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Phylogeography of *Hynobius yatsui* (Amphibia: Caudata) in Kyushu, Japan

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The salamander *Hynobius yatsui* from southwestern Japan was formerly regarded as a small-sized group of *H. naevius*, but has recently been resurrected as a distinct species. We investigated the phylogeography of *H. yatsui* from Kyushu Island using partial sequences of the mitochondrial cytochrome *b* gene. We detected 49 haplotypes in 181 individuals from 24 localities covering the entire geographic distribution of this species on Kyushu. These haplotypes were grouped into two lineages, a northern lineage from northern and central regions, and a southern lineage from the southern region; no haplotypes were shared between the lineages. We surmise that the divergence of the two lineages was induced by volcanic activity that started in the Beppu-Shimabara Graben, between the areas occupied by these two lineages. From the results of a nested clade phylogeographical analysis, we surmise that the high intrapopulation genetic variation observed in the non-volcanic Kyushu Mountains was generated by alternation of contiguous range expansion or long-distance dispersal, and isolation. The current distribution and the observed complicated genetic structures of *H. yatsui* in Kyushu seem to have been affected first by volcanic activities since the late Pliocene, but subsequent climatic oscillations during the Pleistocene may also have some effects, although it is impossible at this time to differentiate the effects of these two factors.

Key words: *Hynobius yatsui*, *Hynobius naevius*, mtDNA, nested clade, phylogeographical analysis, volcanic activity

INTRODUCTION

Among small salamanders of the genus *Hynobius* from East Asia, more than half the known species are endemic to Japan. *Hynobius naevius*, a lotic breeder in mountain streams, was considered widespread in the southwestern region of the country (Sato, 1943; Matsui and Misawa, 1996). However, recent studies in northern and central Kyushu (Tominaga et al., 2003; Sakamoto et al., 2005) have revealed the presence of two genetically, morphologically, and ecologically distinct groups, i.e., the large-sized group (=Type A of Tominaga et al., 2003; Group A of Tominaga et al., 2005a, b; Group I of Sakamoto et al., 2005; Clade 1+2 of Tominaga et al., 2006) and the small-sized group (=Type B of Tominaga et al., 2003; Group B of Tominaga et al., 2005a, b; Group II of Sakamoto et al., 2005; Clade 3+4 of Tominaga et al., 2006), that warrant recognition as separate species. Tominaga and Matsui (2008) recently resurrected the name *H. yatsui* for the small-sized group, which occurs from the Chubu and Kinki Districts of Honshu through Shikoku to Kyushu.

Tominaga et al. (2005a) conducted an extensive allozyme analysis of *H. naevius* (including *H. yatsui*) throughout its distribution in Japan and delimited the ranges of the two species. These authors hypothesized that crust movement in the Pliocene first genetically differentiated the two species. At the same time, they surmised that *H. yatsui* (as the small-sized group) was separated between Kyushu and Shikoku by the formation of a barrier at the present Bungo Strait. More recently, Tominaga et al. (2006), in phylogenetic analyses of this species using mitochondrial 12S and 16S rRNA sequences, found two deeply diverged clades in each of the two species (as *H. naevius* sensu lato) and associated the divergences with the configuration of southwestern Japan in the late Miocene-Pliocene. They found two clades in *H. yatsui*, one composed of populations from central Shikoku eastwards, and the other from western Shikoku and Kyushu. Further, the Kyushu populations were split into northern and southern groups. In this way, the distribution

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range was delimited and a differentiation time was estimated for *H. yatsui*, but details of the evolutionary history within each clade remained unclear.

In the distribution of *H. yatsui* and *H. naevius*, the island of Kyushu is unique, not only in the sympatry of the two species, but also in its geohistory, which is characterized by intense volcanic activity and crust movements. However, the influence of this geohistory on the differentiation of animals has scarcely been surveyed using modern molecular techniques (Nishi and Sota, 2007). Kyushu can be roughly divided into the northern, central, and southern regions on the basis of geological, geographical, and topographical differences (Machida et al., 2001) (Fig. 1). Of these, the northern region has been more stable than the central and southern regions, which contain volcanic grabens (the Beppu-Shimabara and Kagoshima Grabens, respectively). The latter regions have been affected by severe volcanic activity over a wide area, except for the non-volcanic Kyushu Mountains, since the late Miocene. Along the Beppu-Shimabara Graben, extending from east to west across the central region, extensive volcanic activity occurred twice (approximately 6.0-5.0 and 2.0-0.5 MYA; Machida et al., 2001). In the southern region, the northern portion is occupied by the non-volcanic Kyushu Mountains, while the southern part is split into a non-volcanic eastern area and a volcanic western area, with the Kagoshima Graben in between. In the volcanic area, activity started from the west in the Pliocene and moved eastward to the Kagoshima Graben, which was formed in the early Pleistocene. Thus, the volcanic activity on Kyushu can be roughly divided geohistorically into one active period from the late Miocene to the Pliocene and another from the Pleistocene to the present (Fig. 1).

Nested clade phylogeographical analysis (NCPA=NCA;

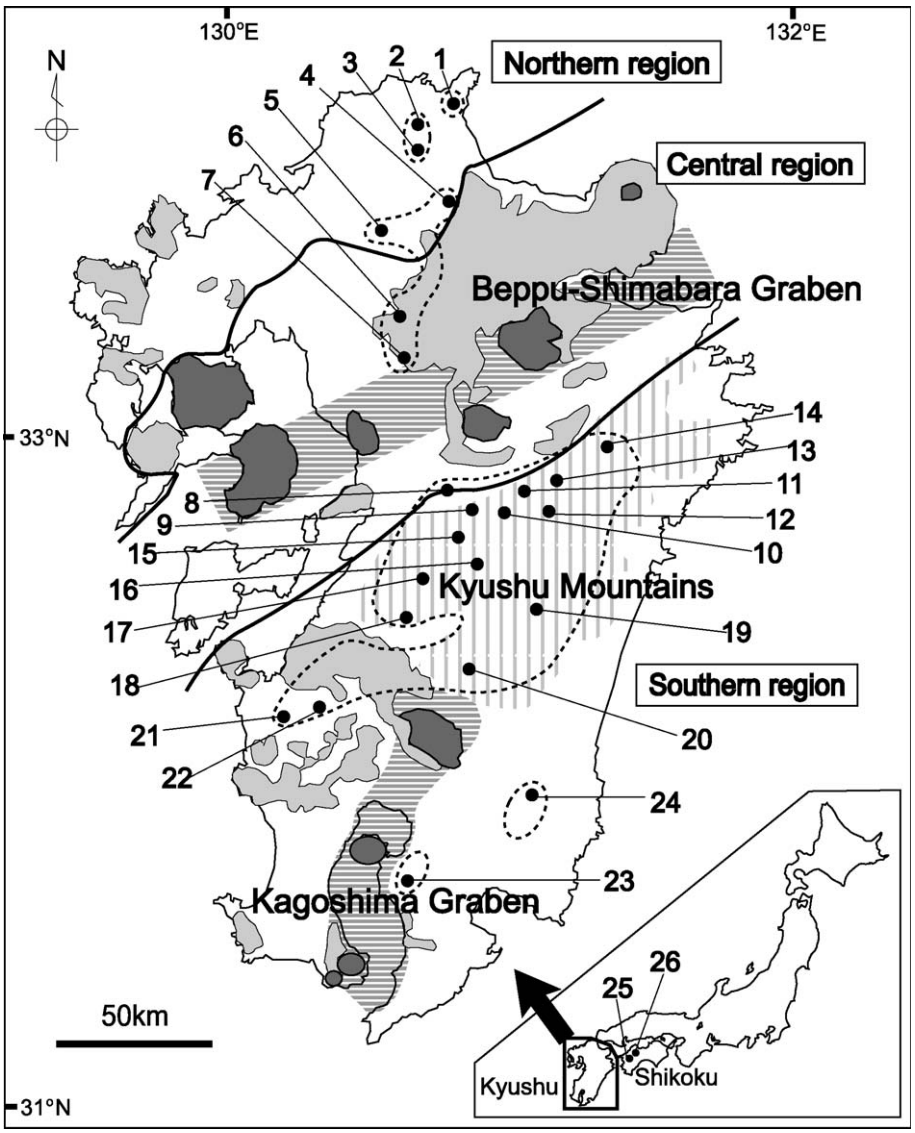

Fig. 1. Map of Kyushu, Japan, showing sampling localities for *Hynobius yatsui*. For sample numbers, refer to Table 1. Light shading, volcanic rock from the Miocene to the Pliocene (Nagao et al., 1995); dark shading, volcanic rock from the Pleistocene to the present (Nagao et al., 1995); horizontal hatching, volcanic grabens; vertical hatching, Kyushu Mountains; broken line, known distribution range.

Templeton, 2004) is an effective method for detecting associations between geographic location and haplotype frequency and infers evolutionary processes that have influenced current genetic structure (Templeton et al., 1995; Templeton, 1998). Thus, studies using NCPA have recently been conducted for many animal lineages, e.g., beetles (Smith and Farrell, 2005); fishes (Pruett et al., 2005); salamanders (Matsui et al., 2007); frogs (Veith et al., 2003); lizards (Jesus et al., 2005); and mammals (Piertney et al., 2005, Li et al., 2005). Simple application of the method without other complementary analyses has recently been criticized (Paulo et al., 2002; Masta et al., 2003), and some authors have even rejected the validity of this method (Panchal and Beaumont, 2007; Petit, 2008). Panchal and Beaumont (2007) proposed to use fully automated software to reduce the subjective decisions made by traditional NCPA, and, using their own automated software, demonstrated that NCPA tends to give a high frequency of false positives. However, Templeton (2008) rebutted these criticisms and insisted that the non-validated implementation of NCPA by Panchal and Beaumont (2007) had caused the high false-positive rate in their simulation. Because of this uncertainty, the new method has scarcely been utilized so far.

To infer differentiation processes in *H. yatsui*, which inhabits a wider area in Kyushu than the larger-sized *H. naevius* (Tominaga et al., 2005a, b), we implemented a NCPA using the mitochondrial cytochrome *b* (cyt *b*) gene, which is believed to evolve faster than the 12S and 16S rRNA genes used by Tominaga et al. (2006) and the control region (CR=D-loop) gene (Matsui et al., 2007). Finally, by comparing inferences from NCPA with known past environmental changes, we tried to estimate differentiation processes in *H. yatsui* within Kyushu.

MATERIALS AND METHODS

Samples

From 1992 to 2006, we collected 181 *H. yatsui* individuals from 24 sampling sites encompassing the entire distribution of this species in Kyushu (Fig. 1, Table 1). For outgroup sequences, we used two populations of the same species from Shikoku. The choice of these populations was based on the results of Tominaga et al. (2006). The specimens are stored at Kyoto University or in the private collection of M. Sakamoto (data available on request).

Amplification and sequencing

We homogenized frozen or ethanol-preserved tissue samples in 0.6 mL of STE buffer containing 10 mM Tris/HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA, pH 8.0. Proteinase K (0.1 mg/mL) was added to the homogenate, and proteins were digested for 12 h at 36°C. We treated the solution with phenol and chloroform/isoamyl alcohol and precipitated DNA with ethanol. We dried the DNA precipitates, dissolved them in 1 mL TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and subjected 3 μL to polymerase chain reaction (PCR). For PCR, we used the primers L14239 (5'-TCTCAGCTTGATGAAATTTTGGCTC-3') and H14935 (5'-GGAAATATCATTCTGGTTGAAT-3') for cyt *b*. The numbering system follows the sequence of *Andrias davidianus* (AJ492192) (Zhang et al., 2003).

Amplified DNA segments were purified by 15% polyethylene glycol (PEG). Sequencing reactions were done with the PCR primers, using the ABI Big Dye terminator version 3 ready mix. Sequencing was performed on an ABI 3100 PRISM sequencer (Applied Biosystems, Foster City, CA). The 51 new sequences obtained in this study were deposited in GenBank under accession

Table 1. Sample numbers, localities, haplotypes, and GenBank accession numbers for *Hynobius yatsui* sequenced in this study.

Sample number	Sampling locality	Haplotype (N)	DDBJ accession number
1	Kokura-ku, Kitakyushu-shi, Fukuoka Pref.	A5 (2)	AB297506
		A6 (2)	AB297507
		A7 (4)	AB297508
		A8 (1)	AB297509
		A9 (2)	AB297510
		A10 (1)	AB297511
2	Yahata-ku, Kitakyushu-shi, Fukuoka Pref.	A1 (1)	AB297502
		A4 (1)	AB297505
		A11 (5)	AB297512
		A12 (2)	AB297513
		A13 (2)	AB297514
3	Nogata-shi, Fukuoka Pref.	A2 (3)	AB297503
		A3 (2)	AB297504
4	Aka-mura, Fukuoka Pref.	A15 (2)	AB297516
5	Asakura-shi (formerly Amagi-shi), Fukuoka Pref.	A15 (8)	AB297516
		A16 (2)	AB297517
6	Hoshino-mura, Fukuoka Pref.	A14 (1)	AB297515
		A15 (7)	AB297516
7	Yamaga-shi (formerly Kikuka-machi), Kumamoto Pref.	A15 (7)	AB297516
		A17 (2)	AB297518
8	Yamato-cho (formerly Yabe-machi), Kumamoto Pref.	D2 (12)	AB297534
9	Yamato-cho (formerly Yabe-machi), Kumamoto Pref.	B3 (3)	AB297530
		D3 (8)	AB297535
		D4 (1)	AB297536
		D5 (2)	AB297537
10	Yamato-cho (formerly Seiwa-son), Kumamoto Pref.	D3 (1)	AB297535
11	Yamato-cho (formerly Soyo-cho), Kumamoto Pref.	D11 (1)	AB297544
		D13 (6)	AB297545
12	Gokase-cho, Miyazaki Pref.	D3 (7)	AB297535
13	Takachiho-cho, Miyazaki Pref.	B4 (1)	AB297522
		D3 (1)	AB297535
		D7 (1)	AB297539
14	Saeki-shi (formerly Ume-machi), Oita Pref.	B4 (10)	AB297522
15	Yatsushiro-shi (formerly Izumi-mura), Kumamoto Pref.	B2 (1)	AB297531
		D3 (3)	AB297535
		D6 (1)	AB297538
		D7 (1)	AB297539
16	Shiba-son, Miyazaki Pref.	D3 (3)	AB297535
		D8 (1)	AB297540
		D9 (1)	AB297541
		D14 (1)	AB297547
17	Itsuki-mura, Kumamoto Pref.	C1 (2)	AB297519
		C2 (1)	AB297520
		C3 (4)	AB297521
		D3 (2)	AB297535
		D17 (1)	AB297549
18	Sagara-mura, Kumamoto Pref.	B1 (1)	AB297532
		C1 (1)	AB297519
		D3 (5)	AB297535
		D10 (1)	AB297542
		D12 (1)	AB297543
19	Kijyo-cho, Miyazaki Pref.	D3 (1)	AB297535
		D15 (2)	AB297547
20	Kobayashi-shi (formerly Suki-son), Miyazaki Pref.	B5 (3)	AB297523
		D1 (1)	AB297533
		D16 (1)	AB297548
		D18 (1)	AB297550
21	Satsuma-cho (formerly Miyanojyo-cho), Kagoshima Pref.	D1 (9)	AB297533
22	Satsuma-cho, Kagoshima Pref.	D1 (2)	AB297533
23	Kanoya-shi, Kagoshima Pref.	B6 (10)	AB297524
24	Miyazaki-shi (formerly Tano-cho), Miyazaki Pref.	B7 (1)	AB297525
		B8 (2)	AB297526
		B9 (1)	AB297527
		B10 (4)	AB297528
		B11 (1)	AB297529
25	Uchiko-cho (formerly Oda-cho), Ehime Pref.	E1 (1)	AB297551
26	Kumakogen-cho (formerly Omogo-mura), Ehime Pref.	F1 (1)	AB297552

numbers AB297502–AB297552.

Sequence alignment and analysis

We analyzed partial cyt *b* sequences in Sequence Analysis 3.7 (Applied Biosystems). The sequence for each individual was determined in both directions, and sequences were aligned by using ClustalX 1.8 (Thompson et al., 1997). We calculated both nucleotide and haplotype diversities in DnaSP 4 (Rozas et al., 2003).

We performed phylogenetic analyses of DNA sequences by maximum parsimony (MP) using PAUP* 4.0b10 (Swofford, 2002). MP phylogenies were estimated with the heuristic search algorithm for each tree-building methodology. We used 100 random-taxon-addition replicates for all analyses to minimize the effect of entry sequence order on the topology of the resulting cladogram. Branch confidence in the MP tree was assessed with 1,000 nonparametric bootstrap pseudoreplicates, each consisting of 100 random-taxon-addition heuristic searches (Felsenstein, 1985; Felsenstein and Kishino, 1993; Hedges, 1992). Nodes with bootstrap values (bs) of 70% or greater were regarded as sufficiently resolved, and those with values between 50 and 70% as weakly supported (Huelsenbeck and Hillis, 1993).

Bayesian analyses using the Markov-chain Monte-Carlo technique (MCMC) were also performed by using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). We selected the settings for the DNA substitution model that best fitted the data by hierarchal likelihood ratio tests (hLRTs) using MrModeltest Version 2.2 (Nylander, 2004) and chose the HKY+G model. We initiated four independent analyses with a random starting tree that ran for 5 million generations. We used the program TRACER (Rambaut and Drummond, 2007) to determine when the log likelihood of sampled trees reached a stationary distribution. Because apparent stationarity of MCMC runs was reached at no later than 500,000 generations, we conservatively discarded the first 1.0 million generations from each run as "burn-in" and sampled one of every 100 generations from the remaining 4.0 million generations to calculate the posterior probability (bpp) for each branch in the Bayesian tree. Bipartition posterior probabilities of 95% or greater were considered significant support (Larget and Simon, 1999; Huelsenbeck et al., 2001).

Phylogeographical and population genetic analyses

To deduce the forces (e.g., recurrent gene flow, fragmentation, range expansion) acting to generate the geographical pattern of the genetic distribution in *H. yatsui*, we first constructed haplotype networks using DNA sequences for 181 individuals with TCS Version 1.8 (Clement et al., 2000), based on the algorithm given by Templeton et al. (1992). Ambiguous connections (loops) in the network were resolved by using predictions from coalescent theory (Crandall and Templeton, 1993; Nordborg, 2001; Rosenberg and Nordborg, 2002). Next, we nested both observed and inferred haplotypes following the rules modified by Posada and Crandall (2001). We then calculated NCPA distance statistics based on geographical coordinates and their significances by comparison with a null distribution derived from 10,000 random permutations of clades against sampled populations using GeoDis Version 2.2 (Posada et al., 2000). Finally, we used the inference key (version dated 14 July 2004) of Templeton (2004) to interpret the summary statistics.

Demographic parameters were estimated from a mismatch distribution analysis of pairwise differences (Rogers and Harpending, 1992) to further verify some of the inferences of NCPA, such as demographic expansion. The fit of the observed data to a model of either sudden population expansion (Rogers and Harpending, 1992) or spatial expansion (Schneider and Excoffier, 1999; Excoffier, 2004) was tested by using ARLEQUIN Version 3.1 (Excoffier et al., 2005). In addition to the mismatch distribution analysis, Fu's tests of neutrality (Fu, 1997) were performed for sequences within each higher-level nested group (2- to 4-step clades). Significant negative values of Fu's statistics can be interpreted as a signature of demographic expansion. The statistics were calculated with ARLEQUIN Version 3.1, and their significances were assessed through 10,000 simulations.

Estimation of divergence time

We produced estimates of divergence times for *H. yatsui* by using BEAST Version 1.4.7 (Drummond and Rambaut, 2007). Analyses were run for 10 million generations, with the first one million generations discarded as burn-in, and parameter values were sampled every 1,000 generations. Parameter estimates and convergence were checked by using Tracer Version 1.4 (Rambaut and Drummond, 2007). We used geohistorical information related to *H. yatsui* to calibrate the molecular clock. As described above, two extensive episodes of volcanic activity along the Beppu-Shimabara Graben appear to have occurred 6-5 MYA and 2-0.5 MYA (Machida et al., 2001). We considered that the start of the latter activity separated the northern and southern lineages (see Results) of *H. yatsui*, and set the normally distributed estimate prior to the divergence between the two lineages to 2±0.1 MYA. We estimated dates of divergence only for well-supported clades (bpp≥95% and bs≥ 70%) in the phylogenetic tree obtained using the GTR+I+G model (partitioned by codon position) and the relaxed molecular clock method (Drummond et al., 2006) with an "uncorrelated lognormal" distribution of evolutionary rates among lineages.

RESULTS

Sequence variation

From all 181 ingroup individuals from Kyushu, we generated a 637-bp fragment of the mitochondrial cyt *b* gene, of which a total of 65 positions were polymorphic and 40 were phylogenetically informative. Within the ingroup, alignment of cyt *b* sequences revealed a total of 49 unique haplotypes. Of these, 17 were present in samples from the

Table 2. Summary of haplotype diversity (*h*) and nucleotide diversity (π).

Sample number	N	*h*±SD	π±SD
1	12	0.864±0.072	0.00532±0.00060
2	11	0.782±0.107	0.00348±0.00089
3	5	0.600±0.175	0.00094±0.00028
4	2	0	0
5	10	0.356±0.159	0.00056±0.00025
6	8	0.250±0.180	0.00078±0.00057
7	9	0.389±0.164	0.00061±0.00026
8	12	0	0
9	14	0.648±0.116	0.00348±0.00099
10	1	–	–
11	7	0.286±0.196	0.00090±0.00062
12	7	0	0
13	3	1.000±0.272	0.00523±0.00203
14	10	0	0
15	6	0.800±0.172	0.00419±0.00195
16	6	0.800±0.172	0.00209±0.00070
17	10	0.822±0.097	0.00844±0.00164
18	9	0.722±0.159	0.00567±0.00225
19	3	0.667±0.314	0.00209±0.00099
20	6	0.800±0.172	0.00900±0.00197
21	9	0	0
22	2	0	0
23	10	0	0
24	9	0.860±0.120	0.00253±0.00047

northern and central regions, and 32 in samples from the southern region. No shared haplotypes were detected between these two regions.

The among-sample nucleotide diversity was significantly lower in the northern and central regions (mean±SD= 0.00526±0.00048) than in the southern region (0.00768± 0.00046; $P<0.05$). In the northern and central regions, the northernmost samples (1 and 2) showed significantly higher nucleotide diversity (0.00348–0.00532) than the remaining samples (3-7, 0.00056–0.00094; $P<0.05$) (Table 2). In the southern region, nucleotide diversity differed greatly among samples, even between closely located samples (e.g., between samples 8 [0] and 9 [0.00348], and among samples 11 [0.00090], 12 [0], and 13 [0.00523]; all $P<0.05$), and was extremely high in several samples from the non-volcanic Kyushu Mountains (samples 17 [0.00844] and 20 [0.00900]), although each of these two samples did not differ from one of the other 23 samples ($P>0.05$).

Phylogenetic relationships

The two phylogenetic analyses for the cyt *b* sequence fragments yielded only slightly different topologies, and only the Bayesian tree is shown in Fig. 2. Haplotype groups A, B, C, and D correspond to haplotypes in nested clades 3-1, 3-2, 3-3, and 3-4, respectively (see below; Fig. 3). The main relationships were as follows:

1) Samples from Kyushu formed a monophyletic group with respect to samples from Shikoku in both the Bayesian and MP trees (bpp and bs=100%). Two samples from Shikoku did not form a group, but one (western Shikoku) grouped with the Kyushu clade while the other (central Shikoku) was the sister group to Kyushu+western Shikoku.

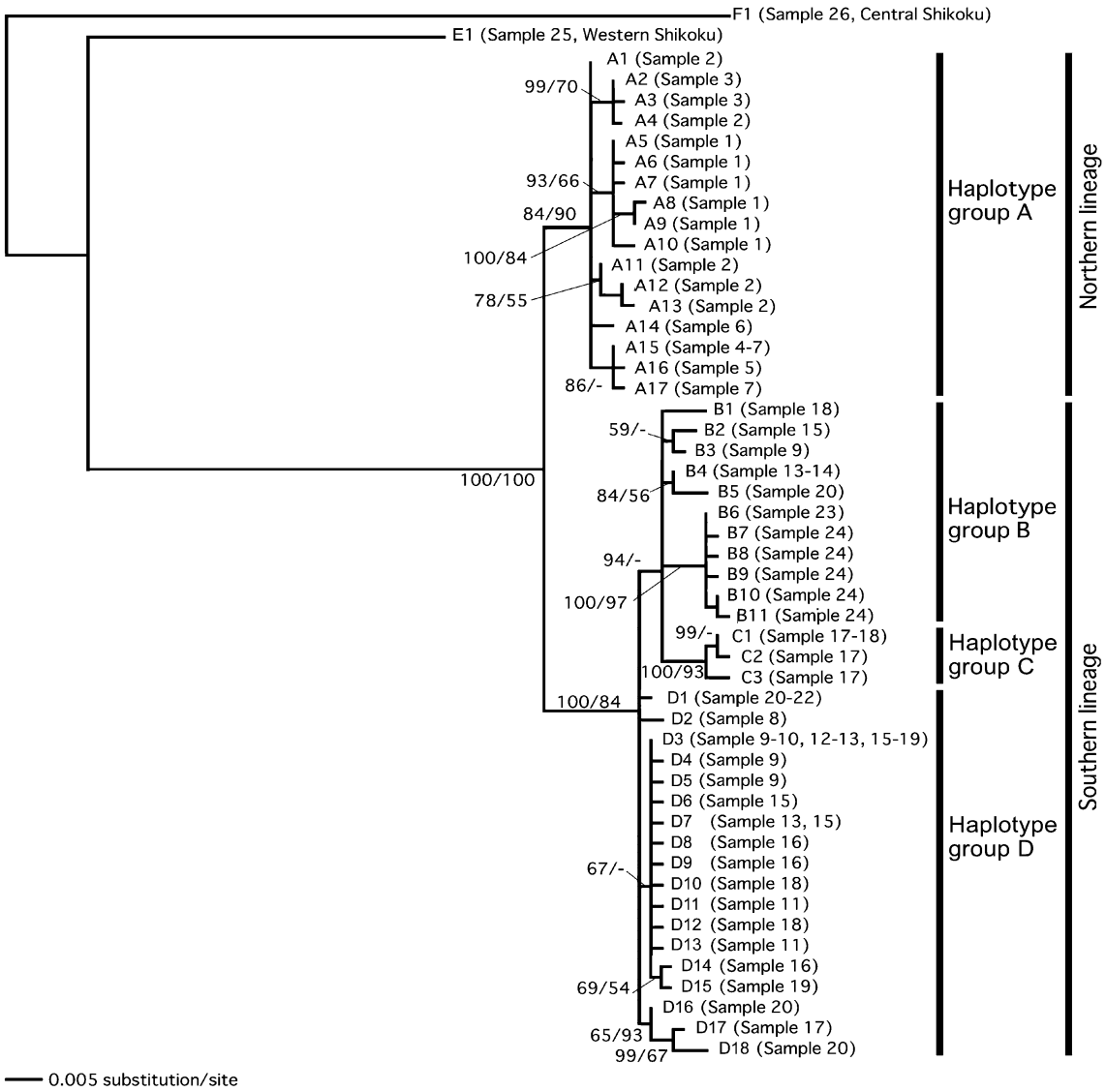

Fig. 2. Bayesian tree showing phylogenetic relationships among haplotypes of *Hynobius yatsui* from Kyushu inferred from partial cyt *b* sequences, rooted with Shikoku samples 25 and 26. Nodal values indicate Bayesian posterior probabilities and bootstrap value (1000 replicates) from maximum-parsimony inference. Haplotype groups A, B, C, and D correspond to haplotypes in nested clades 3-1, 3-2, 3-3, and 3-4, respectively (Fig. 3).

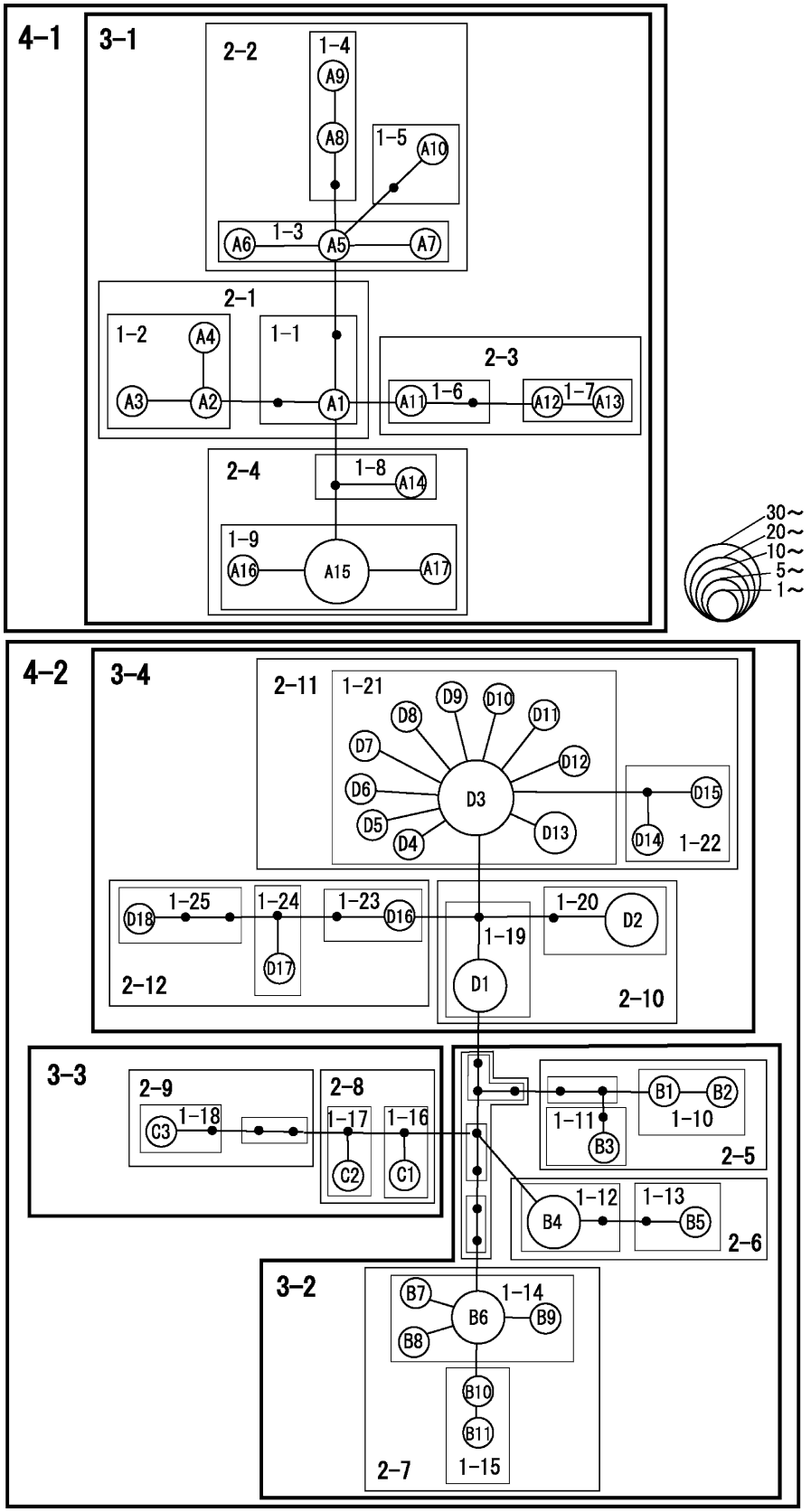

Fig. 3. Statistical parsimony network and nested design for cyt *b* haplotypes of *Hynobius yatsui* from Kyushu. Haplotypes belonging to the same clade level are boxed. Haplotype groups A, B, C, and D correspond to clades 3-1, 3-2, 3-3, and 3-4, respectively. The size of each open circle represents haplotype frequency.

2) Kyushu samples were divided into two clusters, one including samples from the northern and central regions (hereafter called the northern lineage, samples 1–7 [haplotype group A in Fig. 2]) and the other from the southern region (southern lineage, samples 8–24 [haplotype groups B, C and D in Fig. 2]).

3) Monophyly of the southern lineage was strongly supported (bpp=100 and bs=84%), but monophyly of the northern lineage was supported only by the MP tree (bpp=84% and bs=90%).

4) Relationships of samples within each of the two lineages were largely unresolved. As an exception, monophyly was confirmed in part of sample 1 (bpp=100% and bs=84%), and part of samples 2 and 3 (bpp=99% and bs=70%) in the northern lineage, and part of samples 17 and 18 (bpp=100% and bs=93%), and samples 23 and 24 (bpp=100% and bs=97%) in the southern lineage.

Nested clades in *H. yatsui*

The nested clades for the haplotypes of *H. yatsui* are shown in Fig. 3. The TCS program could not link the northern and southern haplotypes within the limit of 95% statistical parsimony. On the basis of three-step clades, one and three haplotype groups were recognized for the northern and southern haplotypes, respectively (Figs. 3, 4). Of the three southern groups, haplotype group B (=clade 3-2) occurred in both the eastern and western parts of the Kyushu Mountains and further south, and was split into three two-step clades. Haplotype group C (=clade 3-3) was monophyletic, restricted to the western part of the Kyushu Mountains, and was split into two two-step clades, while haplotype group D (=clade 3-4) widely occurred in the Kyushu Mountains (Fig. 4), and further to the southwest. It was split into three two-step clades (Fig. 3).

Nested contingency analysis revealed a significant association between clades and sampling locations for clades 1-14, 2-6, 2-7, 2-10, 2-11, 3-1, 3-2, 3-4, and 4-2 (Table 3), in which the geographical distance analysis also showed significant distance measures (D_c, D_n, or I-T). Inferences on population structure and historical influences for these clades (Table 4) are:

1) For one-step clade 1-14, which is monophyletic and endemic to the southeastern region, far south of the Kyushu Mountains (samples 23 [haplotype B6] and 24 [haplotypes B7–B9]), contiguous range expansion was suggested.

2) Clade 2-6, which consisted of haplotypes from two disjunct regions, one from the northern part of the Kyushu Mountains (samples 13 and 14 [=clade 1-12: haplotype B4]) and the other from the southern part of the Kyushu Mountains (sample 20 [=clade 1-13: haplotype B5]), was esti-

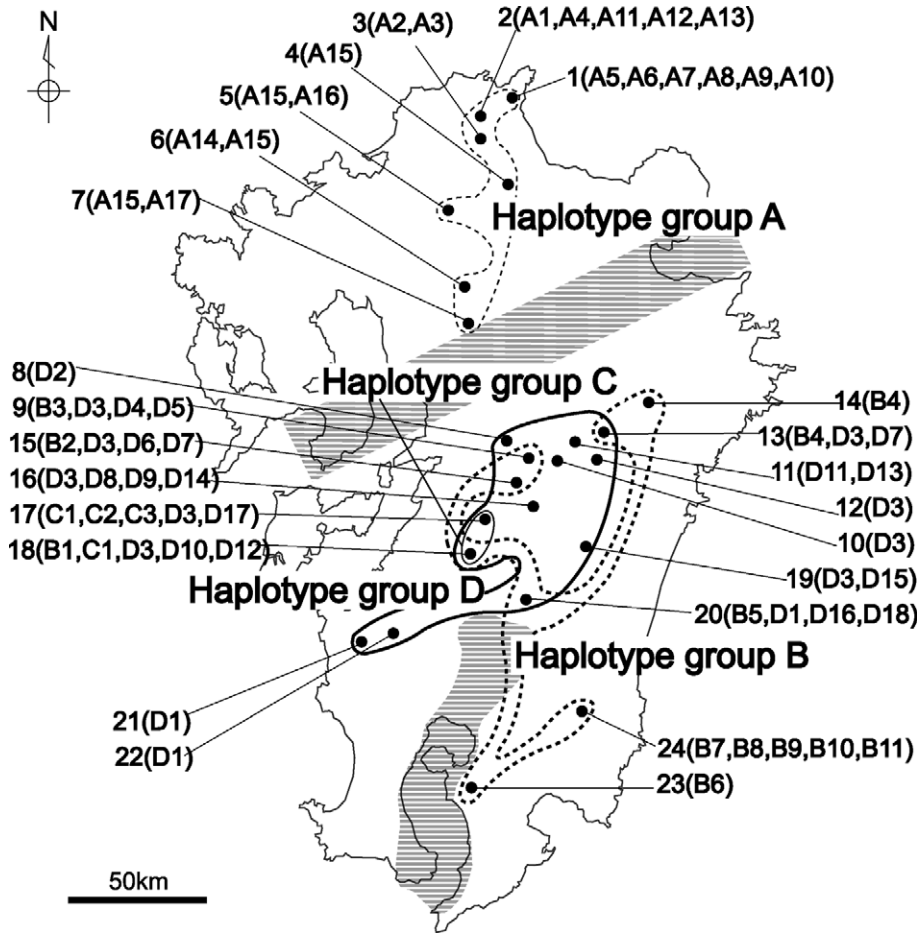

Fig. 4. Distribution of haplotypes and haplotype groups in *Hynobius yatsui* from Kyushu. For sample numbers, refer to Table 1 and Fig. 2. Horizontal hatching, grabens.

Table 3. Nested contingency analysis of geographical associations for the cytochrome *b* data. Clades not showing genetic or geographic variation were excluded.

Clade	Permutation χ^2 statistic	Probability
1-2	6.000	0.180
1-9	8.141	0.307
1-10	0.444	1.000
1-14	14.000	0.000[a]
1-21	110.325	0.215
1-22	3.000	0.344
2-1	2.917	0.309
2-4	2.719	0.334
2-5	1.143	1.000
2-6	14.000	0.005[a]
2-7	7.540	0.015[a]
2-8	4.000	0.234
2-10	24.000	0.000[a]
2-11	23.906	0.014[a]
2-12	3.000	1.000
3-1	147.312	0.000[a]
3-2	82.000	0.000[a]
3-3	5.000	0.401
3-4	118.147	0.000[a]
4-2	116.912	0.000[a]
Total cladogram	180.000	0.000[a]

[a]Significant at the 0.05 level.

Table 4. Demographic inferences from the nested clade phylogeographical distance analysis (Templeton, 2004).

Clade	Inference chain	Inference
Clade 1-14	1-2-11-RE-12-No	Contiguous range expansion.
Clade 2-6	1-19-No	Allopatric fragmentation.
Clade 2-7	1-2-3-5-6-7-8-Yes	Restricted gene flow/ dispersal but with some long-distance dispersal over intermediate areas not occupied by the species; or past gene flow followed by extinction of intermediate populations.
Clade 2-10	1-19-20-2-3-5-15-21-No	Insufficient evidence to discriminate between long-distance movements of the organism and the combined effects of gradual movement during a past range expansion and fragmentation.
Clade 2-11	1-2-11-RE-12-No	Contiguous range expansion.
Clade 3-1	1-2-11-RE-12-No	Contiguous range expansion.
Clade 3-2	1-19-20-2	Inconclusive outcome.
Clade 3-4	1-2-3-4-No	Restricted gene flow with isolation by distance.
Clade 4-2	1-2-3-4-No	Restricted gene flow with isolation by distance.
Total cladogram	1-19-No	Allopatric fragmentation.

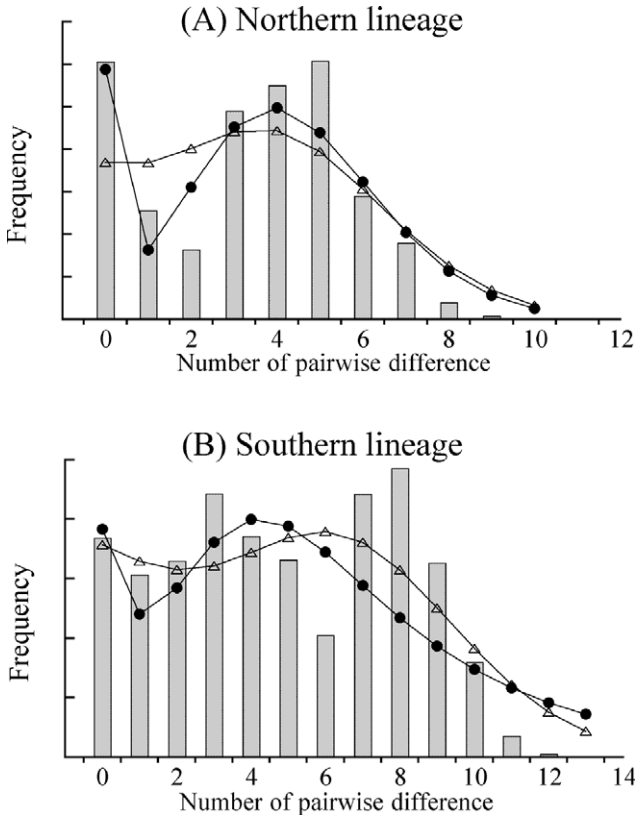

Fig. 5. Mismatch distributions of pairwise sequence differences between individuals from pooled data for each main lineage (A, northern lineage; B, southern lineage). The observed data (bars) were fitted to models of sudden population expansion (open triangles) or spatial expansion (closed circles).

mated to have experienced allopatric fragmentation.

3) Clade 2-7, which is monophyletic and includes clade 1-14 (samples 23 [haplotype B6] and 24 [B7–B9]) and clade 1-15 (sample 24 [haplotypes B10 and B11]) from southern Kyushu, was inferred to have undergone restricted gene flow/dispersal. In addition, some long-distance dispersal over intermediate areas not occupied by the species or past gene flow followed by extinction of intermediate populations was suggested for this clade.

4) Clade 2-10, consisting of haplotypes from remotely disjunct localities (sample 8 [haplotype D2] from north of the Kyushu Mountains [=clade 1-20] and samples 20 from the southern end of the Kyushu Mountains, and 21 and 22 from west of the Kagoshima Graben [haplotype D1=clade 1-19]), was estimated to have been formed either by long-distance movements, or by the combined effects of gradual movement during a past range expansion and fragmentation.

5) Clade 2-11, including clade 1-21 (samples 9 [haplotypes D3–D5], 10 [D3], 11 [D12 and D13], 12 [D3], 13 [D3 and D7], 15 [D3, D6, and D7], 16 [D3, D8, and D9], 17 [D3], 18 [D3, D10, and D11], and 19 [D3]) and clade 1-22 (samples 16 [haplotype D14] and 19 [D15]), widely occurring in the Kyushu Mountains, was inferred by NCPA to have experienced contiguous range expansion.

6) At the three-step clade level, a significant association was detected in clade 3-1 (the entire distribution of the northern lineage=haplotype group A), and was related to contiguous range expansion.

7) Clade 3-2 (haplotype group B in the southern lineage) had an inconclusive outcome.

8) Clade 3-4 (haplotype group D in the southern lineage), widely occurring in and west of the Kyushu Mountains, was inferred to have undergone restricted gene flow with isolation by distance.

Table 5. Demographic parameters estimated by mismatch distribution analysis (Rogers and Harpending, 1992) and Fu's tests of neutrality (Fu, 1997). τ, time parameter in a generation; θ_0, pre-expansion population size; θ_1, post-expansion population size; *SSD*, sum of squared deviations; θ, population size; *M*, number of migrants exchanged with other demes; NS, $P>0.05$; *, $P<0.05$.

Clade	Population expansion model				Spatial expansion model				
	τ	θ_0	θ_1	*SSD*	τ	θ	*M*	*SSD*	Fu's *Fs*
North (4-1)=A (Clade 3-1)	5.002	0.004	7.881	0.019^{NS} (P=0.29)	4.187	0.228	4.510	0.007^{NS} (P=0.89)	–4.65653*
South (4-2)	7.574	0.000	9.690	0.007^{NS} (P=0.60)	3.125	3.709	6.092	0.011^{NS} (P=0.45)	–10.63779**
B (Clade 3-2)	8.504	0.000	9.487	0.022^{NS} (P=0.27)	5.320	2.535	2.946	0.018^{NS} (P=0.62)	0.83819
C (Clade 3-3)	6.211	0.002	6.895	0.144^{NS} (P=0.16)	5.319	0.001	2.708	0.098^{NS} (P=0.40)	1.45828
D (Clade 3-4)	2.740	0.002	5.969	0.013^{NS} (P=0.32)	2.466	0.001	6.981	0.009^{NS} (P=0.54)	–8.39534**
Clade 2-1	1.480	0.000	99999	0.010^{NS} (P=0.81)	1.479	0.001	99999	0.010^{NS} (P=0.69)	–0.91387
Clade 2-2	2.908	0.002	10.670	0.010^{NS} (P=0.62)	2.600	0.084	14.737	0.011^{NS} (P=0.59)	–1.03256
Clade 2-3	2.992	0.000	3.472	0.043^{NS} (P=0.32)	2.600	0.001	3.260	0.023^{NS} (P=0.51)	1.15208
Clade 2-4	3.000	0.000	0.469	0.001^{NS} (P=0.65)	0.548	0.019	2.670	0.000^{NS} (P=0.84)	–1.72496*
Clade 2-5	4.01	0.000	4.143	0.126^{NS} (P=0.16)	3.469	0.000	2.830	0.093^{NS} (P=0.21)	1.53329
Clade 2-6	4.072	0.000	0.474	0.169^{NS} (P=0.05)	3.489	0.001	0.712	0.084^{NS} (P=0.13)	2.77429
Clade 2-7	1.078	0.000	99999	0.019^{NS} (P=0.16)	1.078	0.001	99999	0.019^{NS} (P=0.12)	–2.59756*
Clade 2-8	4.121	0.000	1.877	0.256^{NS} (P=0.11)	3.488	0.001	1.348	0.160^{NS} (P=0.14)	1.71605
Clade 2-9	–	–	–	–	–	–	–	–	–
Clade 2-10	4.059	0.000	2.272	0.267^{NS} (P=0.11)	3.488	0.001	1.494	0.1738* ($P<0.05$)	4.65143
Clade 2-11	0.951	0.002	99999	0.002^{NS} (P=0.48)	0.950	0.004	99999	0.002^{NS} (P=0.32)	–11.19876**
Clade 2-12	4.492	0.002	99999	0.089^{NS} (P=0.55)	4.495	0.001	99999	0.089^{NS} (P=0.56)	0.13353

9) At the four-step clade level, clade 4-2 (the entire distribution of the southern lineage) was inferred to have undergone restricted gene flow with isolation by distance.

10) The total cladogram, including the southern and northern lineages, was estimated to have arisen through allopatric fragmentation.

The mismatch distributions for pooled data of each of the main lineages showed a bimodal distribution and fitted both the sudden population expansion model and the spatial expansion model (Fig. 5A, B). This held for most other clades, except for Clade 2-10, which fitted only the sudden population expansion model (Table 5). Significant negative values of Fu's statistics were detected in clades 4-2, 4-1 (=3-1), 3-4, 2-4, 2-7, and 2-11.

Estimates of divergence times

When the divergence time of the northern and southern lineages was set at around 2 MYBP, diversification within the southern lineage was estimated to have begun around 0.97 (range=0.52–1.49) MYA. Part of samples 17 and 18 were thought to have begun divergence at 0.24 (range= 0.04–0.49) MYA, and samples 23 and 24 at 0.22 (range= 0.06–0.40) MYA in the southern lineage. In the northern lineage, part of samples 2 and 3 were estimated to have started divergence at 0.13 (range=0.01–0.29) MYBP.

DISCUSSION

Phylogeny of *H. yatsui* in Kyushu

Geohistorically, Kyushu has experienced severe volcanic activity, as mentioned above. Activity along the Beppu-Simabara Graben in the central region is thought to have occurred twice, once from 6.0–5.0 MYA and again from 2.0–0.5 MYA; another episode of volcanic activity, known in the southern region, is estimated to have started from the west in the Pliocene (>1.8 MYA) and moved eastward to the Kagoshima Graben, which was formed in the early Pleistocene (1.8–1.0 MYA: Machida et al., 2001). The Kyushu Mountains between these two grabens are non-volcanic.

The samples of *H. yatsui* from Kyushu used in this study formed a monophyletic group with full statistical support (bpp=100%, bs=100%). Samples from Shikoku did not form a monophyletic group, in accordance with the results of Tominaga et al. (2006), in which populations of *H. yatsui* (as the small-sized group of *H. naevius*, Kyushu-B) from Kyushu formed a distinct clade with a population from among the Shikoku populations (as Shikoku B) that was split from the remaining Shikoku populations.

The relationships among the Kyushu populations highlight a major division between the northern (samples 1–7) and southern (samples 8–24) lineages, which have discrete distributions separated by the Beppu-Shimabara Graben (Fig. 6). These two lineages had a mean (±SE) p distance of 2.64±0.49%, and their monophyly was well supported, at least in the MP tree (bs>84%).

We surmise that the second round of volcanic activity along the Beppu-Simabara Graben that started around 2.0 MYA caused the divergence of *H. yatsui*. This is because the first is too old, whereas the second conforms to the estimates by Tominaga et al. (2006), who estimated the time of the first genetic differentiation within *H. yatsui* (as the small-sized *H. naevius*) to be 3.4–2.8 MYA and that between western Shikoku and Kyushu to be 2.9–2.3 MYA. The common ancestor of the two lineages probably invaded Kyushu and expanded its distribution before the second period of volcanic activity (see below).

Population phylogeography and ages of divergence

Several researchers have pointed out discrepancies between NCPA conclusions and existing prior knowledge of a species' history (Paulo et al., 2002; Masta et al., 2003). Panchal and Beaumont (2007) recently demonstrated that NCPA tends to give a high frequency of false positives. In their simulation with panmictic data, Panchal and Beaumont (2007) detected two spurious inference codes (restricted

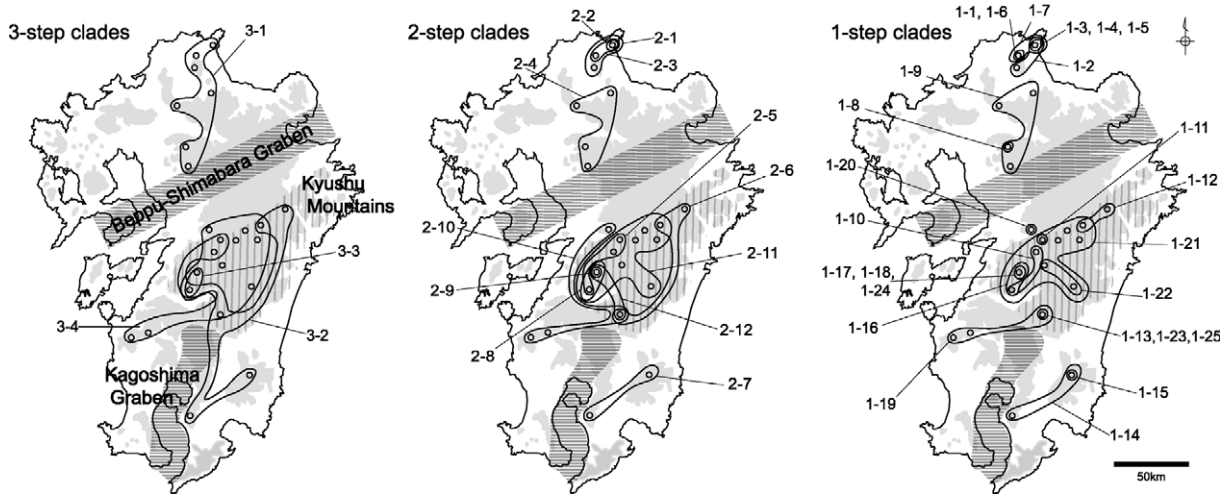

Fig. 6. Distribution of 1-step, 2-step, and 3-step clades of *Hynobius yatsui* on Kyushu. Horizontal hatching, grabens; vertical hatching, Kyushu Mountains; lightly shaded areas, 200 m a.s.l.

gene flow with isolation by distance and contiguous range expansion) that have been commonly reported in recent publications. Panchal and Beaumont (2007) used fully automated software in their analyses. However, it has become clear that their simulations to test NCPA were problematic, because NCPA using the new software was not implemented properly in their simulations (Templeton, 2008). Thus, the performance of automated software (ANeCA) is still uncertain. We used orthodox NCPA and carefully interpreted the results, taking into consideration results from other analyses (phylogenetic analyses, nucleotide diversity, mismatch analysis, Fu's *F*s, and age estimation); however, we admit that there may still be pitfalls in this method, and our ideas presented below provide a working hypothesis for understanding the processes that shaped the phylogeography of *H. yatsui*.

Although the relationships of haplotypes within each of the lineages were not well resolved in the phylogenetic tree (Fig. 2), the results of NCPA clarified the presence of four two-step clades in the northern lineage and three three-step clades in the southern lineage (Figs. 3, 4). As is clear from Figs. 2 and 3, the results of the phylogenetic analyses and NCPA are only slightly congruent; only clades1-2, 1-4, 2-7, and 3-3 recognized in NCPA proved to be monophyletic in the phylogenetic tree. Thus we focused mainly on the results of NCPA to infer population phylogeography.

After the formation of the Bungo Strait separated the common ancestor of *H. yatsui* between Kyushu and western Shikoku (Tominaga et al., 2005a, 2006), the ancestral Kyushu population seems to have had a wide distribution on the island at some point, except for regions with volcanic rocks from the Miocene to the Pliocene. However, the ancestral population seems to have been split into the northern and southern regions by the second volcanic episode, along the Beppu-Shimabara Graben (see above) around 2.0 MYA. Because no haplotypes are shared between the two lineages, we suspect that their isolation has continued since then. This is supported by the allozyme analysis of Tominaga et al. (2005a).

Affected by this volcanism, the ancestor of the northern lineage would have experienced a severe range reduction and been confined to the northern area. This is supported by the fact that samples from northernmost area (samples 1 and 2) showed higher nucleotide diversity than the remaining samples of the northern lineage (samples 3–7). With the subsequent subsidence of volcanic activity, the ancestral northern lineage would have expanded its range both toward the south and farther to the north as inferred by NCPA (Inference 6), mismatch analysis, and Fu's *F*s. The significantly lower mean values of nucleotide diversity observed in samples from the more southern area and the significantly negative value of Fu's *F*s for clade 2-4 suggest that range expansion in the southern area of the northern lineage has been more intense than in the northern area.

The ancestor of the southern lineage mainly inhabited the non-volcanic Kyushu Mountains and would have been less affected by volcanic activity along the Beppu-Shimabara Graben than the northern lineage. It differentiated into three haplotype groups (B, C, and D) as the result of restricted gene flow with isolation by distance (Inference 9). Haplotype group B (clade3-2) was located in the interior of the network. This indicates that this haplotype group is older than haplotype groups C and D. The three nested clades (2-5 [B1–B3], 2-6 [B4 and B5], and 2-7 [B6–B11]) in haplotype group B are now observed allopatrically in remote areas (the northeastern and southwestern areas of the Kyushu Mountains, and far south of the Kyushu Mountains). Because an insignificant value of Fu's *F*s was observed in clade3-2, and an interior 2-step clade (ancestral clade) is now absent within haplotype group B, we surmise that this haplotype group was originally distributed in a wide area of southern Kyushu, and that each of the three nested clades (2-5, 2-6, and 2-7) has accumulated mutations independently, although the NCPA results were inconclusive (Inference 7). Two populations (samples 14 and 23) that present only this haplotype group and are located in a peripheral or isolated area of the distributional range of the species, have values of nucleotide and haplotype diversities equaling zero. This suggests that these populations have undergone severe local bottleneck events in the past, and that the present rates of migration from

other populations to these populations are probably very low because of the populations' geographic locations.

Haplotype group D, which is now similarly widely distributed in the Kyushu Mountains, would have experienced restricted gene flow by isolation (Inference 8). However, because the results of the phylogeographical analyses (NCPA, mismatch distribution analysis, Fu's *F*s) indicate that range expansions occurred after the event of restricted gene flow in clade 5-3 (inference 5 for clade 2-11; see below), we consider the original distributional range of haplotype group D to have been limited to western Kyushu, and that restricted gene flow for clade 5-3 might have occurred only in this region.

The ancestor of haplotypes B4 and B5 likely was subdivided allopatrically into the northeastern and the southwestern populations and successively gave rise to haplotypes B4 and B5 (Inference 2). In contrast, ancestral haplotype D1 likely invaded to the north along the western edge of the Kyushu Mountains and gave rise to haplotype D2 (Inference 4). In more inland areas of the Kyushu Mountains, the common ancestor of haplotypes D3–D15 would have contiguously expanded its range (Inference 5).

Haplotypes B6–B11 are unique to southernmost Kyushu and form a monophyletic group. We estimate that these haplotypes are derived from an invader from the Kyushu Mountains, and infer them to have differentiated by dispersal (Inference 3) and subsequent contiguous range expansion (Inference 1). We estimate that these differentiations occurred after the mid-Pleistocene, because genetic exchange with more northern populations would have been prevented by the volcanic activity north of the Kagoshima Graben that occurred by that time (Machida et al., 2001). Therefore, haplotypes of the southernmost Kyushu populations would have become unique as a consequence of isolation from other populations. We believe that in this way, the high intrapopulation genetic variation observed in the non-volcanic Kyushu Mountains was generated by alternation between contiguous range expansion or long-distance dispersal and isolation.

Factors that affected population differentiation

Although very limited in number, all ages of diversification and divergence estimated above (later than around 0.97 MYA) correspond to periods of repeated climate change during the Pleistocene, in addition to small-scale volcanic activity until 0.5 MYA. Thus, climate change and glacial/interglacial cycling in this period, together with continued crustal movements, may have affected divergence subsequent to the major dichotomy in *H. yatsui* from Kyushu.

Extant populations of *H. yatsui* in Kyushu live at high elevations (mean elevation=731 m above sea level) and in a cool (mean annual temperature=11.6°C) and moist (mean annual precipitation=2311 mm) environment (Tominaga et al., 2005b). If the ancestral *H. yatsui*, like its modern descendants, was adapted to a cool climate in relatively high mountain regions, it could have inhabited the lowlands of mountain border regions only during cool glacial stages. Therefore, we surmise that range expansion occurred during cold stages via routes along mountain border areas at lower elevations, while differentiation occurred in warm stages when the climate limited the distribution to mountainous regions.

The current distribution and the observed complicated genetic structure of *H. yatsui* in Kyushu, first affected by volcanic activity after the late Pliocene, may also have been affected by subsequent climatic oscillations during the Pleistocene, although it is impossible at this time to differentiate the effects of the two factors. Volcanic activity has been reported to have affected various animals, e.g., beetles (Moya et al., 2004, Emerson et al., 2006), fishes (Mateos, 2005), and reptiles (Austin et al., 2004, Brehm et al., 2003, Ciofi et al., 2006, Gubitz et al., 2000, Jesus et al., 2005), but as far as we are aware, only one case has been reported for anuran amphibians (Mulcahy and Mendelson, 2000). For urodelans, the attempt by Nishikawa et al. (2005) to explain phylogenetic divergence in the *H. boulengeri-H. stejnegeri* complex on Kyushu by volcanic activity was not successful. Thus, *H. yatsui* in Kyushu is the first example of an urodelan amphibian whose genetic divergence was promoted by volcanic activity. In contrast, effects of Pleistocene climatic oscillations have been reported for many animals, including amphibians (e.g., Carstens et al., 2005; Crespi et al., 2003; Hoffman and Blouin, 2004; Matsui et al., 2007; Veith et al., 2003), and these oscillations affected animals in different ways in regions non-glaciated during glacial periods. Species adapted to warm climates retreated to refugia, resulting in vicariance and isolation among populations (Avise, 1992), while species adapted to a cool climate at higher elevations expanded their distributions to lower regions (Crespi et al., 2003). *Hynobius yatsui* may represent the latter case, because this species is currently found in cool, wet mountains and seems to be adapted to a cool climate (see above); glaciation periods may have allowed its populations to expand on Kyushu, where there were no glaciers or periglacial influence (Koaze, 2006). In addition, other *Hynobius* species with larger body sizes occur in Kyushu (Tominaga et al., 2003, 2005ab; Sakamoto et al., 2005), and ecological interactions with congeneric species (Crespi et al., 2003) may be related to population differentiation in this species (Nishikawa et al., 2007). It is necessary to test our hypotheses by studying the differentiation patterns of other salamanders in Kyushu, as well as their ecological relationships with *H. yatsui*.

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