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Mitochondrial Cytochrome b Phylogeny and Historical Biogeography of the Tohoku Salamander, *Hynobius lichenatus* (Amphibia, Caudata)

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The Tohoku salamander, *Hynobius lichenatus* Boulenger, 1883, is a lentic breeding species widespread throughout montane regions of northeastern Japan. To explore intraspecific genetic variation and infer evolutionary history of *H. lichenatus*, we performed mitochondrial DNA analysis (complete 1141 bp sequences of the mitochondrial cytochrome b gene) using 215 adult and larval individuals collected from 75 localities, encompassing known distributional range of the species. *Hynobius lichenatus* proved to be monophyletic, including three well-supported and geographically structured clades (Clade I from northern Kanto, Clade II from southern Tohoku, and Clade III from northern Tohoku). These clades, respectively, comprise several subclades, and show genetic distances as large as those seen between different species of *Hynobius*. Results of population statistic analyses indicate that all clades and most subclades have maintained high genetic diversity and demographic stability over long periods. Molecular dating indicates divergence in *H. lichenatus* concords with topographic evolution of northeastern Japan from late Miocene to early Pleistocene, suggesting that paleogeographic events in this region, such as orogenesis, sea level change, and volcanic activity, have been crucial for shaping genetic patterns and diversity in this species. *Hynobius lichenatus* greatly differs from many other animal species from northeastern Japan in its much older periods and the pattern of genetic differentiation, and is suggested as an old faunal element in this region.

Key words: *Hynobius lichenatus*, genealogy, biogeography, mitochondrial DNA, cytochrome b, salamander

INTRODUCTION

Hynobius lichenatus Boulenger, 1883 is a lentic breeding small salamander widely occurring throughout montane regions of northeastern Japan. Several studies have been conducted on the morphology (Inukai and Makino, 1933; Sato, 1943; Aoki, 1977; Maruyama, 1977; Hasumi and Iwasawa, 1987a, b, 1988, 1993), isozymes (Matsui, 1987), Southern blotting (Kuro-o et al., 1992), and mitochondrial DNA (mtDNA) (Matsui et al., 2007) of this species.

In morphological studies, great variations have been detected among and within populations, and there was no clear pattern of geographic variation, with overlap of population variations in many morphological characters, although most of these studies were based on individuals from small numbers of populations. In addition, Hasumi and Iwasawa (1987a) suggested that environmental factors during developmental stages affected these variations.

In describing *H. hidamontanus*, Matsui (1987) studied phylogenetic relationships among seven *Hynobius* species, including *H. lichenatus*, using isozymes. The study resolved *H. lichenatus* to contain three major genetic groups whose distribution indicated a geographic structure. The study also revealed strong genetic differentiations between populations of this species, which suggest their distinct species status. Using Southern blotting, Kuro-o et al. (1992) studied phylogenetic relationships among eight *Hynobius* species, including *H. lichenatus*, and confirmed Matsui's (1987) results. In analyzing intraspecific genetic variation of *H. tokyoensis* using mitochondrial cytochrome b (Cytb) gene, Matsui et al. (2007) included *H. lichenatus* as an outgroup, and also ascertained results of the above two studies.

These studies suggested cryptic genetic diversity within *H. lichenatus*. However, because they did not focus on intraspecific variation of *H. lichenatus*, the studies suffered from limited geographical sampling with only a small number of individuals, leaving the necessity for more detailed surveys. To explore intraspecific variation in this species, we investigated its mtDNA genealogical structure throughout its distribution range. Recent molecular phylogenetic studies of Japanese small salamanders based on mtDNA analysis revealed cryptic diversity within species (e.g., Tominaga et al., 2006; Matsui et al., 2007; Yoshikawa et al., 2008), and estimated underlying evolutionary processes. Therefore, using mtDNA markers for *H. lichenatus* is expected to be effective for elucidating intraspecies cryptic diversity. Furthermore, *H. lichenatus* is the only amphibian species whose range is limited to northeastern Japan; the present study may therefore also be important to the understanding

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of amphibian biogeography in this region. The goals of this study are (1) to construct a robust intraspecific mtDNA genealogy, which will help infer population relationships and investigate cryptic diversity, and (2) to infer evolutionary history in *H. lichenatus*.

MATERIALS AND METHODS

Sampling

A total of 215 samples including larvae and adults of *H. lichenatus* were collected from 75 localities throughout its distribution range (Fig. 1, Table 1). Based on phylogenetic relationships reported by Matsui et al. (2007, 2008), we chose two closely related species *H. tokyoensis* Tago, 1931 (AB266640) and *H. nigrescens* Stejneger, 1907 (AB548378) for comparison, and a more distantly related species *H. retardatus* Dunn, 1923 (AB363609) and *Salamandrella keyserlingii* Dybowski, 1870 (AB363573) as an outgroup for phylogenetic analysis.

Sequencing

Total genomic DNA was extracted from liver or muscle tissues frozen at –80°C or preserved in 99% ethanol using standard phenol-chloroform extraction procedures (Hillis et al., 1996). The complete sequence of the mitochondrial Cytb gene was obtained using polymerase chain reaction (PCR) amplification with the primers described in Table 2. PCR amplification conditions included initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 52°C for 45 s, and 72°C for 75 s, and was completed by a final extension at 72°C for 7 min. Amplified PCR products were electrophoresed on 2.0% agarose gel and viewed under UV light with ethidium bromide to check for correct fragment

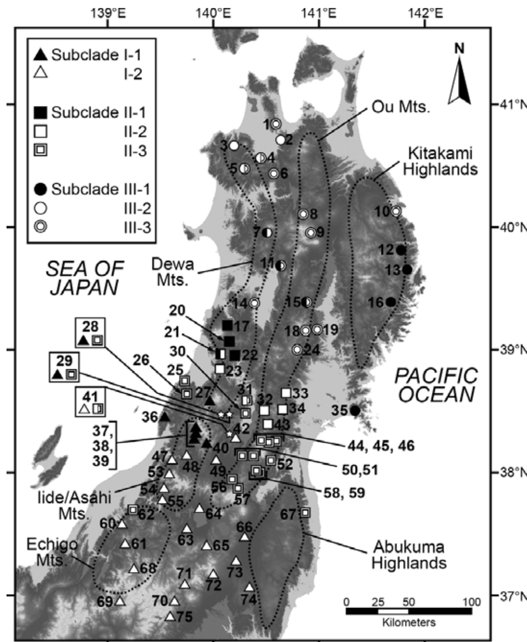

Fig. 1. Map of sampling localities of *Hynobius lichenatus*. Numbers correspond to the sampling point number given in Table 1. Labels indicate specific localities mentioned in the text. Map shading indicates topographic relief.

Table 1. Sampling locality, number of individuals (*n*) and haplotypes (H). Sampling point number corresponds to the sampling map labels in Fig. 1.

Sampling Point	Sampling locality	*n*	H (*n*)
1	Daishaka, Aomori-shi, Aomori Pref.	5	h1, h2 (4)
2	Namioka, Aomori-shi, Aomori Pref.	3	h3 (3)
3	Ajigasawa-machi, Nishitsugaru-gun, Aomori Pref.	5	h4 (2), h5 (3)
4	Tateko-yama, Hirosaki-shi, Aomori Pref.	3	h6, h7 (2)
5	Nishimeya-mura, Nakatsugaru-gun, Aomori Pref.	3	h8, h9, h10
6	Ikarisawa, Ikarigaseki, Hirakawa-shi, Aomori Pref.	3	h11(2), h12
7	Moriyoshi, Kitaakita-shi, Akita Pref.	6	h13, h14, h15 (2), h16, h17
8	Hachimantai, Kazuno-shi, Akita Pref.	1	h18
9	Hachimantai, Hachimantai-shi, Iwate Pref.	3	h11, h19, h20
10	Noda-mura, Kunohe-gun, Iwate Pref.	2	h21, h22
11	Tazawa-ko, Senboku-shi, Akita Pref.	3	h23, h24 (2)
12	Iwaizumi-cho, Shimohei-gun, Iwate Pref.	3	h25, h26, h27
13	Kebaraichi, Miyako-shi, Iwate Pref.	3	h28 (2), h29
14	Omagari, Daisen-shi, Akita Pref.	3	h30, h31 (2)
15	Wakasennin, Kitakami-shi, Iwate Pref.	2	h32, h33
16	Tsuchibuchi, Tono-shi, Iwate Pref.	2	h27 (2)
17	Chokai-san, Akita Pref.	1	h34
18	Isawa-ku, Oshu-shi, Iwate Pref.	4	h35 (2), h36, h37
19	Mizusawa, Oshu-shi, Iwate Pref.	3	h35, h37, h38
20	Mamurogawa-machi, Mogami-gun, Yamagata Pref.	1	h39
21	Tazawa-gawa, Sakata-shi, Yamagata Pref.	5	h40, h41, h42 (2), h43
22	Sakegawa-mura, Mogami-gun, Yamagata Pref.	1	h44
23	Kiyokawa, Sakata-shi, Yamagata Pref.	1	h45
24	Kurikoma-yama, Ichinoseki-shi, Iwate Pref.	2	h35 (2)
25	Kamo, Tsuruoka-shi, Yamagata Pref.	1	h46
26	Sakanoshita, Tsuruoka-shi, Yamagata Pref.	2	h47, h48
27	Nishikawa-machi [1], Nishimurayama-gun, Yamagata Pref.	3	h49(3)
28	Nishikawa-machi [2], Nishimurayama-gun, Yamagata Pref.	8	h50, h51, h52 (2), h53, h49 (3)
29	Nishikawa-machi [3], Nishimurayama-gun, Yamagata Pref.	8	h49 (3), h53, h54, h55, h56, h57
30	Sanesawa-gawa, Sagae-shi, Yamagata Pref.	1	h58
31	Tominami-gawa, Murayama-shi, Yamagata Pref.	4	h59, h60, h61 (2)
32	Shiramizu-gawa, Higashine-shi, Yamagata Pref.	5	h62, h63(4)
33	Kami-machi, Kami-gun, Miyagi Pref.	3	h64, h65, h66
34	Taiwa-cho, Kurokawa-gun, Miyagi Pref.	3	h67, h68, h69
35	Mano, Ishinomaki-shi, Miyagi Pref.	3	h27(3)
36	Sasagawa, Murakami-shi, Niigata Pref.	6	h70(4), h71, h72
37	Miomote-gawa [1], Murakami-shi, Niigata Pref.	4	h73, h74, h75, h76
38	Miomote-gawa [2], Murakami-shi, Niigata Pref.	1	h77
39	Miomote-gawa [3], Murakami-shi, Niigata Pref.	1	h78
40	Oguni-machi, Nishiokitama-gun, Yamagata Pref.	3	h79, h80, h81
41	Yamanobe-machi [1], Higashimurayama-gun, Yamagata Pref.	8	h82 (5), h52 (2), h83
42	Yamanobe-machi [2], Higashimurayama-gun, Yamagata Pref.	4	h84 (4)
43	Omoshiro-yama, Yamagata-shi, Yamagata Pref.	3	h63 (3)
44	Fudo-sawa, Yamagata-shi, Yamagata Pref.	2	h52, h85
45	Zao-dam, Yamagata-shi, Yamagata Pref.	1	h58
46	Kawasaki-machi, Shibata-gun, Miyagi Pref.	3	h52, h55, h86
47	Torisaka-yama, Tainai-shi, Niigata Pref.	1	h87
48	Sekikawa-mura, Iwafune-gun, Niigata Pref.	1	h88
49	Nagai-shi, Yamagata Pref.	3	h89, h90, h91
50	Oishida-yama, Kaminoyama-shi, Yamagata Pref.	1	h92
51	Zao-kogen, Kaminoyama-shi, Yamagata Pref.	2	h52, h93
52	Shichikashuku-machi, Katta-gun, Miyagi Pref.	2	h52, h94
53	Shimosanko, Shibata-shi, Niigata Pref.	3	h95, h96, h97
54	Gozu, Agano-shi, Niigata Pref.	4	h95, h98 (2), h99
55	Aga-machi, Higashikanbara-gun, Niigata Pref.	1	h100
56	Sekine, Yonezawa-shi, Yamagata Pref.	5	h101, h102, h103, h104, h105
57	Touge, Yonezawa-shi, Yamagata Pref.	5	h102 (2), h106 (3)
58	Iizaka-machi [1], Fukushima-shi, Fukushima Pref.	2	h52, h107
59	Iizaka-machi [2], Fukushima-shi, Fukushima Pref.	1	h52
60	Shitada, Sanjyo-shi, Niigata Pref.	3	h108, h109 (2)
61	Irihirose, Uonuma-shi, Niigata Pref.	1	h109
62	Kamiotani, Kamo-shi, Niigata Pref.	1	h110
63	Showa-mura, Onuma-gun, Fukushima Pref.	3	h111 (3)
64	Yamato-machi, Kitakata-shi, Fukushima Pref.	3	h112 (2), h113
65	Shimogo-machi, Minamiaizu-gun, Fukushima Pref.	1	h114
66	Ouse-machi, Koriyama-shi, Fukushima Pref.	2	h115 (2)
67	Haramachi-ku, Minamisoma-shi, Fukushima Pref.	3	h116, h117, h118
68	Ginzandaira, Uonuma-shi, Niigata Pref.	1	h119
69	Minakami-machi, Tone-gun, Gunma Pref.	4	h119 (2), h120 (2)
70	Nikko-shi (former Kuriyama-mura), Tochigi Pref.	3	h111 (3)
71	Nakamiyori, Nikko-shi, Tochigi Pref.	3	h121 (3)
72	Nasushiobara-shi, Tochigi Pref.	4	h111 (2), h122, h123
73	Shirakawa-shi (former Tashiro-mura), Fukushima Pref.	3	h115 (2), h124
74	Tanagura-machi, Higashishirakawa-gun, Fukushima Pref.	3	h111, h125 (2)
75	Nikko-shi, Tochigi Pref.	1	h126
Hynobius tokyoensis	Hachioji-shi, Tokyo met. govern.	1	AB266640
H. nigrescens	Kami-machi, Kami-gun, Miyagi Pref.	1	AB548378
H. retardatus	Ebetsu-shi, Hokkaido Pref.	1	AB363609
Salamandrella keyserlingii	Kushiro-shi, Hokkaido Pref.	1	AB363573

Table 2. List of primers used in this study.

Primer name	Sequence (5′ to 3′)	Position	Source
HYD_Cytb_F1	CYAAYCCTAAAGCWGCAAAATA	external forward	Matsui et al. (2008)
cytb_R1_cynops	AARTAYGGGTGRAADGRRAYTTTRTCT	internal reverse	This study
cytb_F2_cynops	CAYTTYYTGYTMCCATTYYTAATTGCAGG	internal forward	This study
salamander_cytb_R_N2	YTYTCAATCTTKGGYTTACAAGACC	external reverse	Matsui et al. (2008)

size. PCR products were subsequently purified using 13% polyethylene glycol (PEG) purification procedures. Cycle sequencing reactions of purified PCR products were performed with ABI Prism BigDye Terminator Ver. 3.1 Cycle Sequencing Kit (Applied Biosystems), in both directions with the same primers as used for PCR amplification. Cycle sequencing reaction products were cleaned by ethanol precipitation following manufacturer's protocol and these cleaned products were analyzed with an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Continuous gene sequences were assembled and edited manually using Chromas Pro version 1.34 (Technelysium Pty Ltd., Tewantin, Australia) and aligned using CLUSTALX2 version 2.1 (Larkin et al., 2007). All generated sequences have been deposited in GenBank under the accession numbers AB266669–AB266671 and AB750781–AB750992.

Phylogenetic analysis

We performed maximum likelihood (ML) and Bayesian inference (BI) analyses to estimate phylogenetic relationships. ML trees were generated with Treefinder ver. March 2011 (Jobb, 2011). The best-fit substitution models were determined for individual codon positions with Kakusan4 (Tanabe, 2011), using the Akaike Information Criterion (AIC) (Akaike, 1974). Nodal support was estimated with 1000 bootstrap replicates and nodes were considered to be strongly supported if they had bootstrap values (BS) of 70% or greater (Huelsenbeck and Hillis, 1993). BI trees were generated with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The best-fit substitution models were determined with Kakusan4 (Tanabe, 2011), using the Bayesian Information Criterion (BIC) (Schwarz, 1978). BI analyses were performed using three heated and one cold Metropolis coupled Markov Chain Monte Carlo (MCMC) for 10 million generations, sampling every 100 generations. Suitable burn-in and convergence of parameters were determined using Tracer ver. 1.5 (Rambaut and Drummond, 2009). Although all samples showed convergence after 800,000 generations, we conservatively discarded the first 15% of trees (1.5 million generations) as burn-in. After burn-in, trees of two independent runs were combined in a single majority consensus topology, and we considered nodes to be strongly supported if they had Bayesian posterior probabilities (BPP) values 95% or greater (Leaché and Reeder, 2002). Based on clades identified from the phylogenetic tree, we calculated the genetic distances (uncorrected pairwise distances) between clades using MEGA 5.05 (Tamura et al., 2011).

Population analysis

Using the main clades and subclades identified from the phylogenetic tree, population statistics were performed using ARLEQUIN ver. 3.5.1.3 (Excoffier and Lischer, 2010). Genetic diversity within main clades and subclades was measured by haplotype diversity (h; Nei, 1987) and nucleotide diversity (π; Nei and Tajima, 1981). To examine deviations from neutrality, Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) neutrality tests were performed. The significance of both neutrality tests was examined with 10,000 replicates.

Divergence time estimation

For divergence time estimation, we selected 16 Cytb sequences from the *H. lichenatus* datasets that represent each clade and subclade on the phylogenetic tree. We also added four other species used in the phylogenetic analysis. To estimate divergence times, we used Bayesian method using BEAST ver. 1.6.2 (Drummond and Rambaut, 2007). As no known calibration points exist, we used two different rates of hynobiids mitochondrial DNA evolution: Weisrock et al.'s (2001) rate of 1.28% sequence divergence per MY (0.64% per MY per lineage: Calibration I) and Yoshikawa et al.'s (2008) rate of 1.36% per MY (0.68% per MY per lineage: Calibration II). BEAST analyses were performed using the relaxed uncorrelated lognormal molecular clock (Drummond et al., 2006), under a HKY + G model of sequence evolution, and the best topology obtained from the BI analyses was used as a starting tree. We used default prior distributions for all other parameters and ran the analyses for 20 million generations, sampling every 1000 generations. Suitable burn-in and convergence of parameters were determined using Tracer ver. 1.5 (Rambaut and Drummond, 2009), and the first 3 million generations were discarded as burn-in.

RESULTS

Phylogenetic analysis

The total alignment for the complete Cytb gene sequence (1141 base pairs) yielded 126 haplotypes, 300 variable sites, and 240 parsimony informative sites. Private haplotypes dominate the Cytb gene dataset, with 111 of the 126 haplotypes corresponding to one locality.

For ML and BI analyses, the best-fit model of sequence evolution was determined for individual codon positions (1st, 2nd and 3rd). This yielded GTR + G (1st), J2 + G (2nd) and J3 + G (3rd) for ML analysis, and SYM + G (1st), HKY85 + G (2nd) and J3 + G (3rd) for BI analysis. The ML and BI trees (Figs. 2 and 3) had similar topologies, and strongly indicated the monophyly of *H. lichenatus* by high support values (BS = 97 and BPP = 100). However, relationships between *H. lichenatus* and two closely related species (*H. tokyoensis* and *H. nigrescens*) were unresolved due to low support values.

Within *H. lichenatus*, three main clades (Clade I, II, and III), each with several subclades, were recognized (Figs. 2 and 3). All clades and subclades were strongly supported by high BS and BPP. Clade I consists of northern Kanto populations, Clade II of southern Tohoku populations, and Clade III of northern Tohoku population samples (Fig 1). The ML and BI trees indicated deep phylogeographic structure among these main clades that showed allopatric/parapatric distribution except at localities 28, 29, and 41 (Fig. 1). At these localities, haplotypes of Clades I and II were shared sympatrically. The mean pairwise genetic distances were 7.9% (range = 7.2–8.5%) between Clades I and II, 7.4% (6.8–8.0%) between Clades I and III, and 6.4% (5.8–6.9%) between Clades II and III.

Clade I includes two allopatric subclades (Subclade I-1 and I-2) that have a mean pairwise genetic distance of 3.2% (range = 2.8–3.6%). Clade II includes three subclades (II-1, II-2, and II-3); however, the relationship between these subclades is unresolved due to low support values. Haplotypes belonging to two of these three subclades co-occurred at localities 21, 31, and 41. The mean pairwise genetic distances were 2.1% (range = 1.7–2.4%) between Subclades II-1 and II-2, 4.2% (range = 3.7–4.7%) between Subclades II-1 and II-3, and 3.7% (range = 3.2–4.1%) between Subclades II-2 and II-3. Clade III includes three subclades (III-1, III-2, and III-3) whose relationships are unresolved.

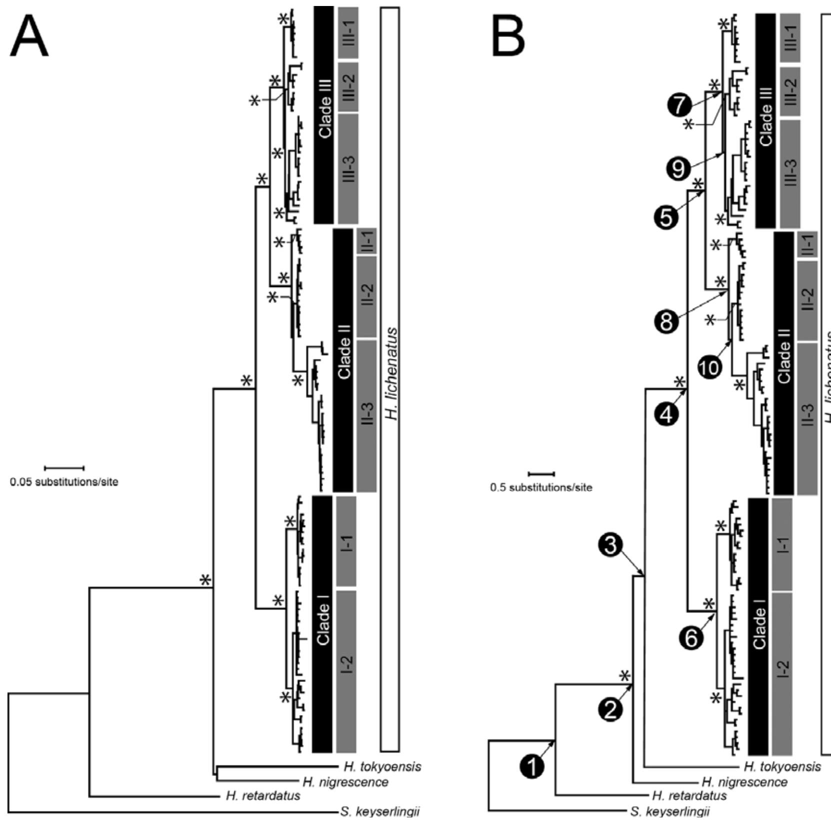

Fig. 2. Phylogenetic trees of *Hynobius lichenatus* from **(A)** maximum likelihood (ML) and **(B)** Bayesian inference (BI) methods. Asterisks on the branches indicate maximum likelihood bootstrap value (only values > 70% are shown) and Bayesian posterior probability (only values > 95% are shown). Node numbers in black circles indicate divergence splits from BEAST analysis (Table 4). The clades and subclades correspond to those shown in Fig. 1.

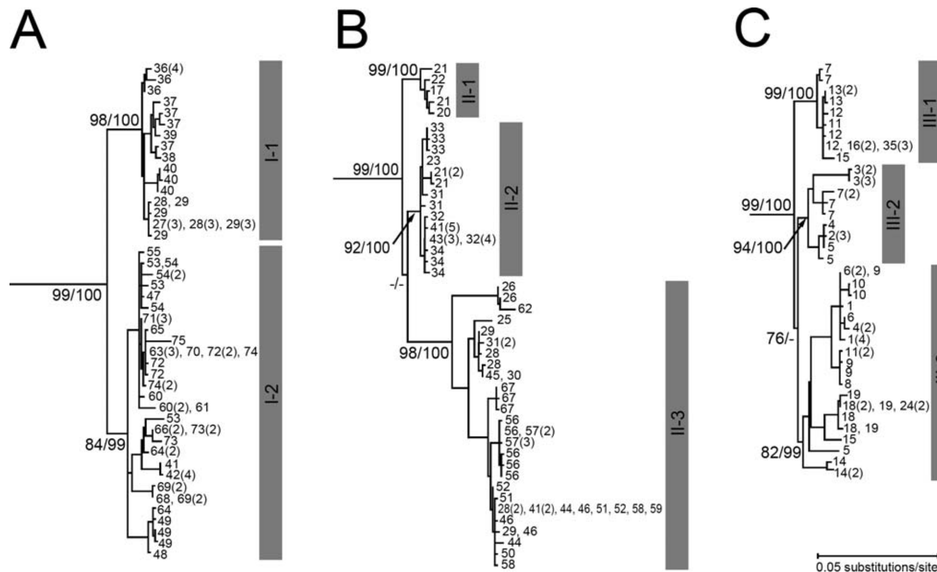

Fig. 3. Phylogenetic relationships within the major clades of *Hynobius lichenatus* identified in Fig. 2: **(A)** Clade I, **(B)** Clade II and **(C)** Clade III. Tree topology and branch lengths are based on ML tree. Numbers on the branches indicate ML bootstrap value (left, only values ≥ 70% are shown) and BI posterior probability (right, only values ≥ 95% are shown). Tip labels are sampling localities and number of individuals (see Fig. 1, Table 1). The clades and subclades correspond to those shown in Fig. 1.

Haplotypes belonging to two of these three subclades coexisted at localities 4, 5, 7, 11, and 15. The mean pairwise genetic distances were 2.7% (range = 2.3–3.0%) between Subclades III-1 and III-2, 2.4% (range = 2.1–2.7%) between Subclades III-1 and III-3, and 2.7% (range = 2.4–3.1%) between Subclades III-2 and III-3.

Population analysis

Measures of genetic diversity are shown in Table 3. Overall, the data showed high haplotype and nucleotide diversities for each of the main clades and subclades. In Clade I, Subclade I-1 had genetic diversity lower than Subclade I-2. In Clade II, Subclade II-2 had the lowest genetic diversity among the three subclades, and in Clade III, Subclade III-1 had the lowest diversity among the three subclades recognized. Results of the neutrality test were not significant for all main clades and subclades, except for Subclades II-2 and III-1 (Table 3). Subclade II-2 showed a significant value for Fu's F_S, but Tajima's D value was not significant. Subclade III-1 showed significant value for both neutrality tests.

Divergence time

Molecular dating indicated that *H. lichenatus* diverged between the middle Miocene and early Pleistocene. The divergence between *H. lichenatus* and two closely related species (*H. tokyoensis* and *H. nigrescens*) occurred at roughly 17–18 million years before present (MYBP), although the estimates of each node were wide in range with substantial overlaps between the 95% credibility intervals (Table 4). Within *H. lichenatus*, the divergence between Clade I and the ancestor of Clades II and III occurred at approximately 8.72 or 9.28 MYBP using the Calibration II and Calibration I, respectively. Subsequently, the divergence between Clades II and III occurred at approximately 6.55–6.98 MYBP. Within each clade, Clade I split into two subclades at approximately 2.70–2.87 MYBP, Clade II into three subclades at approxi-

Table 3. Genetic diversity indices and results of neutrality tests. Number of individuals (*n*), number of haplotypes (Hn), haplotype diversity (*h*) and its standard deviation (SD), nucleotide diversity (π) and its SD, Tajima's *D*, and Fu's *F*s (significance denoted by asterisks; * denotes < 0.05 and ** denotes < 0.02).

Clades and Subclades	*n*	Hn	*h* ± SD	π ± SD	Tajima's *D*	Fu's *F*s
Clade I	81	44	0.9676 ± 0.0091	0.020492 ± 0.010091	–0.53889	–3.69557
Subclade I-1	28	16	0.8862 ± 0.0502	0.006427 ± 0.003451	–1.06847	–2.62276
Subclade I-2	53	28	0.9550 ± 0.0156	0.013074 ± 0.006592	–0.95801	–2.34841
Clade II	72	45	0.9664 ± 0.0114	0.023954 ± 0.011763	0.11985	–4.42136
Subclade II-1	5	5	1.0000 ± 0.1265	0.005960 ± 0.003954	–0.84004	–0.87453
Subclade II-2	25	14	0.8933 ± 0.0456	0.003512 ± 0.002023	–1.01535	–4.67632**
Subclade II-3	42	26	0.9373 ± 0.0282	0.012143 ± 0.006176	–0.88558	–3.63898
Clade III	62	37	0.9751 ± 0.0082	0.023135 ± 0.011398	–0.02878	–1.97418
Subclade III-1	15	9	0.8476 ± 0.0878	0.002571 ± 0.001599	–1.77339*	–2.90203*
Subclade III-2	15	9	0.9238 ± 0.0440	0.014240 ± 0.007543	1.23287	2.47648
Subclade III-3	32	19	0.9536 ± 0.0200	0.016541 ± 0.008370	0.05031	0.33778

Table 4. Estimates of divergence time. Nodes correspond to the 10 labeled nodes on the BI tree in Fig. 2B. Calibrations I and II used 1.28% and 1.36% sequence divergences per MY, respectively.

	Calibration I			Calibration II		
Node	Mean	95% lower	95% upper	Mean	95% lower	95% upper
1	37.2768	25.3108	51.5221	35.0050	23.6580	48.0781
2	18.2679	12.9047	23.7284	17.1294	12.3492	22.3791
3	17.8186	12.5328	23.3777	16.6852	11.7030	21.6846
4	9.2801	6.7988	12.0432	8.7218	6.4860	11.2687
5	6.9810	5.0517	8.9381	6.5548	4.8849	8.4579
6	2.8738	1.6927	4.1476	2.7098	1.6408	3.9537
7	2.8648	1.9928	3.8521	2.6871	1.8731	3.5620
8	3.6297	2.5284	4.8294	3.4180	2.3835	4.5477
9	2.6767	1.8639	3.6215	2.5100	1.7154	3.3226
10	3.6255	2.5188	4.8265	3.4139	2.3835	4.5507

mately 3.41–3.62 MYBP, and Clade III into three subclades at approximately 2.51–2.86 MYBP (Table 4).

DISCUSSION

Phylogeny and genetic structure

The level of mtDNA Cytb genetic differentiation and structure found in *H. lichenatus* reveal the presence of three deeply divergent clades that show a general geographical congruence with three regions of northeastern Japan. The relationships among these three main clades are fully resolved with high support, although the relationships between subclades in Clades II and III could not be resolved with sufficient support. The observed geographic structure of these three main clades slightly differs from those reported previously using nuclear DNA (nDNA) markers (Matsui, 1987; Kuro-o et al., 1992). In these studies, the northern Tohoku clade, corresponding to Clade III in this study ,splits first, unlike our result in which Clade I (northern Kanto clade) splits first from the remaining two clades (Figs. 2 and 3). This discordance may be ascribable to different divergence patterns between mitochondrial and nDNA markers.

Uncorrected pairwise distances found among three major clades were about 7% in *H. lichenatus*. Matsui et al. (2007) pointed out that few sequence diversity data were available for species of *Hynobius*. This situation still holds and direct comparison of the present results of *H. lichenatus* with the other species in terms of the genes sequenced is not easy. From analyses of Cytb gene and control region genes, Matsui et al. (2007) recognized two clades (A and B, with recognizable subclades only in B) in *H. tokyoensis*. When their original data were recalculated for only Cytb gene, a mean (± SE) pairwise distance of 5.7 ± 0.6% was obtained between the two clades. Similar recalculation resulted in means of the pairwise distances between *H. lichenatus* and *H. nebulosus* + *H. tokyoensis* at 12.9 ± 0.7%, and between *H. tokyoensis* and *H. nebulosus* at 11.3 ± 0.8%. This limited information indicates the level of differentiation between the three clades of *H. lichenatus* to be slightly smaller than that between different species of *Hynobius*, but larger than that found between two clades in *H. tokyoensis*. Genetic distances among subclades within each major clade (about 2–4%) were larger than those obtained for *H. tokyoensis* (about 0.9–1.7% for Clade B in Matsui et al. [2007]). This high genetic diversity suggests the possibility of the presence of cryptic species within *H. lichenatus*.

Larval individuals carrying haplotypes from Clades I and II were found to coexist at three localities (28, 29, and 41 in Fig 1). Unfortunately, adult individuals were not obtained in these localities, and future analyses using nuclear markers would reveal whether or not hybridization occurs between the two clades.

At the clade level, both haplotype and nucleotide diversities were high in *H. lichenatus*. However, degree of divergence differed among clades. Among subclades in Clade I, Subclades I-1and I-2, with insignificant results in the neutrality test, seem to have long-stable genetic structures. Likewise, Subclades II-1 and II-3, exhibiting insignificant results in neutrality test, seem to have the structures like subclades in Clade I. In contrast, Subclade II-2, which occupies the northernmost range of Clade II, and showing significant Fu's *F*s value, seems a tendency of recent population expansion. Among subclades of Clade III, Subclade III-1, occurring easternmost region of the clade, and showing significant value for both neutrality tests, is thought to clearly indicate a recent population expansion. In contrast, Subclades III-2 and III-3, with insignificant Fu's *F*s or Tajima's *D* seem to have long-stable genetic structures. Overall, genetic diversity indices show high values and results of neutrality tests show significant negative values, except for some subclades in *H. lichenatus*. These results indicate long-term stability of demographic structure in this species.

Divergence time and historical biogeography

Northeastern Honshu, where *H. lichenatus* occurs, was under the sea except for the current Kitakami and Abukuma Highlands (Sato, 1994; Suzuki, 1989; Yamaji and Sato, 1989) before 15 and 12 MYBP in the middle Miocene, when land formation through volcanic activities began (Koike et al., 2005). The ancestral stock of *H. lichenatus*, probably confined to southwestern Japan, would have migrated northward during these periods. Our estimation suggested the divergence of this species from its sister species occurred in these ages, and huge volcanic activities may have induced

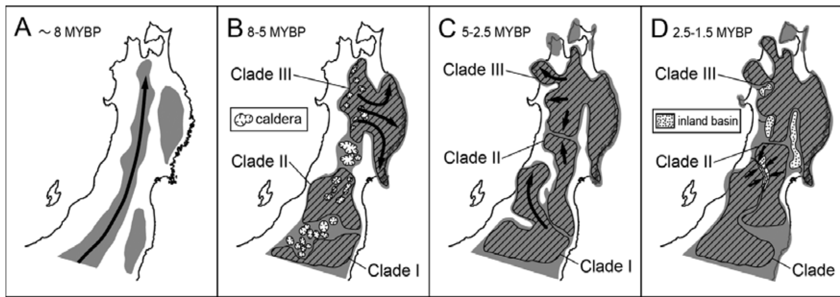

Fig. 4. Paleogeographic map of northeastern Japan, showing estimated dispersal (bold arrow) and estimated distribution (shaded zone) of each clade of *Hynobius lichenatus*. Gray zones indicate estimated land areas. **(A)** is based on Koike et al. (2005), **(B)** is modified from Sato (1994), and **(C)** and **(D)** are based on Koike et al. (2005), Machida et al. (2006), and Nagasawa (1997).

vicariance of the ancestral lineages.

Around 8 MYBP in the late Miocene, most of the current regions of the Ou Mountains started to uplift and became land (Koike et al., 2005; Fig. 4A), and ancestral *H. lichenatus* seems to have expanded its range there. However, regions other than the current Ou Mountains were still under the sea during this period, and the Kitakami/Abukuma Highlands were isolated islets. Therefore, range expansion of ancestral *H. lichenatus* would have been confined to regions around the Ou Mountains. It was estimated that divergence of Clade I and the others occurred around 9 MYBP, and Clades II and III diverged around 7 MYBP. The presence of huge calderas at the northern edge and central part of the Ou Mountains is suggested at 8–3.5 MYBP (Sato, 1994; Fig. 4B), and these calderas are close to the boundary of Clades I and II, and of Clades II and III, respectively. These regions were narrowed through introgression of the sea in this period, and volcanic activities in such narrow land regions may have caused vicariance of habitats and genetic differentiation among the three clades. By 5 to 4 MYBP in the early Pliocene, the Ou Mountains and the Kitakami Highlands were connected by land, where Clade III began invasion (Fig. 4B).

Around 3–2.5 MYBP, the orogenesis of the Dewa-Echigo Mountains began (Nagasawa, 1997; Machida et al., 2005). Clade I seems to have expanded its range from the southern Ou Mountains to the Echigo-Iide/Asahi Mountains, and Clade II from the central Ou Mountains to the Dewa Mountains. However, the region between the Iide/Asahi and the Dewa mountains remained undersea (Amano and Sato, 1989; Nagasawa, 1997), which would have prohibited connection of the two clades (Fig. 4C). Active uplifts in the Dewa-Echigo Mountains in this period would have promoted genetic differentiations within Clades I and II.

Thereafter, uplift in each of the mountains proceeded along with the marine recession, followed by formation of inland basins with marshy areas among mountains and expansion of lands (Suzuki, 1989; Koike et al., 2005: Fig. 4-D). Deposition in inland basins widened land areas where each clade seems to have expanded its distribution range. Clades I and II would have secondarily contacted in this region, resulting in sympatric distribution of the two clades as seen in the sampling sites 28, 29, and 41 by the late Pliocene, until which age the deposition in inland basins continued (Suzuki, 1989).

In this way, diversification of *H. lichenatus* is thought to have been shaped by geohistorical events, such as orogenesis, sea level changes, and volcanic activity, within northeastern Honshu from the late Miocene to the early Pleistocene. Previous studies on genetic variation of animals occurring in northeastern Japan (e.g., *Apodemus* field mice [Suzuki et al., 2004]; *Salvelinus* trouts [Yamamoto et al., 2004]; *Carabus* ground beetles [Sota et al., 2001]) indicate much younger ages of differentiations than in *H. lichenatus*, mostly during the mid Pliocene and the last glacial ages, although older differentiation ages are estimated in freshwater fishes such as *Oryzias* Medaka (4.7 MYBP: Takehana et al., 2003) and *Lefua* loaches (2 MYBP: Mihara et al., 2005). Similarly, recent phylogenetic studies of Japanese clawed salamander (*Onychodactylus japonicus* [Houttuyn, 1782] indicate its very old differentiation in northeastern Honshu (Yoshikawa et al., 2008, 2012).

For these reasons, *H. lichenatus* and *O. japonicus* are thought to represent comparatively ancient faunal elements in this region, unlike many other animal species. This suggest that amphibians are important faunal elements in considering zoogeography and geohistory of northeastern Japan. Unfortunately, geohistrical knowledge from the mid-Miocene to Pliocene in this region is still meager, and as it is difficult to discuss the zoogeography of those ages, future intensive studies in the field of geology are urgently required.

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