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Genetic and morphological variation analyses of *Dryophytes japonicus* (Anura, Hylidae) with description of a new species from northeastern Japan

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Abstract

Japanese tree frog, *Dryophytes japonicus*, formerly known as *Hyla japonica*, is known to include several geographic groups recognized in mitochondrial phylogeny. By analyzing genetic and morphological variations in a large number of individuals of *Dryophytes*, we studied their taxonomic relationships. A mitochondrial DNA phylogeny was consistent with previous studies in that a high molecular divergence existed between populations from northeastern Japan and Sakhalin (Clade A) and those from southwestern Japan and Korea (Clade B). Nuclear DNA analyses based on SNP data also support such separation, whereas hybrid populations were found at some localities near the border of mitochondrial clades in Honshu Island, forming a hybrid zone. The width of hybrid zone was estimated to be narrow (approx. 25 km) and the migration rates into/beyond it were relatively low. Those results indicate that two genetic groups have long been parapatrically maintained with a narrow hybrid zone in Honshu Island. We examined syntypes of *Hyla japonica* and designated lectotypes. In adult morphology, the clades could be differentiated mainly by the pattern of rear of thigh, and the lectotypes proved to be Clade B. From these results, we describe the frogs of Clade A as a new species, *D. leopardus* **sp. nov.**, distinct from *D. japonicus*.

Key words: northeastern Japan, *Dryophytes japonicus*, thigh pattern, mitochondrial DNA phylogeny, nuclear DNA phylogeny

Introduction

Japanese tree frog, *Dryophytes japonicus* (Günther) long known as *Hyla* is thought to be a wide-ranging species in northeast Asia, distributed in Japan, Korea, northeastern China, eastern Mongolia, and Russian Far East, but its uniformity as a single taxon has been repeatedly challenged. After Günther (1859 "1858") first recognized this species as a distinct variety, *Hyla arborea* var. *japonica*, based on Japanese specimens, three additional forms have been reported from the distributional area of this species (*H. stepheni* Boulenger from Korea, *H. sodei-campi* Kostin from northeastern China, and *H. arborea ussuriensis* Nikolski from Russian Maritime Territory), but their validity has been rejected by some authors (e.g. Shannon 1956, Yang 1962). Subsequently, Kuramoto (1980) found a cryptic species, *H. suweonensis* Kuramoto, in Korea. Its distinctness from sympatric *D. japonicus* is widely accepted, although some authors have doubted its specific validity since it is genetically quite close to a Chinese hylid, *D. immaculatus* (Boettger) (Dufresnes *et al.* 2016). Yet, for the frogs which is now identified as *D. japonicus*, no surveys on morphological variation have been conducted except for several local comparisons (e.g. Yang 1962, Matsui 1979, Jang *et al.* 2011).

On the other hand, recent progress of molecular analyses has provided new insights to the geographic variation of this frog (Nishioka *et al.* 1990, Lee *et al.* 1999, Jang *et al.* 2011, Li *et al.* 2015, Dufresnes *et al.* 2016). Especially, Dufresnes *et al.* (2016) reported two major mitochondrial lineages (Clades A and B) in *D. japonicus*, and regarded them as distinct species mainly based on the genetic distances (Dxy=0.104 in approx. 900 bps of cytochrome b region) and estimated divergent time (~5 Mya). According to their results, *D. japonicus* in northeastern Japan

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and Sakhalin could be separated from that in southwestern Japan and the continent. However, their results were mainly based on mtDNA phylogeny alone. Although such separation seemed to be consistent with the cladogram of allozyme analysis by Nishioka *et al*. (1990), Dufresnes *et al.* (2016) failed to show similar evidences from nuclear sequences, because of the low confidential values of phylogenetic tree and only slight variations in network tree.

We, thus, analyzed the genetic structures of *D. japonicus* based on the whole genome through the single-nucleotide polymorphic analyses (SNPs), using the populations of Japan and Korea, where Dufresnes *et al.* (2016) found large variations in mtDNA. Further, we conducted thorough morphological comparison using the specimens collected from these areas. Through those analyses, we reached the conclusion that the two major genetic groups found in Japan should be treated as different species, and established a new species.

Materials and method

Molecular genetic analyses: We analyzed (1) the mitochondrial cytochrome b (cyt b) gene for elucidating intra- and inter- population relationships using a large number of samples (dataset 1), (2) the mitochondrial 12S rRNA, tRNA Valine, 16S rRNA, and cyt b genes for clarifying phylogenetic relationships among genetic groups found in the dataset 1 (dataset 2), and (3) MIGseq analysis, an approach to obtain single-nucleotide polymorphism (SNP) data from across the nuclear genome (dataset 3). For vouchers and sampling localities, see the Supplementary Table 1 deposited in Figshare [DOI: 10.6084/m9.figshare.28067339].

MtDNA analyses: We extracted total DNA from ethanol-preserved muscle tissue using standard phenol–chloroform extraction procedure, and conducted amplifications of regions chosen by the polymerase chain reaction (PCR). The PCR primers used for amplification and sequencing are shown in the Supplementary Table 2 [DOI: 10.6084/m9.figshare.28067339]. We retrieved sequence data used in Dufresnes *et al.* (2016) to assign our genetic grouping with theirs, choosing their samples covering the whole part of their tree (A1–A4 and B1–B5). Following Duellman *et al.* (2016), we chose five congeneric species as hierarchical outgroups, and *Hyla chinensis* Günther as the outermost outgroup. Sequences were aligned by the Clustal W option of Bio Edit 7.2.5.0. All newly obtained sequences were submitted to GenBank (accession numbers LC861226–LC861327).

(1) Phylogenetic inferences

For both mtDNA datasets, ML analyses were performed using RAxMLv.8.2.13 (Stamatakis 2014), and BI analyses were conducted using MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001). The optimum substitution models, selected by Kakusan4 (Tanabe 2011) based on the Akaike information criterion, were GTR+G in dataset 1 and dataset 2 (all for 12S rRNA, 16S rRNA, tRNA Valine, and cyt b). In Bayesian analysis, we ran 20 million generations, sampled a tree every 100 generations, and discarded the initial 25% as burn-in. We used Tracer version 1.7.2 (Rambaut *et al.* 2018) to check the likelihood convergence. Node supports were estimated by 1000 bootstrapping replicates using ML, and posterior probabilities (BPP) for each branch in the Bayesian tree. We regarded tree topologies with bootstrap values (BS) > 70% as sufficiently supported (Hillis & Bull 1993). For the BI analysis, we considered BPP > 0.95 as significant support (Leaché & Reeder 2002).

(2) Species delimitation tests

We applied two species delimitation tests which rely on different methods to search the candidates of species boundaries within *D. japonicus*. One is Assemble Species by Automatic Partitioning (ASAP, Puillandre *et al*. 2021) based on genetic distances. For this method, we processed aligned sequences of ingroups (*D. japonicus* only) of dataset 1 (cytb) and dataset 2 (12S rRNA, tRNA Valine, 16S rRNA, and cytb) in ASAP web (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with default settings and p-distance among each sequence. The other is Bayesian implementation of the Poisson Tree Processes (bPTP, Zhang *et al*. 2013) based on model analyses. For this method, we processed the estimated BI tree from both datasets as input files in bPTP webserver (https://species.h-its.org) applying 100 Markov chain Monte Carlo (MCMC) runs of 500 000 generations each, sampling every 1000 generations, implementing a burn-in of 10%. *Hyla chinensis* and five *Dryophytes* species except for *D. japonicus* were specified as outgroup taxa.

MIG-seq analyses: For MIG-seq analyses, we used two samples of *D. immaculatus*, two samples of *D. suweonensis*, and 37 samples of *D. japonicus*. We extracted total DNA following the procedure shown in mtDNA analysis, and sent them to the genetic analyses service of Bioengineering Lab. Co., Ltd. (MGI, DNBSEQ-G400 system). From the raw sequences obtained, we cut primer and adapter sequences from each read using Trimmomatic

0.36 (Bolger *et al.* 2014). We also eliminated the reads with quality score less than 30, and unified the read length by excluding pair reads less than 50 bp and cutting sequences after the 50th site. The quality-filtered reads were then used as input data for SNP detection with Stacks 1.35 (Catchen *et al.* 2011) following the procedure shown in Matsui *et al.* (2019). The stacking process were done in three datasets; all samples (dataset 3a), *D. japonicus* only (dataset 3b), and populations of *D. japonicus* around the hybrid zone (dataset 3c; see below). The setting of basic parameters are as follows; m = 5, M = 5, and N = 1 in 'ustacks' option, n = 2 in 'cstacks' option, r = 0.70 and p = 10 in 'populations' option. The output formats were PHYLIP (dataset 3a), STRUCTURE (dataset 3b), and GENEPOP (dataset 3c).

(1) Phylogenetic inference

From the dataset 3a including outgroups, we eliminated invariable sites, and estimated the maximum likelihood tree using RAxML-NG (Kozlov *et al.* 2019) with the evolutionary model GTR+G with the corrections of acquisition bias (Leaché *et al.* 2015). We estimated node supports by 1000 bootstrapping replications. We used *D. suweonensis* and *D. immaculatus* as outgroups to root the tree.

(2) Genetic structure analysis

From the dataset 3b, we used STRUCTURE 2.3.4 (Pritchard *et al.* 2000) for number of clusters assumed (K) of 1 to 6 with 10 million MCMC iterations, sampled a tree every 100 generations, and discarded the initial 10% as burn-in. We estimated Evanno's deltaK value (Evanno *et al.* 2005) by STRUCTURE HARVESTER (Earl & von Holdt 2012). Especially in the analysis under K=2, we estimated the inherited fraction (Q-value) of the genome from two lineages. Hereafter, the Q-values for each individual in the eastern and western lineages are referred to as Q_{east} and Q_{west}, respectively.

For the data in STRUCTURE format, we also conducted the principal component analysis (PCA) using the package 'adegenet' (Jombart 2008) of R 3.0.3 (R Core Team 2014), and plotted the principal component score of each sample for the first, the second, and the third axes.

(3) Geographic cline analysis

Genetic clines for SNPs were fitted using the Metropolis-Hastings MCMC algorithm implemented in the package HZAR v2.5 (Derryberry *et al*. 2014) and the extent of mismatch between them was checked. We used 19 samples from Chubu, Kinki, Chugoku, and Shikoku District including samples from hybrid zone at western Kinki (Supplementary Table 3; DOI: 10.6084/m9.figshare.28067339), and estimated a cline using the Q_{east} score for each individual as determined in our STRUCTURE analysis (K=2). Although two populations from Wakayama Prefectures (sites 69 and 70) located close to this area, we excluded them from this analysis because they might belong to another hybrid zone in the Kii Peninsula. To collapse sample localities into the 1-dimensional axis, we designed the site 41 (where Q_{east} and Q_{west} were closest to 0.500) as the tentative center of the contact zone. We measured the minimum geographic distance of each locality from the site. If a population was located on the western side of the line, the distance was expressed as a negative value and vice versa (Supplementary Table 3). We estimated several parameters, including width (w), center (c), delta (d, distance between the center and the tail), and tau (t, slope of the tail). We fit three possible combinations of trait intervals [pMin, pMax] (fixed to 0 and 1, observed values, and estimated values) and five possible combinations of fitting tails (none, left only, right only, mirror tails, and both tails estimated separately) as described by Derryberry *et al*. (2014) using the Metropolis-Hasting algorithm in R 3.1.0 (R Core Team 2014). We compared these 15 models to a null model with no clinal transition. We ran three independent chains for 100 000 and assessed them for convergence and stability by visualizing the MCMC traces. We then discarded 10% generations as burn-in. We performed model selection using corrected AIC (AICc) based on maximum-likelihood estimate cline curves.

(4) Estimation of migration rates

To elucidate the maintenance mechanism of the hybrid zone, we estimated more recent migration rates (within the past few generations) among parental lineages and hybrid swarms using BayesAss v3.0 (Wilson and Rannala 2003). We used 19 samples analyzed in (3), and classified them into three genetic classes (Pure Eastern, Hybrid swarm, and Pure Western). We regarded five samples located close to the mtDNA boundary (neither Q_{east} nor Q_{west} exceeded 0.9) as the hybrid swarm. We ran the program with 1 000 000 MCMC iterations, sampling every 1000 generations following 10% generations of burn-in. Mixing parameters for migration rates, allele frequencies, and inbreeding coefficients were set to 0.35, 0.90, and 0.10, respectively, to set the acceptance rates of each parameter around 40%, following the BayesAss Edition 3.0 user's manual (Rannala 2007) recommending to keep them between 20% and 60%. We used Tracer version 1.7.2 (Rambaut *et al.* 2018) to check the likelihood convergence.

Morphological analyses: For morphological analyses, we used 781 adult specimens of *D. japonicus* from Japan and Korea. Vouchers and locality names of the specimens are deposited as the supplementary Table 1 in Figshare (DOI: 10.6084/m9.figshare.28067339). Most specimens were fixed in 10% formalin and later preserved in 70% ethanol and stored at the Aichi University of Education (AUEZ), Graduate School of Human and Environmental Studies, Kyoto University (KUHE), and National Museum of Nature and Science, Tokyo (NSMT). Some Korean specimens were loaned from California Academy of Science (CAS) and Osaka Museum of Natural History (OMNH). Sex and maturity of specimens were determined by observation of gonads and secondary male sexual characters, such as presence of nuptial pad on finger and presence of vocal sac. Females with mature ovaries and/or convoluted oviducts were regarded as adults.

Adult morphology: We chose 200 samples (100 males and 100 females; 40 samples from each of five mtDNA genetic groups). For the specimen choice, we used a distribution map of each mtDNA lineage obtained in the genetic survey, and picked up specimens collected from each area. We tried to choose as many collection sites as possible to avoid geographical sampling bias (Supplementary file 1). We took the following body measurements to the nearest 0.1 mm with dial calipers under a binocular dissecting microscope when necessary, mainly following Matsui (1984); (1) snout-vent length (SVL), (2) head length (HL); (3) snout-nostril length (S-NL); (4) nostril-eyelid length (N-EL); (5) nostril-upper labial length (N-LL); (6) snout length (SL); (7) eye length (EL, including eyelid); (8) tympanum-eye length (T-EL); (9) horizontal tympanum diameter (TDh); (10) vertical tympanum diameter (TDv); (11) head width (HW); (12) internarial distance (IND); (13) intercanthal distance (ICD); (14) interorbital distance (IOD); (15) upper eyelid width (UEW); (16) first finger length (1FL, from distal edge of inner palmar tubercle to the tip of finger); (17) lower arm and hand length (LAL); (18) inner palmar tubercle length (IPTL); (19) hand length (HAL, from proximal edge of palmar tubercle to the tip of the third finger); (20) third finger disc width (3FDW); (21) first toe length (1TL, from distal edge of inner metatarsal tubercle); (22) inner metatarsal tubercle length (IMTL), (23) fourth toe disc width (4TDW); (24) hindlimb length (HLL); (25) thigh length (THIGH); (26) tibia length (TL); (27) foot length (FL).

Color pattern of thigh: As a candidate of new diagnostic character, we examined the color patterns of the rear of thigh. We separated the specimens into four categories (Fig. 1); (A) large white blotches present, (B) small white dots present, (C) few or no white patterns, but black patterns with clusters of melanophores present, (D) no obvious patterns on the rear of thigh.

Examination of syntypes of *Hyla japonica*: In the original description of *Hyla arborea* var. *japonica*, Günther (1859 "1858") mentioned three specimens (two adult and one half-grown specimens), donated from the Leyden Museum. As far as we are aware in the British Museum, they are applicable to BMNH 1844.2.22.107, containing three specimens. We temporally called them 107A, 107B, 107C in ascending order of body size, observed the color pattern, and measured SVL, HL, HW, EL, and TL.

Results

Molecular analysis

MtDNA: We obtained 809 bp of mitochondrial cyt b (dataset 1), of which 305 bp were variable and 222 bp were parsimony informative.

(1) Phylogenetic inferences

Phylogenetic analyses employing ML and BI methods yielded nearly identical topologies, and only the ML tree is presented in Fig. 2A. *Dryophytes japonicus*, forming a monophyletic group against other congeneric species, included two major clades, which is apparently corresponding with Clade A and Clade B in Dufresnes *et al.* (2016). In clade A, two subclades were recognized; North Japan clade: samples from Sakhalin, Hokkaido, and northeastern part of Honshu including Sado Island; Central Japan clade: samples from central part of Honshu. In the clade B, three subclades were recognized; Chugoku-Shikoku clade: samples from western part of Honshu and Shikoku; Kyushu clade: samples from Kyushu and adjacent islands; Korea clade: samples from Tsushima Island (Japan), Korea, China, Mongolia, and Russian mainland. The relationship among three subclades remained unknown.

We chose each two representatives from five genetic groups found above, and obtained 3,405 bp of 12S rRNA, tRNA Valine, 16S rRNA, and cyt b (dataset 2), of which 780 bp were variable and 484 bp were parsimony

informative. The genetic distances among the taxa examined are shown in Table 1. Completely identical topologies were obtained between phylogenetic analyses employing ML and BI methods, and the ML tree is presented in Fig. 2D (left). Phylogenetic relationships were fundamentally identical to those obtained in the analyses of cyt b alone, but monophyletic relationship between *D. japonicus*, *D. immaculatus*, and *D. suweonensis* against other congeneric species was supported.

(2) Species delimitation tests

Both of two tests in ASAP (dataset 1 and 2) resulted in the species delimitation regarding three subsets which were assigned to North Japan clade, Central Japan clade, and Chugoku-Shikoku+Kyushu+Korea clades (Supplementally Tables 4a, b; DOI: 10.6084/m9.figshare.28067339). The bPTP test on dataset 2 resulted in the same delimitation as ASAP, whereas the bPTP test on dataset 1 supported the delimitation of 31 subsets (Supplementally Table 5a, b; DOI: 10.6084/m9.figshare.28067339).

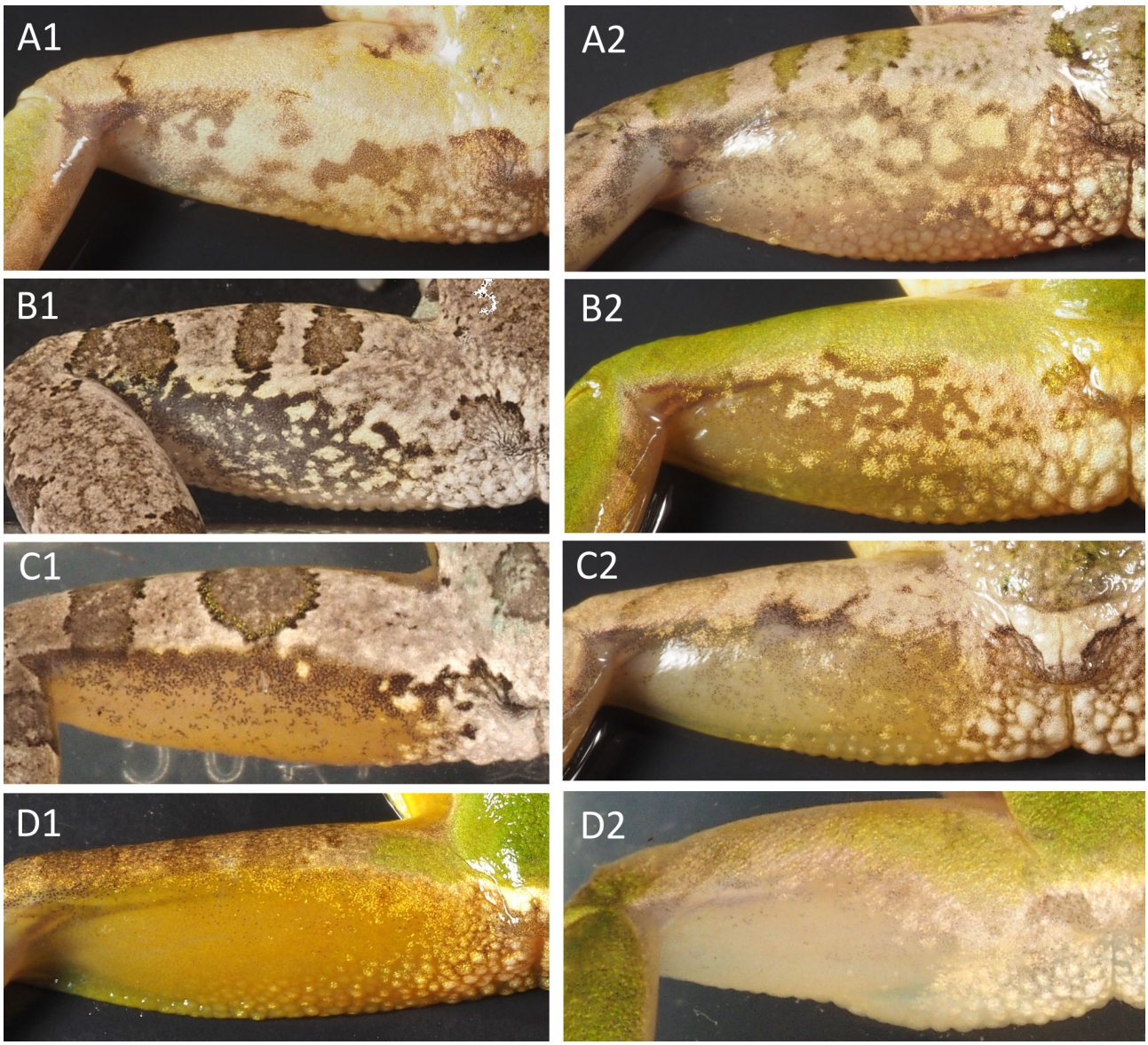

FIGURE 1. Patterns of the rear of thigh; A. Large white blotches (A1: AUEZ4073, A2: AUEZ4087 from Niigata Pref.), B. Small white dots (B1: AUEZ4095, B2: AUEZ4018 from Niigata Pref.), C. Few or no white patterns, but black patterns with clusters of melanophores present (C1: AUEZ4021 from Kyoto Pref., C2: AUEZ4091 from Aichi Pref.), D. No obvious patterns (D1: AUEZ4093 from Fukuoka Pref., D2: AUEZ4002 from Aichi Pref.).

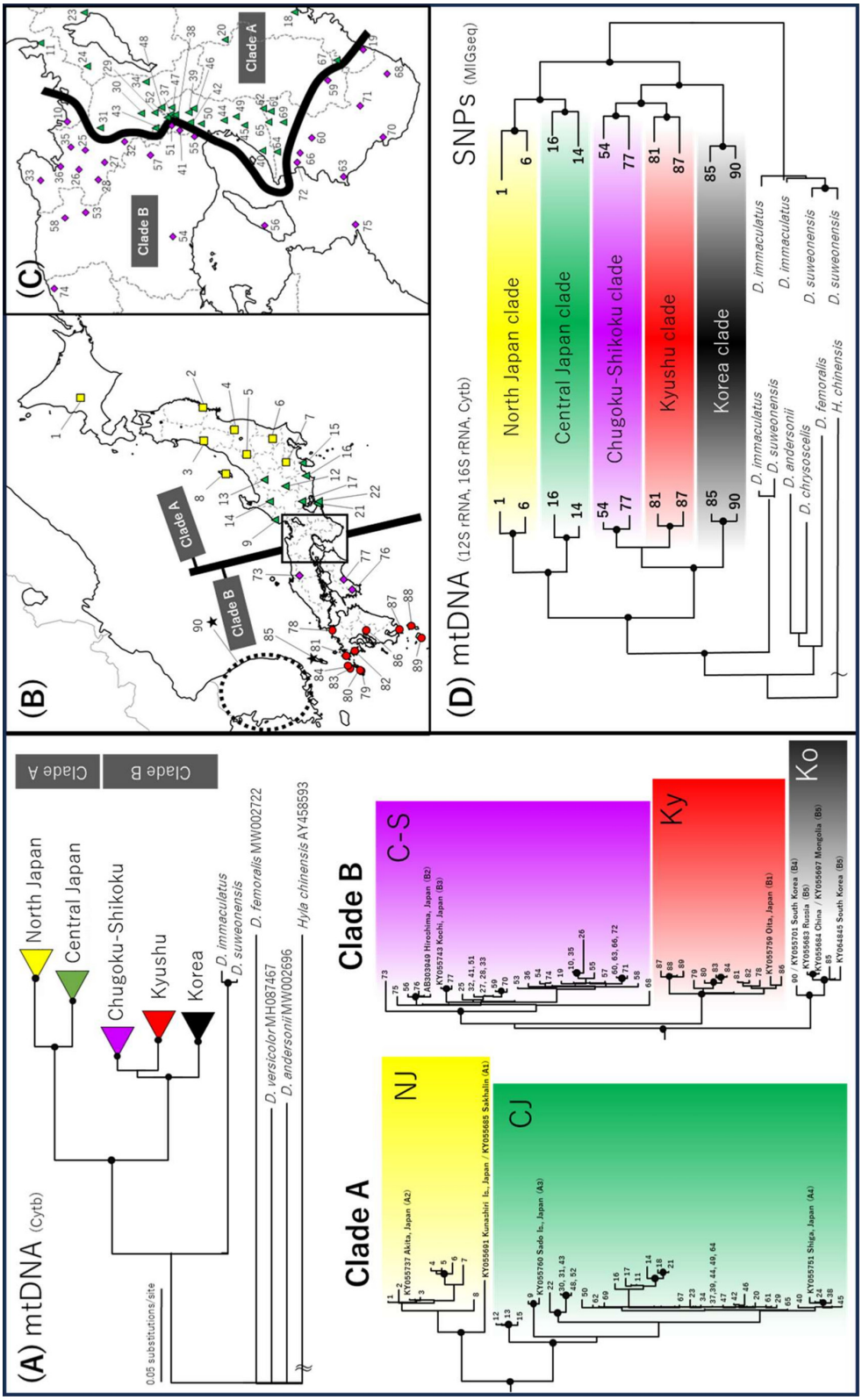

FIGURE 2. (A) ML tree based on sequence of mitochondrial cytb genes for samples of *Dryophytes*. Nodes with black circles satisfy the enough support values (>70% in bootstrap support for ML inference and >95% in Bayesian posterior probability). Accession numbers are shown for the data obtained from GenBank. Number of each sample corresponds with those shown in the supplementary Table 1 deposited in Figshare (DOI: 10.6084/m9.figshare.28067339). (B, C) Maps of Japan and Korea showing our sampling localities of mitochondrial analyses. Squares, triangles, diamonds, circles, and stars indicate North Japan, Central Japan, Chugoku-Shikoku, Kyushu, and Korea clades, respectively. (D) ML trees based on sequence of mitochondrial 12S, 16S rRNA, and cytb genes (left) and variable sites picked up from SNPs data (right).

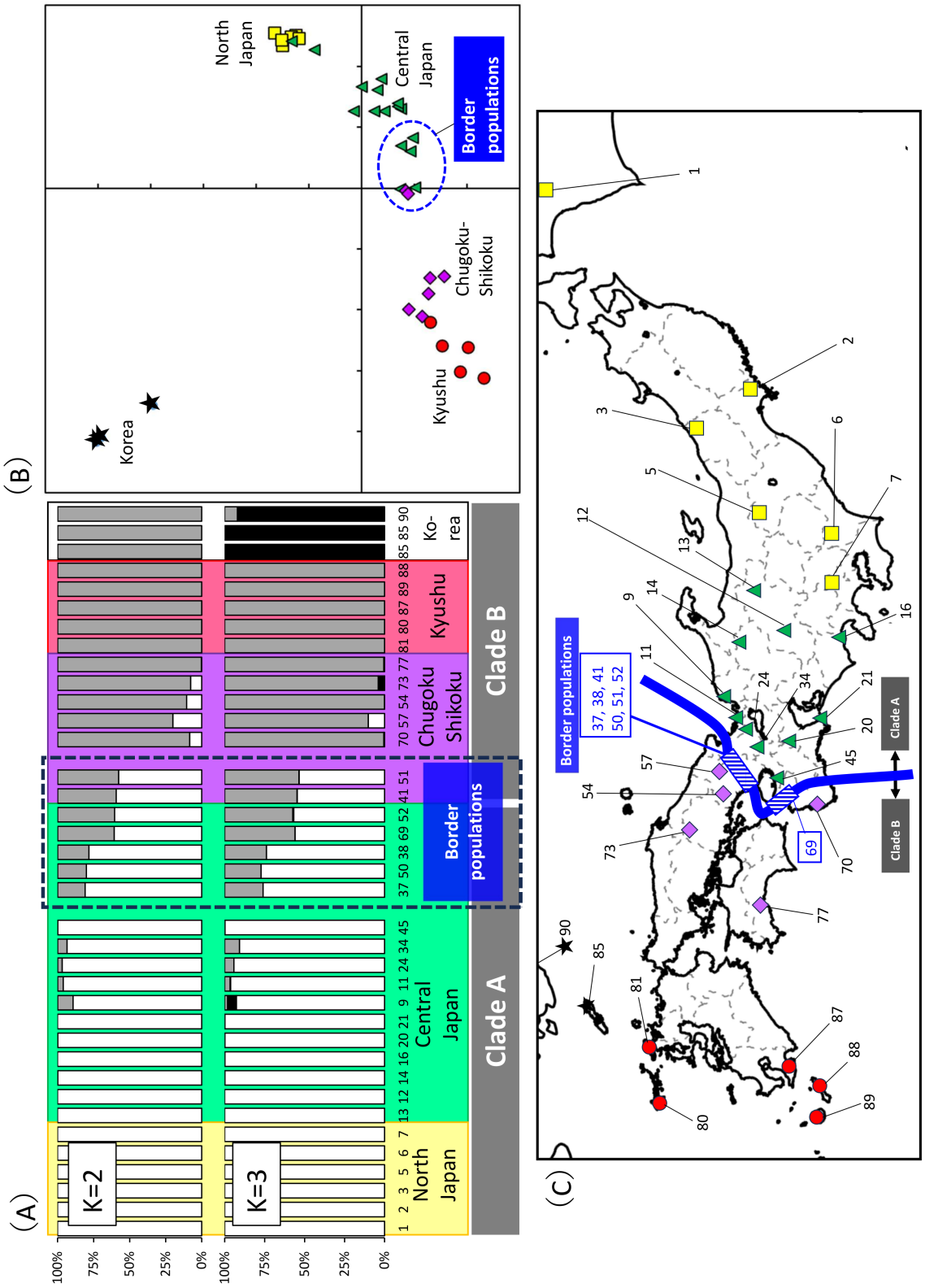

FIGURE 3. Results of MIG-seq (Multiplexed ISSR genotyping by sequencing), focusing on the isolation between *Dryophytes* in East and West Japan. (A) Genetic structure in each individual of mitochondrial groups of *Dryophytes*. Two and three primary genetic demes (K = 2, 3) identified by the STRUCTURE analysis. Samples in the dashed box were the individuals from the border of Clade A and B. (B) Plot of first against second principal scores of 397 alleles derived from SNPs data. Squares: North Japan clade, triangles: Central Japan clade, diamonds: Chugoku-Shikoku clade, circles: Kyushu clade, stars: Korea clade. (C) Map of samples examined in MIG-seq analyses. The line drawn in the middle of Honshu indicates the mitochondrial border of Clade A and B, and the samples on this line were shown in box.

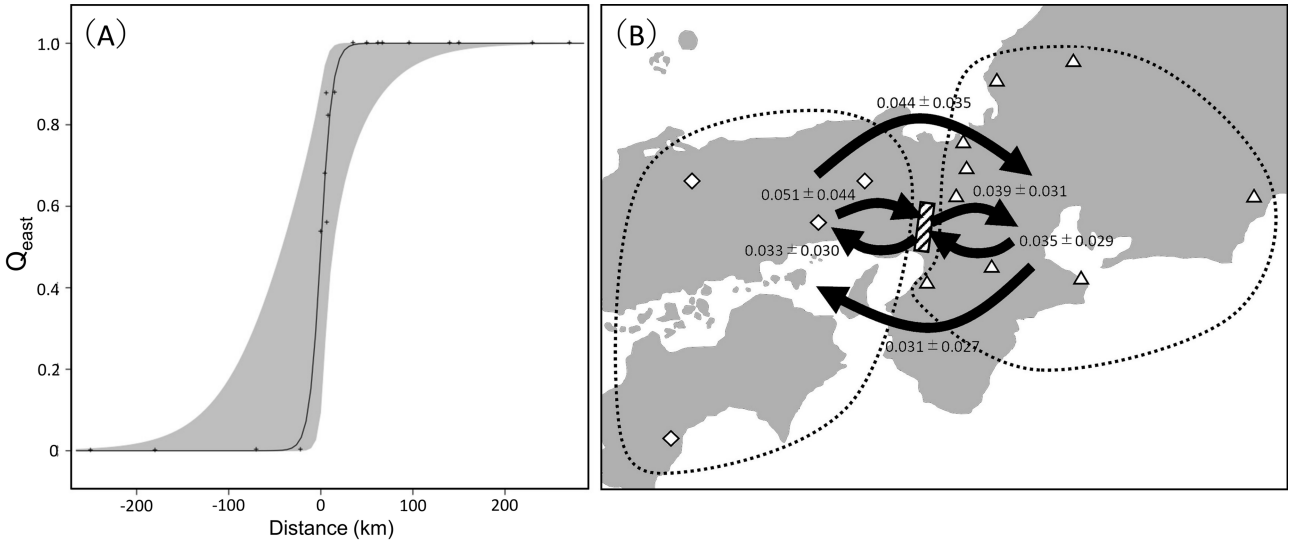

FIGURE 4. (A) Results of the genetic cline analyses based on SNPs. Crosses indicate the observed value for each sample. The solid curves indicate maximum-likelihood estimates of the clines, and the gray zone attached to the cline curve indicates 95% CI. (B) Migration rates among three genetic classes based on BayesAss analysis. The range of diagonal lines indicates the area defined as hybrid zone in the BayesAss analysis.

TABLE 1. Uncorrected p-distances (%) among mtDNA lineages of *Dryophytes* for 16S rRNA (upper right) and cyt b (lower left).

			D. j.				
	D. i.	*D. s.*	NJ	CJ	C-S	Ky	Ko
D. immaculatus	—	0.4	8.3	8.4	8.3	8.1	8.9
D. suweonensis	0.6	—	8.3	8.4	8.1	8.1	8.7
D. japonicus North Japan clade (Hokkaido)	15.3	15.0	—	3.5	5.8	5.8	5.3
D. japonicus Central Japan clade (Shizuoka)	14.1	13.9	6.7	—	6.0	6.0	6.1
D. japonicus Chugoku-Shikoku clade (Hiroshima)	13.1	12.7	11.3	9.2	—	1.7	3.1
D. japonicus Kyushu clade (Kagoshima)	14.2	14.1	11.0	9.4	3.1	—	3.1
D. japonicus Korean clade (Korea)	13.9	13.5	11.4	10.0	3.2	4.4	—

MIG-seq analyses:

(1) Phylogenetic inferences

After elimination of invariable sites of dataset 3a, we obtained 1,049 SNPs. The tree obtained from the sequence data was fundamentally identical to mtDNA trees (Fig. 2D right), but in clade B, two Japanese subgroups (Chugoku-Shikoku and Kyushu clades) formed a sister group against the continental one (Korea clade).

(2) Genetic structure analysis

Among 37 individuals of *D. japonicus* of dataset 3b, 1,605 genomic SNP loci were scored and subjected to estimation of genetic structure. As a result, Evanno's deltaK was highest at K = 2, followed by K =3, and we showed those two results in Fig. 3. The isolation between Clade A and B was apparent in both results, except for the samples on the mitochondrial border of the two clades, which seemed to be their hybrid populations. Further, in the result of K = 3, the isolation between the Korea subclade and others was obvious.

Also, in the result of principal component analysis (PCA) of dataset 3b, the separation between Clade A and B was apparent in the first axis, although samples from the mitochondrial border were plotted at the middle of them. In Clade A, samples of North Japan and Central Japan subclades basically formed distinct clusters, although two samples of Central Japan subclade (12, 13) were much closer to the cluster formed by the samples of North Japan subclade. In Clade B, samples of Korea subclades formed a cluster, apparently remote from others. Plots of

Chugoku-Shikoku and Kyushu subclades seemed to form a single cluster. The proportion of contribution for the first to fifth axis was 9.8%, 6.6%, 5.6%, 4.2%, and 3.9%, respectively.

(3) Geographic cline analysis

The HZAR analysis based on Q_{east} data indicated a steep clinal pattern of variation through the hybrid zone, and the null model without any clinal variation had higher AICc values than those of other cline. The best model at an AICc of 5.16 was the model in which pMin and pMax were fixed to 0 and 1 without any fitting of tails. The geographic cline analysis estimated that the center and width of the cline were 0.01 km [estimated variation (the range of parameter values that are within two log likelihood units of the maximum likelihood for a provided character vector of parameters): −47.4 to 14.6] from the base point and 25.1 km (estimated range: 15.5–160.0), respectively (Fig. 4A).

(4) Estimation of migration rates

Nuclear gene flows in the recent generation were estimated to be symmetric (Fig. 4B). The migration rate from pure eastern lineage to the hybrid swarm (0.035±0.029) was approximately similar with that from pure western group to the hybrid swarm (0.051±0.044). The values were also approximately similar with those from hybrid swarm to pure eastern lineage (0.039±0.031) and pure western lineage (0.033±0.030). The migration rates from pure eastern lineage to pure western lineage (0.031±0.027) and vice versa (0.044±0.035) did not differ from values shown above.

Morphometric comparison

Choice of specimens for morphometric analyses: Through the comparisons among 20 representatives from five groups (North Japan, Central Japan, Chugoku-Shikoku, Kyushu, and Korea groups), no significant differences of SVL were detected (ANOVA, $p>0.05$) in males and females.

Ratios of each character to SVL: Through Dunn's multiple comparisons of the ratio of 29 measurements to SVL among five groups (North Japan, Central Japan, Chugoku-Shikoku, Kyushu, and Korea groups), we found 14 and 21 pairs of significant difference in males and females, respectively. Among them, five and 13 were detected between two major clades in *D. japonicus*; Clade A (North Japan and Central Japan groups) and Clade B (Chugoku-Shikoku, Kyushu, and Korea groups). North Japan males had larger relative values of HW and IMTL than in Kyushu males, while they also had smaller relative values of 1TL than in Korean males. Central Japan males had larger relative values of LAL and smaller relative values of 1TL than in Korean males. North Japan females had larger relative values of HW, 1FL, and HAL than in Kyushu females, while they also had larger relative values of N-LL, 1FL, HAL, and LAL than in Korean females. Central Japan females had larger relative values of LAL than in Kyushu females while they also had larger relative values of N-LL, TDh, IPTL, LAL, and FL than in Korean females. It is notable that such differences between major clades were detected only in the pairs relating with the Kyushu or the Korea groups, and no significant differences were found in the pairs including the Chugoku-Shikoku group.

Between two groups in the clade A in East Japan (North Japan and Central Japan), only a single case of significant difference was detected (North Japan males had larger relative values of IMTL than in Central Japan males). Between two groups in the clade B in West Japan (Chugoku-Shikoku and Kyushu), no significant differences were detected. However, between those groups and the Korea group, there were many significant differences. Korean males had larger relative values in 1TL and smaller relative values in LAL and HLL than in Chugoku-Shikoku males, while it also had larger relative values in HW and smaller relative values in LAL, HLL, TL than in Kyushu males. Korean females had larger relative values in IOD and smaller relative values in EL, TDh, and LAL than in Chugoku-Shikoku females, while it also had larger relative values in IOD and THIGH than in Kyushu females.

CANDISC analysis: We conducted CANDISC analysis for five groups (Fig. 5; North Japan, Central Japan, Chugoku-Shikoku, Kyushu, and Korea groups). In both sexes, CANDISC analysis revealed that the plot range of the Korea group was separated from the other four groups on the first axis (CAN1). Most Japanese groups overlapped their range even in CAN2, but the North Japan group and the Kyushu group shared few (males) or no (females) plot ranges. The Central Japan and the Chugoku-Shikoku groups share an almost identical range, which is in the middle between the ranges of the North Japan and the Kyushu groups.

TABLE 2. Measurements in *Dryophytes*. SVL (mean ± 2SE, in mm) and medians of ratios (r) of other characters to SVL, followed by ranges in parentheses. See text for character abbreviations.

males	North Japan	Central Japan	Chugoku-Shikoku	Kyushu	Korea
N	20	20	20	20	20
SVL	30.5±1.0 (26.9–35.0)	30.2±1.1 (25.2–34.2)	29.5±1.0 (25.9–34.3)	30.9±0.9 (26.1–33.6)	30.2±1.1 (26.5–35.0)
rHL	34.9 (30.8–40.3)	34.2 (32.5–36.4)	34.5 (32.7–37.9)	33.9 (32.3–36.5)	33.9 (32.2–37.5)
rS-NL	4.1 (3.3–5.1)	4.0 (2.9–5.4)	3.9 (2.9–4.6)	3.8 (3.3–4.3)	4.1 (3.1–5.6)
rN-EL	6.4 (5.2–7.1)	6.5 (5.4–7.5)	6.0 (5.0–8.0)	6.4 (5.4–8.9)	6.9 (5.3–8.7)
rN-LL	7.9 (6.7–8.8)	7.7 (6.4–8.3)	8.0 (6.5–9.2)	7.6 (6.7–8.4)	7.5 (3.5–8.2)
rSL	13.0 (8.1–14.2)	13.1 (6.4–15.5)	13.5 (11.9–14.5)	13.0 (11.4–14.1)	13.3 (12.3–17.4)
rEL	13.6 (10.9–15.6)	13.4 (11.7–15.7)	14.1 (12.0–15.6)	13.7 (12.3–15.1)	13.7 (11.9–14.8)
rT-EL	1.5 (1.2–2.1)	1.7 (1.2–2.3)	1.7 (1.3–2.9)	1.6 (1.3–2.2)	1.6 (1.0–1.9)
rTDv	5.4 (4.5–6.8)	5.4 (4.7–6.3)	5.3 (4.3–7.1)	4.9 (4.1–5.8)	5.3 (3.7–9.2)
rTDh	5.2 (4.1–6.0)	5.4 (4.0–6.0)	5.1 (4.2–6.3)	5.1 (3.8–5.8)	4.9 (3.4–6.3)
rHW	39.2 (34.6–42.2)	38.8 (36.1–41.4)	38.7 (35.2–41.0)	37.2 (35.6–40.0)	39.2 (34.6–42.2)
rIND	7.4 (6.9–8.6)	7.2 (6.5–8.3)	7.8 (6.2–8.8)	7.3 (6.7–8.7)	7.4 (6.3–8.3)
rICD	20.1 (18.8–20.7)	19.5 (18.3–22.7)	19.8 (18.6–22.0)	19.6 (17.6–21.6)	20.3 (19.1–21.9)
rIOD	10.8 (9.4–12.6)	10.3 (9.1–12.5)	10.5 (9.1–11.6)	10.4 (8.6–11.7)	10.9 (9.3–12.7)
rUEW	8.8 (7.5–10.4)	8.8 (7.1–10.5)	9.6 (7.0–10.8)	8.9 (8.1–9.9)	8.9 (8.0–10.5)
r1FL	9.6 (7.6–11.4)	9.2 (7.6–10.6)	9.3 (8.3–10.1)	9.8 (7.2–10.9)	10.1 (8.1–11.1)
rIPTL	5.6 (4.8–9.6)	5.4 (4.6–6.2)	5.4 (4.2–6.6)	5.5 (4.4–7.1)	5.2 (4.1–6.4)
r3FDW	4.4 (3.3–5.4)	4.2 (3.7–4.9)	4.6 (3.3–5.6)	4.3 (3.6–5.3)	4.7 (3.2–5.7)
rLAL	29.9 (27.2–33.0)	28.5 (25.0–31.6)	29.3 (26.0–33.1)	29.6 (23.8–31.3)	29.1 (26.9–31.5)
rHAL	10.0 (7.6–12.4)	9.9 (7.9–11.8)	9.8 (8.1–11.2)	10.5 (8.6–12.8)	11.1 (9.6–12.3)
r1TL	5.3 (4.6–6.0)	4.4 (4.0–5.9)	4.7 (3.7–5.6)	4.6 (3.4–5.7)	4.8 (3.6–6.2)
rIMTL	3.9 (3.1–4.8)	3.7 (3.0–4.5)	4.0 (3.2–4.5)	4.1 (3.2–5.1)	4.0 (2.9–4.7)
r4TDW	50.6 (46.7–54.9)	50.7 (46.0–54.5)	51.1 (46.0–56.0)	51.3 (43.7–55.2)	48.3 (44.9–51.5)
rHLL	149.9 (139.5–165.1)	150.1 (139.6–160.3)	152.4 (140.7–162.9)	153.9 (138.4–167.9)	145.1 (135.7–154.8)
rTHIGH	47.7 (44.7–52.3)	47.1 (44.9–49.8)	48.4 (44.8–52.8)	46.8 (43.6–52.2)	47.7 (44.2–52.6)
rTL	45.9 (43.6–51.2)	46.9 (41.0–51.0)	48.0 (43.2–51.2)	48.0 (43.3–52.0)	45.5 (41.9–48.5)
rFL	45.3 (41.6–49.8)	44.7 (40.9–49.3)	45.6 (39.7–50.7)	45.7 (40.7–49.2)	44.5 (37.7–48.5)

.....continued on the next page

TABLE 2. (Continued)

females	North Japan	Central Japan	Chugoku-Shikoku	Kyushu	Korea
N	20	20	20	20	20
SVL	32.9±1.4 (27.6–42.4)	33.2±1.3 (29.2–38.1)	31.8±0.8 (27.8–34.9)	32.6±0.9 (29.6–36.2)	31.7±1.1 (27.9–37.0)
rHL	34.1 (31.7–36.5)	34.4 (31.2–37.2)	34.5 (32.8–38.0)	33.7 (31.8–37.2)	33.4 (31.4–36.8)
rS-NL	4.2 (3.0–5.0)	3.9 (2.9–4.9)	4.1 (3.0–5.0)	3.7 (2.8–5.3)	4.1 (2.5–4.9)
rN-EL	6.4 (5.7–8.1)	6.2 (5.1–8.2)	6.7 (4.6–7.8)	6.4 (5.5–7.6)	6.7 (5.7–7.8)
rN-LL	8.1 (7.2–9.1)	7.9 (7.1–8.8)	8.0 (7.0–8.7)	8.1 (7.2–8.8)	7.1 (6.1–7.8)
rSL	13.6 (8.0–15.6)	13.2 (8.1–14.4)	13.4 (7.7–14.5)	12.9 (11.2–14.4)	13.0 (11.2–14.3)
rEL	13.9 (11.8–15.4)	13.6 (11.7–16.0)	14.4 (12.4–16.3)	13.7 (12.0–15.4)	13.0 (11.4–15.1)
rT-EL	1.6 (1.2–2.2)	1.6 (1.0–1.9)	1.6 (1.2–2.5)	1.5 (1.0–2.2)	1.7 (0.3–2.4)
rTDv	5.3 (3.2–6.1)	5.3 (4.0–6.3)	5.4 (4.1–7.1)	5.0 (4.2–5.9)	4.7 (3.3–6.1)
rTDh	5.2 (3.2–6.5)	5.2 (4.3–6.4)	5.4 (4.5–7.4)	4.9 (3.4–6.1)	4.4 (3.7–6.3)
rHW	38.8 (35.8–41.7)	38.4 (36.1–41.1)	37.8 (34.7–41.5)	37.1 (34.9–39.2)	36.9 (33.0–41.1)
rIND	7.8 (6.6–9.5)	7.6 (6.3–9.3)	7.4 (6.6–8.1)	7.4 (6.8–8.9)	7.4 (6.5–8.6)
rICD	19.6 (17.6–21.7)	19.7 (18.2–22.2)	19.9 (18.4–21.1)	19.8 (17.4–21.3)	19.6 (17.3–21.2)
rIOD	10.3 (8.7–11.8)	10.6 (8.8–12.5)	9.8 (8.9–11.4)	10.0 (8.5–10.6)	10.7 (9.5–12.4)
rUEW	8.8 (7.2–10.6)	8.9 (8.1–10.4)	9.3 (7.8–10.9)	9.1 (7.4–10.8)	8.7 (7.1–10.0)
r1FL	10.1 (8.7–11.5)	9.8 (8.4–11.6)	9.5 (7.8–11.5)	9.1 (7.9–10.4)	9.2 (8.0–10.6)
rIPTL	5.6 (4.0–6.4)	5.9 (4.8–9.3)	5.4 (4.8–6.5)	5.7 (4.1–9.3)	5.1 (3.4–6.9)
r3FDW	4.5 (3.7–5.1)	4.6 (3.9–5.8)	4.5 (3.5–5.6)	4.9 (3.5–5.7)	4.4 (2.4–5.7)
rLAL	30.9 (28.7–34.1)	29.7 (28.8–32.3)	29.0 (26.6–33.2)	29.0 (26.3–31.1)	28.8 (26.4–31.1)
rHAL	10.3 (8.4–12.1)	10.7 (8.3–12.0)	10.0 (8.9–12.9)	10.5 (8.7–13.3)	10.2 (8.9–11.7)
r1TL	5.0 (4.2–5.7)	5.1 (4.2–6.7)	4.8 (3.8–5.9)	4.7 (3.7–5.5)	5.1 (4.0–6.0)
rIMTL	3.9 (3.0–4.6)	4.2 (3.1–5.9)	4.0 (2.6–4.8)	4.6 (2.9–5.2)	4.0 (2.7–4.6)
r4TDW	51.2 (48.4–55.6)	52.1 (48.6–54.2)	50.0 (43.0–55.9)	49.3 (44.7–52.5)	47.8 (43.7–50.4)
rHLL	149.5 (144.7–166.9)	153.3 (141.6–169.3)	152.1 (138.1–164.4)	151.0 (139.5–158.9)	149.9 (139.1–157.8)
rTHIGH	47.6 (45.1–53.7)	47.8 (44.2–54.4)	47.9 (42.0–53.3)	46.2 (42.1–51.3)	49.0 (43.9–51.6)
rTL	46.3 (43.3–51.0)	47.4 (44.0–50.8)	46.8 (42.4–50.7)	47.2 (43.9–51.8)	46.0 (41.5–49.5)
rFL	46.4 (41.4–50.0)	46.8 (43.4–50.8)	45.2 (41.3–51.1)	45.4 (40.3–49.3)	44.3 (41.4–48.0)

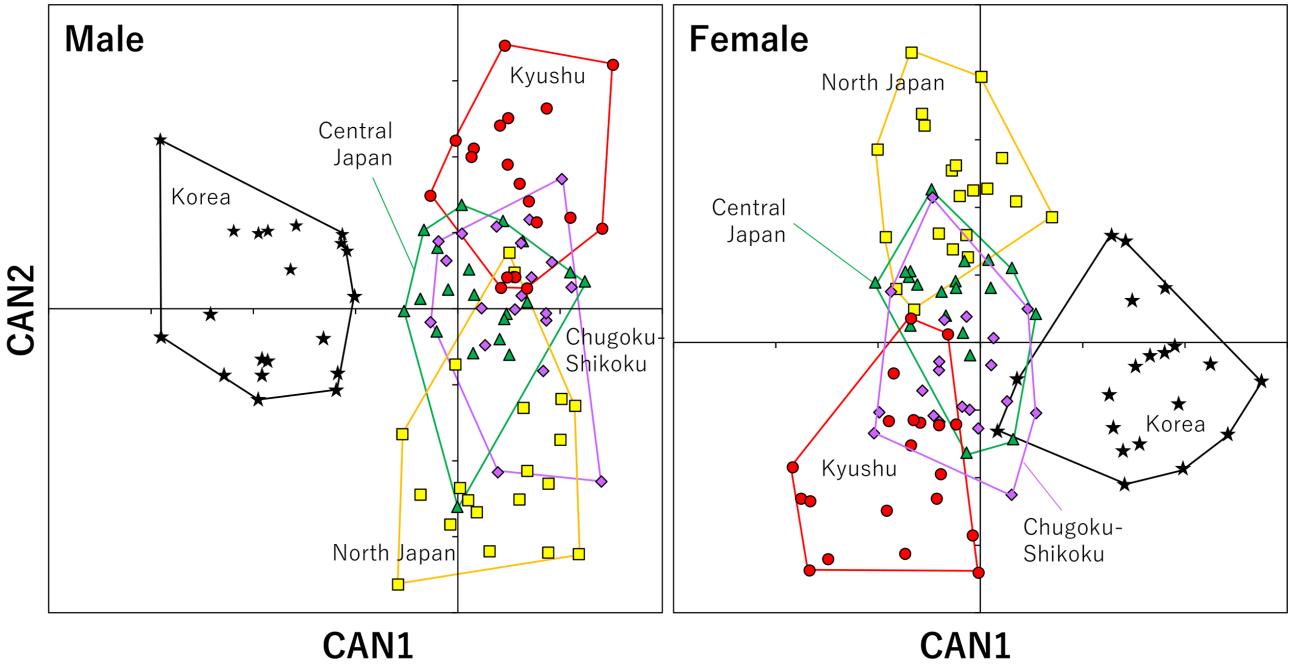

FIGURE 5. Plot of first against second canonical 29 morphological variates from CANDISC for male (left) and female (right) samples of *Dryophytes japonicus*. Squares: North Japan group, triangles: Central Japan group, diamonds: Chugoku-Shikoku group, circles: Kyushu group, stars: Korea group.

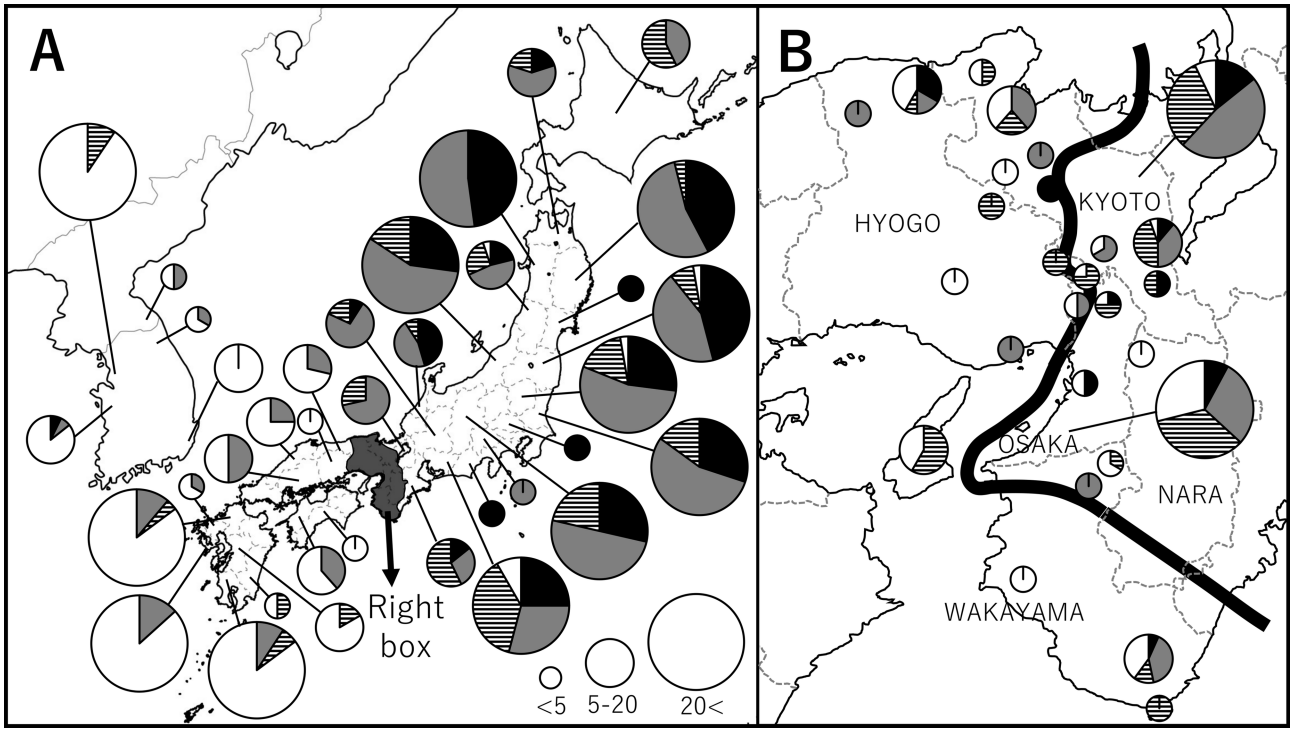

FIGURE 6. Map of examined area showing the result of color pattern of rear of thigh. Data was summarized with prefectural or province level (A) except for Kansai District, central Japan (B), where data of each locality is shown. The line shown in B indicates the mitochondrial border line. Black: category A (large white blotches), Gray: category B (small white dots), Stripe: category C (few or no white patterns, but black patterns with clusters of melanophores present), White: category D (no obvious patterns).

In males, the eigenvalues of the first (CAN1) and second (CAN2) axes accounted for 3.357 (proportion: 0.614) and 1.385 (proportion: 0.253), respectively. On the first axis, the highest absolute magnitude of the standardized canonical discriminant coefficients was 2.287 of LAL, followed by HW (-1.344), and HLL (1.197). On the second axis, SVL (–1.179), TL (-0.936), and IMTL (0.692) were high contributors. In females, the eigenvalues of the first (CAN1) and second (CAN2) axes accounted for 2.840 (proportion: 0.499) and 1.947 (proportion: 0.342), respectively. On the first axis, the highest absolute magnitude of the standardized canonical discriminant coefficients was 1.494 of LAL, followed by N-LL (1.225), and THIGH (-1.137). On the second axis, HLL (2.526), HAL (-1.445), and FL (-1.209) were high contributors.

Color patterns of the rear of thigh: For the color patterns of the rear of thigh, there seemed to be an apparent geographic tendency between East and West Japan (Fig. 6A). In East Japan, most specimens had patterns (category A, B, and C) on the rear of thigh. On the other hand, in West Japan and Korea, most specimens lacked patterns of the rear of thigh (category D). Although patterns B and C were also seen in West Japan and Korea, patterns A were seldom seen there. Those tendencies could be approximately assigned to Clade A (East Japan) and Clade B (West Japan and Korea). However, the difference between genetic clades was not clear in the Kinki region, where they meet (Fig. 6B).

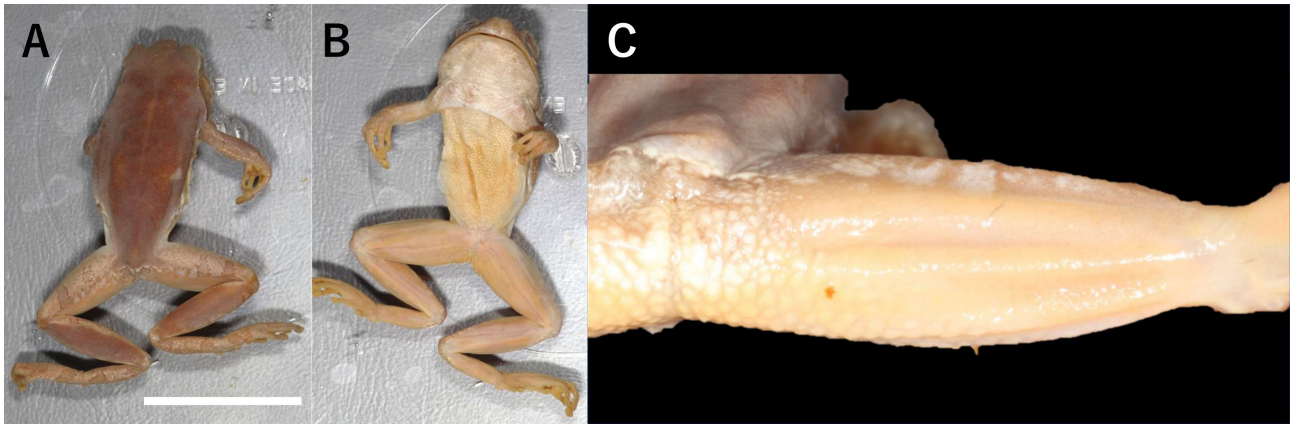

FIGURE 7. Dorsal (A) and ventral (B) views and the rear of thigh of the lectotype of *H. japonica* (=*Dryophytes japonicus*) (BMNH 1844.2.22.107B). Scale bar =20 mm.

Examination of syntypes and designation of lectotypes of *Hyla japonica*: Measurements of syntypes of *H. japonica* are shown in Table 3. BMNH 1844.2.22.107B and BMNH 1844.2.22.107C were supposed to be adult females, because their SVL (32.2 mm and 33.1 mm, respectively) were much larger than the minimum SVL of both sexes of our samples (Table 2, 3), but no apparent secondary sexual characters of male (e.g. outer vocal sacs) were seen. BMNH 1844.2.22.107A was probably a male, considering its weak outer vocal sac, but since its SVL (27.7 mm) was smaller than or relatively close to the minimum SVL of both sexes of our samples (Table 2, 3), we supposed that this might be a young individual, and probably applicable to "half-grown" sample mentioned by Günther (1859 "1858"). Here, we designate BMNH 1844.2.22.107B (Fig. 7) as the lectotype of *H. japonica*. Other two samples are designated as paralectotypes. The rear of thigh of these three specimens lacked any color patterns (Fig. 7C), and could be assigned to "pattern D" in our category.

TABLE 3. Measurements (mm) of the lectotype and paralectotypes of *Dryophytes japonicus*.

		Sex	SVL	HL	HW	EL	TL
BM44-2-22-107A	Paralectotype	Male	27.0	9.5	10.2	4.2	13.5
BM44-2-22-107B	Lectotype	Female	32.2	11.0	11.8	4.6	15.3
BM44-2-22-107C	Paralectotype	Female	33.1	11.5	12.0	4.8	16.4

This observation made us certain that the lectotypes and the paralectotypes of *H. japonica* were collected from southwestern Japan, where “pattern D” is dominant. On this basis, we concluded that the genetic group of southwestern Japan is the true *H. japonica*, and describe the group of northeastern Japan as a new species here.

Systematics

Dryophytes leopardus Shimada et Matsui sp. nov.

(English name: East Japan tree frog)
(Japanese name: Higashi-Nihon-Ama-gaeru)
(Fig. 8)
urn:lsid:zoobank.org:act:107E307B-3F40-4C3D-8F5A-4E4E890C9AC0

Hyla viridis?: Hallowell 1861, p. 500. "Simoda, Japan" (=Shimoda, Shizuoka, Japan)
Hyla arborea (part): Hilgendorf 1880, p. 120. "Yeso" (=Hokkaido, Japan)
Hyla arborea var. *japonica*: Bedriaga 1889, p. 475, 487. "Tokyo" and "Yezo" (=Hokkaido, Japan)
Hyla japonica: Camerano 1879, p. 895. "Tokio (Giappone)" (=Tokyo, Japan)
Hyla arborea japonica: Sclater 1892, p. 32. "Yezo, Japan" (=Hokkaido, Japan); "Kiga, Japan" (=Hamamatsu, Shizuoka, Japan)
Hyla stepheni (part): Nikolski 1905, p. 401. Jesso (=Hokkaido, Japan)
Hyla new taxon A: Dufresnes *et al.* 2016
Dryophytes japonicus (part): Duellman *et al.* 2016, p. 23

Holotype. KUHE 66109 (former AUEZ 3999), an adult male from Yoshiwara, Toyota City, Aichi Prefecture, Japan (35°00'56'' N, 137°05'38'' E, 10 m asl), collected on 25 October 2023 by Tomohiko Shimada.

Paratypes. KUHE 66111 (former AUEZ 4001) one adult male and KUHE 66110, 66112–66113 (former AUEZ 4000, 4002–4003), three adult females, data same as the holotype.

Referred specimens. KUHE 8812, 29190–29191, 37629, Aomori Prefecture; KUHE 29240, 29244, 29246, 32922, 32925, Iwate Prefecture; KUHE 39830, Miyagi Prefecture; KUHE 40056, T3124, Akita Prefecture; KUHE 13131–13132, 13383, 18480, 18485, Yamagata Prefecture; KUHE 43347, Fukushima Prefecture; KUHE 36528, Tochigi Prefecture; KUHE 28075–28076, Ibaraki Prefecture; KUHE 36146, 38829–38830, 38832, 39551–39552, 39554–30555, AUEZ 4026, 4036, 4039–4040, 4048, 4068, 4076, 4089–4091, 4099, Niigata Prefecture; KUHE 7950, 33017, Ishikawa Prefecture; KUHE 16099, 30510–30511, 30513, 42971–42973, 43010, AUEZ 3760–3761, Gifu Prefecture; KUHE 37382, 39976, Shizuoka Prefecture; AUEZ 2616, Aichi Prefecture; AUEZ 13455–13456, 25559, 42928–42929, 42934, 42936, 42938, 42941, Mie Prefecture, KUHE 24152, 25433, 33109, 34913, 39940, 41350–41351, Shiga Prefecture; KUHE 43235–43236, Kyoto Prefecture.

Etymology. The specific epithet is a Greek (and Latin) noun denoting a leopard, alluding to the color pattern of the rear of thigh observed in this species.

Diagnosis. A moderate-sized species of the genus *Dryophytes*, with adult SVL 25–35 mm in males and 27–42 mm in females. It shares most morphological characters with its sister species, *D. japonicus*, but usually differs in having the black and/or white patterns on the rear of thigh, and they definitely differ in mitochondrial and nuclear genome characters. It differs from other congeneric species in East Asia, *D. suweonensis* and *D. immaculatus*, in the presence of finger webbing and more developed toe webbing, as well as in the pattern of the rear of thigh.

Description of holotype (in millimeters). Snout-vent length (SVL) 30.9; body robust; head short, wider (HW 12.2, 39.4%SVL) than long (HL 10.3, 33.4%SVL); snout truncate, tip rounded in dorsal outline; projecting beyond lower jaw, slightly rounded in lateral profile; canthus sharp; lore vertical, concave; nostril below canthus, midway between tip of snout (S-NL 1.5, 4.9%SVL) and anterior margin of upper eyelid; internarial distance (IND 2.5, 8.0%SVL) subequal to nostril to eye (N-EL 2.3, 7.3%SVL); eye large, length (EL 4.2, 13.5%SVL) 1.8 times eye-nostril distance, equaling to snout length (SL 4.2, 13.5%SVL); interorbital (IOD 3.0, 9.7%SVL) wider than width of upper eyelid (UEW 2.7, 8.7%SVL) and internarial distance; pineal spot invisible; tympanum large and distinct, nearly circular (TDv=TDh 1.9, 6.0%SVL), about half eye diameter; vomerine teeth in indistinctly oval, small, and slightly oblique raised series (each of 3 teeth), the center posterior to line connecting posterior margins of choanae, connected with each other, but widely separated from choanae; tongue narrow anteriorly, moderately notched, without papilla; a pair of internal vocal sacs and vocal openings on corners of mouth. Forelimb stout (forelimb length 18.6, 60.1%SVL; LAL 15.0, 48.6%SVL); webbing poorly but distinctly developed; finger length formula: I<II<IV<III (Fig. 8C), second finger as long as third; finger tips with round adhesive discs having circummarginal grooves; disk of third finger largest (3FDW 1.23, 4.0%), two thirds of tympanum diameter; indistinct palmar tubercles and supernumerary tubercles present; subarticular tubercles prominent, circular; indistinct nuptial pads on dorsal, medial, and ventral surfaces of first finger extending from its base to subarticular tubercle, covered with

minute yellow asperities; inner palmer tubercle distinct, oval (IPTL 1.7, 5.5%SVL), subequal to tympanum diameter; indistinct skin fold present in outer side of forelimb, starting from disk of fourth finger to elbow. Hindlimb long (HLL 45.8, 148.6%SVL), about 2.5 times the length of forelimb; tibia (TL 13.8, 44.7%SVL) shorter than foot (FL 13.8, 44.8%SVL); heels do not touch with each other when limbs are held at right angles to body; tibiotarsal articulation of adpressed limb reaching posterior corner of eye; toe tips with round adhesive discs having circummarginal grooves; toe length formula I<II<III<V<IV; third toe subequal to fifth; toes moderately webbed, formula I 1–2 II 0–$2_{1/2}$ III 0–2 IV 2–1 V (Fig. 8D); excision of membrane between two outer toes reaching middle subarticular tubercle of fourth when toes in contact; webs thick, not crenulate; subarticular tubercles prominent, rounded; inner metatarsal tubercle distinct, oblong (IMTL 1.2, 4.0%SVL), equaling to 3FDW, less than half length of first toe (1TL 3.5, 11.2%SVL); outer metatarsal tubercle small but distinct; indistinct tarsal fold present, connecting tibiotarsal articulation to inner metatarsal tubercle. Two distinct skin folds connecting both arms present on pectoral region, starting from anterior and posterior edges of arms, respectively. Head and dorsum smooth; no dorsolateral fold; a supratympanic fold from eye, curving to axilla; ventral and ventrolateral side of trunk and ventral side of thigh coarsely granular.

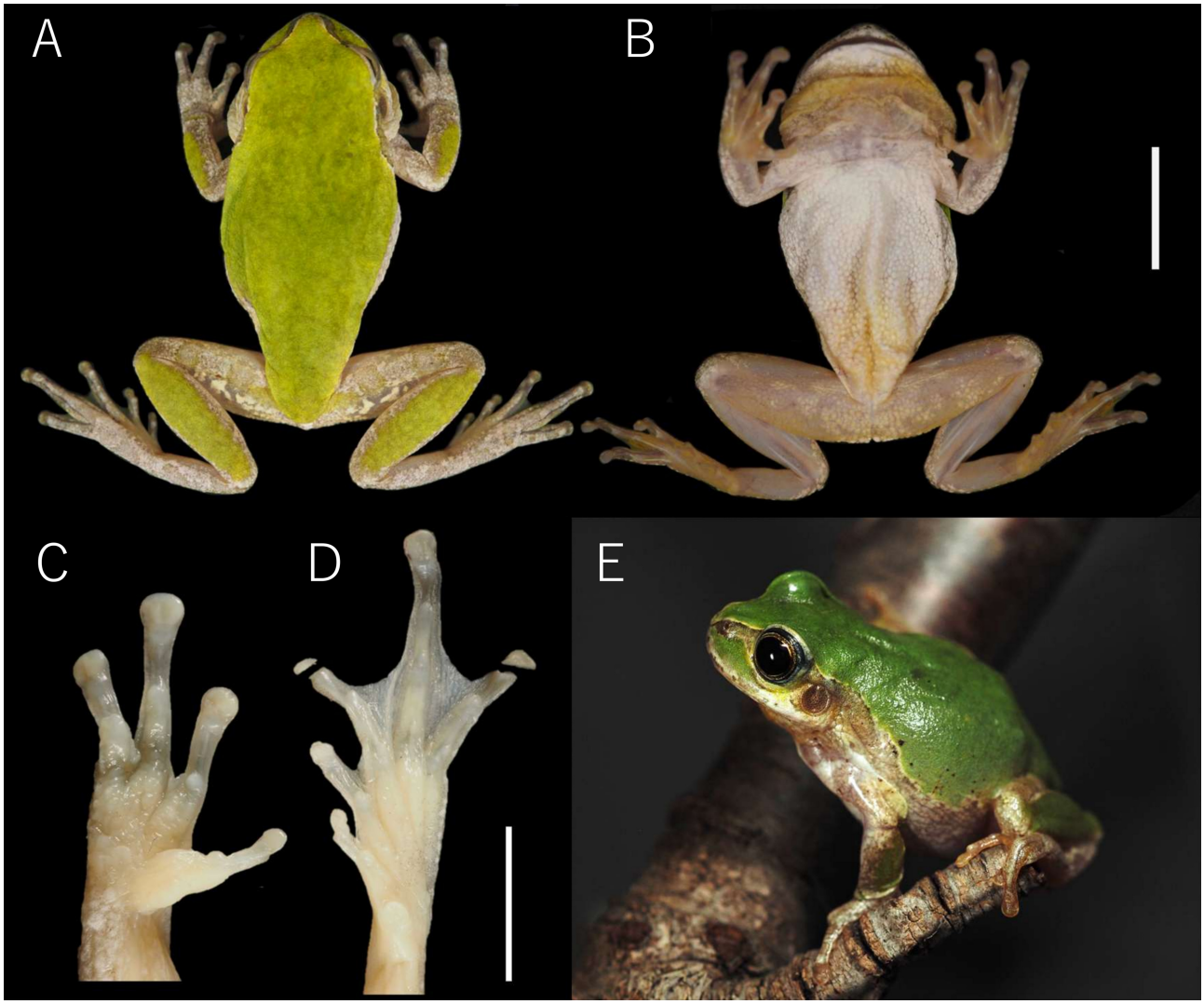

FIGURE 8. Dorsal (A) and ventral (B) views of the whole body, ventral view of left hand (C) and foot (D), and a lateral view in life (E) of the holotype of *Dryophytes leopardus* **sp. nov.** (KUHE 66109). Scale bar = 10 mm (A, B)/5 mm (C, D).

Color in life. Dorsum green fringed with a narrow yellowish white line on canthus, eyelid, supratympanic fold, and lateral side of trunk; a dark brown band running below canthus and supratympanic fold, vaguely expanding to lateral side of trunk; lores green below canthus; upper lip white with dark dots; upper half of tympanum surrounded by the band below supratympanic fold; dorsal side of thighs marked with dark crossbars; rear of thighs marked with

several white blotches with reticulated pattern formed by cluster of pigmentation; anterior half of ventrum white; vocal sac and posterior half of ventrum yellowish white; ventral surfaces of thigh and tibia yellowish pink.

Variation. Females are significantly larger in SVL (mean ± SD = 33.0 ± 3.2 mm, n = 40) than males (30.4 ± 2.0 mm, n = 40; t-test, $P < 0.01$). Size of N-LL, IND, and some characters of distal part of forelimbs (1FL, HAL, 3FDW, 1TL, and FL), all relative to SVL, tended to be greater in females than males (Mann-Whitney U-test, two-tailed, $P < 0.05$). Similar sexual differences tended to be seen in 4TDW and LAL, but p-values were slightly larger than 0.05 ($p=0.052$ and 0.058, respectively). Males tended to have more developed toe webbing than females. In the second and the third toes, outer edge of web reach at toe disk in males, but reach at the first articulation in females. In the fourth toes, two phalanges are free from inner webs in males, while three phalanges are free in females.

Although the center of vomerine teeth lays posterior to the line connecting the posterior borders of the choanae in the holotype, it lays anterior the line in a part of paratypic specimens (i.e. KUHE 66110), and the latter status seems to be common in this species (Matsui & Matsui, 1982).

Eggs and larvae. In the topotypic population, Aichi Prefecture, eggs laid at a time ranged from 109–161 (mean ± SD = 124 ± 25, n = 4) (Motohiro Nakamura, personal communication). Eggs each 1.2 mm in diameter and light brown in animal hemisphere. Eggs are usually laid in small clumps. A total of eight tadpoles in stages 31–35 (total length [TOTL] = 25.8–38.3 [mean ± SD = 33.5 ± 3.8] mm, head body length [HBL] = 11.1–14.0 [mean ± SD = 12.3 ± 1.0] mm), and three in stages 36–41 (TOTL 33.8–40.9 [mean = 37.4] mm, HBL=14.5–15.3 [mean = 14.8] mm), from the type locality were closely examined. Head and body slightly flattened above, spheroidal below; head body width (HBW) maximum slightly anterior to level of spiracle 54–68% (median = 62%) of HBL; head body depth (HBD) 89–108% (median = 97%) of HBW; snout rounded; eyes dorsolateral, visible from below; nostril open, dorsal, rim raised, midway between tip of snout and eye; internarial 29–58% (median = 49%) of interorbital. Oral disk anteroventral, emarginate, width 31–43% (median = 39%) of HBW; marginal papillae on upper labium with wide gap; lower labium with a continuous row of papillae, submarginal papillae present near corners; denticles 2(2)/3 (Fig. 9D) or 2(1–2)/3; beaks with black outer margins; outer surface smooth; margin finely serrate; upper beak weekly convex medially; neither beak divided. Spiracle sinistral, tube pointing upward and backward, almost completely attached to body wall. Anal tube dextral, attached to ventral fin; loops of gut visible ventrally in young larvae. Tail moderately long and lanceolate, both margins weakly convex, tapering gradually to slightly rounded tip; tail length 178–219% (median = 197%; only specimens with non-damaged tail) of HBL, maximum depth 30–36% (median = 36%) of length; dorsal fin origin at midpoint of body, deeper than ventral fin except near tail tip and body; ventral fin origin continuous to vent; caudal muscle moderately strong, maximum tail width 36–51% (median = 44%) of HBW; muscle depth at anterior one-third of tail 34–45% (median = 41%) of tail depth, steadily narrowed posteriorly, shallower than either fin in distal half of tail. Neuromasts on body surface are not discernible. In life dorsal and lateral body brown, spotted with clusters of dark pigmentations; venter white, scattered with dark gray on throat; tail scattered with black spots (Fig. 9A–C).

Karyotype. Diploid chromosome 2n = 24, with six large and six small pairs, that are homomorphic and lacking sexual difference (Seto 1964). Chromosomes Nos. 1 and 2 in the larger group and Nos. 8 and 9 in the smaller group are metacentric, while Nos. 3, 4, 5 in the larger group and Nos. 7, 10, 11, and 12 in the smaller group are submetacentric. The large chromosome No. 6 is subtelocentric, having satellites at the tip of the short arms. Although nucleolus organizer region (NOR) is supposed to exist on No. 6 as is usual in *Dryophytes* frogs (Anderson 1991), an additional examination is needed for this character because there are no reports which could be exactly assigned to this species.

Call. We analyzed mating calls of ten males, recorded at the topotypic population, Aichi Prefecture, Japan at an air temperature of 16.8°C on 1 June 2023 by T. Shimada. Calls (N=10; acoustic parameters of each individual were estimated from average of five continuous notes) consisted of a series of notes each emitted at an interval (between the beginnings of two successive notes) of 0.29 ± 0.01 (0.25– 0.32) s (Fig. 10). Each note was composed of 17.3 ± 0.57 (13–20) short pulses and lasted for 0.10 ± 0.00 (0.07–0.12) s. The fundamental frequency was 1.68 ± 0.08 (1.55–1.88) kHz and the dominant frequency was 3.48 ± 0.12 (3.30–3.80) kHz. There are weak frequency and intensity modulations and some clear harmonics. Although we collected those data from males calling alone, it is known that a pair of males often call alternately, and the intervals of notes become longer in such cases compared with those emitted alone (Aihara *et al*., 2006). The pulse duration is known to correlate positively with male body size, while the dominant frequency negatively correlate with it (Takahashi *et al.* 2016).

Comparisons. This species shares most morphological characters with its sister species, *D. japonicus*. However, the typical specimens of the new species is conspicuous in having a pigmented pattern of the rear of thigh (Fig. 1), which is not popular in *D. japonicus* and other congeneric species of East Asia (Fig. 6; Fei & Ye 2016). Yet, this diagnosis is not perfect because there is a pattern (category C) which is sometimes appear both in *D. japonicus* and the new species. Further, this diagnosis cannot totally be applied for the populations close to the specific border (Kinki region). Although several morphometric differences were detected among local groups of those species, no significant differences were found between *D. leopardus* **sp. nov.** and its adjacent populations of *D. japonicus* (Chugoku-Shikoku group) (See "Ratios of each character to SVL" in Morphometric comparison of Results section). *Dryophytes immaculatus* and *D. suweonensis* differ from the new species in lacking finger webbing (vs. rudimentary finger webbing present) and less developed toe webbing with its excision reaching the line connecting dorsal ends of the middle subarticular tubercle of fourth toe and distal subarticular tubercle of fifth toe (vs. excision of webbing not reaching the line; Kuramoto, 1980; Borzée *et al.* 2020).

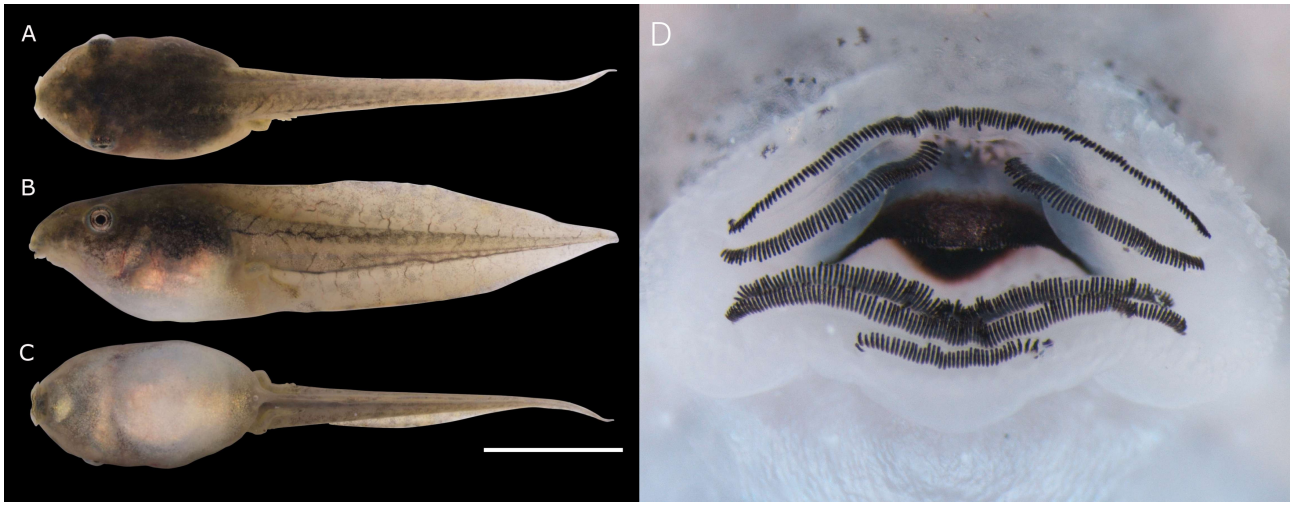

FIGURE 9. Dorsal (A), lateral (B), and ventral (C) views and the oral disc (D) of a tadpole of *Dryophytes leopardus* **sp. nov.** in stage 37 of Gosner (1960), collected on 18 June 2014 at the topotypic population (Toyota city, Aichi Pref., Japan). Scale bar = 10 mm.

From the New World congeners, the new species, *D. leopardus* **sp. nov.**, differs morphologically in the following manner (data from Beheld & King,1979 and Duellman, 2001): The new species has nearly smooth dorsal skin [vs. skin granulated in *D. gratiosus* (Le Conte)] and has finger webbing [vs. no webbing between fingers in *D. plicatus* (Brocchi) and *D. walkeri* (Stuart)]. In the new species, lores green below canthus and upper lip white with dark dots [vs. dark-edged light spot beneath eye in *D. arenicolor* (Cope), *D. avivoca* (Viosca), *D. versicolor* (Le Conte, 1825), and *D. chrysoscelis* (Cope)], and a narrow yellowish white line fringing on canthus and eyelid, and a narrow light line and a dark brown band running below canthus and supratympanic fold, vaguely expanding to lateral side of trunk [vs. white-edged lavender stripe on side in *D. andersonii* (Baird), a light-edged purple to black stripe from snout through eye and along side in *D. eximius* (Baird), *D. arboricola* (Taylor), and *D. wrightorum* (Taylor), white stripe along upper jaw and side of body in *D. squirellus* (Daudin), and sharply defined light stripe along upper jaw and side of body in *D. cinereus* (Schneider)]. In the new species, color pattern of rear of thigh is variable, and large or small white dots present, and if absent, black patterns with clusters of melanophores present [vs. uniformly brown in *D. plicatus* (Brocchi), yellow spots on dark brown in *D. euphorbiaceus* (Günther) and *D. bocourti* (Mocquard), gray to reddish-brown; yellow to white spots on dark in *D. femoralis* (Daudin), and uniformly tan in *D. walkeri* (Stuart)].

Range. Northeastern Japan (Hokkaido, Honshu, and some adjacent islands) and Sakhalin. Hokkaido region: Hokkaido Prefecture (including Rishiri Is., Yagishiri Is., Teuri Is., Kunashiri Is. and Shikotan Is.). Tohoku region: Aomori, Iwate, Miyagi, Akita, Yamagata (including Tobi Is.), and Fukushima Prefectures. Kanto region: Tochigi, Gunma, Ibaraki, Chiba, Saitama, and Kanagawa Prefectures (including Eno Is.) and Tokyo Metropolis. Chubu region: Niigata, (including Sado Is. and Awa Is.), Toyama, Ishikawa, Fukui (except for western tip, where mtDNA of *D. japonicus* was found), Yamanashi, Nagano, Gifu, Shizuoka, Aichi (including Saku Is.), and Mie Prefectures

(including Toshi Is., Sakate Is., and Suga Is.) (except for southern tip, where mtDNA of *D. japonicus* was found) . Kinki region: Shiga, Osaka, Kyoto, and Nara Prefectures. Although most part of Osaka Prefecture is supposed to be occupied by this species, the hybrid zone with *D. japonicus* exists at the northern area (Minoo City, Toyono Town, Nose Town) (See Fig. 2). In Kyoto Prefecture, *D. leopardus* **sp. nov.** is restricted to southeastern half, and the middle to northwestern half are supposed to be *D. japonicus* or (if any) hybrid populations of those two species (See Fig. 2). In Nara Prefecture, we genetically examined only a sample from the southern tip, which had mtDNA of *D. japonicus*, but judging from genetic identification of surrounding prefectures, it is natural to consider that at least northern Nara population is *D. leopardus* **sp. nov**. In Wakayama Prefecture, most populations seem to belong to *D. japonicus*, but we found mtDNA of *D. leopardus* **sp. nov.** at the northern and eastern tips. At least, one of them (Koya Town) is supposed to be a hybrid population, and we need further studies to examine if pure populations of *D. leopardus* **sp. nov.** exist in this prefecture. Outside of Japan, it is only distributed in southernmost part of Sakhalin (Kuzmin & Maslova 2003).

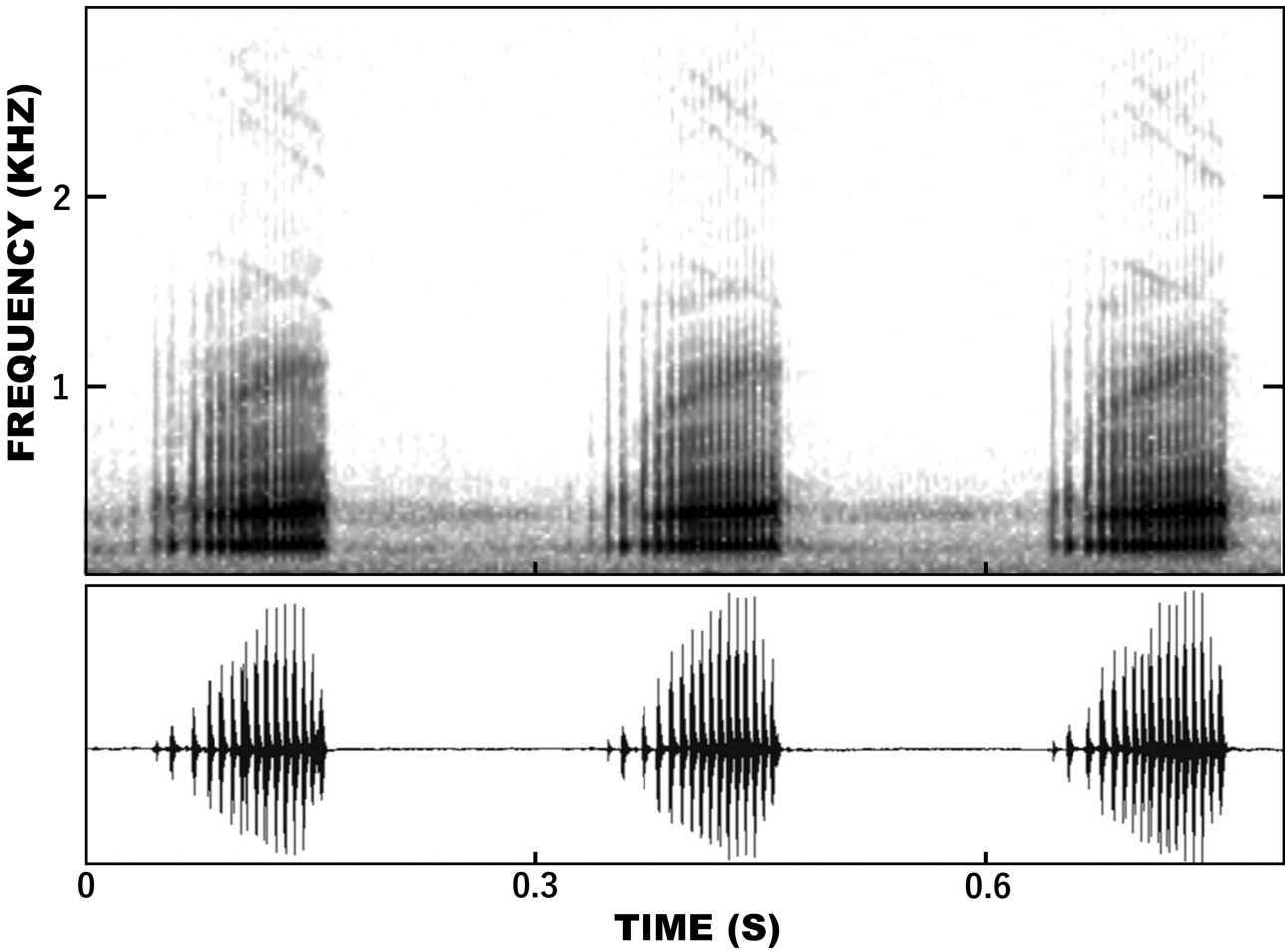

FIGURE 10. Advertisement call of *Dryophytes leopardus* **sp. nov.** from the topotypic population (Toyota city, Aichi Prefecture, Japan), recorded at an air temperature of 16.8°C, showing sonogram (top) and wave form (bottom).

Natural History. *Dryophytes leopardus* **sp. nov.** inhabits widely in plains and low mountains, but is sometimes seen in highland. Its altitudinal range occupies from shoreline (Oga city, Akita Pref.; Kimura *et al.* 2017) up to at least alt. 1,150 m (Minamimaki village, Nagano Pref.; Shimada's personal observation). It uses various types of habitats, such as paddy fields, forests, wetlands, and gardens. In Honshu Is., it emits calls during most part of spring and summer [late April to middle August; Yamamoto 2012; Shimada *et al.* 2013], but some of them might include rain calls, and ovipositional activities itself are supposed to finish before high summer (Shimada *et al.* 2013). The timing of reproductive start is similar in Hokkaido Is. (late April; Tokuda 2011), but is reported to be later in Sakhalin and Kunashiri Is. (late May to early June; Basarukin 1982, 1984). It breeds in various types of still water such as rice fields, ponds, ditches, wetlands, and sometimes small artificial tanks. Although Basarukin (1984)

reported that females lay each single egg separately in Sakhalin, this remark should be re-checked because eggs laid in small batches are popular in other populations. Eggs are often attached to vegetation such as weeds, roots, and aquatic plants, but those laid just on muddy bottoms are seen as well.

Conservation status. *Dryophytes japonicus* including *D. leopardus* **sp. nov.** is listed as Least Concern (LC) in IUCN category (Kuzmin *et al*. 2017). It is not listed in the Japanese Red List by the Ministry of Environment, but in Tokyo Metropolis, populations assigned to *D. leopardus* **sp. nov.** are treated as Endangered (urban area), Vulnerable (northern and southern Tama), and Near Threatened (western Tama) by the local government. Populations in Rishiri and Yagishiri Is. in Hokkaido are listed as the threatened local populations by the local government, although the former might have already been extinct (K. Kazama and M. Kazama, personal communications).

Discussion

Recently, genetic distances of mtDNA and/or the divergent time estimated from it tend to become a common tool to discuss the boundary of closely related species. However, the variation of such a small set of genes is often poorly indicative of the true evolutionary history of populations (Dufresnes & Jablonski, 2022), and it is sometimes risky to obtain taxonomic conclusion relying on mtDNA alone. For example, Dufresnes & Litvinchuk (2022) reviewed the diversity of anurans in eastern part of Palearctic region, and proposed the taxonomic alteration of numerous known taxa including ten Japanese species (i.e. *D. japonicus*, *Glandirana rugosa*, *Zhangixalus schlegelii*, *Buergeria buergeri*, *B. japonica*, *Microhyla okinavensis*, *Fejervarya sakishimensis*, *Rana japonica*, *R. sakuraii*, and *Bufo japonicus*) based mainly on mtDNA phylogeny, but such taxonomic decision should be done through multi-angled analyses, and hasty alteration of taxonomic system would cause more confusions (Shimada, 2022). Actually, in the case of *G. rugosa*, the species boundary proposed by them was totally rewritten by subsequent authors (Shimada *et al*. 2022) based on nuclear genome.

From this point of view, we investigated morphological and molecular variations found in *D. japonicus*, especially focusing on the border area of genetic groups, and surveyed if separation at the species level proposed by Dufresnes *et al.* (2016) and Dufresnes & Litvinchuk (2022) is badly needed. Our mtDNA tree basically agrees with that obtained in Dufresnes *et al.* (2016) and the cladogram of Nishioka *et al*.'s (1990) allozyme analysis. The genetic distance between two major clades (Clade A and Clade B) were approximately 6% in 16S rRNA and 10% in Cytb, which could be judged to be interspecific level in Dufresnes & Litvinchuk's (2022) criteria. The species delimitation tests on mtDNA phylogeny also supported the specific separation of these two clades (and even further division of Clade A). Through detailed sampling, we found that the geographic border of those clades exists in the line connecting southern area of Fukui Pref., middle area of Kyoto Pref., northern area of Osaka Pref., northern area of Wakayama Pref., southern area of Nara Pref., and southern area of Mie Pref.

We further compared these mitochondrial results with the genetic structures detected through SNPs analyses based on whole genome. The ML and Bayes trees did not contradict with the mtDNA tree, and the split of two major clades were supported in STRUCTURE analysis in K=2 (Fig. 3). However, in the area near mtDNA border (northern area of Osaka Pref. and northern area of Wakayama Pref.), some hybrid populations were found. Similarly, in PCA analyses, a relatively large genetic gap was recognized between Clades A and B except for some hybrid populations plotted between them. We regarded this area as the hybrid zone between Clade A and B, and conducted the analyses of migration rates and the shape of the cline. The migrations into/beyond the hybrid zone were analytically detected, but were proved to be kept in low level (migration rates=0.02–0.04). The width of hybrid zone was estimated to be 25 km, which is similar to or narrower than most cases of interspecific hybrid zones reported based on anuran nuclear loci (i.e. Fukutani *et al.* 2023; Dufresnes *et al.* 2020a, b; van Riemsdijk *et al.* 2019). The genetic cline was quite steep indicating that hybrid events are restricted to the area quite close to the boundary, and genetic penetration are not prevailing in the area remote from the hybrid zone. Through the estimation of divergence time, Dufresnes *et al*. (2016) proved that Clade A and B diverged in approx. 5 MYA. Although we are not sure if the boundary of those genetic groups has been kept stable at the present location for such a long period, it is notable that such an old pair of sister group is parapatrically maintained without genetic fusion even now, despite slight gene flow through the hybrid zone. *Dryophytes japonicus* is one of the commonest species in Japanese mainland, distributing in wide range of environment from lowland to highland (Matsui & Maeda, 2018), and no geographic gaps seem to exist around the hybrid zone (Japan Wildlife Research Center 2010). This suggests that there are relatively strong but incomplete

prezygotic and/or postzygotic isolation mechanisms preventing the gene flow beyond the hybrid zone. Such status might not satisfy the conventional biological species concept (Mayr 1942), but fully matches with evolutionary species concept (Wiley 1978), which is also accepted by many zoologists (Stankowski & Ravinet, 2021). The interspecific hybridizations are not rare in *Dryophytes* [e.g. *D. japonicus* vs. *D. suweonensis* (Borzée *et al.* 2020); *D. versicolor* and some extinct lineages of *Dryophytes*, resulted in *D. chrysoscelis* (Halloway *et al.* 2006)], and it is apparent that we cannot apply the rigorous definition of the biological species concept. Based on such views, we concluded that genetic gap between treefrogs from northeastern and southwestern Japan is significant and that they should be recognized as distinct species.

For this taxonomic alteration, we must identify the type specimens of this species. The specimens used in the original description of *Hyla arborea* var. *japonica* (=*Dryophytes japonicus*) (Günther, 1859 "1858") were collected by Philipp Franz von Siebold during his stay in Japan (1822-1828), but no exact collection localities were recorded. Although he spent most time in Japan in Nagasaki (Kyushu) where southwestern species exist, he once visited Edo (=Tokyo) where northeastern species is seen. Therefore, like many amphibians described based on his collection (e.g. *Hynobius naevius*; Tominaga and Matsui 2007, *Glandirana rugosa*; Shimada *et al*., 2022), it is difficult to specify the type locality of *Hyla arborea* var. *japonica* from literature or records. However, through the morphological observations, we found that the syntypes lacked any patterns on the rear of thigh, although the coloration of other body parts was still preserved (Fig. 7). In our morphological categories, such states of the rear of thigh are assigned to "pattern D", which are common in southwestern species, but are rare in northeastern species (Fig. 6). Based on this observation, it seems natural to identify the syntypes to the southwestern species, and we described the northeastern species as a new species, *D. leopardus* **sp. nov.** We also designated the lectotype from syntypes of *D. japonicus* to prevent further taxonomic confusions.

Morphological comparison between *D. leopardus* **sp. nov.** and *D. japonicus* has seldom been done except for the local and preliminary observation (i.e. Matsui, 1979). Through the comparisons using body proportions, we found several interspecific differences, but all of them were detected in the pairs of remotely located groups (North/Central Japan groups of *D. leopardus* **sp. nov.** vs. Kyushu/Korea groups of *D. japonicus*), and no interspecific differences were found between the groups directly touching with each other (Central Japan group of *D. leopardus* **sp. nov.** vs. Chugoku-Shikoku group of *D. japonicus*). Similarly, in CAN plot, three remotely located groups (North Japan group of *D. leopardus* **sp. nov.** and Kyushu/Korea group of *D. japonicus*) had different plot patterns, but two groups touching with the specific border (Central Japan group of *D. leopardus* **sp. nov.** and Chugoku-Shikoku group of *D. japonicus*) overlapped its plot range with both species. Judging from those results, there seems to be a clinal variation in body proportion between northeastern and southwestern Japan, but we can analytically detect it only when we compare the easternmost and westernmost populations. At present, it is difficult to list any characters to distinguish *D. japonicus* and *D. leopardus* **sp. nov.** as a specific level, except for the color pattern of the rear of thigh.

To elucidate the mechanism to maintain such a species pair with quite similar morphology, studies on the isolation mechanism is needed. For *D. japonicus*, Kawamura *et al.* (1990) studied postzygotic isolation between Korean and Japanese populations. Further, Kuramoto (1980) found variations in acoustic characters between Korean and Japanese populations, and such differences in the character concerning mating behaviors might result in prezygotic isolation. However, both studies used populations from southwestern Japan (=*D. japonicus*) as a representative of Japanese population, and no comparisons between *D. japonicus* and *D. leopardus* **sp. nov.** has been conducted. Detailed studies on the reproductive isolation mechanism across the specific border are needed in future.

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