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Taxonomic Reinvestigation of *Hynobius lichenatus*: Description of a New Species and Resurrection of a Previously Described Species from Eastern Japan

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Abstract: A new species of the genus *Hynobius* from the central part of Tohoku District, Japan, is described. *Hynobius lichenatus* was divided into three groups based on morphological and molecular analyses, and the boundaries of these groups were delineated. The central part of the Tohoku group was described as *Hynobius senzanensis* sp. nov., whereas populations from the southern part of Tohoku, the northern part of Kanto, and the eastern part of Hokuriku were resurrected as *Hynobius unanngso*. Morphometric comparisons revealed that *H. lichenatus* possesses significantly shorter vomerine teeth length in both sexes compared with the other two species. Additionally, *H. lichenatus* exhibited a significantly longer tail length compared with *H. unanngso*. Although the new species and *H. unanngso* shared similar morphology, they differed significant in the width of their vomerine teeth, and did not form a sister group as closest relatives. Following this taxonomic reassessment, *H. lichenatus* is now limited to the Tohoku area (comprising Aomori, Iwate, Akita, and Miyagi Prefectures). The boundary between *H. lichenatus* and the new species is located in the southern part of Akita Prefecture, along the Japan sea side, and the eastern part of Miyagi Prefecture, along the Pacific Ocean side. This study provides insights into the distribution ranges of the three species and a re-evaluation of their habitat status is crucial for their conservation.

Keywords: Cryptic Species, Mitochondrial DNA, Ou Mountain Range, Species Boundary, Tohoku District

1. Introduction

The genus *Hynobius* Tschudi, 1838, is the most diverse genus within Hynobiidae, encompassing 66 described species and ranging in distribution from Japan to eastern China [1]. In Japan, 48 endemic species constitute 72.7% of the total *Hynobius* diversity [1]. The taxonomy of *Hynobius* has been complex and dynamic owing to early taxonomic studies primarily relying on external morphological features [2, 3]. Such features are often insufficient for establishing phylogenetic relationships from a genetic perspective. Research involving molecular markers has revealed that the diversity of *Hynobius* species has been underestimated, largely owing to the presence of cryptic species [4, 5]. Consequently, the number of recognized Japanese

Hynobius species has increased by more than twofold in the past two decades [1, 6].

The Tohoku salamander, *Hynobius lichenatus*, was initially described in Aomori Prefecture and is found in the Tohoku District (mainly around the Ou Mountain Range), as well as Tochigi, Gunma, and Niigata Prefectures, in Japan [7, 8]. A prior study identified three genetically three distinct groups within this species: the northern Tohoku (Group III), central Tohoku (Group II), and southern Tohoku (Group I) groups, based on mitochondrial DNA analyses [8]. However, this molecular analysis encompassed samples from the entire distribution range of *H. lichenatus*, albeit with an insufficient number of sampling localities to delineate the boundaries of these three groups. Moreover, morphological comparisons among these groups were not conducted. Therefore, additional morphological and molecular analyses of *H. lichenatus* are necessary to assess the taxonomic status of the three groups.

In herpetology, Frost and Hillis [9] advocate for employing both phylogenetic and evolutionary species concepts. Additionally, Dubois [10] stipulated that essential features should be directly derived from characteristics observed in specimens rather than indirectly inferred when describing a new species. Hence, we adopted the three species concepts (phylogenetic, evolutionary, and morphological) to assess the taxonomic status of each group. First, we delineated the precise species boundaries of the three groups by incorporating additional samples from uncollected areas in a previous study [8]. Second, we evaluated morphological distinctions among the three groups though morphometric measurements and observational characteristics. Finally, we determined the taxonomic status and detailed distribution areas of the three groups.

2. Materials and Methods

2.1. Molecular Analysis

For phylogenetic analysis, DNA samples were collected from personal property or fields during February 1991 to April 2022 (Tables 1–3; Figure 1). We obtained a single tailbud embryo from each paired egg sac or tissue samples from larvae during field sampling. Tissue samples collected tissues from the field were preserved in 99.5% ethanol. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Amplification of the mitochondrial cytochrome *b* gene (630 base pairs) was conducted using ExTaq (TaKaRa, Tokyo, Japan) with primers L14010 (5′-TAHGGWGAHGGATTWGAWGCMACWGC-3′) and H14778 (5′-AARTAYGGGTGRAADGRRAYTTTR TCT-3′) [11]. The PCR reaction mixture (total volume: 10 µl) consisted of 1.0 µl of 10×Ex Taq Buffer, 0.8 µl of 25 mM dNTP mix, 0.5 µl of each forward and reverse primer (10 pM), 0.05 µl of Taq polymerase, 6.15 µl of distilled deionized water, and 1.0 µl of template DNA. The PCR protocols were implemented using a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA) as follows: an initial 3 m denaturing step at 94°C; followed by 40 cycles of 30 s at 94°C, 45 s at 56°C, and 90 s at 72°C; culminating in a final 10 m extension at 72°C. The PCR products were purified using illustra™ ExoProStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) and subsequently sequenced using BigDye® Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The acquired DNA sequences were deposited in the DNA Data Bank of Japan (Tables 1–3). The alignment of DNA sequences was performed using MEGA X [12]. Phylogenetic analyses of the aligned sequences, incorporating several *Hynobius* species and *Salamandrella keyserlingii* as the outgroup (Table 3), were conducted through Bayesian inference (BI) and maximum likelihood (ML) estimation. We determined the best-fit nucleotide substitution model based on the Bayesian information criterion (BIC) [13] and corrected Akaike's information criterion (AICc) [14] using jModelTest 2 [15]. The selected model was the Hasegwa-Kishino-Yano (HKY) model (gamma distribution with invariant sites) for both BI and ML. Bayesian and ML trees were constructed using MrBayes 3.2 [16] and MEGA X [12], respectively. For Bayesian analyses, two independent Markov Chain Monte Carlo runs were conducted for 2,000,000 generations, with a sample frequency of 100. During Bayesian analysis, the stationarity of the likelihood scores of sampled trees was assessed using Tracer version 1.7 (http://tree.bio.ed.ac.uk/software/tracer/), and the initial 25% of generations were discarded as burn-in. Monophyly was evaluated based on posterior probability (PP) and bootstrap (BS) values, according to the criteria established by Huelsenbeck and Rannala [17] as well as Hillis and Bull [18]: monophyletic group = PP ≥ 0.95 and BP ≥ 70.

Table 1. *List of Hynobius lichenatus samples used in molecular analyses. Population number corresponds to localities in Figure 1, which indicate where individuals were collected.*

Population	Sampling Locality	Accession number / Label in Figure 2
1	Mutsu City (former Kawauchi Town), Aomori Prefecture	LC776097 / L01
2	Yokohama Town, Aomori Prefecture	LC776098 / L02
3	Hiranai Town, Aomori Prefecture	LC776099 / L03
4	Aomori City (former Aomori City), Aomori Prefecture	LC776100 / L04
5	Goshogawara City (former Kanagi Town), Aomori Prefecture	LC776101 / L05
6	Nakadomari Town (former Kodomari Village), Aomori Prefecture	LC776102 / L06
7	Shichinohe Town (former Shichinohe Town), Aomori Prefecture	LC776103 / L07
8	Sannohe Town, Aomori Prefecture	LC776104 / L08
9	Fukaura Town (former Iwasaki Village), Aomori Prefecture	LC776105 / L09
10	Kuji City (former Kuji City), Iwate Prefecture	LC776106 / L10
11	Hachimantai City (former Matsuo Village), Iwate Prefecture	LC776107 / L11

Population	Sampling Locality	Accession number / Label in Figure 2
12	Takizawa City, Iwate Prefecture	LC776108 / L12
13	Shizukuishi Town, Iwate Prefecture	LC776109 / L13
14	Shiwa Town, Iwate Prefecture	LC776110 / L14
15	Hanamaki City (former Towa Town), Iwate Prefecture	LC776111 / L15
16	Oshu City (former Esashi City), Iwate Prefecture	LC776112 / L16
17	Sumita Town, Iwate Prefecture	LC776113 / L17
18	Ofunato City (former Sanriku Town), Iwate Prefecture	LC776114 / L18
19	Kosaka Town, Akita Prefecture	LC776115 / L19
20	Odate City (former Hinai Town), Akita Prefecture	LC776116 / L20
21	Kitaakita City (former Ani Town), Akita Prefecture	LC776117 / L21
22	Fujisato Town, Akita Prefecture	LC776118 / L22
23	Noshiro City (former Futatsui Town), Akita Prefecture	LC776119 / L23
24	Mitane Town (former Kotooka Town), Akita Prefecture	LC776120 / L24
25	Oga City (former Oga City), Akita Prefecture	LC776121 / L25
26	Gojome Town, Akita Prefecture	LC776122 / L26
27	Katagami City (former Showa Town), Akita Prefecture	LC776123 / L27
28	Akita City (former Akita City), Akita Prefecture	LC776124 / L28
29	Akita City (former Yuwa Town), Akita Prefecture	LC776125 / L29
30	Senboku City (former Nishiki Village), Akita Prefecture	LC776126 / L30
31	Senboku City (former Kakunodate Town), Akita Prefecture	LC776127 / L31
32	Daisen City (former Kyowa Town), Akita Prefecture	LC776128 / L32
33	Daisen City (former Ota Town), Akita Prefecture	LC776129 / L33
34	Daisen City (former Nangai Village), Akita Prefecture	LC776130 / L34
35	Misato Town (former Rokugo Town), Akita Prefecture	LC776131 / L35
36	Yokote City (former Omori Town), Akita Prefecture	LC776132 / L36
37	Yokote City (former Masuda Town), Akita Prefecture	LC776133 / L37
38	Yuzawa City (former Inakawa Town), Akita Prefecture	LC776134 / L38
39	Yuzawa City (former Minase Village), Akita Prefecture	LC776135 / L39
40	Higashinaruse Village, Akita Prefecture	LC776136 / L40
41	Yurihonjo City (former Iwaki Town), Akita Prefecture	LC776137 / L41
42	Yurihonjo City (northern part of former Honjo City), Akita Prefecture	LC776138 / L42
43	Yurihonjo City (former Ouchi Town), Akita Prefecture	LC776139 / L43
44	Minamisanriku Town (former Shizugawa Town), Miyagi Prefecture	LC776140 / L44
45	Aomori City (former Namioka Town), Aomori Prefecture	AB750781 / AL01
46	Ajigasawa Town, Aomori Prefecture	AB750790 / AL02
47	Hirosaki City (former Hirosaki City?), Aomori Prefecture	AB750794 / AL03
48	Nishimeya Village, Aomori Prefecture	AB750797 / AL04
49	Hirakawa City (former Ikarigaseki Village), Aomori Prefecture	AB750802 / AL05
50	Hachimantai City (former Ashiro Town?), Iwate Prefecture	AB750811 / AL06
51	Noda Village, Iwate Prefecture	AB750813 / AL07
52	Iwaizumi Town, Iwate Prefecture	AB750818 / AL08
53	Tono City (former Tono City), Iwate Prefecture	AB750829 / AL09
54	Miyako City (former Miyako City), Iwate Prefecture	AB750822 / AL10
55	Kitakami City, Iwate Prefecture	AB750826 / AL11
56	Ichinoseki City (former Ichinoseki City), Iwate Prefecture	AB750847 / AL12
57	Oshu City (former Isawa Town), Iwate Prefecture	AB750833 / AL13
58	Oshu City (former Mizusawa City), Iwate Prefecture	AB750836 / AL14
59	Kitaakita City (former Moriyoshi Town), Akita Prefecture	AB750803 / AL15
60	Kazuno City, Akita Prefecture	AB750809 / AL16
61	Senboku City (former Tazawako Town), Akita Prefecture	AB750809 / AL17
62	Daisen City (former Omagari City), Akita Prefecture	AB750825 / AL18
63	Ishinomaki City (former Ishinomaki City), Miyagi Prefecture	AB750888 / AL19

Table 2. *List of Hynobius senzanensis sp. nov. samples used in molecular analyses. Population number corresponds to localities in Figure 1, which indicate where individuals were collected.*

Population	Sampling Locality	Accession number / Label in Figure 2
64	Yurihonjo City (southern part of former Honjo City), Akita Prefecture	LC776141 / S01
65	Yurihonjo City (former Higashiyuri Town), Akita Prefecture	LC776142 / S02
66	Yurihonjo City (former Yuri Town), Akita Prefecture	LC776143 / S03
67	Yurihonjo City (former Nishime Town), Akita Prefecture	LC776144 / S04
68	Yurihonjo City (former Yashima Town), Akita Prefecture	LC776145 / S05
69	Nikaho City (former Kisakata Town), Akita Prefecture	LC776146 / S06
70	Ugo Town, Akita Prefecture	LC776147 / S07
71	Yuzawa City (former Yuzawa City), Akita Prefecture	LC776148 / S08
72	Yuzawa City (former Ogachi Town), Akita Prefecture	LC776149 / S09
73	Tsuruoka City (Haguro Town), Yamagata Prefecture	LC776150 / S10

Population	Sampling Locality	Accession number / Label in Figure 2
74	Tsuruoka City (Kushibiki Town), Yamagata Prefecture	LC776151 / S11
75	Tsuruoka City (Atsumi Town), Yamagata Prefecture	LC776152 / S12
76	Shinjo City, Yamagata Prefecture	LC776153 / S13
77	Mogami Town, Yamagata Prefecture	LC776154 / S14
78	Tozawa Village, Yamagata Prefecture	LC776155 / S15
79	Okura Village, Yamagata Prefecture	LC776156 / S16
80	Nakayama Town, Yamagata Prefecture	LC776157 / S17
81	Shirataka Town, Yamagata Prefecture	LC776158 / S18
82	Nanyo City, Yamagata Prefecture	LC776159 / S19
83	Takahata Town, Yamagata Prefecture	LC776160 / S20
84	Osaki City (former Iwadeyama Town), Miyagi Prefecture	LC776161 / S21
85	Shikama Town, Miyagi Prefecture	LC776162 / S22
86	Osato Town, Miyagi Prefecture	LC776163 / S23
87	Aoba Ward, Sendai City, Miyagi Prefecture	LC776164 / S24
88	Yamamoto Town, Miyagi Prefecture	LC776165 / S25
89	Date City (former Yanagawa Town), Fukushima Prefecture	LC776166 / S26
90	Soma City, Fukushima Prefecture	LC776167 / S27
91	Kawauchi Village, Fukushima Prefecture	LC776168 / S28
92	Yurihonjo City (former Chokai Town?), Akita Prefecture	AB750830 / AS01
93	Mamurogawa Town, Yamagata Prefecture	AB750838 / AS02
94	Nishikawa Town, Yamagata Prefecture	AB750856 / AS03
95	Sakegawa Village, Yamagata Prefecture	AB750844 / AS04
96	Sakata City (former Hirata Town), Yamagata Prefecture	AB750839 / AS05
97	Shonai Town (former Tachikawa Town), Yamagata Prefecture	AB750845 / AS06
98	Tsuruoka City (former Tsuruoka City), Yamagata Prefecture	AB750848 / AS07
99	Sagae City, Yamagata Prefecture	AB750870 / AS08
100	Yamanobe Town, Yamagata Prefecture	AB750909 / AS09
101	Murayama City, Yamagata Prefecture	AB750871 / AS10
102	Higashine City, Yamagata Prefecture	AB750875 / AS11
103	Yamagata City, Yamagata Prefecture	AB750916 / AS12
104	Kaminoyama City, Yamagata Prefecture	AB750930 / AS13
105	Yonezawa City (east), Yamagata Prefecture	AB750950 / AS14
106	Kami Town (former Miyazaki Town?), Miyagi Prefecture	AB750880 / AS15
107	Taiwa Town, Miyagi Prefecture	AB750883 / AS16
108	Kawasaki Town, Miyagi Prefecture	AB750924 / AS17
109	Shichikashuku Town, Miyagi Prefecture	AB750934 / AS18
110	Fukushima City (north), Fukushima Prefecture	AB750954 / AS19
111	Minamisoma City (former Haramachi City), Fukushima Prefecture	AB750970 / AS20
112	Kamo City, Niigata Prefecture	AB750960 / AS21

Table 3. *List of Hynobius unanngso samples and sequences of 11 species used in molecular analyses. The 11 species are described in the population column. Population number corresponds to localities in Figure 1, which indicate where individuals were collected.*

Population	Sampling Locality	Accession number / Label in Figure 2
113	Tsuruoka City (Asahi Village), Yamagata Prefecture	LC776169 / U01
114	Oe Town, Yamagata Prefecture	LC776170 / U02
115	Asahi Town, Yamagata Prefecture	LC776171 / U03
116	Iide Town, Yamagata Prefecture	LC776172 / U04
117	Yonezawa City (west), Yamagata Prefecture	LC776173 / U05
118	Fukushima City (south), Fukushima Prefecture	LC776174 / U06
119	Otama Village, Fukushima Prefecture	LC776175 / U07
120	Motomiya City (Motomiya Town), Fukushima Prefecture	LC776176 / U08
121	Inawashiro Town, Fukushima Prefecture	LC776177 / U09
122	Kitashiobara Village, Fukushima Prefecture	LC776178 / U10
123	Bandai Town, Fukushima Prefecture	LC776179 / U11
124	Sukagawa City (former Naganuma Town), Fukushima Prefecture	LC776180 / U12
125	Tenei Village, Fukushima Prefecture	LC776181 / U13
126	Nishigo Village, Fukushima Prefecture	LC776182 / U14
127	Aizuwakamatsu City (former Aizuwakamatsu City), Fukushima Prefecture	LC776183 / U15
128	Minamiaizu Town (former Tajima Town), Fukushima Prefecture	LC776184 / U16
129	Tainai City (former Kurokawa Village), Niigata Prefecture	LC776185 / U17
130	Gosen City (former Muramatsu Town), Niigata Prefecture	LC776186 / U18
131	Akiha Ward (former Niitsu City), Niigata City, Niigata Prefecture	LC776187 / U19
132	Uonomua City (former Koide Town), Niigata Prefecture	LC776188 / U20
133	Minamiuonuma City (former Yamato Town), Niigata Prefecture	LC776189 / U21
134	Tokamachi City (former Matsunoyama Town), Niigata Prefecture	LC776190 / U22
135	Shioya Town, Tochigi Prefecture	LC776191 / U23

Population	Sampling Locality	Accession number / Label in Figure 2
136	Katashina Village, Gunma Prefecture	LC776192 / U24
137	Numata City (former Tone Village), Gunma Prefecture	LC776193 / U25
138	Nishikawa Town, Yamagata Prefecture	AB750851 / AU01
139	Oguni Town, Yamagata Prefecture	AB750901 / AU02
140	Nagai City, Yamagata Prefecture	AB750927 / AU03
141	Yamanobe Town, Yamagata Prefecture	AB750915 / AU04
142	Kitakata City (former Yamato Town), Fukushima Prefecture	AB750966 / AU05
143	Showa Village, Fukushima Prefecture	AB750961 / AU06
144	Shimogo Town, Fukushima Prefecture	AB750967 / AU07
145	Koriyama City, Fukushima Prefecture	AB750968 / AU08
146	Shirakawa City (former Taishin Village), Fukushima Prefecture	AB750988 / AU09
147	Tanagura Town, Fukushima Prefecture	AB750990 / AU10
148	Murakami City (former Sanpoku Town), Niigata Prefecture	AB750893 / AU11
149	Murakami City (former Asahi Village), Niigata Prefecture	AB750895 / AU12
150	Tainai City (former Nakajo Town), Niigata Prefecture	AB750925 / AU13
151	Sekikawa Village, Niigata Prefecture	AB750926 / AU14
152	Shibata City (former Shibata City), Niigata Prefecture	AB750936 / AU15
153	Agano City (former Sasakami Village), Niigata Prefecture	AB750941 / AU16
154	Aga Town (former Kanose Town?), Niigata Prefecture	AB750942 / AU17
155	Sanjo City (former Shitada Village), Niigata Prefecture	AB750956 / AU18
156	Uonuma City (former Irihirose Village), Niigata Prefecture	AB750959 / AU19
157	Uonuma City (former Yunotani Village), Niigata Prefecture	AB750972 / AU20
158	Nasushiobara City (former Kuroiso City?), Tochigi Prefecture	AB750984 / AU21
159	Nikko City (former Kuriyama Village), Tochigi Prefecture	AB750978 / AU22
160	Nikko City (former Fujihara Town), Tochigi Prefecture	AB750979 / AU23
161	Nikko City (former Nikko City?), Tochigi Prefecture	AB750992 / AU24
162	Minakami Town (former Minakami Town?), Gunma Prefecture	AB750975 / AU25
Hynobius abei	Kyotango City, Kyoto Prefecture	LC225433 /*H. abei*
Hynobius mikawaensis	Shinshiro City, Aichi Prefecture	LC225429 /*H. mikawaensis*
Hynobius nigrescens	Kami Town, Miyagi Prefecture	LC558260 /*H. nigrescens*
Hynobius owariensis	Nagoya City, Aichi Prefecture	LC436440 /*H. owariensis*
Hynobius sengokui	Iwaki City, Fukushima Prefecture	AB266633 /*H. sengokui*
Hynobius setoi	Fukubecho Takae, Tottori City, Tottori Prefecture	LC605085 /*H. setoi*
Hynobius setouchi	Takebecho Shimokoume, Okayama City, Okayama Prefecture	LC436426 /*H. setouchi*
Hynobius takedai	Hakui City, Ishikawa Prefecture	LC225430 /*H. takedai*
Hynobius tokyoensis	Hachioji City, Tokyo	AB266640 /*H. tokyoensis*
Hynobius vandenburghi	Nakamachi, Nara City, Nara Prefecture	AB266668 /*H. vandenburghi*
Salamandrella keyserlingii	Russia	NC_008082 /*S. keyserlingii*

2.2. Morphological Analysis

In this study, we revised the nomenclature for the three genetic groups proposed in a previous study [8] as follows: the northern Tohoku group (is referred to as Group III), the central Tohoku group (as Group II), and the southern Tohoku group (as Group I). We conducted sampling from February 2018 to April 2022, collecting 121 *H. lichenatus* individuals. These comprised 32 males and 11 females) from the northern Tohoku group, representing 11 populations [Pops. 1 (one female), 4 (one female), 5 (two males and two females), 12 (two males), 13 (nine males), 15 (five males), 16 (two males), 17 (one male and one female), 28 (one female), 46 (six males and three females), 50 (three males and two females), 54 (one male), 62 (one male)]; 29 males and 13 females from the central Tohoku group representing nine populations [Pops. 68 (two males and four females), 78 (two males and one female), 87 (16 males and five females), 90 (one female), 91 (one male), 92 (two males and two females), 93 (two males), 95 (three males), 105 (one male)]; with 26 males and 10 females from the southern Tohoku group, represented by six populations [Pops. 118 (nine males), 119 (one female), 127 (one female), 132 (14 males), 145 (two males and eight females), 158 (one male)] (Tables 1–3; Figure 1).

After being anesthetized using ethyl 3-aminobenzoate methanesulfonate salt (Sigma-Aldrich®, St. Louis, MO, USA) diluted 1000 times with water [19], the collected individuals were measured and subsequently returned to their capture sites, except for candidate type specimens. Prior to being returned, photographs were taken to document of the dorsal, ventral, and lateral sides of all individuals against a black background. Additionally, tissue samples were obtained from the tail tips of each individual and preserved in 99.9% ethanol as evidence of their collection.

All individuals were subjected to 22 measurements using a vernier caliper, as recommended in a previous study [20]. These measurements were as follows: snout–vent length (SVL), trunk length (TRL), axilla-groin distance (AGD), head length (HL), tail length (TAL), median tail width (MTAW), median tail height (MTAH), vomerine teeth length (VTL), vomerine teeth width (VTW), head width (HW), forelimb length (FLL), hindlimb length (HLL), second finger length (2FL), third finger length (3FL), third toe length (3TL), fifth toe length (5TL), internarial distance (IND), interorbital distance (IOD), upper eyelid length (UEL), snout length (SL), upper eyelid width (UEW), and

lower jaw length (LJL). Additionally, we noted the presence of distinct markings (e.g., white dots, yellow spots, or black dots) on the dorsal side of the body (DMDB), distinct white spots on the ventral side of the body (DWSV), distinct white spots on the lateral sides of the body (DWSL), brownish (or yellowish) lines on the dorsal (BLDT) and ventral (BLVT) sides of the tail, and distinct gular mottling (DGM) for each individual. We also counted the number of costal folds between the adpressed limbs (CFBALN) and the number of costal grooves (CGN). Regarding CGN, we employed the counting method described in a previous study [21].

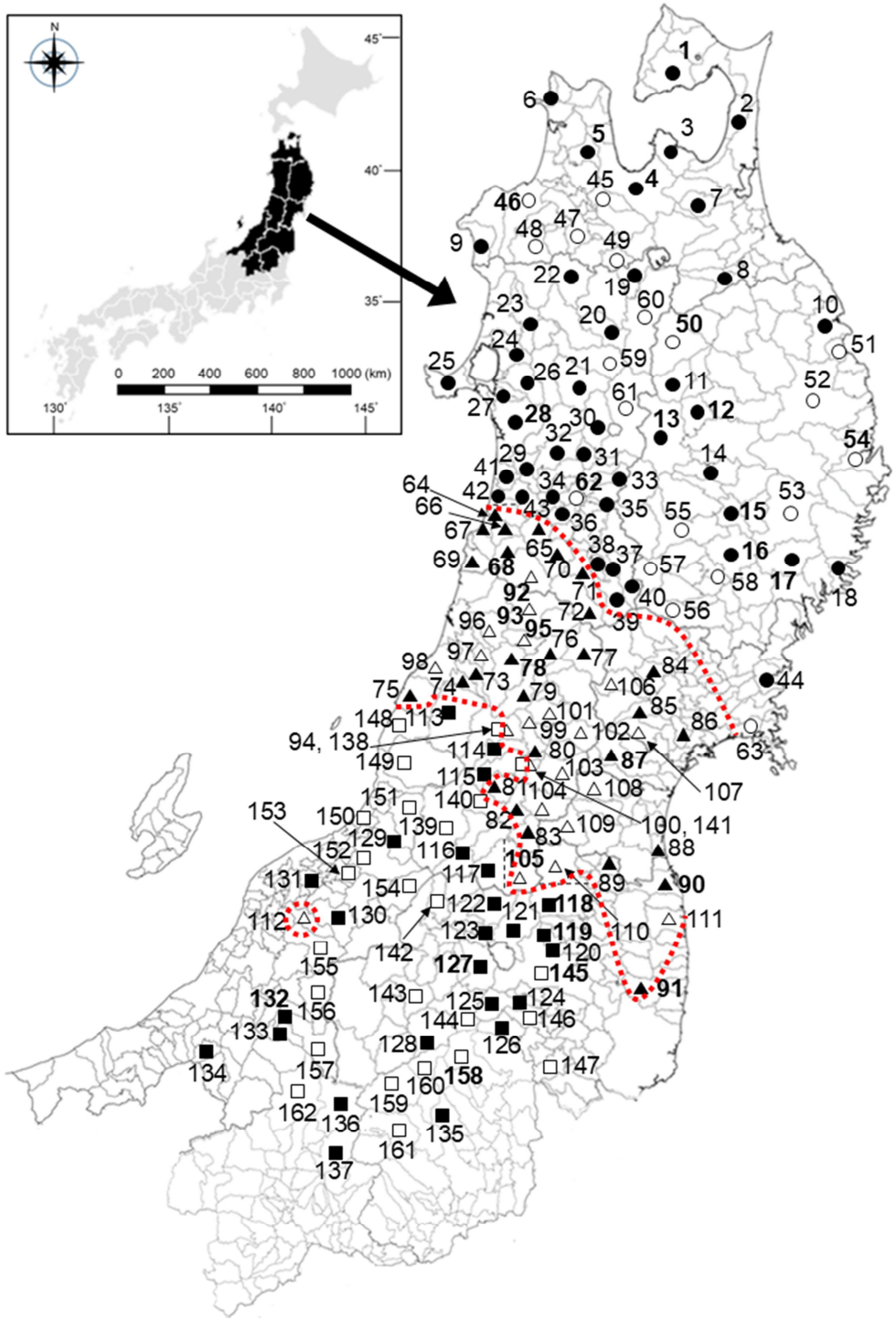


Figure 1. *Sampling points for individuals of three Hynobius species studied in this research. Enlarged area includes the Tohoku District with Niigata, Tochigi, and Gunma Prefectures, where the three species are found. Closed symbols represent the three species sequenced in this study: Hynobius lichenatus (closed circles), Hynobius senzanensis sp. nov. (closed triangles), and Hynobius unanngso (closed squares). Open symbols correspond to the three species sequenced by Aoki et al. (2013): H. lichenatus (open circles), H. senzanensis sp. nov. (open triangle), and H. unanngso (open squares). Bold locality numbers indicate the sampling points for individuals used in morphological comparisons. Displayed municipality ranges (including cities, towns, and villages) adhere to the older version of municipality demarcations in Japan, aiming to provide as clear a representation of each species' conservation status as possible.*

Statistical analyses were performed using R [22]. We assessed normality using Shapiro–Wilk test and tested for homoscedasticity using Bartlett's test when data followed a normal distribution. For populations with equal variances, we used Tukey–Kramer tests, and for populations with unequal variances, we conducted Games–Howel tests. In cases where data did not follow a normal distribution and variances among populations were unequal, we used Steel–Dwass tests. To assess overall morphological variation among the three groups, we conducted canonical discriminant analysis using SVL and standardized values for the 21 measurements.

2.3. Measurement of Holotype and Topotype Specimens

When measuring the holotype and topotype specimens, we referred to a previous study [20] and performed 47 measurements. These included SVL, TRL, left axilla-groin distance (LAGD), right axilla-groin distance (RAGD), HL, TAL, MTAW, MTAH, basal tail width (BTAW), basal tail height (BTAH), left vomerine teeth length (LVTL), right vomerine teeth length (RVTL), VTW, HW, maximum head width (MXHW), left forelimb length (LFLL), left hindlimb length (LHLL), right forelimb length (RFLL), right hindlimb length (RHLL), left first finger length (L1FL), left second finger length (L2FL), left third finger length (L3FL), left fourth finger length (L4FL), right first finger length (R1FL), right second finger length (R2FL), right third finger length (R3FL), right fourth finger length (R4FL), left first toe length (L1TL), left second toe length (L2TL), left third toe length (L3TL), left fourth toe length (L4TL), left fifth toe length (L5TL), right first toe length (R1TL), right second toe length (R2TL), right third toe length (R3TL), right fourth toe length (R4TL), right fifth toe length (R5TL), IND, IOD, left upper eyelid length (LUEL), right upper eyelid length (RUEL), left snout length (LSL), right snout length (RSL), left upper eyelid width (LUEW), right upper eyelid width (RUEW), left lower jaw length (LLJL), and right lower jaw length (RLJL). These measurements were taken after fixation in 10% formalin and subsequent transfer to 70% ethanol.

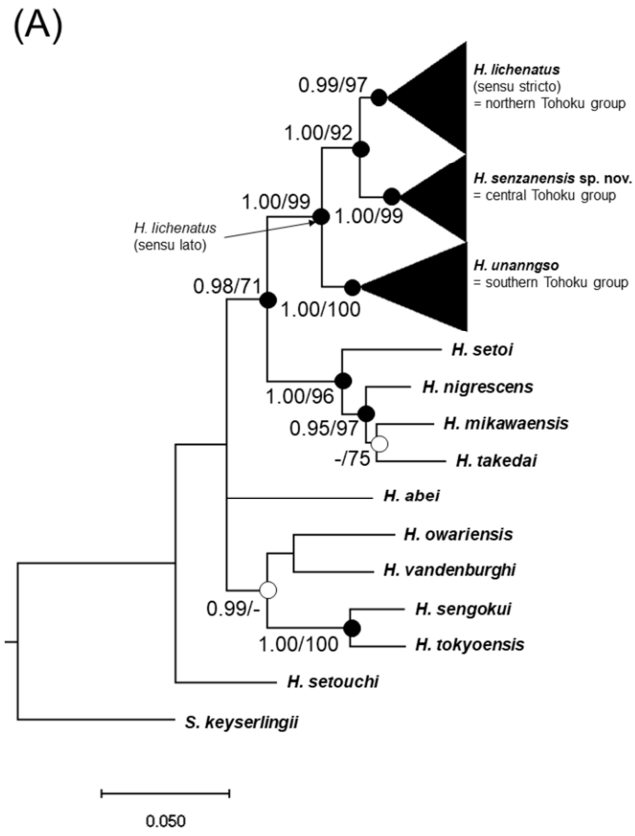
3. Results

3.1. Molecular Analysis

Molecular phylogenetic trees based on BI and ML exhibited marked similarity. The monophyly of three distinct groups, named the northern Tohoku, central Tohoku, and southern Tohoku groups of *H. lichenatus* (*sensu lato*), was strongly supported by PP and BS, as consistent with findings in a previous study [8] (Figure 2A). Furthermore, the monophyly of each of the three species was strongly supported by PP and BS, aligning with the outcomes of the aforementioned study [8] (Figure 2A). However, the phylogenetic relationships within these groups remained unclear, deviating from the results in the previous study [8] (Figure 2B–D). In particular, the central Tohoku group exhibited two lineages, but the monophyly of the clade that included S24 (shaded in Figure 2C) lacked support from both BI and ML analyses. Additionally, the monophyly of another clade received support solely from BI (Figure 2C).

3.2. Morphological Analysis

Morphological measurements for the three groups are shown in Table 4, with significant values among three groups and between sexes listed in Table 5. Significant differences were observed among males of the northern and central Tohoku groups in 10 morphological characters (Table 5). Nine morphological characters showed significant differences between males of the northern Tohoku and southern Tohoku groups (Table 5). Additionally, six morphological characters exhibited significant differences between males of the central Tohoku and southern Tohoku groups (Table 5). Among females, five morphological characters differed significantly between those of the northern and central Tohoku groups (Table 5), whereas five morphological characters showed significant differences between females of the northern Tohoku and southern Tohoku groups (Table 5). Finally, two morphological characters exhibited significant differences between females of the central Tohoku and southern Tohoku groups (Table 5). Canonical discriminant analyses in both sexes revealed distinct differences among the three groups, with nonoverlapping score distributions (Figure 3).



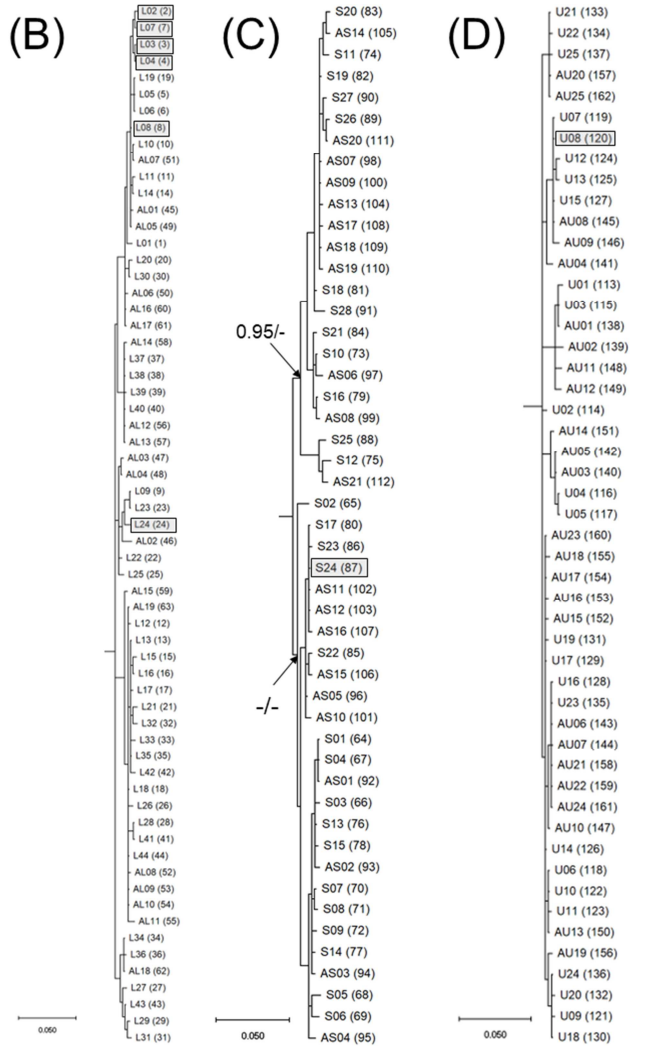


Figure 2. *Phylogenetic tree generated though Bayesian inference (BI) using a 630-base-pair (bp) segment of the cytochrome b gene. Salamandrella keyserlingii served as the outgroup. Four separate trees indicate the phylogenetic relationships of (A) pond-breeding Hynobius species distributed in central to eastern Japan, (B) relationships within Hynobius lichenatus (= northern Tohoku group), (C) relationships within Hynobius senzanensis sp. nov. (= central Tohoku group), and (D) relationships within Hynobius unanngso (= southern Tohoku group). Scale bars represent the genetic distance (expected changes per site). Numbers positioned near the nodes represent posterior probabilities (PP) for Bayesian inference and bootstrap values (BS) for maximum likelihood (ML) estimation. Values in parentheses following haplotype names correspond to population localities, as indicated in Tables 1–3 and Figure 1. Labels enclosed by shaded boxes indicate samples collected from the type localities of H. senzanensis sp. nov. and H. unanngso, and samples from the candidate type locality of H. lichenatus.*

The results of morphological observations are shown in Table 6. In males of the northern Tohoku group, the majority typically exhibited > 1.0 CFBALN (27/32 = 84.4%), DMDB (28/32 = 87.5%), and BLDT (27/32 = 84.4%), and none had BLVT = (32/32 = 100%), with a notable absence of DGM (27/32 = 84.4%) (Table 6). Females of the northern Tohoku group always had DMDB (11/11 = 100%), almost always had BLDT (10/11 = 90.9%), DWSV (10/11 = 90.9%), and DWSL (10/11 = 90.9%), typically had ≥ 0.0 CFBALN (9/11 = 81.8%) and 12 CGN (9/11 = 81.8%), and never had BLVT (11/11 = 100%) and DGM (11/11 = 100%) (Table 6). In males of the central Tohoku group, the majority usually had ≥ 1.0 CFBALN (25/29 = 86.2%), frequently exhibited DMDB (23/29 = 79.3%), never had BLVT (29/29 = 100%) (Table 6). Among females of the central Tohoku group, a large proportion usually had ≤ 1.5 CFBALN (11/13 = 84.6%), frequently had 12 CGN (10/13 = 76.9%), whereas none had BLVT (13/13 = 100%) and DGM (13/13 = 100%) (Table 6). Males of the southern Tohoku group typically had > 0.5 CFBALN (23/26 = 88.5%), frequently exhibited DMDB (20/26 = 76.9%), usually lacked DGM (22/26 = 84.6%), and never had BLVT (26/26 = 100%) (Table 6). Regarding females of the southern Tohoku group, they consistently had DWSV (10/10 = 100%) and DWSL (10/10 = 100%), typically had < 1.5 CFBALN (8/10 = 80.0%) and DMDB (8/10 = 80.0%), and never had BLVT (10/10 = 100%) and DGM (10/10 = 100%) (Table 6).

Based on the results of molecular and morphological analyses, the central Tohoku and southern Tohoku groups of *H. lichenatus* meet the criteria for distinct species under the three species concepts. Consequently, we have described the central Tohoku group as a new species *Hynobius senzanensis* sp. nov. Furthermore, we have resurrected the southern Tohoku group of *H. lichenatus* as *Hynobius unanngso*, as previously described in a prior study [23]. Finally, we have redescribed the northern Tohoku group as the true *H. lichenatus*.

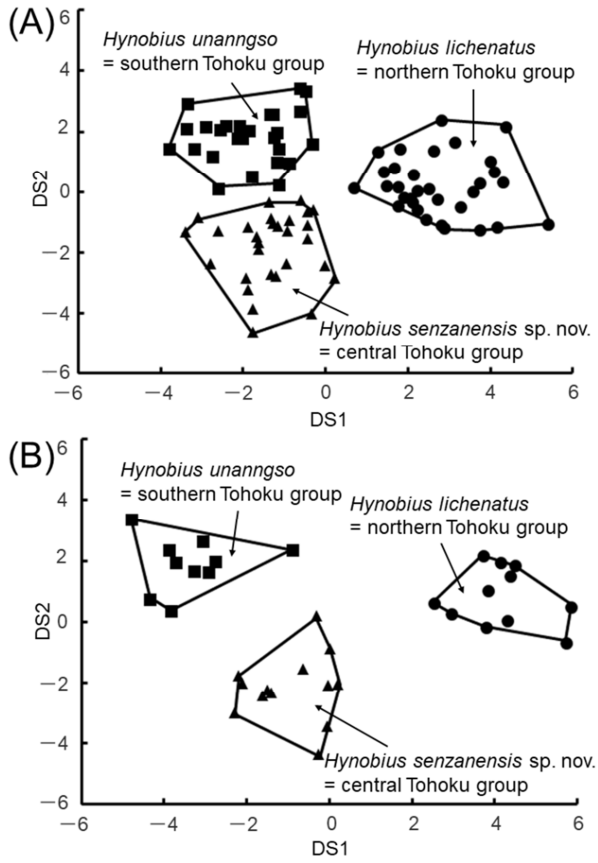


Figure 3. *Two-dimensional plots of canonical discriminant analyses involving 22 measurements in (A) males and (B) females. X and y axes show discriminant score 1 (DS1) and discriminant score 2 (DS2), respectively. Contribution ratios of DS1 and DS2 are 66.9% and 33.1% in males and 75.1% and 24.9% in females, respectively. Refer to the Materials and Methods section for definitions of morphological traits.*

Table 4. *Measurement (mm) of SVL and character ratios (R = %SVL) of TRL to LJL (ranges are shown in parentheses). Refer to the Materials and Methods section for definitions of morphological characters.*

	Hynobius lichenatus		***Hynobius senzanensis* sp. nov.**		***Hynobius unanngso***	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Trait	*n* = 32	*n* = 11	*n* = 29	*n* = 13	*n* = 26	*n* = 10
SVL	59.2±3.63	62.4±2.47	59.9±5.51	60.0±3.52	61.0±8.17	57.2±3.34
	(52.0-66.8)	(57.3-65.8)	(50.0-71.5)	(54.8-65.5)	(47.3-74.5)	(53.0-63.9)
RTRL	75.2±1.20	76.0±0.85	75.8±0.93	75.6±1.16	75.6±2.02	75.4±1.33
	(72.2-77.6)	(74.8-77.5)	(74.0-77.6)	(73.3-77.6)	(72.9-83.2)	(73.3-77.3)
RAGD	52.9±1.45	55.5±1.80	52.4±1.46	53.7±1.43	52.5±1.81	54.1±2.22
	(50.3-55.7)	(53.2-59.4)	(50.2-56.6)	(51.8-56.9)	(48.4-55.1)	(50.8-57.2)
RHL	25.5±1.20	24.5±0.97	25.0±1.14	24.6±1.10	25.5±1.15	25.3±1.22
	(23.3-28.1)	(22.6-26.3)	(21.8-27.7)	(23.2-26.4)	(22.1-27.6)	(22.9-27.5)
RTAL	91.7±7.18	87.1±5.63	89.3±9.11	82.7±10.48	84.1±5.14	81.3±2.44
	(68.4-107.5)	(76.5-95.3)	(65.0-107.2)	(60.2-96.8)	(75.1-96.3)	(75.2-83.5)
RMTAW	6.3±0.87	5.8±0.82	6.3±0.66	6.6±0.60	6.2±0.71	6.1±0.72
	(4.7-9.7)	(4.7-7.3)	(4.9-7.2)	(5.3-7.5)	(4.8-7.2)	(4.9-7.6)
RMTAH	11.4±0.99	9.6±0.92	11.5±1.27	10.7±1.13	11.2±1.24	10.6±1.04
	(8.0-13.1)	(8.2-10.9)	(8.5-13.6)	(8.0-12.1)	(8.3-13.6)	(8.0-11.5)
RVTL	3.3±0.32	3.2±0.43	3.7±0.44	3.8±0.42	3.8±0.35	3.7±0.52
	(2.7-4.2)	(2.6-4.0)	(2.9-4.6)	(3.2-4.4)	(3.1-4.4)	(3.1-4.7)
RVTW	5.3±0.31	4.8±0.77	5.6±0.38	5.5±0.32	5.9±0.40	5.9±0.29
	(4.8-5.9)	(2.7-5.5)	(5.0-6.4)	(5.0-6.0)	(4.9-6.5)	(5.4-6.3)
RHW	17.5±0.63	16.3±0.77	17.9±0.75	17.0±0.94	17.3±0.67	17.4±0.62
	(16.4-19.1)	(14.9-17.6)	(16.6-20.1)	(14.9-18.5)	(16.2-18.9)	(16.2-18.0)
RFLL	30.5±1.25	27.2±0.75	29.4±2.00	26.3±1.78	30.0±1.13	28.1±0.74
	(28.0-32.9)	(26.1-28.3)	(24.1-33.0)	(23.4-28.1)	(28.2-32.2)	(27.0-29.4)
RHLL	34.3±1.23	33.0±1.05	34.0±2.12	31.8±1.64	34.1±1.38	32.5±1.18
	(31.7-36.7)	(30.2-34.4)	(29.3-36.9)	(29.2-34.1)	(29.7-36.1)	(31.3-34.7)
R2FL	6.4±0.56	5.6±0.54	5.9±0.75	5.4±0.76	5.9±0.49	5.9±0.31
	(4.9-7.5)	(4.5-6.2)	(4.2-7.8)	(4.2-6.6)	(4.9-6.9)	(5.4-6.4)
R3FL	5.0±0.53	4.8±0.74	4.4±0.66	4.6±0.60	4.6±0.59	4.9±0.33
	(3.8-6.7)	(3.4-5.8)	(2.9-5.9)	(3.3-5.8)	(3.1-5.7)	(4.3-5.3)
R3TL	8.7±0.60	8.1±0.67	8.2±0.69	8.0±0.75	8.2±0.54	8.8±0.79
	(7.4-10.3)	(7.5-9.5)	(6.7-9.3)	(6.8-9.3)	(7.1-9.1)	(7.7-10.2)
R5TL	1.5±0.97	1.8±0.77	2.1±0.72	2.0±0.80	2.0±0.62	2.3±0.54
	(0.0-3.2)	(0.2-2.8)	(0.9-3.4)	(0.8-3.4)	(0.6-3.6)	(1.3-2.9)
RIND	6.2±0.52	5.9±0.44	6.3±0.41	5.8±0.32	6.1±0.42	5.9±0.37
	(5.2-7.2)	(5.3-6.7)	(5.2-7.0)	(5.3-6.4)	(5.1-6.9)	(5.0-6.3)
RIOD	6.8±0.46	6.3±0.36	7.1±0.37	6.5±0.37	7.1±0.38	6.4±0.31
	(5.9-8.3)	(5.8-6.3)	(6.4-7.9)	(6.0-7.4)	(6.3-7.9)	(6.4-0.31)
RUEW	3.5±0.31	3.4±0.22	3.5±0.26	3.4±0.30	3.4±0.26	3.3±0.25
	(2.7-4.3)	(3.0-3.8)	(3.1-4.1)	(2.8-3.8)	(2.7-3.8)	(3.1-3.8)
RSL	7.9±0.45	7.3±0.46	7.4±0.58	7.3±0.24	7.8±0.38	7.3±0.38
	(7.0-8.8)	(6.5-8.1)	(5.3-8.2)	(7.0-7.9)	(7.1-9.0)	(6.7-8.1)
RUEL	4.9±0.25	4.6±0.27	4.9±0.30	4.7±0.23	4.6±0.33	4.7±0.26
	(4.3-5.4)	(4.0-5.0)	(4.3-5.4)	(4.4-5.1)	(4.0-5.4)	(4.4-5.2)
RLJL	14.9±0.79	13.8±0.58	15.0±0.83	14.6±0.72	14.4±0.89	14.3±1.27
	(13.0-16.6)	(13.0-14.5)	(13.5-16.8)	(13.3-16.0)	(12.8-16.1)	(12.7-16.5)

Table 5. *Significant values of the 22 morphological traits among three species and between sexes. LIC, SEN, UNA, are abbreviations of Hynobius lichenatus (= northern Tohoku group), Hynobius senzanensis sp. nov. (= central Tohoku group), and Hynobius unanngso (southern Tohoku group), respectively. Refer to the Materials and Methods section for definitions of morphological characters.*

Trait	**Male**			**Female**			**Male vs. Female**		
	LIC vs. SEN	**LIC vs. UNA**	**SEN vs. UNA**	**LIC vs. SEN**	**LIC vs. UNA**	**SEN vs. UNA**	**LIC**	**SEN**	**UNA**
SVL	NS	NS	NS	NS	P < 0.01	NS	P < 0.01	NS	NS
RTRL	NS	NS	NS	NS	NS	NS	P < 0.05	NS	NS
RAGD	NS	NS	NS	NS	NS	NS	P < 0.0001	P < 0.01	P < 0.05
RHL	NS	NS	NS	NS	NS	NS	P < 0.05	NS	NS
RTAL	NS	P < 0.0001	P < 0.05	NS	P < 0.05	NS	NS	P < 0.05	NS
RMTAW	NS	NS	NS	P < 0.05	NS	NS	NS	NS	NS
RMTAH	NS	NS	NS	P < 0.05	NS	NS	P < 0.0001	NS	NS
RVTL	P < 0.001	P < 0.0001	NS	P < 0.01	P < 0.05	NS	NS	NS	NS
RVTW	P < 0.05	P < 0.0001	P < 0.05	P < 0.05	P < 0.001	P < 0.05	P < 0.05	NS	NS
RHW	P < 0.05	NS	P < 0.01	NS	P < 0.05	NS	P < 0.0001	P < 0.01	NS
RFLL	P < 0.05	NS	NS	NS	NS	P < 0.05	P < 0.0001	P < 0.0001	P < 0.0001

Trait	Male			Female			Male vs. Female		
	LIC vs. SEN	LIC vs. UNA	SEN vs. UNA	LIC vs. SEN	LIC vs. UNA	SEN vs. UNA	LIC	SEN	UNA
RHLL	NS	NS	NS	NS	NS	NS	P < 0.001	P < 0.01	P < 0.01
R2FL	P < 0.01	P < 0.01	NS	NS	NS	NS	P < 0.001	NS	NS
R3FL	P < 0.01	P < 0.05	NS	NS	NS	NS	NS	NS	NS
R3TL	P < 0.01	P < 0.01	NS	NS	NS	NS	P < 0.05	NS	P < 0.01
R5TL	P < 0.01	P < 0.05	NS	NS	NS	NS	NS	NS	NS
RIND	NS	NS	NS	NS	NS	NS	NS	P < 0.001	NS
RIOD	P < 0.05	P < 0.05	NS	NS	NS	NS	P < 0.001	P < 0.0001	P < 0.0001
RUEW	NS	NS	NS	NS	NS	NS	NS	NS	NS
RSL	P < 0.05	NS	P < 0.05	NS	NS	NS	P < 0.001	NS	P < 0.01
RUEL	NS	P < 0.01	P < 0.01	NS	NS	NS	P < 0.01	NS	NS
RLJL	NS	NS	P < 0.05	P < 0.01	NS	NS	P < 0.0001	NS	NS
P < 0.05	5	3	4	3	3	2	4	1	1
P < 0.01	4	3	2	2	1	0	2	3	3
P < 0.001	1	0	0	0	1	0	4	1	0
P < 0.0001	0	3	0	0	0	0	5	2	2
Total	10	9	6	5	5	2	15	7	6

Table 6. *Characteristics among the three Hynobius. Values represent the number of individuals exhibiting characters, with the respective percentages (rounded to one decimal place) shown in parentheses. Refer to the Materials and Methods section for definitions of morphological traits.*

Character	Condition	*Hynobius lichenatus*		*Hynobius senzanensis* sp. nov.		*Hynobius unanngso*	
		Male	Female	Male	Female	Male	Female
		n **= 32**	***n*** **= 11**	***n*** **= 29**	***n*** **= 13**	***n*** **= 26**	***n*** **= 10**
DMDB	Absent	4 (12.5%)	0 (0%)	6 (20.7%)	5 (38.5%)	6 (23.1%)	2 (20.0%)
	Present	28 (87.5%)	11 (100%)	23 (79.3%)	8 (61.5%)	20 (76.9%)	8 (80.0%)
DWSV	Absent	18 (56.3%)	1 (9.1%)	10 (34.5%)	7 (53.8%)	14 (53.8%)	0 (0%)
	Present	14 (43.8%)	10 (90.9%)	19 (65.5%)	6 (46.2%)	12 (46.2%)	10 (100%)
DWSL	Absent	10 (31.3%)	1 (9.1%)	11 (37.9%)	6 (46.2%)	14 (53.8%)	0 (0%)
	Present	22 (68.8%)	10 (90.9%)	18 (62.1%)	7 (53.8%)	12 (46.2%)	10 (100%)
BLDT	Absent	5 (15.6%)	1 (9.1%)	14 (48.3%)	8 (61.5%)	16 (61.5%)	6 (60.0%)
	Present	27 (84.4%)	10 (90.9%)	15 (51.7%)	5 (38.5%)	10 (38.5%)	4 (40.0%)
BLVT	Absent	32 (100%)	11 (100%)	29 (100%)	13 (100%)	26 (100%)	10 (100%)
	Present	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
DGM	Absent	27 (84.4%)	11 (100%)	18 (62.1%)	13 (100%)	22 (84.6%)	10 (100%)
	Present	5 (15.6%)	0 (0%)	11 (37.9%)	0 (0%)	4 (15.4%)	0 (0%)
CGN	11	11 (34.4%)	2 (18.2%)	10 (34.5%)	3 (23.1%)	9 (34.6%)	4 (40.0%)
	12	21 (65.6%)	9 (81.8%)	19 (65.5%)	10 (76.9%)	15 (57.7%)	4 (40.0%)
	13	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (7.7%)	2 (20.0%)
CFBALN	3.5	1 (3.1%)	0 (0%)	1 (3.5%)	0 (0%)	0 (0%)	0 (0%)
	3.0	2 (6.3%)	0 (0%)	2 (6.9%)	0 (0%)	3 (11.5%)	0 (0%)
	2.5	3 (9.4%)	0 (0%)	3 (10.4%)	0 (0%)	1 (3.9%)	0 (0%)
	2.0	14 (43.8%)	0 (0%)	11 (37.9%)	2 (15.4%)	5 (19.2%)	1 (10.0%)
	1.5	7 (21.9%)	0 (0%)	4 (13.8%)	1 (7.7%)	5 (19.2%)	1 (10.0%)
	1.0	5 (15.6%)	2 (18.2%)	4 (13.8%)	2 (15.4%)	9 (34.6%)	5 (50.0%)
	0.5	0 (0%)	6 (54.6%)	1 (3.5%)	1 (7.7%)	2 (7.7%)	3 (30.0%)
	0.0	0 (0%)	1 (9.1%)	1 (3.5%)	1 (7.7%)	1 (3.9%)	0 (0%)
	-0.5	0 (0%)	2 (18.2%)	1 (3.5%)	2 (15.4%)	0 (0%)	0 (0%)
	-1.0	0 (0%)	0 (0%)	1 (3.5%)	1 (7.7%)	0 (0%)	0 (0%)
	-1.5	0 (0%)	0 (0%)	0 (0%)	2 (15.4%)	0 (0%)	0 (0%)
	-2.0	0 (0%)	0 (0%)	0 (0%)	1 (7.7%)	0 (0%)	0 (0%)

4. Taxonomy

4.1. Central Tohoku Group

Hynobius senzanensis sp. nov.

ZooBank LSID:

urn:lsid:zoobank.org:act:E2BAEB21-FDE0-4EA3-A268-7C981B93AC64

(Figures 4–5)

Synonymy: *Hynobius lichenatus*: Aoki et al. [8] (2013: 168–172: clade II); Itagawa et al. [24] (2017: 37–46); Fujiwara [25] (2020: 47–57).

Holotype. An adult male (specimen number: AKPM-AM-40) from Sakuragaoka, Aoba Ward, Sendai City, Miyagi Prefecture, Tohoku, Japan [38° 18’ N, 140° 51’ E; elevation = 50 m above sea level (a.s.l.); in all cases, datum = WGS84], collected by Ayumi Fujiwara on March 13, 2022. This specimen is stored in the Akita Prefectural Museum: 52, Kanaashi-niozaki-ushiroyama, Akita City, Akita Prefecture, 010-0124, Japan.

Paratype. An adult female (specimen number: YCM-RA608) from the same locality as the holotype, collected by Ayumi Fujiwara on March 16, 2022, an adult male from Uchimachi, Mamurogawa Town, Yamagata

Prefecture (specimen number: YCM-RA609), collected by Hirotaka Sugawara on June 14, 2022, and an adult female from Higashitamano, Soma City, Fukushima Prefecture, collected by Makoto Kuraishi on April 18, 2022 (specimen number: YCM-RA610). These specimens were deposited in the Yokosuka City Museum of Natural History: 95 Fukadadai, Yokosuka City, Kanagawa Prefecture, 238-0016, Japan. An adult male from Yashimamachi, Yurihonjo City, Akita Prefecture (specimen number: AKPM-AM-41), collected by Ryuichi Sugawara on June 15, 2022. This specimen is stored in the Akita Prefectural Museum. To avoid the overcollection of this species, further details regarding these specimens are available only by contacting the Yokosuka City Museum or Akita Prefectural Museum.

Diagnosis. Adult individuals of both sexes have exhibited the following characters: fifth toe of hindlimb always present; shallow U-shaped or V-shaped vomerine teeth series; CGN 12 or 11; BLVT never present; and color of iris dark brown or lighter brown. Male individuals possessed the following characters: SVL ≥ 50.0 mm; RTRL ≥ 74.0%; RAGD ≥ 50.0%; RHL ≥ 22.0% (rarely < 22.0%); RTAL ≥ 75.0% (rarely < 75.0%); RMTAW ≥ 5.5% (rarely < 5.5%); RMTAH ≥ 10.0% (rarely < 10.0%); RVTL ≥ 3.0% (rarely < 3.0%); RVTW ≥ 5.0%; RHW ≥ 17.0% (rarely < 17.0%); RFLL ≥ 26.0% (rarely < 26.0%); RHLL ≥ 31.0% (rarely < 31.0%); R2FL ≥ 4.8% (rarely < 4.8%); R3FL ≥ 3.8% (rarely < 3.8%); R3TL ≥ 7.0% (rarely < 7.8%); R5TL ≥ 1.1% (rarely < 1.1%); RIND ≥ 5.9% (rarely < 5.9%); RIOD ≥ 6.7% (rarely < 6.7%); RUEW ≥ 3.2% (rarely < 3.1%); RSL ≥ 7.0% (rarely < 7.0%); RUEL ≥ 4.5% (rarely < 4.5%); RLJL ≥ 14.0% (rarely < 14.0%); DMDB frequently present; DWSV often present; DWSL often present; BLDT sometimes absent; DGM often absent; and CFBALN usually > 0.5. Female individuals had the following characters: SVL ≥ 55.0 mm (rarely < 55.0 mm); RTRL ≥ 74.0% (rarely < 74.0%); RAGD ≥ 52.0% (rarely < 52.0%); RHL ≥ 23.3% (rarely < 23.3%); RTAL ≥ 76.0% (rarely < 76.0%); RMTAW ≥ 6.0% (rarely < 6.0%); RMTAH ≥ 9.0% (rarely < 9.0%); RVTL ≥ 3.3% (rarely < 3.2%); RVTW ≥ 5.0%; RHW ≥ 16.5% (rarely < 16.5%); RFLL ≥ 23.5% (rarely < 23.5%); RHLL ≥ 30.0% (rarely < 30.0%); R2FL ≥ 4.7% (rarely < 4.7%); R3FL ≥ 4.0% (rarely < 4.0%); R3TL ≥ 7.2% (rarely < 7.2%); R5TL ≥ 1.0% (rarely < 1.0%); RIND ≥ 5.5% (rarely < 5.5%); RIOD ≥ 6.0%; RUEW ≥ 3.0% (rarely < 3.0%); RSL ≥ 7.0%; RUEL ≥ 4.5% (rarely < 4.5%); RLJL ≥ 13.7% (rarely < 13.7%); DMDB often present; DWSV sometimes present; DWSL sometimes absent; BLDT frequently absent; DGM never present; and CFBALN usually < 2.0.

Description of holotype. Moderately large individual: HL larger than HW; TAL shorter than SVL; VTL shorter than VTW; FLL shorter than HLL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; expanded cloaca; webbing between digits absent; four digits on each forelimb, order of length II > III > IV > I on both sides; five toes on each hindlimb, order of length III > IV > II > I > V on both sides; V-shaped vomerine teeth series; skin smooth and shiny; distinct grayish marking on dorsum present; DWSV and DWSL present; BLDT absent; BLVT absent; DGM absent. Holotype had the following measurements (in mm): SVL = 61.8, TRL = 46.3, LAGD = 31.4, RAGD = 31.8, HL = 15.5, TAL = 54.5, MTAW = 3.4, MTAH = 6.1, BTAW =7.6, BTAH = 6.1, LVTL = 2.5, RVTL = 2.3, VTW = 3.5, HW = 11.0, MXHW = 11.4, LFLL = 20.1, RFLL = 19.5, LHLL = 21.3, RHLL = 20.8, L1FL = 1.1, L2FL = 3.8, L3FL = 3.3, L4FL = 1.3, R1FL = 1.4, R2FL = 4.1, R3FL = 3.6, R4FL = 1.6, L1TL = 1.9, L2TL = 3.6, L3TL = 5.2, L4TL = 4.1, L5TL = 1.4, R1TL = 2.0, R2TL = 3.5, R3TL = 4.8, R4TL = 3.8, R5TL = 1.5, IND = 3.9, IOD = 4.5, LUEW = 1.8, RUEW = 1.8, LSL = 4.7, RSL = 4.5, LUEL = 3.1, RUEL = 2.9, LLJL = 8.5, RLJL = 8.3, CGN = 12.

Comparisons. Individuals of *H. senzanensis* sp. nov. exhibited statistically significant differences from *H. lichenatus* in the following length measurements: RVTL, RVTW, RHW, RFLL, R2FL, R3FL, R3TL, R5TL, RIOD, and RSL in males; RMTAW, RMTAH, RVTL, RVTW, and RLJL in females. In males except for RFLL, R2FL, R3FL, R3TL, and RSL, these measurements were significantly longer in *H. senzanensis* sp. nov. than in *H. lichenatus*. Notable, the new species displays R2FL in males and RVTL in females frequently at ≤ 6.2% (21/29 = 72.4%) and ≥ 3.5% (10/13 = 76.9%), respectively, whereas *H. lichenatus* typically exhibits R2FL in males and RVTL in females frequently at > 6.2% (24/32 = 75.0%) and usually < 3.5% (9/11 = 81.8%). The new species coexists in sympatry with *Hynobius nigrescens*; however, they are morphologically distinct across various life stages, including egg sacs, larvae, juveniles, and adults [26, 27].

Variation. Morphometric measurements and observations are presented in Tables 4 and 6, respectively. Significant values of all measurements between sexes are listed in Table 5. Males differed significantly from females in the following length measurements: RAGD, RTAL, RHW, RFLL, RHLL, RIND, and RIOD; these measurements, except for RAGD, were significantly longer in males than in females (Table 5). The dorsum is uniformly yellowish–brown, darkish–brown, or blackish–brown. The venter is lighter than the dorsum. DMDB rarely absent (6/29 = 20.7%) in males and occasionally absent in females (5/13 = 38.5%). DWSV occasionally absent in males (10/29 = 34.5%) and sometimes present in females (6/13 = 46.2%). DWSL occasionally absent in males (11/29 = 37.9%) and sometimes absent in females (6/13 = 46.2%); BLDT sometimes absent in males (14/29 = 48.3%) and occasionally present in females (5/13 = 38.5%); DGM occasionally present in males (11/29 = 37.9%), CGN occasionally 11 (10/29 = 34.5%) in males and rarely 11 in females (3/13 = 23.1%), and CFBALN rarely has < 1.0 CFBALN (4/29 = 14.0%) in males and rarely < -1.0 (3/13 = 23.1%) in females. The iris is dark brown or lighter brown. When preserved, the dorsal coloration tends to fade to dark gray, and BLDT become unclear after preservation.

Distribution. Based on our field surveys and previous studies [8, 28, 29, 30, 31, 32], the species is known from the following locations: Kurihara (only including the former Uguisuzawa and Ichihasama Towns and Hanayama Village), Osaki (only including the former Furukawa City and Naruko,

Iwadeyama, Sanbongi, Matsuyama, and Kashimadai Towns), Sendai, Natori, and Shiroishi Cities, as well as Kami (only including the former Miyazaki and Onoda Towns), Shikama, Taiwa, Osato, Matsushima, Rifu, Iwanuma, Watari, Yamamoto, Marumori, Kawasaki, Zao, and Shichikashuku Towns, in addition to Ohira Village, Miyagi Prefecture; Yurihonjo (only including the former Honjo City, and Nishime, Yuri, Higashiyuri, Yashima, and Chokai Towns), Nikaho (including the former Nikaho, Konoura, and Kisakata Towns), Yokote (only including the former Omonogawa Town), and Yuzawa (only including the former Yuzawa City and Ogachi Town) Cities, Akita Prefecture; Sakata (only including the former Sakata City and Yawata and Hirata Towns), Tsuruoka (only including the former Tsuruoka City and Atsumi Town), Shinjo, Obanazawa, Higashine, Tendo, Yamagata, Kaminoyama, Yonezawa Cities, as well as Yuza, Shonai (only including the former Tachikawa Town), Mamurogawa, Kaneyama, Mogami, Nakayama, Yamanobe, and Takahata Towns, in addition to Sakegawa and Tozawa Villages, Yamagata Prefecture; and Fukushima (only including the former Fukushima City), Date (only including the former Yanagawa and Ryozen Towns), Minamisoma (only including the former Haramachi City and Kashima Town) Cities, as well as Kori, Kunimi, and Namie Towns, in addition to Iitate and Kawauchi Villages, Fukushima Prefecture. Only one individual was recorded from Kamo City, Niigata Prefecture [8].

Etymology. The specific name "*senzanensis*" is derived from "Senzan", which represents the combined area of Sendai City including the type locality and Yamagata City. The new species is primarily found in and around the Senzan region. The suggested standard Japanese name for this species is Senzan Sanshouo.

Natural History. The dominant vegetation type at the type locality consists of mixed forest comprising live oak (*Quercus*) and Japanese red pine (*Pinus densiflora*). Larvae of this species have distinct black dots on lateral sides of the tail, and they lack claws on the tips of their digits and toes. During the early developmental stages of the larva, a single pair of balancers is present. The egg sacs of this species take on a coiled shape with striations and are typically attached to fallen branches or leaves in puddles, ponds, or swamps located at forest edges from mid-February to early June.

Remarks. The new species forms the sister group with *H. lichenatus*.

Figure 4. *Holotype of Hynobius senzanensis sp. nov. (AKPM-AM-40, adult male, 61.8 mm SVL) of (A) dorsal, (B) ventral, and (C) lateral views.*

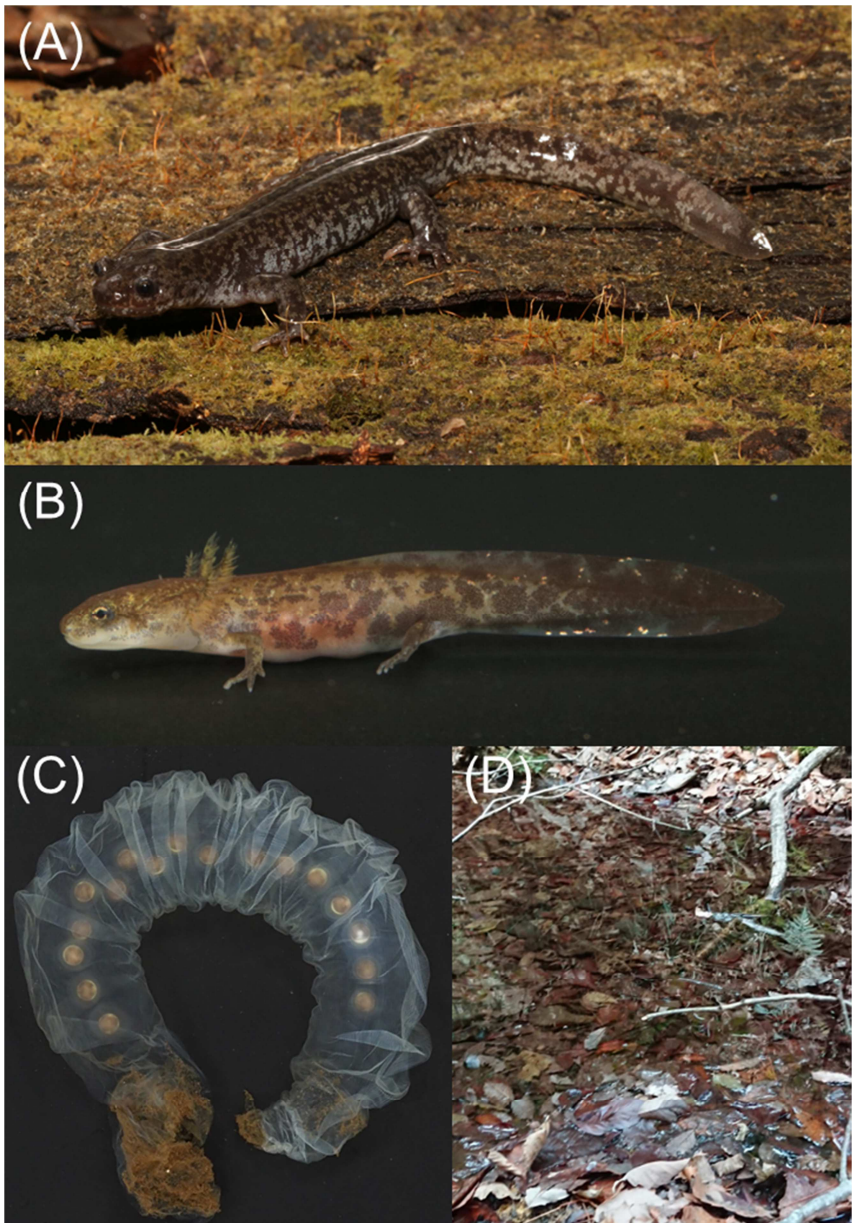


Figure 5. *Hynobius senzanensis sp. nov.: (A) adult, (B) larva, (C) egg sacs, and (D) habitat at the type locality.*

4.2. Southern Tohoku Group

Hynobius unanngso Tago, 1931
(Figure 6)

Synonymy: *Hynobius lichenatus* Iizuka [33] (1964: 19–21); Tanabe [34] (1984: 15–18); Tanabe [35] (1986: 1–4); Okayama [36] (1991: 1–2); Kanai [37] (1997: 61–63); Hirose and Kanai [38] (1999: 1–2); Saito [39] (1995: 39–45); Saito [40] (2003: 275–287); Aoki et al. [8] (2013: 168–172: clade I); Hayase et al. [41] (2016: 1–4).

Holotype. An adult male (specimen number: TIU903) from Iwane Village, Adachi County, Fukushima Prefecture, Tohoku, Japan, probably collected by Katsuya Tago. This specimen is stored in the Tokyo Imperial University, Science College Museum (= Tokyo University at present). According to a curator of the museum, the type specimens of this species may have already been lost.

Diagnosis. Adult individuals of both sexes possessed the following characters: fifth toe of hindlimb always present; shallow U-shaped or V-shaped vomerine teeth series; CGN 12 or 11 (rarely 13); BLVT never present; and color of iris dark brown or lighter brown. Male individuals possessed the following characters: SVL ≥ 48.0 mm (rarely < 48.0 mm); RTRL ≥ 73.4% (rarely < 73.4%); RAGD ≥ 49.5% (rarely < 49.5%); RHL ≥ 24.0% (rarely < 24.0%); RTAL ≥ 77.0% (rarely < 77.0%); RMTAW ≥ 5.0% (rarely < 5.0%); RMTAH ≥ 9.5% (rarely < 9.5%); RVTL ≥ 3.2% (rarely < 3.2%); RVTW ≥ 5.3% (rarely < 5.3%); RHW ≥ 16.3% (rarely < 16.3%); RFLL ≥ 28.5% (rarely < 28.5%); RHLL ≥ 30.0% (rarely < 30.0%); R2FL ≥ 5.0% (rarely < 5.0%); R3FL ≥ 3.8% (rarely < 3.8%); R3TL ≥ 7.4% (rarely < 7.4%); R5TL ≥ 1.2% (rarely < 1.2%); RIND ≥ 5.5% (rarely < 5.5%); RIOD ≥ 6.5% (rarely < 6.5%); RUEW ≥ 3.0% (rarely < 3.0%); RSL ≥ 7.4% (rarely < 7.4%); RUEL ≥ 4.1% (rarely < 4.1%); RLJL ≥ 13.0%

(rarely < 13.0%); DMDB frequently present; DWSV sometimes present; DWSL sometimes present; BLDT often absent; DGM usually absent; CFBALN usually ≥ 0.5. Female individuals had the following characters: SVL ≥ 53.0 mm; RTRL ≥ 74.0% (rarely 74.0%); RAGD ≥ 51.0% (rarely 51.0%); RHL ≥ 24.0% (rarely < 24.0%); RTAL ≥ 76.0% (rarely < 76.0%); RMTAW ≥ 5.2% (rarely < 5.2%); RMTAH ≥ 10.0% (rarely < 10.0%); RVTL ≥ 3.0%; RVTW ≥ 5.5% (rarely < 5.5%); RHW ≥ 16.5% (rarely < 16.5%); RFLL ≥ 27.0%; RHLL ≥ 31.3%; R2FL ≥ 5.5% (rarely < 5.5%); R3FL ≥ 4.5% (rarely < 4.5%); R3TL ≥ 8.0% (rarely < 8.0%); R5TL ≥ 1.5% (rarely < 1.5%); RIND ≥ 5.5% (rarely < 5.5%); RIOD ≥ 6.0% (rarely < 6.0%); RUEW ≥ 3.0%; RSL ≥ 6.9% (rarely < 6.9%); RUEL ≥ 4.5% (rarely < 4.5%); RLJL ≥ 13.0% (rarely < 13.0%); DMDB usually present; DWSV always present; DWSL always present; BLDT often absent; DGM always absent; CFBALN almost always ≤ 1.5.

Description of topotype. A male (specimen number: AKPM-AM-42) from Iwaneshojiishi (former Iwane Village), Motomiya City, Fukushima Prefecture, collected by Hirotaka Sugawara on March 30, 2016. This specimen is stored in the Akita Prefectural Museum. An individual with HL larger than HW; TAL shorter than SVL; VTL shorter than VTW; FLL shorter than HLL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; not expanded cloaca; webbing between digits absent; four digits on each forelimb, order of length II > III > I > IV on both sides; five toes on each hindlimb, order of length III > IV > II > I > V on both sides; shallow V-shaped vomerine teeth series; skin smooth and shiny; brownish marking on dorsum present; DWSV and DWSL present; BLDT absent; BLVT absent; DGM absent. The holotype had the following measurements (in mm): SVL = 52.7, TRL = 39.7, LAGD = 27.1, RAGD = 26.1, HL = 13.4, TAL = 41.1, MTAW = 3.0, MTAH = 5.2, BTAW = 5.9, BTAH = 4.8, LVTL = 1.7, RVTL = 1.8, VTW = 3.1, HW = 9.6, MXHW = 9.9, LFLL = 16.2, RFLL = 16.3, LHLL = 17.7, RHLL = 17.2, L1FL = 1.7, L2FL = 3.2, L3FL = 1.9, L4FL = 1.0, R1FL = 1.2, R2FL = 2.9, R3FL = 2.0, R4FL = 1.0, L1TL = 1.6, L2TL = 3.2, L3TL = 4.6, L4TL = 3.7, L5TL = 1.2, R1TL = 1.5, R2TL = 3.0, R3TL = 4.0, R4TL = 3.4, R5TL = 1.2, IND = 2.8, IOD = 3.8, LUEW = 1.5, RUEW = 1.6, LSL = 4.0, RSL = 4.2, LUEL = 2.7, RUEL = 2.5, LLJL = 7.2, RLJL = 6.8, and CGN = 12.

Comparisons. This species shows statistical differences in the following length measurements compared with those of *H. lichenatus*: RTAL, RVTL, RVTW, R2FL, R3FL, R3TL, R5TL, RIOD, and RUEL in males and SVL, RTAL, RVTL, RVTW, and RHW in females; the lengths of these measurements, except RVTL, RVTW, R5TL, and RIOD in males and RVTL, RVTW, and RHW in females, are significantly shorter in *H. unanngso*. In general, the RTAL, RVTL, and RVTW of *H. unanngso* males are ≤ 87.5% (21/26 = 80.8%), ≥ 3.6% (21/26 = 80.8%), and ≥ 5.6% (21/26 = 80.8%), respectively. However, the RTAL, RVTL, and RVTW of *H. lichenatus* males are generally > 87.5% (26/32 = 81.3%), almost always < 3.6% (29/32 = 90.6%), and frequently < 5.6% (23/32 = 71.9%), respectively. The SVL and RVTW of *H. unanngso* females are generally < 60 mm (8/10 = 80%) and almost always ≥ 5.5% (9/10 = 90%), respectively. However, the SVL and RVTW of *H. lichenatus* females are almost always ≥ 60 mm (10/11 = 90.9%) and almost always < 5.5% (9/10 = 90%), respectively. Although *H. unanngso* is not the closest species to *H. senzanensis* sp. nov., is morphologically similar to *H. senzanensis* sp. nov. than to *H. lichenatus*, which is the closest species to *H. senzanensis* sp. nov. (Figure 2). This species shows statistical differences in the following length measurements compared when to *H. senzanensis* sp. nov.: RTAL, RVTW, RHW, RSL, RUEL, and RLJL in males and RVTW and RFLL in females; the lengths of these measurements, except RVTW and RSL in males and RVTW and RFLL in females, are significantly shorter in *H. unanngso*. The resurrected species coexists in sympatry with *H. nigrescens*; however, they are morphologically distinct across various life stages, including egg sacs, larvae, juveniles, and adults [26, 27].

Variation. Morphometric measurements and observations are presented in Tables 4 and 6, respectively. Significant values of all measurements between sexes are listed in Table 5. Males differed significantly from females in the following length measurements: RAGD, RFLL, RHLL, R3TL, RIOD, and RSL; these measurements, except for RAGD and R3TL, were significantly longer in males (Tables 4–5). The dorsum is uniformly yellowish–brown, grayish–brown, darkish–brown, or blackish–brown. The venter is lighter than the dorsum. Variations in the observed characters are as follows: DMDB rarely absent in males (6/26 = 23.1%) and females (2/10 = 20.0%); DWSV (12/26 = 53.8%) and DWSL (12/26 = 53.8%) sometimes present in males; BLDT sometimes present in males (10/26 = 38.5%) and females (4/10 = 40%); DGM rarely present in males (4/26 = 15.4%); CGN rarely 13 in males (2/26 = 7.7%) and females (2/10 = 20%); CFBALN rarely ≤ 0.5 in males (3/26 = 11.6%) and ≥ 1.5 in females (2/10 = 20.0%). When preserved, the dorsal coloration tended to fade to dark gray, and BLDT become unclear after preservation.

Distribution. Based on our field surveys and previous studies [8, 30, 37, 42], the species is known from the following locations; Tsuruoka (only including the former Haguro Town and Asahi Village), Nagai, and Yonezawa Cities, as well as Nishikawa, Oe, Asahi, Yamanobe, Shirataka, Oguni, and Iide Towns, Yamagata Prefecture; Motomiya (only including the former Motomiya Town), Koriyama, Sukagawa (only including the former Naganuma Town and Iwase Village), Shirakawa (only including the former Taishin Village), Kitakata (including the former Kitakata City, Yamato and Shiokawa Towns, and Atsushiokanoh and Takasato Villages), Aizuwakamatsu (only including the former Aizuwakamatsu City) Cities, as well as Inawashiro, Bandai, Aizubange, Nishiaizu, Tanagura, Shimogo, and Minamiaizu Towns, in addition to Kitashiobara, Yugawa, Tenei, and Nishigo Villages, Fukushima Prefecture; Murakami (including the former Murakami City, Sanpoku and Arakawa Towns, and Asahi and Kamihayashi Villages), Tainai (including the former Nakajo Town, and Kurokawa Village), Shibata (only

including the former Shibata City, Toyoura Town, and Kajikawa Village), Niigata (only including the former Niitsu City, Kosudo Town, and Iwamuro Village), Agano (only including the former Suibara and Yasuda Towns, and Sasakami Village), Gosen (including the former Gosen City and Muramatsu Town), Sanjo (only including the former Sanjo City and Shitada Village), Tsubame City (only including the former Bunsui Town), Mitsuke, Nagaoka (only including the former Nagaoka and Tochio Cities, Koshiji and Oguni Towns, and Yamakoshi Village), Kashiwazaki (only including the former Kashiwazaki City), Ojiya, Uonuma (including the former Horinouchi, Kawaguchi, and Koide Towns, and Irihirose, Sumon, Hirokami, and Yunotani Villages), Minamiuonuma (only including the former Yamato and Muika Towns), Tokamachi (only including the former Tokamachi City and Matsunoyama Town), and Joetsu (only including the former Kakizaki and Yoshikawa Towns) Cities, as well as Aga and Tagami Towns, in addition to Sekikawa and Yahiko Villages, Niigata Prefecture; Nasuhiobara (only including the former Kuroiso City and Shiobara Town), Yaita, Nikko (including the former Imaichi and Nikko Cities, and Fujihara, Ashio Town, and Kuriyama Village) Cities, as well as Shioya Town, Tochigi Prefecture; Numata (only including the former Numata City and Tone Village) City, as well as Minakami (only including the former Minakami Town) Town, in addition to Katashina and Kawaba Villages, Gunma Prefecture. Probably, this species is also found in Kamo City, Niigata Prefecture; however, further surveys using genetic markers are needed because only a single individual with the haplotype of *H. senzanensis* sp. nov. was detected in this city [8].

Etymology. The specific name is derived from "Unnansoh", which means salamander in Tohoku District (mainly Akita Prefecture). This species was originally referred to as Tohoku-kasumi-sanshouo in the initial description [23]. However, some species in the aforementioned study incorporate the term "Kasumi" in their Japanese names, although this word is not included in the current standard Japanese names. For example, *Hynobius dunni* is referred to as Oita-kasumi-sanshouo in the study [23], but it is presently known as Oita-sanshouo in Japanese. Currently, kasumi-sanshouo is the standard Japanese name for the clouded salamander (*Hynobius nebulosus*). Therefore, using the name as described in this study [23] may lead to confusion regarding the standard Japanese names for certain species. Consequently, the suggested standard Japanese name for this species is Banetsu sanshouo, with Banetsu referring to the combined area of Fukushima (Ban) and Niigata (Etsu) Prefectures, where the new species is primarily found.

Natural History. The larval and egg sac morphologies of this new species closely resemble those of *H. senzanensis* sp. nov. The breeding season for this species occurs from early March to late May. Surveys conducted on the clutch size of *H. unanngso* have been well documented [33, 34, 36, 38], with the range typically being 14 to 81. The developmental stage of this species has been comprehensively investigated by Sawano [43].

Remarks. The new species forms the sister group with the *H. senzanensis* sp. nov. + *H. lichenatus* clade [8] (Figure 2). There is a possibility that populations from Gunma Prefecture may occasionally exhibit 13 costal grooves [35].

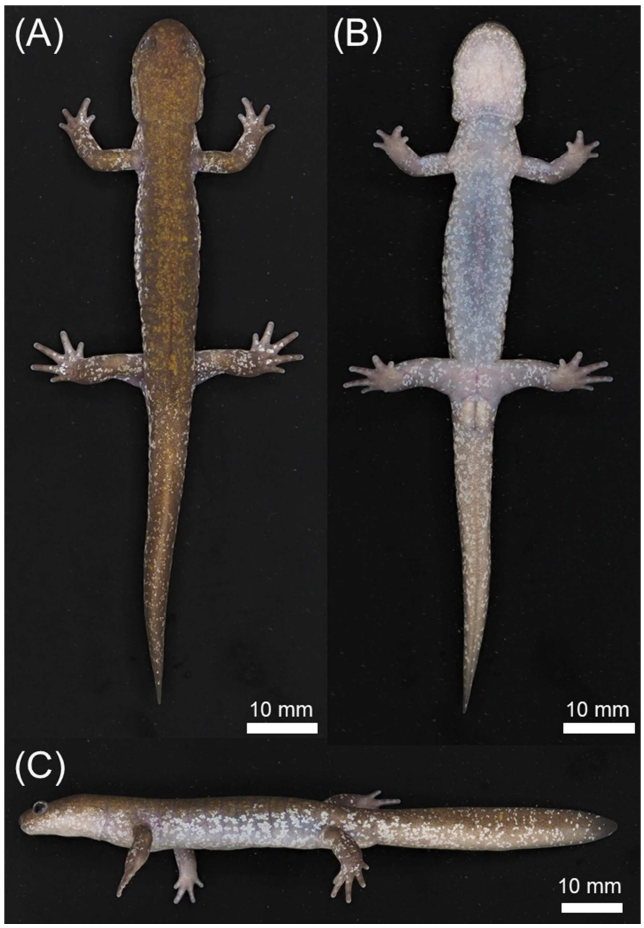


Figure 6. *Topotype of Hynobius unanngso (AKPM-AM-42, adult male, 52.7 mm SVL) of (A) dorsal, (B) ventral, and (C) lateral views.*

4.3. Northern Tohoku Group

Hynobius lichenatus Boulenger, 1883

(Figures 7–8)

Holotype. A juvenile (specimen number: BMNH 1946.9.6.49) from Aomori Prefecture, Japan, collected by George Lewis on May, 1907 [1, 7]. This specimen is stored in the Natural History Museum: Kensington & Chelsea, London, SW7, UK.

Diagnosis. Adult individuals of both sexes have following characters: fifth toe of hindlimb always present; shallow U-shaped or V-shaped vomerine teeth series; CGN 12 or 11; BLVT never present; and color of iris dark brown or lighter brown. Male individuals have following characters: SVL ≥ 55.0 mm (rarely < 55.0 mm); RTRL ≥ 73.0% (rarely < 73.0%); RAGD ≥ 50.5% (rarely < 50.5%); RHL ≥ 24.0% (rarely < 24.0%); RTAL ≥ 85.0% (rarely < 85.0%); RMTAW ≥ 5.0% (rarely < 5.0%); RMTAH ≥ 10.0% (rarely < 10.0%); RVTL ≥ 2.9% (rarely < 2.9%); RVTW ≥ 4.9% (rarely < 4.9%); RHW ≥ 16.5% (rarely < 16.5%); RFLL ≥ 28.5% (rarely < 28.5%); RHLL ≥ 32.5% (rarely < 32.5%); R2FL ≥ 5.5% (rarely <

5.5%); R3FL ≥ 4.0% (rarely < 4.0%); R3TL ≥ 7.5% (rarely < 7.5%); R5TL ≥ 0.2% (rarely < 0.2%); RIND ≥ 5.5% (rarely < 5.5%); RIOD ≥ 6.0% (rarely < 6.0%); RUEW ≥ 3.0% (rarely < 3.0%); RSL ≥ 7.0%; RUEL ≥ 4.5% (rarely < 4.5%); RLJL ≥ 13.5% (rarely < 13.5%); DMDB usually present; DWSV sometimes present; DWSL often present; BLDT usually present; DGM usually absent; CFBALN usually > 1.0. Female individuals have following characters: SVL ≥ 60.0 mm (rarely < 60.0 mm); RTRL ≥ 74.8%; RAGD ≥ 53.5% (rarely < 53.5%); RHL ≥ 23.0% (rarely < 23.0%); RTAL ≥ 77.0% (rarely < 77.0%); RMTAW ≥ 5.0% (rarely < 5.0%); RMTAH ≥ 8.5% (rarely < 8.5%); RVTL ≥ 2.5%; RVTW ≥ 4.0% (rarely < 4.0%); RHW ≥ 15.0% (rarely < 15.0%); RFLL ≥ 26.0%; RHLL ≥ 32.0% (rarely < 32.0%); R2FL ≥ 5.0% (rarely < 5.0%); R3FL ≥ 3.5% (rarely < 3.5%); R3TL ≥ 7.5%; R5TL ≥ 1.0% (rarely < 1.0%); RIND ≥ 5.3%; RIOD ≥ 5.8%; RUEW ≥ 3.0%; RSL ≥ 6.5%; RUEL ≥ 4.0%; RLJL ≥ 13.0%; DMDB always present; DWSV almost always present; DWSL almost always present; BLDT almost always present; DGM never present; CFBALN usually < 1.0.

Description of candidate topotypes. A male from Asamushi-babayama, Aomori City, Aomori Prefecture (specimen number: AKPM-AM-44), collected by Shunsuke Momoi on April 2, 2022. A moderately large individual; HL larger than HW; TAL shorter than SVL; VTL shorter than VTW; FLL shorter than HLL; body almost semicylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; not expanded cloaca; webbing between digits absent; four digits on each forelimb, order of length II > III > I > IV on both sides; five toes on each hindlimb, order of length III > IV > II > I > V on both sides; shallow V-shaped vomerine teeth series; skin smooth and shiny; distinct black spots on dorsum absent; DWSV and DWSL present; BLDT present (it became unclear after preservation); BLVT absent; DGM absent. The candidate topotype had the following measurements (in mm): SVL = 56.9, TRL = 42.4, LAGD = 30.7, RAGD = 30.2, HL = 14.0, TAL = 52.1, MTAW = 2.6, MTAH = 5.1, BTAW = 6.3, BTAH = 5.6, LVTL = 1.8, RVTL = 1.9, VTW = 3.7, HW = 10.7, MXHW = 10.9, LFLL = 18.7, RFLL = 19.4, LHLL = 20.0, RHLL = 20.2, L1FL = 1.6, L2FL = 4.2, L3FL = 3.1, L4FL = 1.3, R1FL = 1.6, R2FL = 3.6, R3FL = 3.0, R4FL = 1.3, L1TL = 1.9, L2TL = 3.6, L3TL = 4.8, L4TL = 4.0, L5TL = 1.6, R1TL = 1.7, R2TL = 3.4, R3TL = 4.8, R4TL = 3.8, R5TL = 1.4, IND = 4.0, IOD = 4.1, LUEW = 1.7, RUEW = 1.8, LSL = 4.6, RSL = 4.4, LUEL = 2.9, RUEL = 2.8, LLJL = 8.1, RLJL = 7.8, and CGN = 12. A male (specimen number: AKPM-AM-43) from Matsudaimachi, Ajigasawa Town, Aomori Prefecture, collected by Ryuichi Sugawara on May 21, 2022. A moderately large individual; HL larger than HW; TAL shorter than SVL; VTL shorter than VTW; FLL shorter than HLL; body almost semicylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; not expanded cloaca; webbing between digits absent; four digits on each forelimb, order of length II > III > IV > I on both sides; five toes on each hindlimb, order of length III > IV > II > I = V on both sides; shallow V-shaped vomerine teeth series; skin smooth and shiny; distinct black spots on dorsum absent; DWSV and DWSL present; BLDT present (as a very bright yellow line); BLVT absent; DGM absent. The candidate topotype had the following measurements (in mm): SVL = 59.6, TRL = 45.0, LAGD = 30.0, RAGD = 29.1, HL = 15.1, TAL = 50.1, MTAW = 2.6, MTAH = 5.1, BTAW = 7.0, BTAH = 4.9, LVTL = 2.1, RVTL = 2.0, VTW = 3.4, HW = 10.7, MXHW = 10.9, LFLL = 17.9, RFLL = 17.9, LHLL = 20.4, RHLL = 19.3, L1FL = 1.0, L2FL = 3.5, L3FL = 2.8, L4FL = 1.5, R1FL = 0.9, R2FL = 3.9, R3FL = 3.1, R4FL = 1.5, L1TL = 1.2, L2TL = 3.7, L3TL = 4.7, L4TL = 3.8, L5TL = 1.2, R1TL = 1.4, R2TL = 3.4, R3TL = 5.3, R4TL = 3.5, R5TL = 1.4, IND = 3.8, IOD = 4.1, LUEW = 1.6, RUEW = 1.5, LSL = 4.9, RSL = 4.6, LUEL = 2.7, RUEL = 2.6, LLJL = 8.6, RLJL = 8.1, and CGN = 12.

Comparisons. This species coexists in sympatry with *H. nigrescens*; however, they are morphologically distinct across various life stages, including egg sacs, larvae, juveniles, and adults [26, 27].

Variation. Morphometric measurements and observations are presented in Tables 4 and 6, respectively. Significant values of all measurements between sexes are listed in Table 5. Males statistically differed significantly from females in the following length measurements: RAGD, RTAL, RHW, RFLL, RHLL, RIND, and RIOD; these measurements, except for RAGD, were significantly longer in males than in females (Table 5). The dorsum is uniformly yellowish–brown, grayish-brown, darkish–brown, or blackish–brown. The venter is lighter than the dorsum. Variations of observed characters are as follows: DMDB rarely absent (4/32 = 12.5%) in males; DWSV sometimes present in males (14/32 = 43.8%) and very rarely absent in females (1/11 = 9.1%); DWSL occasionally absent in males (10/32 = 31.3%) and very rarely absent in females (1/11 = 9.1%); BLDT rarely absent in males (5/32 = 15.6%) and very rarely absent in females (1/11 = 9.1%); DGM rarely present in males (5/32 = 15.6%); and CFBALN rarely < 1.5 in males (5/32 = 15.6%) and rarely > 0.5 in females (2/11 = 18.2%). When preserved, the dorsal coloration tends to fade to dark gray, and BLDT become unclear after preservation.

Distribution. Based on our field surveys and previous studies [8, 28, 44, 45], the species is known from the following locations: Mutsu (only including the former Mutsu City, and Ohata and Kawauchi Towns), Misawa, Aomori (including the former Aomori City and Namioka Town), Towada (including the former Towada City and Towadako Town), Hirakawa (including the former Hiraka Town and Ikarigaseki Village), Kuroishi, Tsugaru (only including the former Kizukuri Town), Goshogawara (only including the former Goshogawara City, Kanagi Town, Shiura Village), and Hirosaki (including the former Hirosaki City, Iwaki Town, and Soma Village) Cities, as well as Yokohama, Hiranai, Shichinohe (only including the former Shichinohe Town), Sannohe, Takko, Owani, Ajigasawa, Fukaura (only including the former Iwasaki Village), Nakadomari, (including the former Nakasato Town and Kodomari Village), Sotogahama (only including the former Kanita Town and Tairadate Village) Towns, in addition to

Kazamaura, Sai, Nishimeya, and Yomogita Villages, Aomori Prefecture; Hachimantai (including the former Ashiro and Nishine Towns and Matsuo Village), Ninohe (including the former Ninohe City and Johoji Town), Kuji (only including the former Kuji City), Takizawa, Morioka (including the former Morioka City and Tamayama Village), Miyako (only including the former Miyako City, Taro Town, Niisato Village), Hanamaki (including the former Hanamaki City and Towa, Ishidoriya, and Osako Towns), Kitakami, Tono (including the former Tono City and Miyamori Village), Kamaishi, Ofunato (including the former Ofunato City and Sanriku Town), Rikuzentakata, Oshu (only including the former Esashi and Mizusawa Cities, and Isawa Town), and Ichinoseki (only including the former Ichinoseki City, Higashiyama, Daito, Senmaya, Fujisawa Towns, and Murone and Kawasaki Villages) Cities, as well as Hirono (only including the former Taneichi Town), Karumai, Ichinohe, Kuzumaki, Iwate, Iwaizumi, Shizukuishi, Shiwa, Nishiwaga (including the Yuda Town and Sawauchi Village), Kanegasaki, Yamada, Otsuchi, Sumita, Hiraizumi Towns, in addition to Kunohe, Noda, Fudai, and Tanohata Villages, Iwate Prefecture; Kesennuma (including the former Kesennuma City and Karakuwa and Motoyoshi Towns), Tome (only including the former Towa and Tsuyama Towns), Ishinomaki (including the former Ishinomaki City and Kitakami, Kahoku, Ogatsu, and Oshika Towns) Cities, as well as Minamisanriku (including the former Utatsu and Shizugawa Towns) and Onagawa Towns, Miyagi Prefecture; Noshiro (including the former Noshiro City and Futatsui Town), Odate (including the former Odate City and Tashiro and Hinai Towns), Kazuno, Kitaakita (including the former Takanosu, Aikawa, Ani, Moriyoshi Towns), Oga (only including the former Oga City), Katagami (only including the former Showa Town), Akita (including the former Akita City and Kawabe and Yuwa Towns), Senboku (including the former Kakunodate and Tazawako Towns and Nishiki Village), Daisen (including the former Omagari City, Kyowa, Nishisenboku, Nakasen, Kamioka, Ota, Senboku Towns, and Nangai Village), Yurihonjo (only including the former Honjo City and Iwaki and Ouchi Towns), Yokote (only including the former Yokote City, Omori, Hiraka, and Masuda Towns, and Daiyu and Sannai Villages), and Yuzawa (only including the former Inakawa Town and Minase Village) Cities, as well as Happo (including the former Hachimori Town and Minehama Village), Fujisato, Kosaka, Mitane (only including the former Yamamoto and Kotooka Towns), Ikawa, Gojome, Misato (including the former Senhata and Rokugo Towns and Sennan Village), and Ugo Towns, in addition to Kamikoani and Higashinaruse Villages, Akita Prefecture.

Etymology. The specific name is derived from lichen = (lichen in Latin) + -atus (suffix in Latin).

Natural History. The larval and egg sac morphologies of this species are similar to *H. senzanensis* sp. nov. The species' breeding season falls between late February and late May.

Remarks. This species forms the sister group with *H. senzanensis* sp. nov. [8] (Figure 2).

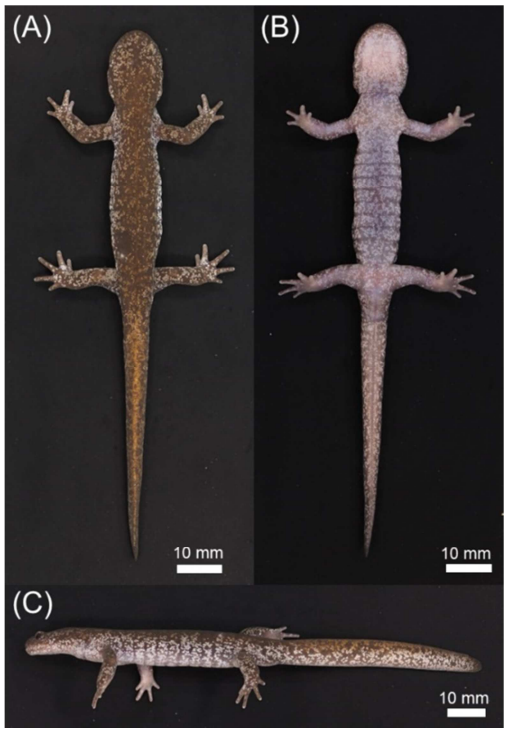


Figure 7. *Candidate topotype of Hynobius lichenatus (AKPM-AM-44, adult male, 56.9 mm SVL) of (A) dorsal, (B) ventral, and (C) lateral views.*

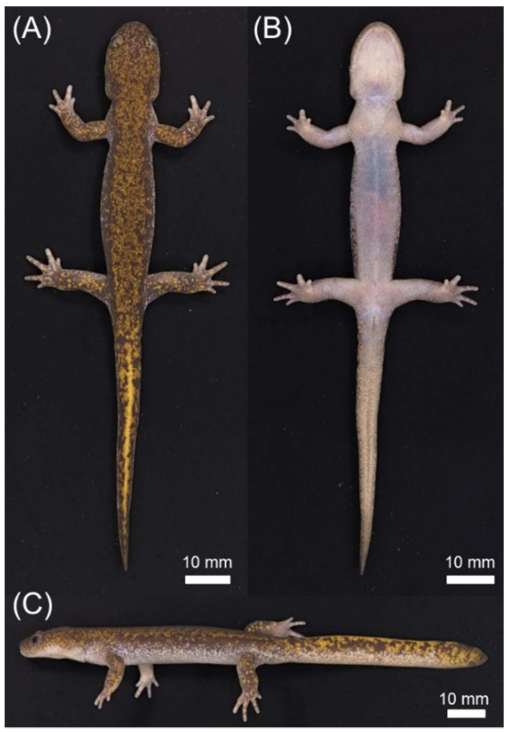


Figure 8. *Candidate topotype of Hynobius lichenatus (AKPM-AM-43, adult male, 59.6 mm SVL) of (A) dorsal, (B) ventral, and (C) lateral views.*

5. Discussion

As previously reported by Aoki et al. [8], *H. lichenatus* has been categorized into three genetic groups, with strong support for the monophyly of these groups. Our analyses, which incorporated samples spanning the entire distribution

of *H. lichenatus*, corroborate these findings (Figure 2). However, the presence of subgroups within each group remains unclear, in contrast to the results obtained by Aoki et al. [8] (Figure 2). Notable, the length of the cytochrome b gene in our analyses was shorter than that reported by Aoki et al. [8], It is possible that the sustainability of monophyletic relationships within each group (subgroup) may be compromised with an increased number of sampling points in subsequent analyses. To resolve the detailed phylogenetic relationships within each group, future studies may require population genetic approaches using microsatellite (SSR) or single nucleotide polymorphism (SNP) markers are necessary.

Before the description of *H. unanngso* by Tago [23], two scientific names were suggested by Shibata [46] based on individuals from Echigo (= Niigata Prefecture): *Hynobius kuramotoe* and *Hynobius echigoensis*. However, these names conflict with Article 12 (12.2.7) of the International Code of Zoological Nomenclature (ICZN) [47], rendering them "nomina nuda". *Hynobius longimanus*, described by Lantz [48], has been considered synonymous with *H. lichenatus* [1, 49]. If *H. longimanus* is indeed synonymous with *H. lichenatus*, it may correspond to *H. unanngso* owing to the proximity of its type locality (= Tochio, Nagaoka City, Niigata Prefecture) to the distribution area of *H. unanngso* (Figure 1). A checklist by Okada [50] supports the hypothesis that *H. longimanus* is synonymous with *H. unanngso*. However, it cannot be ruled out that *H. longimanus* corresponds to *H. nigrescens* based on the original description of *H. longimanus*. As both species inhabit the same area, resolving the taxonomic status of *H. longimanus* would necessitate additional morphological data for both species.

The type locality of *H. lichenatus* is known only from Aomori Prefecture, with limited detailed information available. The holotype of *H. lichenatus* was collected by George Lewis, who likely followed the footsteps of his field sampling when he visited Japan [51]. Lewis collected samples from various localities in Aomori Prefecture, including Mt. Iwaki, Mt. Hakkoda, Asamushi, Soma, Kominato, Shichinohe, Sannohe. DNA analyses of samples from these areas or nearby regions suggest the presence of true *H. lichenatus* (Figure 2). Consequently, it is likely that true *H. lichenatus* corresponds to the northern Tohoku group of *H. lichenatus* (Figure 2). However, a detailed assessment of the type locality of *H. lichenatus* remains challenging, as our analyses did not include adult individuals from all areas. The holotype of *H. lichenatus* has a distinct yellow line on the dorsal side of the tail [7], and some individuals from the eastern part of Aomori Prefecture in the present study exhibited a bright yellow line on the tail's dorsal side. However, number of samples from Aomori Prefecture in this study is insufficient for accurately estimating the type locality of *H. lichenatus*. Furthermore, the holotype of *H. lichenatus* is a juvenile specimen [23], making direct morphological comparisons with adult specimens unfeasible. To accurately estimate the type locality of *H. lichenatus*, further morphological analyses using juvenile specimens from candidate type localities are warranted.

The distribution ranges of *H. senzanensis* sp. nov. and *H. unanngso* partially overlap in the central part of Yamagata Prefecture [8], raising the posiibility of introgression between these two species, similar to the situation observed in *Pelophylax nigromaculatus* and *Pelophylax porosus* [52]. Although our results, along with those of Aoki et al. [8], suggest that the distribution ranges of *H. lichenatus* and *H. senzanensis* sp. nov. do not overlap, their distribution areas are geographically close in the southern part of Akita Prefecture, leaving open the possibility of introgression at the boundary of their ranges.

The three studied species (*H. lichenatus*, *H. senzanensis* sp. nov., and *H. unanngso*) have relatively extensive distribution ranges within Japanese lentic *Hynobius* species: *H. lichenatus* = approximately < 35,000 km^2; *H. senzanensis* sp. nov. = approximately < 17,000 km^2; and *H. unanngso* = approximately < 27,000 km^2. However, it is unclear where each species with endemic alleles, without introgression, is found in the overlapping or boundary areas of these species. To elucidate the extent of introgression between *H. lichenatus* and *H. senzanensis* sp. nov. or *H. senzanensis* sp. nov. and *H. unanngso*, studies employing population genetics or conservation genetics are essential. Such studies should employ genetic markers, such as microsatellites (SSRs) or single nucleotide polymorphisms (SNPs), to facilitate precise conservation efforts.

The Great East Japan Earthquake on March 11, 2011, led to a temporary decline in the number of individuals [24]. However, the current status of these three species across their entire distribution ranges remains uncertain. Following the present description, there is an urgent need to reassess the conservation status of these species, and develop immediate management plans to prevent extinction or drastic population decline.

6. Conclusion

Based on the three species concept used in this study, the Tohoku salamander, *Hynobius lichenatus*, is now classified into three distinct species: *H. lichenatus*, *H. senzanensis* sp. nov., and *H. unanngso*. However, critical questions regarding the detailed genetic structure within each species, potential introgression between species (*H. lichenatus* vs. *H. senzanensis* sp. nov. and *H. senzanensis* sp. nov. vs. *H. unanngso*), and the impact of human-induced genetic pollution remain unanswered. To ensure the future conservation of these species, population genetics studies using genetic markers that provide detailed insight into genetic structure, such as SSRs and SNPs, are imperative.

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