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Distributional Change and Epidemic Introgression in Overlapping Areas of Japanese Pond Frog Species over 30 Years

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***Pelophylax nigromaculatus*, *P. porosus porosus*, and *P. p. brevipoda* are three pond frog species distributed in Japan. Their distributions overlap at two basins in central Japan (*P. nigromaculatus* and *P. p. porosus* in the Matsumoto basin, and *P. nigromaculatus* and *P. p. brevipoda* in the Ina basin), and hybrid descendants have been found in these areas. To clarify the distribution areas and hybrid zones of the frogs, and to understand the mode of introgressive hybridization and its impact on the frog populations, we conducted exhaustive sampling at each basin and performed allozyme and mtDNA analyses of 233 individuals. Analysis using genetic markers clearly detected nine F1 hybrids and 94 hybrid descendants of *P. nigromaculatus* and *P. porosus* from the overlapping areas of both basins. Allozyme and mtDNA data suggest directional hybridization between female *P. p. porosus* and male *P. nigromaculatus* in the Matsumoto basin. Over the past 30 years, the distribution of *P. p. porosus* has been narrowed and fragmented by the invasion of *P. nigromaculatus*, seemingly because of directional hybridization in the Matsumoto basin. In the Ina basin, the "pure" *P. p. brevipoda* (*n* = 8) population was extremely reduced by gene introgression from *P. nigromaculatus*, yet its distribution was barely changed compared to the Matsumoto basin. Consequently, this study shows that *P. porosus* populations are threatened by interspecific hybridization with *P. nigromaculatus*, and that introgressive hybridization damaged *P. porosus* populations by different means in each basin.**

Key words: introgressive hybridization, distributional change, directional hybridization, mtDNA replacement, *Pelophylax nigromaculatus*, *Pelophylax porosus*

INTRODUCTION

Pelophylax nigromaculatus and its relatives *P. porosus porosus* and *P. p. brevipoda* are species (or subspecies) of pond frogs distributed in Japan. They commonly inhabit areas around rice paddies and watersheds in flat lands. *Pelophylax nigromaculatus* is widely distributed across Kyushu, Shikoku, and Honshu (except in the Kanto and Sendai plains) and has been introduced to Hokkaido. *Pelophylax p. porosus* occurs in Eastern Honshu from the Kanto Plain to the Sendai Plain, in central and southern Niigata Prefecture, and in northern and central Nagano Prefecture, and it has also been introduced to Hokkaido. *Pelophylax p. brevipoda* is distributed in Western Honshu, Tokai, central Kinki, San-yo districts, and northeastern Shikoku (Maeda and Matsui, 1999) (Fig. 1). All three species are known to hybridize with each other in overlapping areas (e.g., Moriya, 1959; Nishioka et al., 1992; Sumida and Ishihara, 1997). The distribution of *P. nigromaculatus* and *P. p. brevipoda* overlaps widely, while the distribution of *P. p. porosus* rarely overlaps with that of the others (Fig. 1). Remarkably, in Nagano Prefecture, the distributional areas of the frogs are close to each other. In particular, the Matsumoto and Ina basins are known as hybrid zones of *P. nigromaculatus* and *P. p. porosus* and of *P. nigromaculatus* and *P. p. brevipoda* (Shimoyama, 1986; Nishioka et al., 1992). Although the F1 hybrid male of *P. nigromaculatus* and *P. porosus* is sterile, the hybrid female is fertile (Moriya, 1960), indicating gene introgression between *P. nigromaculatus* and *P. porosus* populations through F1 females.

Introgression is a phenomenon that imports genes from one species (or population) into a gene pool of another through repetitive backcrossing of interspecific hybrids. Introgression occasionally plays an important role in biological events, such as gene pollution, genetic diversification, speciation, adaptive evolution, and extinction, and has thus attracted the attention of researchers in a variety of disciplines, including population genetics and evolutionary and conservation biology (e.g., Rhymer and Simberloff, 1996; Allendorf et al., 2001; Burke and Arnold, 2001; Mooney and Cleland, 2001; Mallet, 2005; Evans et al., 2006; Baack and Rieseberg, 2007; Song et al., 2011). Several studies have

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reported historical, or possibly ongoing, introgressions among ranid frogs, including pond frog populations (e.g., Berger, 1968; Kim et al., 2004). For example, Liu et al. (2010) reported frequent occurrences of mtDNA introgression from *P. plancyi* to *P. nigromaculatus* populations throughout their distributional areas, which cover a wide range of eastern Eurasia. However, difficulties in locating and sampling introgressants and specifying hybrid zones based on morphological observation and/or small numbers of usable loci to diagnose the parent species of an introgressant often prevent surveying introgression details in wild populations (Allendorf et al., 2001). This is also true for the Japanese pond frogs. Although several studies based on allozyme markers have demonstrated the occurrence of introgressions between *P. nigromaculatus* and *P. porosus* in their parapatric distributional areas, such as Hakone, Maibara, Igaueno, Higashiosaka, and Konko (Nishioka et al., 1992; Sumida and Ishihara, 1997), detailed survey of hybrid zones using DNA markers, especially maternal mitochondrial genes, has not been conducted. To date, a variety of knowledge on these frogs has accumulated and covers areas such as diagnostic allozyme loci, possible hybrid zones, differences in breeding period and reproductive behavior, and reproductive abilities of hybrids (Moriya, 1960; Nishioka et al., 1992; Sumida and Ishihara, 1997; Shimoyama, 1986, 2000). More importantly, information is available on the past distribution of the pond frogs in Nagano Prefecture, including the Matsumoto and Ina basins (Shimoyama, 1986), which is the only area where all three Japanese pond frogs occur parapatrically or sympatrically. In addition, the possible occurrence of two distinct hybrid zones (*P. nigromaculatus* × *P. p. porosus* in the Matsumoto basin, and *P. nigromaculatus* × *P. p. brevipoda* in the Ina basin) has been suggested. These two basins seem to be the most relevant areas for studying the hybridization of pond frogs and the impact of hybridization in relation to other biological events, such as gene introgression, outbreeding depression, niche competition, and distributional change.

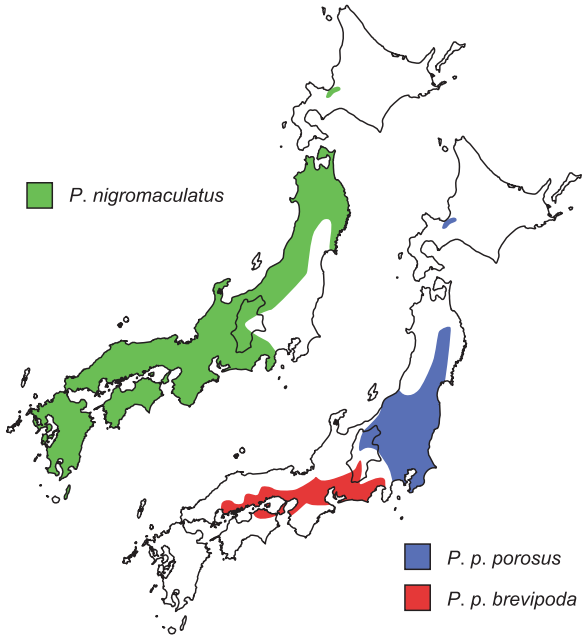

Fig. 1. Distribution map of the three pond frog species in Japan as described by Maeda and Matsui (1999). Nagano Prefecture, where the Matsumoto and Ina basins are located, is shown by a solid line. *Pelophylax nigromaculatus* and *P. p. porosus* were introduced to Hokkaido.

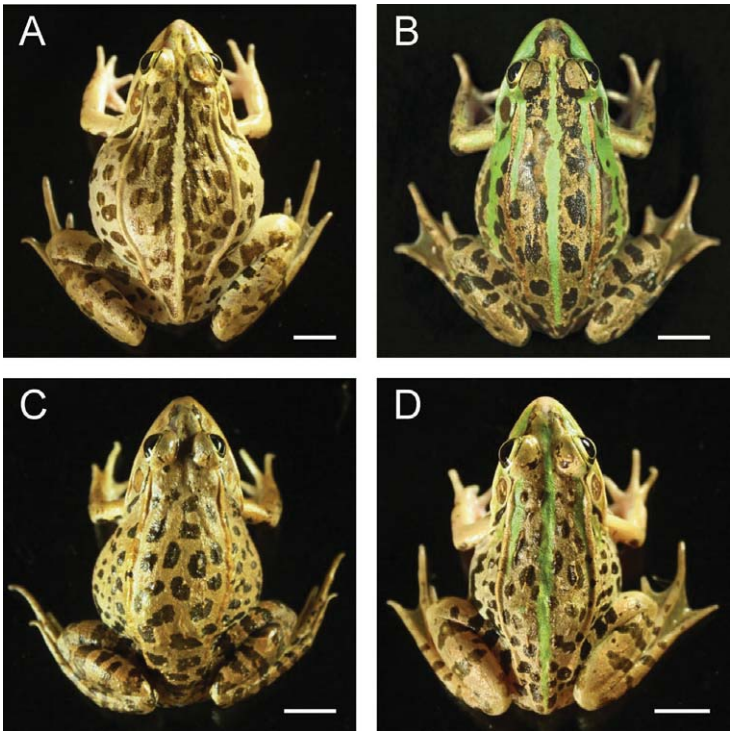

Fig. 2. Dorsal view of the pond frogs and a hybrid individual. **(A)** *P. nigromaculatus*. **(B)** *P. p. porosus*. **(C)** *P. p. brevipoda*. **(D)** F1 hybrid between *P. p. porosus* female and *P. nigromaculatus* male. Scale bars: 1 cm.

To clarify (1) the accurate distribution of three pond frog taxa and their hybrid zone areas in the Matsumoto and Ina basins, and (2) the degree and pattern of two introgressive hybridizations (i.e., *P. nigromaculatus* × *P. p. porosus*, and *P. nigromaculatus* × *P. p. brevipoda*), we performed exhaustive sampling of pond frogs from each basin and conducted morphological observation and allozyme and mtDNA (*cytb* gene) analyses. In addition, we compare the present distributional areas with those of 30 years ago, and discuss the observed distributional change and its cause.

MATERIALS AND METHODS

Sampling localities and specimens

A total of 233 specimens of pond frogs were collected from 30 localities of the Matsumoto and Ina basins in Nagano Prefecture in June 2010, which is their breeding season. First, all specimens were identified on the basis of dorsal morphological characters according to Moriya (1954) and Maeda and Matsui (1999). Dorsal images of representative individuals of *P. nigromaculatus*, *P. p. porosus*, *P. p. brevipoda*, and their hybrid (*P. p. porosus* female × *P. nigromaculatus* male) are shown in Fig. 2. Sampling localities and sample size of each locality are shown in Table 1, Fig. 3A and Supplementary Table S1 online. Localities 1–16 and 17–30 belong to the Matsumoto and Ina basins, respec-

tively. Skeletal muscles and the toes of specimens were sampled for allozyme and mtDNA analyses, respectively.

Allozyme analysis

In addition to the 233 collected individuals, three other individuals corresponding to *P. nigromaculatus*, *P. p. porosus*, and *P. p. brevipoda* (used in Nishioka et al., 1992) were used in the allozyme analysis. Four enzymes, α-GDH, IDH-B, LDH-B, and MDH-B, known as diagnostic loci for distinguishing between *P. nigromaculatus* and *P. porosus* (Nishioka et al., 1992), were examined by horizontal starch gel electrophoresis, following the procedure reported by Nishioka et al. (1980). Detection of each enzyme was performed by the agar overlay method of Brewer (1970) and Harris and Hopkinson (1976) with a slight modification, as described by Nishioka et al. (1992).

DNA extraction and mtDNA sequence analysis

Total DNA was extracted from the clipped toes using DNeasy Tissue Kit (QIAGEN) according to the manufacturer's instructions. Total DNA was used to amplify a partial sequence of the mitochondrial *cytb* gene with a set of primers by polymerase chain reaction (PCR). Primers used were L14850 (5′-TCT CAT CCT GAT GAA ACT TTG GCT C-3′) and H15410 (5′-GTC TTT GTA GGA GAA GTA TGG-3′), designed by Tanaka et al. (1996). PCR mixtures were prepared with the Ex-Taq Kit (TaKaRa) according to the manufacturer's instructions. DNA sequencing was performed with an automated sequencer (ABI 3130xl, Applied Biosystems). Resultant *cytb* sequences and the three other *cytb* sequences from previous studies (*P. nigromaculatus* from Hiroshima, *P. p. prosus* from Tsuchiura, and *P. p. brevipoda*, Maibara; GenBank Accession Numbers AB029937, AB029938, and AB029940, respectively; Sumida et al., 1998, 2000) were aligned using ClustalW (Thompson et al., 1994). Based on the aligned data of 491 nucleotide sites, an unrooted neighbor-joining (NJ) tree was reconstructed using MEGA ver. 4 (Tamura et al., 2007).

RESULTS

Identification and distributional survey based on external morphology

Species (subspecies) and hybrid identification of the 233 specimens based on dorsal characters identified 148 as *P. nigromaculatus*, 38 as *P. p. porosus*, 35 as *P. p. brevipoda*, and 12 as hybrids (Table 1). Distribution of each taxon based on morphological observation is shown in Figs. 3A and 5A.

In the Matsumoto basin, *P. nigromaculatus* was distributed on the shore of Kizaki Lake (population 1) and in the central to southern areas of the basin and watershed of the Sai River (populations 2, 4–7, 9, 12, 13, 15, and 16). *Pelophylax p. porosus* was distributed in six populations in

Table 1. Morphological and genetic characteristics of pond frogs in each locality. N, P, B, and H indicate the characters of *P. nigromaculatus*, *P. p. porosus*, *P. p. brevipoda* and their hybrids, respectively. F1 in the allozyme column indicates individuals with heterozygous alleles of *P. nigromaculatus* and *P. porosus* at all four loci examined, and introgressant indicates individuals that possessed alleles specific to both *P. nigromaculatus* and *P. porosus,* except F1 hybrids.

Locality		Sample size	Morphological character (No. of individuals)				Allozyme (No. of individuals)					*cytb* haplotype (No. of individuals)		
No.	Name		N	P	B	H	N	P	B	F1	Introgressant	N	P	B
1.	Kizaki Lake	6	6	0	0	0	6	0	0	0	0	6	0	0
2.	Ikusaka	4	4	0	0	0	3	0	0	0	1	4	0	0
3.	Ikeda	8	0	8	0	0	0	8	0	0	0	0	8	0
4.	Oshino	6	1	5	0	0	0	4	0	1	1	1	5	0
5.	Akashina	10	10	0	0	0	10	0	0	0	0	10	0	0
6.	Toyoshina	10	9	0	0	1	5	0	0	4	1	3	7	0
7.	Tazawa	6	6	0	0	0	5	0	0	0	1	5	1	0
8.	Nakagaya	5	0	3	0	2	0	3	0	2	0	0	5	0
9.	Shiogura	4	4	0	0	0	4	0	0	0	0	0	4	0
10.	Hora	10	0	10	0	0	0	7	0	1	2	3	7	0
11.	Misato	3	0	3	0	0	0	3	0	0	0	0	3	0
12.	Samizo	10	10	0	0	0	8	0	0	0	2	8	2	0
13.	Shimadachi	9	9	0	0	0	7	0	0	0	2	7	2	0
14.	Kaisei JHS	10	0	9	0	1	1	9	0	0	0	0	10	0
15.	Seba	5	5	0	0	0	5	0	0	0	0	5	0	0
16.	Soga	13	13	0	0	0	13	0	0	0	0	1	12	0
17.	Iinuma	3	3	0	0	0	2	0	0	0	1	3	0	0
18.	Kamihiraide	6	6	0	0	0	4	0	0	0	2	6	0	0
19.	Miyaki	15	13	0	0	2	10	0	2	0	3	13	0	2
20.	Tatsuno JHS	17	7	0	10	0	2	0	5	0	10	17	0	0
21.	Inatomi	16	4	0	10	2	4	0	5	0	7	15	0	1
22.	Higashiminowa	6	6	0	0	0	4	0	0	0	2	6	0	0
23.	Nagaoka	4	2	0	2	0	2	0	0	0	2	4	0	0
24.	Minowa	12	5	0	4	3	1	0	4	1	6	7	0	5
25.	Fukuyo	2	2	0	0	0	0	0	0	0	2	1	1	0
26.	Tabata	11	1	0	9	1	0	0	4	0	7	3	0	8
27.	Hasenakano	3	3	0	0	0	3	0	0	0	0	3	0	0
28.	Higashi-Ina	2	2	0	0	0	2	0	0	0	0	2	0	0
29.	Komagane	7	7	0	0	0	6	0	0	0	1	5	0	2
30.	Shimodaira	10	10	0	0	0	8	0	0	0	2	9	1	0
	Total	233	148	38	35	12	89	33	8	9	94	147	68	18

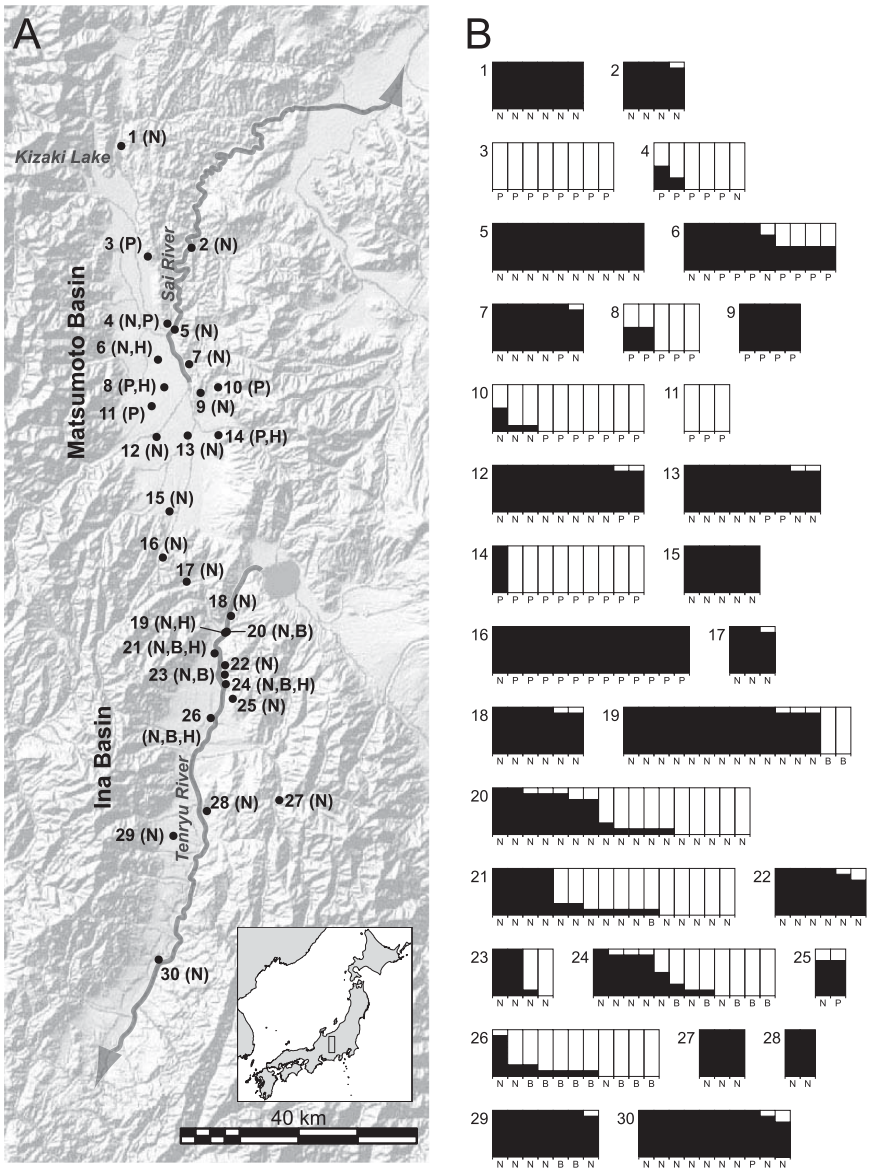

Fig. 3. Sampling localities, distributions, and results of allozyme and *cytb* gene analyses. **(A)** Map showing sampling localities and morphology-based distribution. N, P, B, and H indicate occurrence of *P*. *nigromaculatus*, *P*. *p*. *porosus*, *P*. *p*. *brevipoda*, and hybrid individuals assigned by dorsal morphology, respectively. **(B)** Summary of the results by allozyme and *cytb* analyses. Chart numbers correspond to sampling localities shown in A. The charts show the allelic condition of eight alleles at four analyzed loci, each vertical column in the charts corresponds to each individual, and black and white bars in each column correspond to *P*. *nigromaculatus*- and *P*. *porosus*-specific alleles, respectively. The N, P, and B marks below each column indicate the *cytb* haplotypes specific to *P*. *nigromaculatus*, *P*. *p*. *porosus*, and *P*. *p*. *brevipoda*, respectively.

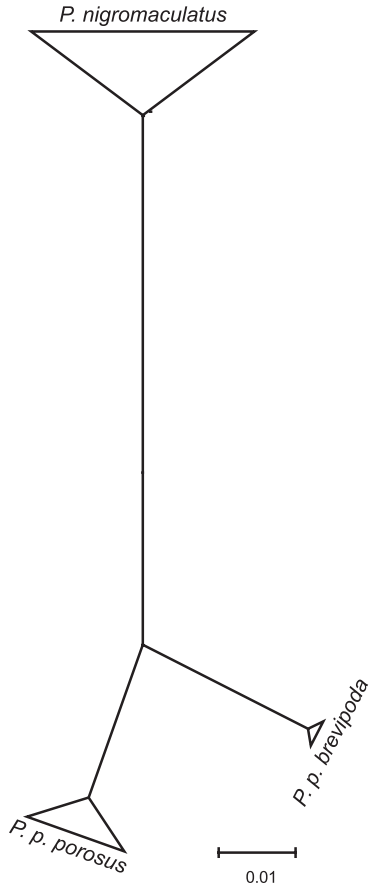

Fig. 4. Unrooted neighbor-joining tree based on a 491-bp segment of the *cytb* gene sequence. Triangle size reflects the numbers of specimens included in each clade.

three isolated areas of the northern (populations 3 and 4), western (populations 8 and 11), and eastern (populations 10 and 14) areas. Hybrid individuals were found in the central parts of the basin (populations 6, 8, and 14). In the Ina basin, *P*. *nigromaculatus* was distributed across a wide range from the northern to southern parts of the basin (populations 17–30), while the distribution of *P*. *p*. *brevipoda* was limited to the northern part of the Tenryu River watershed (populations 20, 21, 23, 24, and 26). Unlike the distribution in the Matsumoto basin, the entire distributional area of *P*. *p*. *brevipoda* overlapped with that of *P*. *nigromaculatus*.

Allozyme analysis

Of the four allozyme loci (α-GDH, IDH-B, LDH-B, and MDH-B) α-GDH and IDH-B are known to have alleles specific to *P*. *nigromaculatus* and *P*. *porosus*, respectively (designated N and P). The LDH-B locus has three alleles specific to *P*. *nigromaculatus*, *P*. *p*. *porosus*, and *P*. *p*. *brevipoda*, respectively (designated N, Pp, and Pb); and the MDH-B locus has three alleles consisting of two intraspecific polymorphic types in *P*. *nigromaculatus* and one type in *P*. *porosus*, respectively (N1, N2, and P) (Nishioka et al., 1992). The results of our allozyme analysis are summarized in Table 1 and Fig. 3B. The chart numbers in Fig. 3B correspond to the sampling localities in Fig. 3A, and the charts show the allelic condition of the eight alleles of the four loci. Vertical columns correspond to individuals, and black and white bars correspond to *P*. *nigromaculatus*- and *P*. *porosus*-specific alleles, respectively (N, P, and B below each column indicate the *cytb* haplotypes specific to *P*. *nigromaculatus*, *P*. *p*. *porosus*, and *P*. *p*. *brevipoda*, respectively). For example, chart 2 in Fig. 3B shows that four individuals were collected from population 2, and three

individuals have only *P*. *nigromaculatus*-specific alleles at all four observed loci (total eight alleles), but one individual has a *P*. *porosus*-specific allele at one locus. Chart 2 also shows that all four individuals from population 2 have the *P*. *nigromaculatus*-specific *cytb* haplotype. Detailed allelic information is displayed in Supplementary Table S1 online.

Out of the 233 specimens, 115 had only *P*. *nigromaculatus*-specific alleles at the four analyzed loci, and 55 frogs possessed homozygous genotypes consisting of only *P*. *porosus*-specific alleles (Supplementary Table S1 online). Based on the allozyme analysis, these individuals can be regarded as "pure" *P*. *nigromaculatus* and *P*. *porosus*. However, the remaining 63 individuals had both *P*. *nigromaculatus* and *P*. *porosus* alleles, and nine of these showed heterozygous genotypes of *P*. *nigromaculatus* and *P*. *porosus* at all four loci (in populations 4, 6, 8, 10, and 24; Fig. 3B). These nine individuals can be regarded as F1 hybrid individuals, for the following two reasons: (1) The F1 hybrid must have heterozygous genotypes of parent species at all loci; and (2) pond frogs with sterile F1 males (F2 of F1 male × F1 female) cannot occur. Consequently, the probability of a hybrid descendant arising with heterozygous alleles at all four loci is very low (6.25% and 0.40% for first and second backcross generations, respectively). The remaining 54 individuals possessing alleles specific to the two species at more than one locus are predicted to be hybrid descendants (i.e., introgressants) emerged via backcrossing of female F1 hybrids.

Of the 148 individuals assigned as *P*. *nigromaculatus* on the basis of dorsal characters, 114 (77.0%) possessed only *P*. *nigromaculatus*-specific alleles ("pure" *P*. *nigromaculatus*), but 34 (23.0%) had alleles from both *P*. *nigromaculatus* and *P*. *porosus* (five F1 hybrids and 29 introgressants). Likewise, of the 38 individuals regarded morphologically as *P*. *p*. *porosus*, 34 (89.5%) were regarded as "pure" *P*. *p*. *porosus*, and the remaining four (10.5%) consisted of one F1 hybrid and three hybrid descendants. Of the 35 frogs initially regarded as *P*. *p*. *brevipoda*, 19 (54.3%) and 16 (45.7%) were revealed to be "pure" *P*. *p*. *brevipoda* and hybrid descendants, respectively. Of the 12 individuals morphologically regarded as hybrids, three (25.0%) and six (50.0%) were revealed to be F1 hybrids or their descendants, and the remaining one (8.3%) and two (16.7%) were shown to be *P*. *nigromaculatus* (mtDNA replaced by that of *P*. *p*. *porosus*) and "pure" *P*. *p*. *brevipoda*, respectively. In total, 63 F1 hybrids and their descendants were detected by allozyme analysis.

Cytb gene analysis

Partial *cytb* regions (491 bp) from the 233 frogs were sequenced. In the alignment matrix of the *cytb* sequences, 76 variable sites were found. Sequence data of observed 23 haplotypes were deposited into GenBank (Accession Nos. AB686619–AB686641). Our NJ tree, reconstructed from the *cytb* data, clearly showed three major clades corresponding to the haplogroups of *P*. *nigromaculatus* (N), *P*. *p*. *porosus* (P), and *P*. *p*. *brevipoda* (B), respectively (Fig. 4). Average uncorrected "p" distances between these haplogroups were 0.085 (N vs. P), 0.090 (N vs. B), and 0.045 (P vs. B), respectively. The *cytb* haplotypes of each individual are shown in Fig. 3B and Supplementary Table S1 online.

Interestingly, some "pure" individuals revealed by allozyme analysis possessed the *cytb* haplotype of another species. Of the 115 "pure" *P*. *nigromaculatus* identified by allozyme analysis, 89 (77.4%) carried the *P*. *nigromaculatus* haplotype, but 24 (20.9%) and two individuals (1.7%) carried *P*. *p*. *porosus* and *P*. *p*. *brevipoda* haplotypes, respectively. Likewise, of 36 "pure" *P*. *p*. *porosus* individuals, one (2.8%) carried the *P*. *nigromaculatus* haplotype, and of 21 "pure" *P*. *p*. *brevipoda* individuals, 13 (68.4%) carried the *P*. *nigromaculatus* haplotype. In total, the mtDNA of 40 individuals was replaced with that of another species. These individuals showed complete mismatch between nuclear genotypes (i.e., allozyme), and mitochondrial haplotypes must have emerged by mtDNA introgression through a number of backcrossing generations and/or self-crossing between

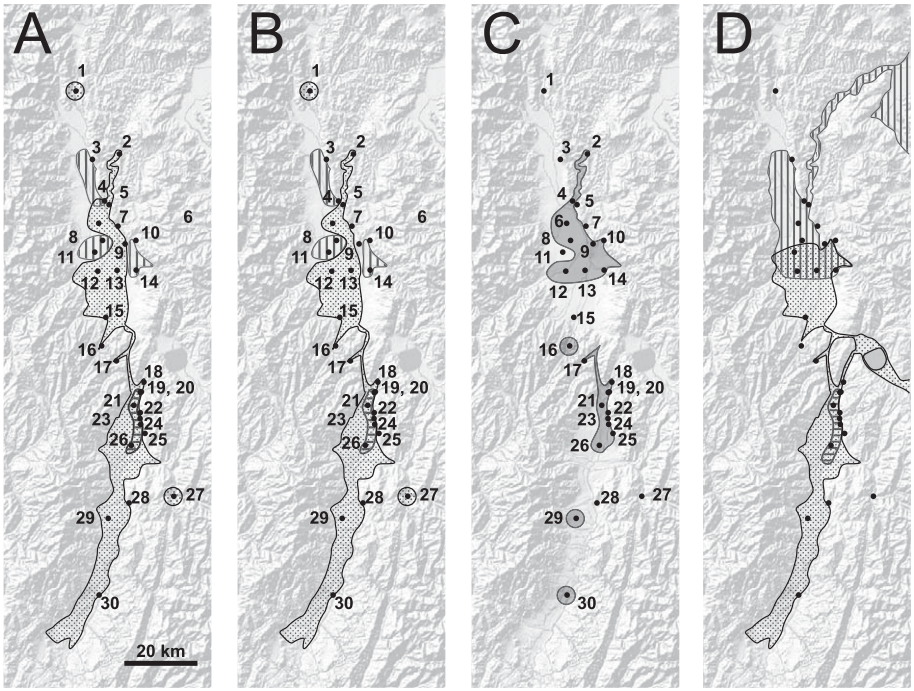

Fig. 5. The distribution maps of pond frogs. **(A)** Present distribution map of pond frog taxa based on morphological observation. **(B)** Distribution map based on allozyme and *cytb* data. **(C)** Distributions of F1 hybrids and hybrid descendants assigned by genetic markers. **(D)** Past distribution based on the morphological survey from 1978–1985 by Shimoyama (1986). In (A), (B), and (D), dotted, vertically-striped, and horizontally-striped areas indicate the distributional areas of *P*. *nigromaculatus*, *P*. *p*. *porosus*, and *P*. *p*. *brevipoda*, respectively. Distribution of hybrids is not shown in (A), (B), and (D), but is illustrated in (C), which shows the areas where F1 hybrids or introgressants were found by genetic markers (shaded).

hybrid descendants. Consequently, species and hybrid identification based on genetic markers indicated that our morphological observation underestimated the occurrence of hybrids and introgressants. But it is clear that distribution maps made on the basis of morphology and genetic markers are nearly congruent (Fig. 5A, B).

Of the F1 hybrids and introgressants (n = 63) revealed by allozyme analysis, 44 (69.8%), 11 (17.5%), and eight (12.7%) carried the *P. nigromaculatus*, *P. p. porosus*, and *P. p. brevipoda cytb* haplotypes, respectively. Furthermore and interestingly, of nine possible F1 hybrids of *P. nigromaculatus* and *P. porosus*, seven carried the *P. p. porosus* haplotype (from the Matsumoto basin) and only two carried the *P. nigromaculatus* haplotype (from the Matsumoto and Ina basins).

DISCUSSION

Distribution and hybridization of *P. nigromaculatus* and *P. p. porosus* in the Matsumoto basin

1. Shift of distributional areas and hybrid zones over the past 30 years

About 30 years ago, from 1978 to 1985, Shimoyama (1986) surveyed the distributions of the three pond frog species in the Matsumoto and Ina basins (Fig. 5D). In his report, the distribution of *P. nigromaculatus* in the Matsumoto basin was limited to the southern area, while the range of *P. p. porosus* was from the northern to central areas of the basin, including the Sai River watershed, and their distributions overlapped in the central area (Fig. 5D). Comparing past and present distributions (Fig. 5B, D), it is obvious that *P. nigromaculatus* extended its distributional areas north, especially around the Sai River. In addition, the distributional areas of *P. p. porosus* have decreased over the past 30 years. *Pelophylax p. porosus* individuals identified on the basis of external morphology are found only in three fragmented areas (populations 3 and 4; 8 and 11; 10 and 14), and "pure" *P. p. porosus* individuals identified by genetic markers have the same distribution pattern (Fig. 5A, B).

The present study detected many hybrid descendants of the two species from the past overlapping areas (Fig. 5C, D). Possible F1 hybrids of *P. nigromaculatus* and *P. p. porosus* were found in populations 4, 6, 8, and 10 (Figs. 3B, 5C), which suggests that the hybridization between these species is ongoing in the present populations. Remarkably, populations 4 and 6 are clearly distant from *P. nigromaculatus* of the past distributional area (Fig. 5D). Furthermore, in populations 2, 5, and 7, where only *P. p. porosus* was distributed in the past, *P. nigromaculatus* was found alongside hybrid descendants. Comparison of past and present distribution and the hybrid areas detected in this study clearly indicates that the hybrid zone of *P. nigromaculatus* and *P. p. porosus* has shifted northward in response to *P. nigromaculatus* invasion.

It is noteworthy that the area around population 16 located in the most southern part of the Matsumoto basin supported the *P. nigromaculatus* population 30 years ago (Fig. 5D), and all 13 individuals collected from the population in this study have only *P. nigromaculatus*-specific alleles and *P. nigromaculatus* external morphology (Figs. 3B, 5A). However, in 12 of the 13 frogs, mitochondrial haplotypes were replaced by those of *P. p. porosus* (Fig. 3B). This observation suggests that *P. p. porosus* was distributed in these areas before Shimoyama's survey (1986); subsequently, *P. nigromaculatus* invaded the population, and mtDNA from *P. p. porosus* was introgressed into the invading *P. nigromaculatus* (referred to as the "ghost of hybrids past" by Wilson and Bernatchez, 1998). Presumably, the situation of population 16 means that the hybrid zone has gradually shifted from the southern area around population 16 to the northern area in the Matsumoto basin.

2. Cause of distributional change

The distribution of *P. nigromaculatus* in the Matsumoto basin has expanded, whereas that of *P. p. porosus* has narrowed and fragmented. A gender bias in the hybridization (directional hybridization), as expected from our allozyme and *cytb* analyses, seems to be a major cause of this distributional change.

Of the eight possible F1 hybrids of *P. nigromaculatus* and *P. p. porosus* of the Matsumoto basin, seven carried the *P. p. porosus* haplotype (Fig. 3B and Supplementary Table S1 online). The abundance ratio of *P. nigromaculatus* to *P. p. porosus* haplotypes in these F1 hybrids is significantly biased toward the *P. p. porosus* haplotypes (binominal test, P = 0.0352). Furthermore, except for the F1 hybrids, 25 out of 34 hybrid descendants carried the *P. p. porosus* haplotypes, and this is also significantly biased toward the *P. p. porosus* haplotype (P = 0.0045). Considering that no differences in viability of reciprocal hybrids of *P. nigromaculatus* and *P. p. porosus* were reported from crossing experiments (Moriya, 1960), and that the maternal inheritance manner of mtDNA is maintained, even in ranid hybrids (e.g., Sumida, 1997), the observed bias toward the *P. p. porosus* mtDNA in the hybrids (including introgressants) seems to be caused by directional hybridization between *P. p. porosus* females and *P. nigromaculatus* males, rather than by postzygotic factors. The occurrence of hybridization in the same direction between these frog taxa was suggested by Sumida and Ishihara (1997). They surveyed a population in the hybrid zone of *P. nigromaculatus* and *P. p. porosus* using allozyme and mtDNA analyses in Kanagawa Prefecture in Japan and found four possible F1 hybrids, all of which possessed the *P. p. porosus* mitochondrial haplotypes. Furthermore, Shimoyama (1999, 2000) reported three breeding features of *P. nigromaculatus* based on his field observations. First, compared with the *P. porosus* male (including both *P. p. porosus* and *P. p. brevipoda*), the *P. nigromaculatus* male has the advantage of clasping *P. porosus* females, because the body size of *P. nigromaculatus* is larger than that of *P. porosus*. Second, the *P. nigromaculatus* male does not distinguish between heterospecific mating calls and conspecific ones. In contrast, it is known that *P. p. brevipoda* can distinguish between different and conspecific mating calls. Consequently, the *P. nigromaculatus* male often joins the mating chorus of *P. p. brevipoda*, but the *P. p. brevipoda* male usually does not join that of *P. nigromaculatus*. Third, the reproductive pair formation of *P. nigromaculatus* is started by males forcibly clasping females, while that in *P. p. brevipoda* is initiated by females approaching males in their territory. The distinguishability and pair formation characters have not been studied in *P. p. porosus*. However, if *P. p. prosus* has the same characters as in *P. p. brevipoda*, these characters will lead to high frequent directional hybrid-

ization between *P. p. porosus* females and *P. nigromaculatus* males, compared with reverse pairs.

Interspecific hybridization in a hybrid zone generally brings disadvantages to both parent species because of wastage of reproductive efforts (except when the resultant hybrids have normal or advanced fitness), and reproductive efforts in females are usually greater than those in males (e.g., Mallet, 2005). Thus, in a hybrid zone where directional hybridization occurs, mother species will suffer more damage than father species (e.g., Konishi and Takata, 2004). Removing females by directional hybridization also leads to less opportunity for normal mating in mother species and brings about serious damage to mother species, especially in a species in which the sex ratio or operational sex ratio are biased toward the male (the latter being the case with Japanese pond frogs; Shimoyama, 1996). Consequently, directional hybridization occasionally causes species replacement by father species in hybrid populations. For example, Konishi and Takata (2004) showed species replacement in hybrid populations caused by a directional hybridization between the Japanese minnow species *Pseudorasbora pumila* and *P. parva*. Similarly, the directional hybridization between *P. nigromaculatus* and *P. p. porosus* detected in the present study seems to be a major factor for the range expansion of the father species (*P. nigromaculatus*) and the range contraction and fragmentation of the mother species (*P. p. porosus*).

Introgression between *P. nigromaculatus* and *P. p. brevipoda* in the Ina basin

In the Ina basin, the distribution areas of *P. nigromaculatus* and *P. p. brevipoda* do not seem to have changed over the past 30 years (Fig. 5B, D), although many hybrid descendants (and a possible F1 hybrid) were found in overlapping populations (northern part, populations 17–26; Fig. 5C). Therefore, in contrast to the Matsumoto basin, the pond frog species in the Ina basin have hybridized with each other without changing their distributions. The difference in the distributional aspect between the two basins more likely comes from the lower frequency of hybridization between *P. nigromaculatus* and *P. p. brevipoda* in the Ina basin than that between *P. nigromaculatus* and *P. p. porosus* in the Matsumoto basin. Between *P. nigromaculatus* and *P. p. brevipoda*, many premating isolation mechanisms (e.g., differences in breeding sites, breeding period, and advertising calls) and other ecological differences that are unknown or not well known between *P. nigromaculatus* and *P. p. porosus* have been reported (e.g., Shimoyama, 1986; Matsui, 1996; Maeda and Matsui, 1999). These isolation mechanisms would have prevented the occurrence of high frequent hybridization between *P. nigromaculatus* and *P. p. brevipoda*. In agreement, only one possible F1 hybrid between *P. nigromaculatus* and *P. p. brevipoda* was found in the Ina basin (population 24), in contrast to the Matsumoto basin where eight possible F1 hybrids of *P. nigromaculatus* and *P. p. porosus* were found (significant difference; $P = 0.0359$, Fisher's exact test). Consequently, the low frequent hybridization seems to have allowed the long-term (at least over 30 years) co-occurrence of these frogs without distributional change.

Pelophylax p. brevipoda is now listed in the Red Data Book as a critically endangered species in Nagano Prefecture (where the Matsumoto and Ina basins are located), and in fact, distribution of *P. p. brevipoda* is limited in the northern part of the basin (Fig. 5B, D). Furthermore, our allozyme and mtDNA analysis detected only eight individuals that can be assigned as "pure" *P. p. brevipoda*. Many individuals with the *P. p. brevipoda* morphology (29/35) are assigned as introgressants derived from backcrosses between *P. p. brevipoda* and hybrids. This observation indicates that large-scale gene introgression occurred between these two species in the Ina basin, seemingly contradicting the low frequent hybridization of these species mentioned above. However, considering the long-term co-occurrence of these species, the presence of many hybrid descendants is of little wonder. Namely, the normal fitness of females of F1 hybrids and the gradual recovery of fitness in hybrid descendants can allow these hybrids to engage in continuous backcrossing and self-crossing of introgressants, and the long term backcrossing (at least 30 years) can accumulate introgressed genes in populations of each species, even if the initial hybrid number is small. Furthermore, small population size generally leads to easy fixation of introgressed genes via random genetic drift (Frankham et al., 2002); the small population size of *P. p. brevipoda* would have enhanced the rampant introgression from *P. nigromaculatus* into *P. p. brevipoda* and caused extreme reduction of the "pure" *P. p. brevipoda* in this basin. In addition, it remains a possibility that the eight individuals regarded as "pure" *P. p. brevipoda* are actually introgressants carrying *P. nigromaculatus* alleles at loci not analyzed here. To figure out the accurate survival and genetic situation of endangered *P. p. brevipoda,* for example, in the context of conservation, an immediate and more detailed survey with additional genetic markers, such as microsatellite loci, seem to be necessary.

It is noteworthy that *P. nigromaculatus*-like individuals with a *P. porosus*-specific allele or *P. p. brevipoda*-specific *cytb* haplotype are found in southern population 29, which is outside the area of *P. porosus* distribution revealed by both Shimoyama (1986) and the present study. This may be the legacy of *P. p. brevipoda* originally distributed in this area.

Two individuals of the *P. p. porosus*-specific haplotype were found in populations 25 and 30 in the Ina basin (Fig. 3B), where the occurrence of *P. p. porosus* has not been known. As a cause of these introgressants, two possible explanations —past occurrence of *P. p. porosus* in this area and artificial transports of *P. p. porosus* (or its hybrids)— can be suggested.

CONCLUSION

In the present study, we surveyed multiple hybrid zones of *P. nigromaculatus* and *P. porosus* in the Matsumoto and Ina basins and showed that different modes of introgressive hybridizations have occurred in each basin. In both basins, *P. porosus* populations are commonly threatened by interspecific-hybridizations and subsequent gene introgressions from *P. nigromaculatus*. Among the basins, however, introgressive hybridizations damaged *P. porosus* populations in different ways. Populations of *P. p. porosus* have been narrowed and fragmented by *P. nigromaculatus* invasion of the Matsumoto basin. In contrast, in the Ina basin, "pure" *P. p. brevipoda* has been decreased by the introgression without

distributional change. We also postulated the cause of these differences by combining our results and information of past distribution as well as other biological features.

In the near future, the distributional area of *P. p. porosus* will be further narrowed in the Matsumoto basin. Furthermore, "pure" *P. p. brevipoda* individuals might completely disappear from the Ina basin due to continuous gene introgression from *P. nigromaculatus* and random genetic drift in scarce populations where many hybrid descendants already exist. The real impact of the introgressive hybridization between pond frog species should become clearer with further continuous monitoring of the pond frogs in these basins.

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