-2a	1
31	Kyoto City, Kyoto Pref.	B-2a	12
32	Kyoto City, Kyoto Pref.	B-2a	1
33	Otsu City, Shiga Pref.	B-2a	1
34	Konan City, Shiga Pref.	B-2a	1
35	Koka City, Shiga Pref.	B-2a	9
36	Higashiomi City, Shiga Pref.	B-2a	1
37	Komono Town, Mie Pref.	B-2a	1
38	Taga Town, Shiga Pref.	B-2a	5
39	Taga Town, Shiga Pref.	A-1a	1
40	Taga Town, Shiga Pref.	A-1a	5
41	Maibara City, Shiga Pref.	A-1a	2
Rana tsushimensis			
	Tsushima City, Nagasaki Pref., Japan		1
Rana sauteri			
	Alishan, Chiayi Country, Taiwan		1

Table 2. Primers used to amplify nuclear genes in this study.

Target	Name	Sequence	Reference
NCX1	NCX1F	ACAACAGTRAGRATATGGAA	Shimada et al. (2011)
	NCX1R1	GCCATATCTCTCCTCGCTTCTTC	This study
POMC	POMC1	GAATGTATYAAAGMMTGCAAGATGGWCCT	Wiens et al. (2005)
	POMC7	TGGCATTTTTGAAAAGAGTCAT	Smith et al. (2005)
RAG1	Rag-1 Meristo1	CAGTTCCTGAGAAAGCAGTACG	Shimada et al. (2008)
	Rag-1 Meristo2	GGCTTTGCTGAAACTCCTTTC	Shimada et al. (2008)

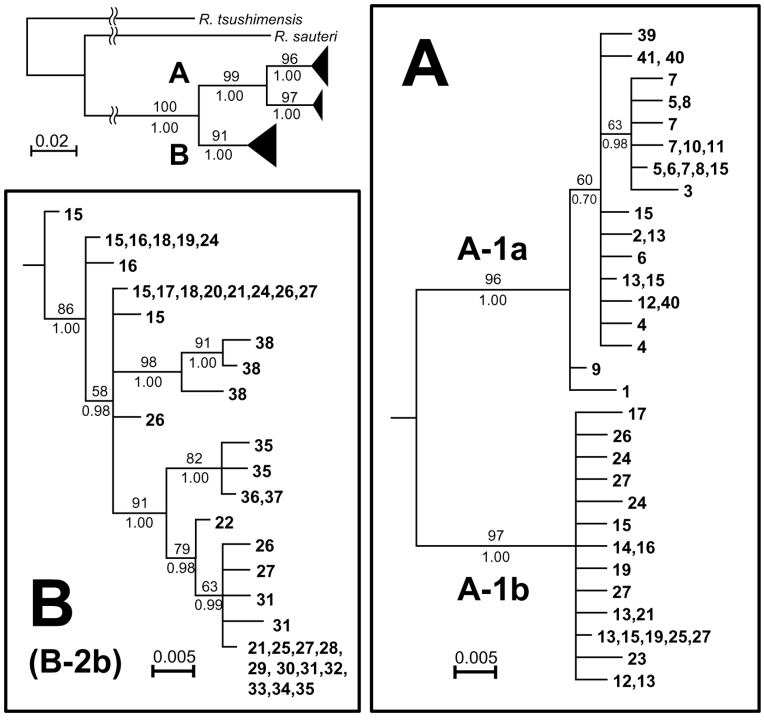

Fig. 2. Bayesian tree of mitochondrial ND1 gene for *Rana t. tagoi* and outgroup taxa. Nodal values indicate bootstrap supports for ML (above) and Bayesian posterior probability (below). For locality information, see Table 1 and Fig. 1.

All these analyses were conducted by using GENALEX 6.41 (Peakall and Smouse, 2006). To estimate population genetic structure, we used STRUCTURE ver. 2.3.3 (Pritchard et al., 2000) with admixture model. The most likely number of clusters was estimated according to the delta K value (Evanno et al., 2005).

RESULTS

Phylogenetic relationships based on mtDNA sequences

We obtained 564 bp of the mitochondrial ND1 gene for all samples, and after combining identical sequences, total 51 sequences were used in the subsequent analysis. Within ingroup sequences, 83 variable sites (vs) and 55 parsimony-informative sites (pi) were included. Newly obtained sequences were deposited in GenBank (AB779781-AB779812). The best substitution models estimated by Kakusan 4 (Tanabe, 2011) for ML and BI were J1 model (Jobb, 2011) with a gamma shape parameter (G) and Hasegawa-Kishino-Yano-1985 (HKY85) + G, respectively.

Phylogenetic analyses based on ML and BI yielded essentially identical topologies (-lnL = 2029.55 and 2290.28, respectively), and only BI tree is shown in Fig. 2. Just same as already reported (Eto et al., 2012), the ingroup was divided into three clades corresponding to Groups A-1a (ML-BS = 97% and BPP = 1.00, respectively), A-1b (96% and 1.00), and B-1a (91% and 1.00) of Eto et al. (2012). Groups A-1a and A-1b formed a clade (99% and 1.00) with closer genetic similarity in between (mean p-distance = 3.7%) than to B-2a (p-distance between A-1a = 6.7% and between A-1b = 6.5%).

Geographically, Groups A-1a and B-2a of the large type occurred parapatrically, with the former distributed in northeastern part and the latter in southwestern part of the sampling area. In contrast, distribution of the small type Group A-1b samples largely overlapped with them in the western side of Lake Biwa (Fig. 1).

Genetic variations in nuclear genes

Samples selected for nDNA analyses were 126 from six locality groups, consisting of localities (Locs.) 7, 13–16, 27, 31, 35, and 38–40 (Fig. 1), and nine topotypic ones from Loc. 1. Among them, a single mitochondrial genetic group was recognized in Locs. 1 (A-1a), 7 (A-1a), 31 (B-2a), and 35 (B-2a), and two groups occurred sympatrically in Loc. 27 (A-1b and B-2a). The boundary areas of multiple groups were located in 13–16 (A-1a and B-2a, with sympatric samples of A-1b) and 38–40 (A-1a and B-2a). For subsequent analyses, we differentiated these mitochondrial lineages in a given locality group (Table 3).

After a haplotype reconstruction using PHASE ver. 2.1, we obtained a total of 13 haplotypes (“a” to “m” in Fig. 3A) in *NCX1* (535 bp; vs = 14, pi = 8), 23 haplotypes (“a” to “w” in Fig. 3B) in *POMC* (552 bp; vs = 24, pi = 12), and 13 haplotypes (“a” to “m” in Fig. 3C) in *RAG1* (454 bp; vs = 14, pi = 11). Each haplotype was deposited in GenBank (AB779768-AB779780, AB779813-AB779848). In some samples (two in *NCX1*, one in *POMC*, and 11 in *RAG1*), we could not reconstruct their haplotypes with significant support (< 0.95). Thus we omitted these samples in the haplotype network analyses, although we used them in the structure analysis by applying missing data value.

Haplotype networks and frequencies of each gene are shown in Fig. 3 and Table 3, respectively. Two haplotype groups were recognized in the network of *NCX1* (Fig. 3A): one of them mainly consisted of the haplotypes specific to samples belonging to Group A-1a and B-2a (e.g., haplotypes “a” and “b”), and another one mainly consisted of haplotypes specific to Group A-1b (e.g., “l” and “m”). The haplotype network of *POMC* (Fig. 3B) also included several haplotype groups, which exhibited following tendencies: haplotypes specific to or frequently observed in Group A-1b samples (e.g., haplotypes “v” and “w”) tended to form a group; haplotypes frequently observed in A-1a and B-2a samples from Locs. 7, 13–16, 27, and 31 (e.g., “a” and “c”) tended to form a group; topotypic samples from Loc. 1 pos-

Table 3. Haplotype frequencies and genetic variabilities at three nuclear genes (*NCX1*, *POMC*, and *RAG1*) among localities and mitochondrial genetic groups of *Rana t. tagoi*. For locality numbers, see Fig. 1 and Table 1.

	Loc.	1	7	13–16			27		31	35	38–40	
	MtDNA	A-1a	A-1a	A-1a	A-1b	B-2a	A-1b	B-2a	B-2a	B-2a	A-1a	B-2a
	n	9	11	20	14	10	16	14	12	9	6	5
NCX1		a0.722	a0.455	a0.342	h0.036	a0.300	b0.031	a0.731	a1.000	a1.000	a0.583	a0.900
		c0.111	b0.091	b0.526	j0.036	b0.650	h0.031	b0.269			g0.250	h0.100
		f0.056	c0.227	c0.079	k0.250	c0.050	i0.031				h0.167	
		m0.111	f0.091	d0.026	l0.071		l0.281					
			h0.136	e0.026	m0.607		m0.625					
	Ho	0.556	0.727	0.632	0.429	0.300	0.467	0.538	–	–	0.667	0.200
	He	0.451	0.707	0.598	0.561	0.485	0.524	0.393	–	–	0.569	0.180
POMC		a0.056	a0.250	a0.700	a0.071	a0.591	c0.063	a0.821	a0.375	a0.300	a0.083	a0.100
		c0.611	c0.250	b0.075	t0.071	b0.227	s0.094	c0.179	c0.208	j0.350	j0.333	j0.400
		q0.278	f0.150	c0.100	u0.071	c0.091	y0.156		o0.083	m0.200	l0.333	k0.200
		r0.065	g0.100	d0.075	v0.214	e0.045	v0.438		p0.333	o0.100	m0.167	l0.100
			h0.100	e0.050	w0.571	v0.045	w0.250			v0.050	n0.083	m0.200
			i0.050									
			j0.050									
			v0.050									
	Ho	0.667	0.900	0.500	0.571	0.700	0.867	0.214	0.750	0.667	0.833	0.600
	He	0.543	0.825	0.486	0.612	0.570	0.700	0.293	0.698	0.716	0.736	0.740
RAG1		a0.214	a0.250	a0.575	a0.167	a0.650	a0.344	a0.179	a0.045	b0.118	a0.250	b0.125
		b0.429	b0.250	b0.150	b0.208	b0.100	b0.063	b0.643	b0.409	c0.294	g0.500	c0.125
		f0.286	d0.063	c0.050	j0.042	c0.050	j0.063	m0.179	c0.182	d0.176	h0.167	g0.375
		m0.071	f0.250	d0.100	l0.125	f0.100	k0.031		i0.045	g0.176	m0.083	h0.375
			j0.063	e0.025	m0.458	i0.050	l0.156		m0.318	h0.118		
			m0.125	j0.050		m0.050	m0.344			m0.118		
				m0.050								
	Ho	0.571	0.750	0.684	0.500	0.600	0.800	0.500	0.727	0.875	0.833	0.500
	He	0.684	0.789	0.597	0.701	0.550	0.720	0.523	0.694	0.820	0.653	0.688

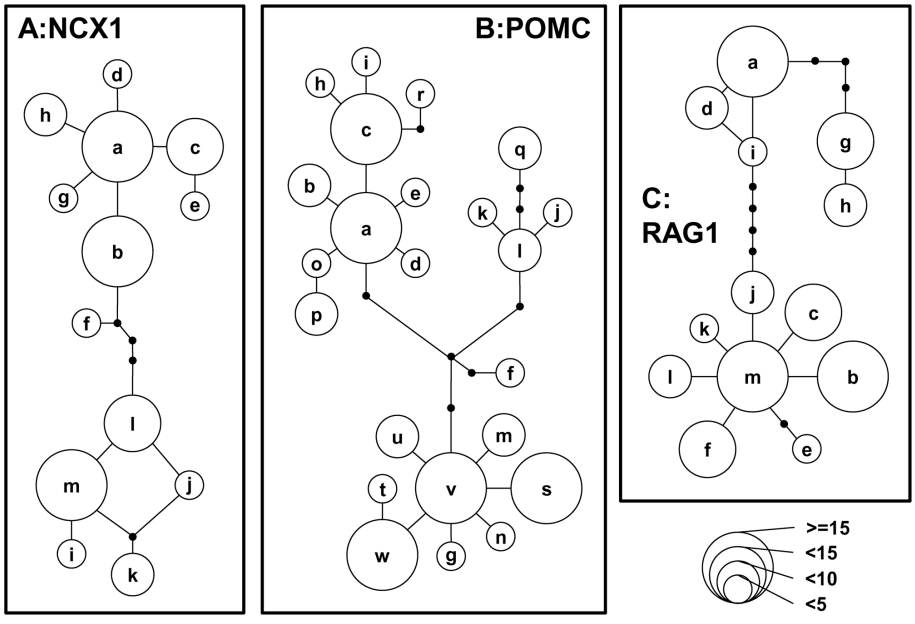

Fig. 3. Statistical parsimony networks of **(A)** *NCX1*, **(B)** *POMC*, and **(C)** *RAG1* haplotypes of *Rana. t. tagoi* from northeastern Kinki region and type locality. Filled circles indicate missing haplotypes. The size of each open circle is proportional to the haplotype frequency.

sessed some unique haplotypes ("q" and "r"), although they largely shared haplotypes ("a" and "c") with A-1a and B-2a samples from the other localities. The haplotype network of *RAG1* (Fig. 3C) included three distinct haplotype groups that did not clearly match the groupings by either mitochondrial genealogy or geographic distribution, but weakly showed the following tendencies: haplotypes frequently observed in samples of Group B-2a from Locs. 27 and 31, and A-1b (e.g., haplotypes "b" and "m") tended to form a group, which also included several haplotypes from other mitochondrial groups or localities; haplotypes specific to samples of A-1a and B-2a from Locs. 35 and 38–40 ("g" and "h") formed a group.

Statistics on the genetic variability of mitochondrial groups from each locality are shown in Table 3. No significant deviation from HW expectations was observed in each gene/locality. The structure analysis was performed for up to k = 10, and resultant barplots for k = 2 to 4 are shown in Fig. 4. The likelihood values reached a plateau after k = 2, and the estimated delta K value was highest at k = 2 (data not shown). At k = 2, two clusters, one including mitochondrial Groups A-1a and B-2a and another corre-

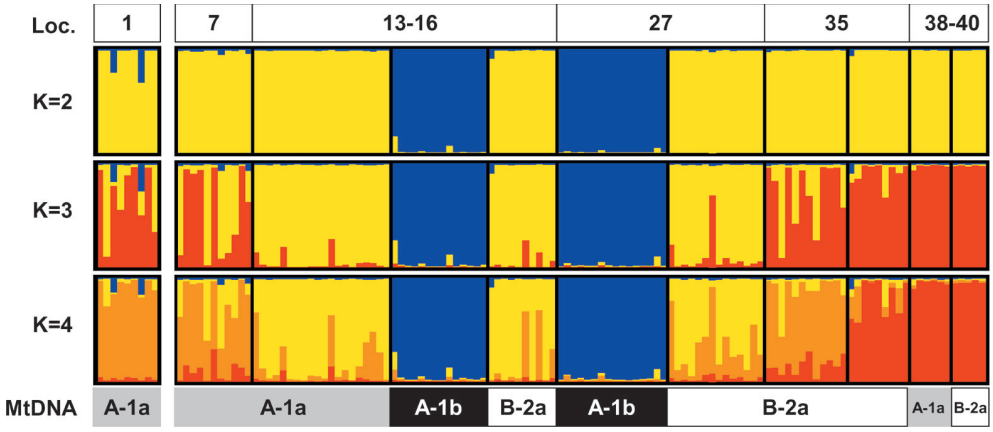

Fig. 4. Assigned genetic clusters of 135 individuals of *R. t. tagoi* from six locality groups in the Kinki and the type locality by structure analysis (k = 2–4). For locality numbers, see Fig. 1 and Table 1.

sponding to Group A-1b were recognized. At k = 3, the cluster of A-1a + B-2a at k = 2 was further divided into two clusters, but the division did not support the separation of two mitochondrial groups. Based on the test of delta K and clustering patterns of each bar plot, the most plausible number of clusters was considered to be two, by which the large type (mitochondrial Groups A-1a and B-2a) and the small type (mitochondrial Group A-1b) were split.

DISCUSSION

Discordance of estimated relationships among genetic markers

As in our previous report (Eto et al., 2012), the results of phylogenetic analyses based on mtDNA did not support morphological delimitation of *R. t. tagoi* from the Kinki region. Obtained genealogy showed that the *R. t. tagoi* large type was not monophyletic, and was split into two highly differentiated lineages. In contrast, the results of nDNA analyses did not support such a mitochondrial relationship, but were congruent with morphological delimitation.

Discordance of results between mt- and n-DNA analyses could be explained by mitochondrial incomplete lineage sorting (ILS) or gene introgression derived from past hybridization among ancestral lineages (Avise, 2000; Ballard and Whitlock, 2004). Because lineage sorting normally progresses rapidly in mtDNA, ILS of mtDNA is rare compared to that of nDNA (Ballard and Whitlock, 2004). However, lineages of *R. tagoi* are thought to have diverged recently from their relatively small genetic divergences (Eto et al., 2012), and therefore the possibility of mtDNA ILS, even at the species level, is not completely rejected (e.g., as a product of budding speciation: Funk and Omland, 2003). In this scenario, the ancestor of the *R. t. tagoi* small type (A-1b) originated as an internal lineage of the large type (A-1a and B-2a). The ancestral populations of A-1b subsequently underwent morphological and ecological differentiation toward the smaller body size, while ancestral A-1a and B-2a populations retained their larger body size.

On the other hand, past mitochondrial introgression among ancestors of each lineage can also explain the discordance of mtDNA and nDNA properties. Based on this hypothesis, hybridization between the ancestral populations of A-1b (or other Clade A lineages) and B-2a had occurred in past, resulting in mtDNA introgression from the former to the latter. After the introgression event, mtDNA in the ancestral populations of B-2a, A-1b, and the introgressed populations of B-2a (ancestral A-1a) independently experienced mutations and resulted in the formation of present relationships.

Mitochondrial ILS and past gene introgression are often difficult to distinguish (Ballard and Whitlock, 2004; Funk and Omland, 2003). In our case, if ILS caused the discordance, the small type (A-1b) should be genetically close to one of the large type lineages (A-1a) not only in mtDNA, but also in nDNA. However, our results actually did not support close relationship of A-1a and A-1b in nDNA, thus not favoring ILS. Nonetheless, however, the ILS scenario may be supported by male-biased gene flow. In such a case, the original nuclear haplotypes and genetic structure of A-1a would have been similar to those of A-1b, but were completely overwritten via male-biased gene flow with B-2a. However, nDNA is fundamentally less likely to be introgressive than mtDNA, and no behavioral data for male-biased dispersal in this species are available at present. Compared with the ILS hypothesis, the past mtDNA introgression hypothesis is less problematic and is considered more plausible.

In addition to discordance of mt- and n-DNA, each nuclear gene also showed more or less discordant patterns on their haplotype networks. Among three nuclear genes, only *NCX1* showed obvious relationships between the haplotype network and the morphotype. This result suggests that the ILS of the remaining two genes (*POMC* and *RAG1*) may have caused discordance among nuclear genes. These results seem to indicate that phylogenetic analyses using direct sequences of nuclear genes may be not efficient in the study of *R. tagoi*, and that population genetic analyses based on frequency data of nuclear genotypes may be more effective (Avise, 2000).

Taxonomic status of two morphotypes of *Rana t. tagoi*

Sympatric occurrence of two types of *Rana t. tagoi* was first reported from Kyoto Prefecture in the Kinki region by Sugahara (1990). Later, Sugahara and Matsui (1992, 1993, 1994, 1995, 1996, and 1997) performed morphological, acoustic, and ecological comparisons, and suggested that these two types were not conspecific, being reproductively isolated from each other. Subsequent genetic survey using mtDNA (Tanaka et al., 1994) clarified remarkably large genetic divergences between the large (corresponding to B-2a in this paper) and the small (A-1b) types from Kyoto. Furthermore, Eto et al. (2012) showed that the large type was further divided into two genetic lineages (A-1a and B-2a), although they were morphologically similar. Eto et al. (2012)

also confirmed that one of them (A-1a) was phylogenetically close to the small type (A-1b). Present result of mtDNA analysis supported these previous studies.

In contrast, our nDNA analyses suggested a closer relationship of A-1a to B-2a than to A-1b. Our structure analysis indicated unlimited gene flow between A-1a and B-2a, and the existence of genetic isolation of A-1b from sympatric A-1a or B-2a was also suggested. These results are congruent with previous results of morphological and ecological studies (Sugahara and Matsui, 1992, 1993, 1994, 1995, 1996, 1997), which indicated that the *R. t. tagoi* large type (A-1a and B-2a) and the small type (A-1b) from the Kinki region are specifically distinct.

In our nDNA analyses, the large type was genetically also close to topotypes of *R. t. tagoi* that have body size intermediate between the large and small groups. These facts suggest that the large type is in fact conspecific with the topotypes, and should be treated as true *R. t. tagoi*, while the small type is a distinct but unnamed species. The reason for the presence of size variation within true *R. t. tagoi* (the large and the medium type) is unknown, but may be related to the sympatry of the large type with the small type (Sugahara and Matsui, 1996), unlike singly occurring medium type.

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