学位論文

Development of molecular markers for the endangered *Babina* species (Anura: Ranidae) and clarification of its phylogenetic position

絶滅危惧無尾類 *Babina* 属（アカガエル科）に関する分子マーカーの開発と系統学的位置の解明

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主論文

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I. General Introduction

The genus *Babina*, a member of Ranidae, initially included only two species, *B. holsti* and *B. subaspera* (Thompson 1912). These species are found only in the Ryukyu Islands, southwest Japan. The range of *B. holsti*, is restricted to the Okinawa and Tokashiki islands, whereas *B. subaspera* is found on the Amami and Kakeroma islands. Within Ranidae, *B. holsti* and *B. subaspera* are the only species to exhibit a unique phenotype, a thumb-like structure medial to the first finger that gives the appearance of five fingers on a hand (Maeda and Matsui 1999; Tokita and Iwai 2010). An additional eight species have been assigned to *Babina* (including *B. okinavana* from the Ryukyu Islands) following several taxonomic revisions (e.g., Frost et al. 2006; Maeda and Matsui 1999). Here, we designated *B. holsti* and *B. subaspera* as *Babina* sensu stricto, and the remaining eight species as *Babina* sensu lato for convenience. Although the monophyly of *Babina* sensu stricto and lato has been highly supported (Chen et al. 2013; Kurabayashi et al. 2010), there is currently no morphological or ecological basis for assignment as the same genus. Some previous studies have reported phylogenetic relationships within Ranidae, including the genus *Babina* (Cai et al. 2007; Frost et al. 2006; Kurabayashi et al. 2010; Pyron and Wiens 2011; Stuart 2008). However, no consensus has been reached on the phylogenetic position of *Babina* and statistical support for any relationship has been low. Additionally, the *Babina* sensu stricto species were not used in these analyses, except in Kurabayashi et al. (2010). Thus, the phylogenetic position and the closest genus to *Babina* remain unclear.

Because of the limited original range of the three Japanese *Babina* species and recent habitat loss as a result of deforestation and development, these species have been

listed as class B1 endangered species in the IUCN Red List of Threatened Species (Kaneko and Matsui 2014; Kuangyang et al. 2014). Additionally, the invasive species *Herpestes auropunctatus* has also been implicated in the decline of native species, including amphibians (Ogura et al. 2002). In *B. holsti*, regional population declines and extinction have occurred around the Motobu Peninsula on Okinawa Island (Toyama 1996). To guide conservation management of these species, there is a need for fine scale genetic information, such as population structure, gene flow, and demographic history. To obtain this information, there is a need for molecular markers for *Babina spp*.

To address the unresolved phylogenetic classification of *Babina* I used a larger mitochondrial (mt) sequence dataset than has been used in past studies. Additionally, because the mt gene arrangement in the neobatrachian group (phylogenetically nested anurans including family Ranidae) is variable and suitable for use as phylogenetic markers, I determined the complete mt genome sequences of three Japanese *Babina* species and used them for phylogenetic inference.

Effective conservation relies on correct identification of the evolutionary significant unit. To achieve this, there is a need for data describing the genetic population structure and gene flow, both of which rely on analysis of genetic markers. The mt gene has been widely used for intraspecies genetic analyses. Therefore, I compared genes within the mt genome to determine which was most appropriate for intraspecies analyses of *Babina* species.

To mitigate the shortcomings of mt genes (e.g., maternal heritage and limited single locus) and obtain fine scale population genetic structure data, researchers commonly use genetic markers that have a high mutation rate and codominance (e.g., microsatellite markers). Thus, I developed microsatellite markers for *Babina* sensu stricto.

II. Mitochondrial genomes of Japanese *Babina* frogs (Ranidae, Anura): unique gene arrangements and the phylogenetic position of genus *Babina*.

Abstract

Genus *Babina* is a member of Ranidae, a large family of frogs, currently comprising 10 species. Three of them are listed as endangered species. To identify mitochondrial (mt) genes suitable for future population genetic analyses for endangered species, I determined the complete nucleotide sequences of the mt genomes of 3 endangered Japanese *Babina* frogs, *B. holsti*, *B. okinavana,* and *B. subaspera* and 1 ranid frog *Lithobates catesbeianus*. The genes of NADH dehydrogenase subunit 5 (*nad5*) and the control region (CR) were found to have high sequence divergences and to be usable for population genetics studies. At present, no consensus on the phylogenetic position of genus *Babina* has been reached. To resolve this problem, I performed molecular phylogenetic analyses with the largest dataset used to date (11,345 bp from 2 ribosomal RNA- and 13 protein-encoding genes) in studies dealing with *Babina* phylogeny. These analyses revealed monophyly of *Babina* and *Odorrana*. It is well known that mt gene rearrangements of animals can provide usable phylogenetic information. Thus, I also compared the mt gene arrangements among *Babina* species and other related genera. Of the surveyed species, only *L. catesbeianus* manifested typical neobatrachian-type mt gene organization. In the *B. okinavana*, an additional pseudogene of tRNA-His (*trnH*) was observed in the CR downstream region. Furthermore, in the *B. holsti* and *B. subaspera*, the *trnH* / *nad*5 block was translocated from its typical position to the CR downstream region, and the translocated *trnH* became a pseudogene. The position of the *trnH* pseudogene is consistent with the

translocated *trnH* position reported in *Odorrana*. Consequently, the *trnH* rearrangement seems to be a common ancestry characteristic (synapomorphy) of *Babina* and *Odorrana*. Based on the "duplication and deletion" gene rearrangement model, a single genomic duplication event can explain the order of derived mt genes found in *Babina* and *Odorrana*.

Introduction

Genus *Babina* is a member of Ranidae, a large family of frogs currently comprising 10 species (see below). Thompson (1912) described the first two species in this genus—*B. holsti* and *B. subaspera*. These two original *Babina* species are famous for their "fifth finger": their first finger is a medially and ventrally fleshy sheath encasing a spine-like elongated 1st metacarpal, giving the appearance of five fingers on a hand. Because this character is unique among amphibians, these species have attracted the attention of many researchers in ecology, developmental biology, and taxonomy fields (e.g., Tokita et al., 2010). *Babina holsti* and *B. subaspera* are endemic to the small Ryukyu Islands, Okinawa and Amami, respectively. Recent environmental destruction has devastated populations of these species; and as a result, *B. holsti* and *B. subaspera* have been listed as a class B1 endangered species in the IUCN Red List of Threatened Species (http://www.iucnredlist.org) and designated as "natural monument species" in Okinawa and Kagoshima Prefectures, respectively. In addition, another *Babina* species, *B. okinavana* is listed as endangered. Prompt measures to conserve these species are awaited, and a recent population genetics study has been performed on *B. subaspera* using microsatellite markers (Iwai et al., 2012), but no usable mitochondrial genomic marker on these species has been obtained.

Since the initial description of genus *Babina* (Thompson, 1912), several taxonomic changes have been made. Consequently, the original *Babina* species have been moved to genus *Rana*, a large ranid taxon, or occasionally to subgenus *Babina* of *Rana* (e.g., Maeda and Matsui, 1999). Recently, Frost et al. (2006) re-realized genus *Babina* and separated it from genus *Rana*. They also assigned 10 species to this genus: 2 species are the original species (designated here as *Babina* sensu stricto) while the others (*Babina*

sensu lato) are from genus *Nidirana* (or subgenus *Nidirana* of genus *Rana*). Although the monophyly of these two *Babina* groups has been suggested (Kurabayashi et al., 2010; Kuraishi and Matsui, unpublished), there is no morphological or ecological foundation (e.g., synapomorphy in *Babina*) that justifies assigning them to the same genus. Further, which ranid group is the sister taxon of genus *Babina* is an unresolved matter in ranid phylogeny. Previous molecular phylogenetic studies have resulted in three distinct phylogenetic relationships for *Babina*. First, Stuart (2008) proposed that *Babina* is a sister taxon of the *Glandirana*, *Hylarana,* and *Sanguirana* clades based on two nuclear genes. By contrast, Cai et al. (2007) showed the close relationship of *Babina* with *Rana* and *Lithobates* based on mitochondrial ribosomal RNA gene data. From the same mitochondrial gene data, Kurabayashi et al. (2010) suggested that *Babina* is the sister group of *Odorrana*. Frost et al. (2006) and Pyron and Wines (2011) also support the sister relationship of *Babina* and *Odorrana*. In every case, the statistical supports of *Babina* nodes were not high, and the *Babina* sensu stricto species was not used in these analyses (excluding Kurabayashi et al., 2010). Further, relatively small molecular data sets were applied in phylogenetic analyses in the previous studies regarding *Babina*. However, no consensus on the phylogenetic position of *Babina* has been reached.

To perform population genetics studies and resolve the phylogenetic problems related to *Babina*, mt genomic information seems to be a usable marker. Vertebrate mitochondrial (mt) DNA comprises closed circular molecules. The mt genomes generally span a length of 16 kbp, but some vary from 15 to 21 kbp (Sano et al., 2005). These mt genomes typically encode 37 genes for 2 ribosomal RNAs (12S and 16S *rrns*), 22 transfer RNAs (*trns*), and 13 proteins (ATPase subunits 6 and 8 (*atp6* and *8*);

cytochrome oxidase subunits I, II, and III (*cox1-3*); cytochrome b apoenzyme (*cob*); and nicotinamide adenine dinucleotide dehydrogenase subunits 1, 2, 3, 4, 5, 6, and 4L (*nad1-6* and *4L*)). Most vertebrate mt genomes contain one long non-coding region (approximately 500 bp–9 kbp; Kurabayashi et al., 2008), a "control region" (CR; also referred to as the D-loop region), which is thought to mediate signaling for regulation of mtDNA replication and transcription (Wolstenholme, 1992; Boore, 1999). The nucleotide substitution rates are widely accepted to be much faster within the mt genes and the CR than in the nuclear encoding genes; and substitution rates are known to vary from one mt gene to another (Kumazawa and Nishida, 1993).

The gene arrangement of the animal mt genomes tends to be conserved among vertebrates, with all 37 genes and the CR organized relatively in the same gene order in almost all species—from teleost fish to eutherian mammals (Anderson et al., 1981; Roe et al., 1985; Tzeng et al., 1992; Boore, 1999). In anurans, the mt gene arrangement of archaeobatrachians (a paraphyletic assemblage of basal anuran families; e.g., Duellman, 2003) is identical to the typical arrangement in vertebrates (vertebrate-type arrangement, see Fig. 1; e.g., San Mauro et al., 2004a). Yet the gene arrangement in the neobatrachian group (phylogenetically nested anurans including family Ranidae) is different. Four *trns* genes are commonly rearranged in the neobatrachian groups (neobatrachian-type arrangement) (Sumida et al., 2001; Zhang et al., 2005), and further gene arrangements have been reported in some natatanurans. Although a few convergent gene rearrangements have been observed in vertebrate mtDNA, many of these derived genomic rearrangements are clearly useful as phylogenetic markers (thus, synapomorphic characters) for the specification of monophyletic groups in natatanurans (e.g., Kurabayashi et al., 2006, 2008; Zhang et al., 2009). Furthermore, and remarkably,

Kurabayashi et al. (2010) showed unexpected gene rearrangement in ranid taxa including *Babina* and suggested the possible phylogenetic utility of the gene arrangement data of these taxa.

In the present study, I determined the complete mtDNA sequences of 3 endangered *Babina* species in Japan (2 sensu stricto species and 1 sensu lato species, *B. okinavana*) to identify mt genes with high sequence divergence and to check the unknown mt gene rearrangements. The former information can be applied in interpopulation genetic analyses of these species, and the latter will furnish possible phylogenetic markers to elucidate the phylogenetic problems related to this genus. I also sequenced the mtDNA of the American bullfrog, *Lithobates catesbeianus*. This species, of which complete mt genomic information has not been obtained, is very common and notorious as as invader species in many countries. Furthermore, *Lithobates catesbeianus* is one of the possible sister taxa of genus *Babina* (Cai et al., 2007). This paper describes the novel mt genomic structures and estimates the possible rearrangement pathway for the observed gene arrangements in these ranid taxa. Based on this information, I discuss the most related genus of *Babina*.

Materials and methods

Frog specimens Since the taxonomic revision of amphibians by Frost et al. (2006), the names and taxonomic ranks of anuran taxa, including those for a multitude of ranid groups, have been vertiginously changed. To prevent unnecessary confusion, I follow the newest system for anuran taxonomy from Frost (2011) in this paper.

The experiments in this study were performed using 3 frog specimens of *Babina*

species *Babina holsti*, *B. subaspera,* and *B. okinavana* collected in the Ryukyu islands, Japan (Okinawa Island, Amami Island, and Iriomote Island, respectively). Another ranid species, *Lithobates catesbeianus*, kept by the strain maintenance team of the Institute for Amphibian Biology, Hiroshima University, was also used.

PCR, subcloning, and sequencing Total DNA was extracted from a clipped toe of each frog specimen using the DNeasy Tissue Kit (QIAGEN). To determine the complete mt genomic sequences of 4 species, normal or long-and-accurate (LA) PCR using Ex- and LA-Taq (Takara-Bio.), respectively, was used according to the manufacturer's instructions (sequences of amplification primers are available in Table 1). The resultant PCR fragments were electrophoresed on 0.7% agarose gel; then target DNAs were purified from excised pieces of gel using GenElute Agarose Spin Column (Sigma). The purified DNA fragments were used for sequencing and subcloning.

The primer-walking method was employed for mtDNA sequencing using an automated DNA sequencer (3130xl Genetic Analyzer, ABI) with the BigDye Terminator Cycle Sequencing Kit (ABI) (sequences of the walking primers are available in Table 1). A PCR fragment (corresponding to the CRs) containing long tandem repeats from which walking primers could not be constructed were subcloned into a pCR 2.1 vector using the TOPO TA Cloning Kit (Invitrogen). To determine the precise sequence of the long tandem repeats in the CR of *B. okinavana*, a series of deleted subclones was constructed from the single resultant clone by the Exonuclease III deletion method (Henikoff, 1987).

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under following accession numbers: AB761264

(for *B. holsti*), AB761265 (for *B. subaspera*), AB761266 (for *B. okinavana*), and AB761267 (for *L. catesbeianus*).

Genetic distance Sequence data were analyzed with GENETIX (Software Development). Genes were identified by comparison with corresponding gene sequences from other ranid species, and in the case of *trns*, by determination of secondary structural features.

The nucleotide sequences of 2 *rrns*, 13 mt protein-coding genes, and the partial CR were separately aligned using MUSCLE (Edgar, 2004). For the CR, it is known that multiple repetitive sequences exist in neobatrachians, such as *Pelophylax nigromaculatus* and *Buergeria buergeri* (Sumida et al., 2001; Sano et al., 2004). Thus, the alignable non-repetitive portions, about 1.6 kbp sequences from the 3' end of the repeat to around CSB III were used for analyses as the partial CRs. Genetic distances of each gene and the partial CR were calculated among 6 ranid species (*Babina holsti*, *B. okinavana*, *B. subaspera*, *Odorrana splendida*, *O. tormota*, and *Lithobates catesbeianus*). Gaps and missing sites were deleted in the calculations. The homogeneity of variance in the distance among different genes and the CR was tested using Bartlett's test. A Kruskal-Wallis test was performed to test for significant differences between the distance of each gene and the CR.

Molecular phylogenetic analyses The phylogenetic analyses of the higher anuran groups were performed with 34 anurans, 8 other amphibians (6 caudate and 2 caecilians), and 4 other vertebrates (a mammal, bird, reptile, and teleost fish) for which complete mt sequences are available (see Fig. 1 legend). The nucleotide sequences of 2

ribosomal RNA and 13 mt protein-coding genes were separately aligned using MUSCLE (Edgar, 2004). To exclude gaps and ambiguous areas, the alignments were revised using Gblocks 0.91b software (Castresana, 2000) with the default parameters. Then, the corrected alignments from 15 genes were combined (11,345 bp). The phylogeny was analyzed with the combined data by maximum likelihood (ML) and Bayesian inference (BI) methods. The ML and BI analyses were respectively performed using TREEFINDER ver. Mar. 2011 (Jobb, 2011) and MrBayes5D (Tanabe, 2008), a modified version of MrBayes3.1.2 (Ronquist and Huelsenbeck, 2003). Different substitution models were applied for each gene partitioned by codon positions in both analyses. The best-fit substitution model for each partition was estimated using Akaike's information criteria implemented in Kakusan4 ver. 4.0 software (Tanabe, 2011). In the BI analysis, the following settings were applied: number of Markov chain Monte Carlo generations, 11 million; sampling frequency, 100; burn-in, 1,001. The robustness of the resultant ML tree was evaluated using bootstrap probabilities calculated from nonparametric bootstrap analyses with 1,000 pseudo-replications, and statistical support of the resultant BI tree was determined based on Bayesian posterior probability (BPP).

Results

Mitochondrial genome organization of the *Babina* species In this study, I determined the complete nucleotide sequences of the mt genomes from three *Babina* species (*B. holsti, B. okinavana,* and *B. subaspera*) and *L. catesbeianus*. Sizes were 19,113 bp, 19,959 bp, 18,525 bp, and 17,682 bp, respectively. As in other animals, the mt genomes of the four ranid species contain 37 genes consisting of 2 *rrns*, 22 *trns,* and

13 protein-coding genes, all of which are similar in length to their counterparts in other anurans (data not shown). In contrast, however, *B. okinavana* (sensu lato group) mtDNA has one pseudogene of mt *trnH* (ps-*trnH*, whose nucleotide similarity with the corresponding gene is 63.5%; Table 2). Similarly, the mt genomes of *B. holsti* and *B. subaspera* (sensu stricto group) possess ps-*trnH* (similarities are 75.8% and 76.2% in *B. holsti* and *B. subaspera*, respectively; Table 2). Additionally, the *Babina* sensu stricto group has ps-*trnE* (similarities are 72.1% and 70.0% in *B. holsti* and *B. subaspera*, respectively; Table 2).

I also identified a major non-coding region downstream of *cob* in *Babina* species and in *L. catesbeianus* (see Fig. 1). Upon determining that this region contained TAS (termination-associated sequence), O_H (Heavy-strand replication origin), and CSB I-III (conserved sequence blocks I, II, and III), common features of the CR, I identified it as the CR. Noncoding sequences of 36–37 bp in length were also found. These were deemed to be of *L*-strand replication origin (O_L) because of their potential to form a hairpin structure characteristic of vertebrate O_L (data not shown) and because their positions between *trnN* and *trnC* in the WANCY *trns* cluster correspond to the typical O_L position (see Fig. 1).

As shown in Fig. 1, the mt gene arrangement of *L. catesbeianus* and *B. okinavana* is similar to that in most vertebrates and consistent with the typical neobatrachian-type arrangement having a LTPF *trn*-cluster. However, in the *B. okinavana* mt genome, there is an additional noncoding region of 551 bp between *nad5* and *nad6*; and ps-*trnH* is additionally found downstream of the CR. In contrast to the former, the mt gene arrangement of the *Babina* sensu stricto group differs from the neobatrachian-type arrangement and shows the unique position of the ps-*trnH*–*trnS* (AGY)–*nad5* gene

region between the CR and the LTPF *trn*-cluster. The translations of *nad5* from its original position (upstream of the CR) to downstream of the CR were reported in several neobatrachian mt genomes (e.g., dicroglossids, rhacophorids, mantellids, and the ranid *Staurois latopalmatus*; see Kurabayashi et al., 2010, Alam et al. 2010). However, the CR downstream position of *trnS* (AGY) and *trnH* or its pseudogene is found only in *Babina* and a few ranid species (*S. latopalmatus* and *Odorrana* spp.; see Kurabayashi et al., 2010).

Genetic distances of ranids I compared the currently available nucleotide sequences of all mt genes (2 *rrns*, 13 protein-coding genes and a concatenated sequence of 22 *trns*) and the CR among 6 ranid species, including *Babina* and their related genera *Odorrana* and *Lithobates*. The genetic distance for each gene and the CR is shown in Fig. 2. Mean values of nucleotide divergence for each gene and the CR range from 10.6% in 12S *rrns* to 30.8% in *atp8*. Bartlett's test showed that the variances of nucleotide divergence are significantly heterogeneous ($P < 0.05$) among genes (and the CR). The Kruskal-Wallis test also rejected the uniformity of nucleotide divergences among the mt genes and the CR ($P < 0.01$). In the ranid taxa analyzed, *atp8*, *nad5*, and the CR showed the highest nucleotide divergence (30.8%, 26.7%, and 25.8%, respectively), and 12S showed the lowest nucleotide divergence (10.6%).

Phylogenetic analyses I analyzed the phylogenetic relationship among anurans using the long mitochondrial sequence data (11,345 bp). The resultant Bayesian (BI) tree is shown in Fig. 3. The ML tree based on the same data set has an identical tree topology. In these trees, neobatrachians form a clade (BP = 100) in which the ranoid

families form a clade (BP = 100). Within the ranoid clade, ranid frogs form a monophyletic group (i.e., family Ranidae, BP = 100). These relationships are consistent with recent molecular phylogenetic studies on anurans (e.g., Igawa et al., 2008; Kurabayashi et al. 2010). In the ranid clade, genus *Babina* forms a monophyletic group (BP = 100). Genus *Odorrana* becomes the sister taxon of *Babina*. The BP support in ML is not high for this node, but BPP is high (BP = 67.8; BPP = 98.8). Genus *Lithobates* becomes the sister taxon to the *Babina* + *Odorrana* clade.

Discussion

Nucleotide diversity in *Babina* mt genes One of the purposes of this study was to find mt genes with high nucleotide divergence, genes which will be useful as genetic markers among populations of *Babina*, including three endangered species (*B. holsti*, *B. subaspera,* and *B. okinavana*). Among the ranid taxa examined here (Fig. 2), 12S and 16S *rrns* and *trns* have small nucleotide divergence (10.6%, 14.2%, and 12.2%, respectively), while those of protein-coding genes and the CR are large. In particular, *atp8*, *nad5*, and the CR have large nucleotide divergence (30.8%, 26.7%, and 25.8%, respectively). Given the very short length of *atp8* (168 bp), *nad5* and the CR seem to be candidates for a suitable polymorphic marker for *Babina* and related ranid taxa.

It is noteworthy that the kinds of mt genes (and the CR) with high nucleotide diversity differ among amphibian taxa. For example, *cox1*, *cox3,* and *cob* have low divergence in the ranid taxa examined here (~17.4–19.7 %), but these genes have high nucleotide substitution rates in plethodontid salamanders and their related taxa (Mueller, 2006). Furthermore, the CR shows low divergence in salamanders and newts (Mueller, 2006;

Kurabayashi et al., 2012), yet the CR is the third-most variable region in *Odorrana* (Kurabayashi et al. 2010). These findings emphasize that performing a preliminary survey of fast-evolving genes and the CR is important for determining phylogenetic and/or population genetic markers for closely related amphibian taxa.

Phylogenetic relationship of *Babina* and related genera As mentioned above, several alternative hypotheses have proposed a sister taxon for genus *Babina*. This study is the first attempt to tackle this problem using all mt sequence data, and our data revealed the sister relationship between *Babina* and *Odorrana* (Fig. 3). Genus *Lithobates,* which has been suggested to have a close relationship with *Babina* (Cai et al. 2007), becomes the sister taxon of the *Babina* + *Odorrana* clade in our analysis. These relationships recovered here are consistent with the results of three previous molecular phylogenetic studies (Frost et al. 2006; Kurabayashi et al. 2010; Pyron and Weins 2011). Although the BP support of the *Babina* and *Odorrana* clade is not sufficient, BPP is high (BP = 67.8; BPP = 98.8). Furthermore, a unique mt gene arrangement seems to support the monophyly of *Babina* and *Odorrana* (see below).

Evolutionary pathway and systematic implications of mt gene rearrangement The present study shows that the mt gene arrangements of *Babina* species differ from those of typical neobatrachians (Fig. 1), and *Babina* sensu lato and stricto groups possess different gene orders. Furthermore, the unique position of *trnH* and its pseudogene (downstream of the CR) is observed in all *Babina* and *Odorrana* (sister taxon of *Babina*) species examined so far. By contrast, mtDNA of *Lithobates*, the sister taxon of the *Babina* + *Odorrana* clade, possesses the typical neobatrachian-type gene

arrangement. These observations seem to indicate that gene rearrangement events occurred and that the unique *trnH* (and its pseudogene) emerged in an ancestral lineage which led to *Babina* and *Odorrana* after the splitting of *Lihobates* (see Fig. 4).

Animal mt genomes lack introns and intergenic spacers, and there are no multicopy genes (with a few exceptions; e.g., Kurabayashi et al. 2008). In such genomes, the occurrence of unregulated gene rearrangement will destroy an essential single-copy gene. Thus, gene rearrangements in animal mt genomes are generally explained by the "Duplication and Deletion model" (Moritz and Brown, 1986; Boore, 1999; San Mauro et al., 2006). In this model, a duplication including multiple genes is caused by a replication slippage or DNA recombination (see Kurabayashi et al. 2008). Then, one of copied genes is deleted through an accumulation of natural mutations. The duplicated CRs and several remnants of the duplicated genes (i.e., ps-*trns*) observed in ranoids, including the *Babina* species examined here, support the occurrence of duplication and deletion events.

According to the gene arrangement mechanism, there seems to be only one duplication event that can explain the divergent gene arrangements between *Babina* sunsu lato and sensu strict groups as well as the unique *trnH* position shared by *Babina* and *Odorrana* as follows. First, the gene region from *trnH* to *trnE* was duplicated in the common ancestral lineage of *Babina* and *Odorrana* (Fig. 4I). From the duplicated genomic condition, the upstream *trnH* gene and the downstream *trnS* (AGY) to *trnE* region would have been deleted in the lineage which previously led to *Odorrana*; then, the present *Odorrana* mt gene arrangement occurred (Fig. 4II). In the common ancestor lineage of *Babina*, the duplicated genomic condition was maintained in the last common ancestor of the sensu stricto and lato groups. In the lineage leading to *B. okinavana*

(sensu lato group), the downstream copied genes were deleted, and only *trnH* remained as the pseudogene (Fig. 4III). Finally, in the lineage leading to *B. holsti* and *B. subaspera* (sensu strict group), the original *trnS* (AGY), *nad5,* and *trnE* were deleted, and the copied *trnH* remained as the pseudogene (Fig. 4IV). Consequently, these species have the translocated *trnS* (AGY), *nad5,* and *trnE* genes as well as an additional *trnH* pseudogene.

If I hypothesize a single duplication event, the gene rearrangements observed in *Babina* and *Odorrana* can easily be explained by only several deletion events. In this context, the derived position of *trnH* and ps-*trnH* (CR downstream) can be regarded as a synapomorphic character of these genera (Fig. 4II-IV). Furthermore, the additional rearranged gene positions (*trnS* (AGY)–*nad5*–*trnE* between the CR and LTPF *trns* cluster) found in *B. holsti* and *B. subaspera* will be synapomorphic with the *Babina* sensu stricto group (Fig. 4IV).

The mt genome arrangement is thought to be a usable phylogenetic marker, especially for the taxa in which it is difficult for phylogeny to be resolved by normal gene sequence data (e.g., Boore and Brown, 1994, 1998; Boore et al., 1995; Macey et al., 2000; Kurabayashi and Ueshima, 2000; Inoue et al., 2003; Mabuchi et al., 2004). The unique *trnH* and ps-*trnH* position shared by *Babina* and *Odorrana* seems to be one such example. There remain many unresolved relationships among ranid genera (e.g., the position of genera *Glandirana*, *Amolops*, *Hylarana*; Cai et al. 2007; Che et al. 2007; Phyron and Wiens 2012), but the mt genomic information may be a powerful tool for resolving these problems.

III. Development and characterization of new microsatellite loci in the Otton frog (*Babina subaspera*) and cross-amplification in a congeneric species, Holst's frog (*B. holsti*).

Abstract

The Otton frog (*Babina subaspera*) and Holst's frog (*B. holsti*) are both endangered sister species belonging to family Ranidae. For extensive genetic and ecological research of these species, I isolated and characterized 8 new microsatellite loci of the Otton frog and validated cross-amplification in Holst's frog along with 8 previously reported loci. The total number of alleles and the expected heterozygosity of newly isolated loci in the Otton frog population ranged from 5 to 12 and from 0.620 to 0.905, respectively. I also confirmed cross-amplification in 4 of the new loci and in all previously reported loci in Holst's frog with the same level of polymorphism as the Otton frog. These findings suggest that these novel loci will be applicable for conservation genetic studies across varying scales.

Introduction

The Otton frog (*Babina subaspera*) and Holst's frog (*B. holsti*) are sister species belonging to family Ranidae, endemic to the Amami and Okinawa Islands in southwestern Japan. Both species display unique morphological and ecological characteristics which are evolutionarily interesting, such as possessing a pseudothumb in the forelimb (Tokita and Iwai 2010; Iwai 2013) and creating a nest for oviposition (Maeda and Matsui 1999). However, due to their small original distribution range,

recent habitat loss by deforestation, and predation from introduced mongooses (Watari et al. 2008), both species are declining and have been listed as class B1 endangered species in the IUCN Red List of Threatened Species (Kaneko and Matsui 2004). They have also been designated as natural assets in Okinawa and Kagoshima prefectures, respectively.

For the effective conservation and ecological study of both species, knowledge of their fine-scale population structure is needed. In previous studies, 8 microsatellite loci for the Otton frog have already been developed (Iwai et al. 2011), yet no microsatellite loci have been reported in Holst's frog. In addition, further development of new microsatellite loci for the Otton frog will contribute to individual-based analyses such as parentage and individual recognition. Here, I describe 8 newly identified microsatellite loci in the Otton frog and their associated cross-amplification tests, along with previously reported loci in Holst's frog.

Materials and methods

Genomic DNA was extracted from the toe pads of two individuals, a Holst's frog and an Otton frog, using NucleoSpin® Tissue (TaKaRa). From the Otton frog, microsatellite loci were isolated as reported by Iwai et al. (2011). Seventy clones containing microsatellites were located using MSATCOMMANDER ver. 0.8.2 (Faircloth 2008), and primer pairs for the amplification of 16 microsatellites were designed using Primer 3 ver. 2.2.3 (Rozen and Skaletsky 2000). PCR amplifications were performed with each of the 16 primer pairs using EmeraldAmp® PCR Master Mix (TaKaRa). Thermal cycling was performed under the following conditions: 95 °C for 5

min, 30 cycles of 95 °C for 15 s; a locus-specific annealing temperature (Table 3) for 30 s and 72 °C for 30 s followed by 15 cycles of 95 °C at 15 s; 53 °C for 30 s; 72 °C for 30 s; and a final extension period of 10 min at 72 °C. I labeled M13 universal primers with HEX, FAM, NED, or PET fluorescent dyes. Fragment sizes were determined using GeneScan™ LIZ 500® (Applied Biosystems) as an internal size standard and genotyped using GeneMapper®4.0 (Applied Biosystems). Finally, 8 loci were screened as polymorphic loci (Table 3).

For each of the 8 published primer pairs, PCR amplification was performed using KOD FX Neo (TOYOBO). Thermal cycling was performed under the following conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 s; a locus-specific annealing temperature (Table 3) for 30 s and 68 °C for 30 s; and a final extension period of 5 min at 68 °C. The forward primers were labeled with fluorescent dyes (see Table 3). Genotyping was performed in the same manner as above.

Results and discussion

In total, 8 of the 16 newly developed microsatellite loci were shown to be polymorphic in the Otton frog, and the number of alleles, observed heterozygosity (H_O), and expected heterozygosity (H_E) were calculated using GenALEx 6.5b3 (Peakall and Smouse 2012). Tests for deficiency in the Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed using ARLEQUIN ver. 3.1 (Excoffier et al. 2005). The null alleles were detected using Microchecker v.2.2.3 (Van Oosterhout et al. 2004) for the non-equilibrium locus. Polymorphic information content (PIC) was calculated using Cervus 3.0 (Kalinowski et al. 2007). The observed allelic diversity

ranged from 5 to 12 alleles. H_O and H_E ranged from 0.500 to 1.000 and 0.620 to 0.905, respectively, and the PIC value ranged from 0.571 to 0.897 (Table 3). After calculating the Bonferroni correlation, no significant LD occurred between any of the loci ($P < 0.00179$), and no significant deviation from HWE was detected ($P < 0.00625$).

In cross-species utilization tests, I verified the practical effectiveness of published and newly developed microsatellite loci for Holst's frog. As a result, 12 of 16 microsatellite loci were shown to be polymorphic in Holst's frog, and the observed allelic diversity ranged from 4 to 20 alleles. H_O and H_E ranged from 0.500 to 1.000 and 0.480 to 0.950, respectively (Table 3). After calculating the Bonferroni correlation, no significant LD occurred between any of the loci ($P < 0.00076$), and only Bas2056 showed significant deviation from HWE ($P < 0.00417$). The mean PIC value of 0.761 in 12 new and published primers was higher than that of 0.747 in 8 published primers only, indicating that the additional primers improved visible polymorphism in the Holst's frog population.

In conclusion, the 8 microsatellite markers identified in this study will be useful for further population genetics studies on the Otton frog with the aim of species conservation. Cross-species utilization tests suggest that most microsatellite markers in the Otton frog are usable for Holst's frog. These microsatellite markers have appropriate properties for use in future population genetics studies on Holst's frog across varying scales, from both inter- and intra-population studies to studies at the individual level.

IV. General discussion

Phylogenetic position of *Babina* within Ranidae

Previous studies have not reached consensus regarding the phylogenetic position of *Babina* within Ranidae (Cai et al. 2007; Frost et al. 2006; Kurabayashi et al. 2010; Pyron and Wiens 2011; Stuart 2008). To resolve this, I performed phylogenetic analysis using mt nucleotide sequences of two ribosomal RNA and 13 protein-coding genes (11,345 bp). The phylogenetic analyses supported the monophyly of *Odorrana* and *Babina*, consistent with the results of three previous studies (Frost et al. 2006; Kurabayashi et al. 2010; Pyron and Wiens 2011). Genus *Lithobates*, which has been suggested to be close to *Babina* (Cai et al. 2007), becomes the sister taxon of the *Babina* – *Odorrana* clade. The *Lithobates* + (*Babina* + *Odorrana*) clade is statistically supported by high BP and BPP values (99.4 and 100, respectively). In the *Babina* + *Odorrana* clade, the BP value was low (67.8) but the BPP value was high (98.8). The unique mt gene arrangements outlined in the following chapter also support the monophyly of *Babina* and *Odorrana*.

Estimated pathway of mt gene rearrangement

The mt gene arrangement can provide useful information for taxa in which it is difficult to resolve phylogenetic classifications using only gene sequence data (e.g., Boore and Brown 1994, 1998; Boore et al. 1995; Inoue et al. 2003; Kurabayashi and Ueshima 2000; Mabuchi et al. 2004; Macey et al. 2000). Of the four mt gene arrangements determined here, the arrangement of *Lithobates catesbeianus* was identical to that of typical neobatrachians, *Babina holsti*, *B. okinavana*, and *B. subaspera* but different from

the neobatrachian-type (Fig. 1). The most remarkable common characteristic in these *Babina* species is that the pseudogene of *trnH* is located downstream of CR. This unique position of *trnH* has also been observed in the *Odorrana* species examined to date. Such an arrangement suggests that gene rearrangement events have transferred *trnH* to a location downstream of CR after the divergence between the lineages of *Babina* + *Odorrana* and *Lithobates* (see Fig. 4).

The rearrangement of mt genes in animals is generally explained by the Duplication and Deletion model (Boore 1999; Moritz and Brown 1986; San Mauro et al. 2006). According to this model, gene rearrangement results from random deletion of original or copied genes produced by a tandem duplication. A single duplication event can easily explain the novel mt gene arrangements observed in *Babina* and *Odorrana* as follows. First, the partial mt gene region from *trnH* to *trnE* of the common ancestral lineage of *Babina* and *Odorrana* was duplicated (Fig. 4I). After this duplication event, the original *trnH* and the copied gene region from *trnS* (AGY) to *trnE* would have been deleted in the lineage which led to *Odorrana* (Fig. 4II). In the same manner, the copied region from *trnS* (AGY) to *trnE* was deleted and the *trnH* copy became the pseudogene in the lineage of *Babina* sensu lato (Fig. 4III), and the original *trnS* (AGY) and *nad5* and the *nad6* copies were deleted and the original *trnE* and the *trnH* copy became the pseudogene in the *Babina* sensu stricto lineage (Fig. 4IV). In this scenario, *Babina* and *Odorrana* share the novel positioning of *trnH* or ps-*trnH*, i.e., a synapomorphic character between these genera.

A mt gene for population genetic studies of *Babina*

Population genetic studies require markers from a DNA region with a high mutation rate.

Whereas mt genes generally have higher mutation rates than nuclear genes, the substitution rates of the mt gene are often variable across amphibians (Hoegg et al. 2004; Igawa et al. 2008; Vences et al. 2002). Thus, I compared the p-distances of each mt gene (2 *rrns*, 13 protein-coding genes, and a concatenated sequence of 22 *trns*) and the CR among six ranid frogs (*Babina holsti*, *B. okinavana*, *B. subaspera*, *Odorrana ishikawae*, *O. tormota*, and *Lithobates catesbeianus*) to identify the gene that is most appropriate for population genetics studies of endangered *Babina* species. Among the gene sequences I examined, the average p-distances of protein-coding genes and the CR (17.2–30.8%) were higher than that of *rrns* and *trn* (10.6–14.2%) (Fig. 2). Within the protein-coding genes and CR, the highest p-distance was for atp8, followed by nad5 and CR. However, the atp8 gene is not appropriate for use as a genetic marker because of the low amount of comparable information (168 bp in sequence length). In conclusion, nad5 is the most suitable marker for population genetic studies of *Babina* species.

Novel microsatellite loci for *B. subaspera*

I identified eight novel microsatellite loci that were polymorphic in *B. subaspera* (Table 1). The N_a, H_O, H_E, and PIC were 5–12, 0.500–1.00, 0.620–0.905, and 0.571–0.897, respectively. For each loci and between any of the loci, there was no significant LD ($P < 0.00179$) and no significant deviation from HWE ($P < 0.00625$) after calculating the Bonferroni correlation. Combining previous and present microsatellite loci, 16 microsatellite loci are available for *B. subaspera*. These microsatellite loci are appropriate in quality and quantity for individual-based analyses, such as parentage and individual recognition.

Cross-species amplification test in *B. holsti*

Microsatellite loci are practical and powerful tools for evaluating the genetic diversity of endangered species. The targets of microsatellite primers that are developed for a species tend to have low polymorphism in non-target species (Primmer et al. 1996). To verify the effectiveness of microsatellite markers developed for *B. subaspera* in *B. holsti*, we performed cross-species amplification using the 16 microsatellite markers described above. We detected 12 polymorphic loci in *B. holsti* from the 16 microsatellite loci (Table 1). The N_a, H_O, H_E, and PIC were 4–20, 0.500–1.00, 0.480–0.950, and 0.450–0.948, respectively. After calculating the Bonferroni correlation, there was no significant LD between any of the loci ($P < 0.00076$) and a significant deviation from HWE ($P < 0.00417$) in Bas2056. The mean PIC value of 12 microsatellite loci (0.761) was higher than that of the eight published microsatellite loci (0.747). Thus, the additional loci increased the quantity of information about polymorphism in the *B. holsti* population.

In summary, I developed both mt and microsatellite markers that can be used for population analyses across varying scales, from historical population demography to individual recognition. These markers are expected to be useful for future studies of Japanese *Babina* species. Furthermore, I resolved the phylogenetic position of *Babina* within Ranidae using derived mt genome information.

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References

Alam MS, Kurabayashi A, Hayashi Y, Sano N, Khan MMR, Fujii T, and Sumida M (2010) Complete mitochondrial genomes and novel gene rearrangements in two dicroglossid frogs, *Hoplobatrachus tigerinus* and *Euphlyctis hexadactylus*, from Bangladesh. Genes Genet Syst 85: 219-232

Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290: 457-465

Boore JL (1999) Survey and summary: animal mitochondrial genomes. Nucleic Acids Res 27: 1767-1780

Boore JL, Brown WM (1994) Mitochondrial genomes and the phylogeny of mollusks. Nautilus 108 (Suppl 2): 61–78

Boore JL, Brown WM (1998) Big trees from little genomes. Mitochondrial gene order as a phylogenetic tool. Curr Opin Genet Dev 8: 668–674

Boore JL, Collins TM, Stanton D, Daehler LL, Brown WM (1995) Deducing the pattern of arthropod phylogeny from mitochondrial DNA rearrangement. Nature 376 : 163–165

Cai H-X, Che J, Pang J-F, Zhao E-M, Zhang Y-P (2007) Paraphyly of Chinese *Amolops* (Anura, Ranidae) and phylogenetic position of the rare Chinese frog, *Amolops tormotus*. Zootaxa 1531: 49–55

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17: 540–552

Che J, Pang J, Zhao H, Wu G-F, Zhao E-M, Zhang Y-P (2007) Phylogeny of Raninae

(Anura: Ranidae) inferred from mitochondrial and nuclear sequences. Mol Phylogenet Evol 43: 1–13

Chen X, Chen Z, Jiang J, Qiao L, Lu Y, Zhou K, Zheng G, Zhai X, Liu J (2013) Molecular phylogeny and diversification of the genus *Odorrana* (Amphibia, Anura, Ranidae) inferred from two mitochondrial genes. Mol Phylogenet Evol 69(3): 1196–202

Duellman WE (2003) Anura (Frogs and toads). In: Hutchins M, Duellman WE, Schlager, N (Eds.), Grzimek's Animal Life Encyclopedia (vol. 6) Amphibians, Second ed. Gale Group Canada, Farmington Hills, MI, pp. 61–68

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792-1797

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary bioinformatics online 1: 47–50

Faircloth BC (2008) Msatcommander: Detection of Microsatellite Repeat Arrays and Automated, Locus-Specific Primer Design. Molecular ecology resources, 8(1): 92–94

Frost DR (2011) Amphibian species of the world: an online reference. American Museum of Natural History, New York, USA. Version 5.5. Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/

Frost DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad CFB, De Sa RO, Channing A, Wilkinson M, Donnellan SC, Raxworthy CJ, Campbell JA, Blotto BL, Moler P, Drewes RC, Nussbaum RA, Lynch JD, Green DM, Wheeler WC (2006) The amphibian tree of life. Bull Am Mus Natl Hist 297: 1–371

Henikoff S (1987) Exonuclease III generated deletions for DNA sequence analysis.

Promega Notes No. 8

Hoegg S, Vences M, Brinkmann H, Meyer A (2004) Phylogeny and comparative substitution rates of frogs inferred from sequences of three nuclear genes. Mol Biol Evol 21(7):1188–1200

Igawa T, Kurabayashi A, Usuki C, Fujii T, Sumida M (2008) Complete mitochondrial genomes of three neobatrachian anurans: A case study of divergence time estimation using different data and calibration settings. Gene 407: 116-129

Inoue JG, Miya M, Tsukamoto K, Nishida M, (2003) Evolution of the Deep-Sea Gulper Eel Mitochondrial Genomes: Large-Scale Gene Rearrangements Originated Within the Eels. Mol Biol Evol 20: 1917–1924

Iwai N (2013) Morphology, function and evolution of the pseudothumb in the Otton frog. Journal of Zoology 289(2): 127–133

Iwai N, Shoda-Kagaya E (2012) Population structure of an endangered frog (*Babina subaspera*) endemic to the Amami Islands: possible impacts of invasive predators on gene flow. Conserv Genet 13: 717–725

Iwai N, Shoda-Kagaya E, Hamaguchi K (2011) Isolation and characterization of eight microsatellite loci in the Otton frog, *Babina subaspera*. Journal of Forest Research, 17(5): 446–448

Jobb G (2011) TREEFINDER version of March 2011. Munich, Germany. Distributed by the author at www.treefinder.de

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular ecology 16(5): 1099–106

Kaneko Y, Matsui M (2004) *Babina holsti* and *Babina subaspera*. The IUCN Red List

of Threatened Species. Version 2014.3. Electronic Database accessible at www.iucnredlist.org.

Kuangyang L, Wenhao C, Kaneko Y, Matsui M (2004) *Babina okinavana*. The IUCN Red List of Threatened Species. Version 2014.3. Electronic Database accessible at www.iucnredlist.org.

Kumazawa Y, M Nishida (1993) Sequence evolution of mitochondrial tRNA genes and deep-branch animal phylogenetics. J Mol Evol 37: 380-398

Kurabayashi A, Sumida M (2009) PCR Primers for the Neobatrachian Mitochondrial Genome. Current Herpetology 28: 1-11

Kurabayashi A, Sumida M, Yonekawa H, Glaw F, Vences M, Hasegawa M (2008) Phylogeny, Recombination, and Mechanisms of Stepwise Mitochondrial Genome Reorganization in Mantellid Frogs from Madagascar. Mol Biol Evol 25: 874-891

Kurabayashi A, Nishitani T, Katsuren S, Oumi S, Sumida M (2012) Mitochondrial genomes and divergence times of crocodile newts: Inter-islands distribution of *Echinotriton andersoni* and the origin of a unique repetitive sequence found in Tylototriton mt genomes. Genes Genet Syst 87: 39-51

Kurabayashi A, Ueshima R (2000) Complete sequence of the mitochondrial DNA of the primitive opisthobranch gastropod *Pupa strigosa*: systematic implication of the genome organization. Mol Biol Evol 17: 266–277

Kurabayashi A, Usuki C, Mikami N, Fujii T, Yonekawa H, Sumida M, Hasegawa M (2006) Complete nucleotide sequence of the mitochondrial genome of a Malagasy poison frog *Mantella madagascariensis*: evolutionary implications on mitochondrial genomes of higher anuran groups. Mol Phylogenet Evol 39: 223–236

Kurabayashi A, Yoshikawa N, Sato N, Hayashi Y, Oumi S, Fujii T, Sumida M (2010)

Complete mitochondrial DNA sequence of the endangered frog *Odorrana ishikawae* (family Ranidae) and unexpected diversity of mt gene arrangements in ranids. Mol Phylogenet Evol 56: 543-553

Mabuchi K, Miya M, Satoh TP, Westneat MW, Nishida M (2004) Gene Rearrangements and Evolution of tRNA Pseudogenes in the Mitochondrial Genome of the Parrotfish (Teleostei: Perciformes: Scaridae). J Mol Evol 59: 287–297

Macey JR, Schulte II JA. Larson A (2000) Evolution and phylogenetic information content of mitochondrial genomic structural features illustrated with acrodont lizards. Syst Biol 49: 257–277

Maeda N, Matsui M (1999) Frogs and Toads of Japan, Rev. Bun-ichi Sogo Shuppan, Tokyo (in Japanese with English abstract)

Moritz C, Brown WM (1986) Tandem duplications of D-loop and ribosomal RNA sequences in lizard mitochondrial DNA. Science 233: 1425–1427

Mueller RL (2006) Evolutionary Rates, Divergence Dates, and the Performance of Mitochondrial Genes in Bayesian Phylogenetic Analysis. Syst Biol 55(2): 289-300

Ogura G, Sasaki T, Toyama M, Takehara K, Nakachi M, Ishibashi O, Kawashima Y, Oda S (2002) Food Habitats of the Feral Small Asian Mongoose (*Herpestes javanicus*) and Impacts on Native Species in the Northern Part of Okinawa Island. Mammalian Science 41:53–62

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics (Oxford, England) 28(19): 2537–2539

Primmer CR, Mqller AP, Ellegren H (1996) A wide-range survey of cross-species microsatellite amplification in birds. Molecular Ecology 5: 365–378

Pyron RA, Wiens JJ (2011) A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol Phylogenet Evol 61: 543-583

Roe BA, Ma DP, Wilson RK, Wong JFH (1985) The Complete Nucleotide Sequence of the *Xenopus Zaevis* Mitochondrial Genome. J Biol Chem 260: 9759-9774

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

San Mauro D, García-París M, Zardoya R (2004) Phylogenetic relationships of discoglossid frogs (Amphibia:Anura:Discoglossidae) based on complete mitochondrial genomes and nuclear genes. Gene 343: 357-366

San Mauro D, Gower DJ, Zardoya R, Wilkinson M (2006) A hotspot of gene order rearrangement by tandem duplication and random loss in the vertebrate mitochondrial genome. Mol Biol Evol 23: 227–234

Sano N, Kurabayashi A, Fujii T, Yonekawa H, Sumida M (2004) Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the bell-ring frog, *Buergeria buergeri* (family Rhacophoridae). Genes Genet Syst 79: 151-163

Sano N, Kurabayashi A, Fujii T, Yonekawa H, Sumida M (2005) Complete nucleotide sequence of the mitochondrial genome of Schlegel's tree frog *Rhacophorus schlegelii* (family Rhacophoridae): duplicated control regions and gene rearrangements. Genes Genet Syst 80: 213-224

Stuart BL (2008) The phylogenetic problem of *Huia* (Amphibia: Ranidae). Mol

Phylogenet Evol 46: 49–60

Sumida M, Kanamori Y, Kaneda H, Kato Y, Nishioka M, Hasegawa M, Yonekawa H (2001) Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata*. Genes Genet Syst 76: 311–325

Sumida M, Kondo Y, Kanamori Y, Nishioka M (2002) Inter- and intraspecific evolutionary relationships of the rice frog *Rana limnocharis* and the allied species R. cancrivora inferred from crossing experiments and mitochondrial DNA sequences of the 12S and 16S rRNA genes. Mol Phylogenet Evol 25: 293–305

Tanabe AS (2008) MrBayes5D. Software distributed by the author at http://www.fifthdimension.jp/

Tanabe AS (2011) Kakusan4 version 4.0. Software distributed by the author at http://www.fifthdimension.jp/

Thompson JC (1912) Prodrome of a description of a new genus of Ranidae from the Loo Choo islands. Herpetological Notices 1: 1-3

Tokita M, Iwai N (2010) Development of the pseudothumb in frogs. Biol Lett 6: 517-520

Toyama M (1996) *Rana* (*Babina*) *holsti* Boulenger, 1982. In: Odate S (ed) Basic data for Rare Wild Aquatic Animals of Japan (III). Japan Fishers Resource Conservation Association, Tokyo

Tzeng CS, Hui1 CF, Shen SC, Huang PC (1992) The complete nucleotide sequence of the *Crossostoma lacustre* mitochondrial genome: conservation and variations among vertebrates. Nucleic Acids Res 20: 4853-4858

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-Checker:

Software for Identifying and Correcting Genotyping Errors in Microsatellite Data. Molecular Ecology Notes 4(3): 535–538

Vences M, Andreone F, Glaw F, Kosuch J, Meyer A, Schaefer H-C, Veith M (2002) Exploring the potential of life-history key innovation: brook breeding in the radiation of the Malagasy treefrog genus *Boophis*. Molecular Ecology 11(8): 1453–1463

Watari Y, Takatsuki S, Miyashita T (2008) Effects of exotic mongoose (*Herpestes javanicus*) on the native fauna of Amami-Oshima Island, southern Japan, estimated by distribution patterns along the historical gradient of mongoose invasion. Biological Invasions 10(1): 7–17

Wolstenholme DR (1992) Animal mitochondrial DNA: structure and evolution. In: Wolstenholme, D.R., Jeon, K.W. (Eds.), Mitochondrial Genomes. Academic Press, New York, pp. 173-216

Zhang J-F, Nie L-W, Wang Y, Hu L-L (2009) The complete mitochondrial genome of the large-headed frog, *Limnonectes bannaensis* (Amphibia: Anura), and a novel gene organization in the vertebrate mtDNA. Gene 442: 119–127

Zhang P, Zhou H, Liang D, Liu YF, Chen YQ, Qu LH (2005) Mitogenomic perspectives on the origin and phylogeny of living amphibians. Syst Biol 54: 391–400

Tables and figures

Table 1. Primers and their sequences used in this study

Name of primers	Priming region	Sequence (5'→3')	Primers used in sequencings				Reference
			B. holsti	*B. okinavana*	*B. subaspera*	*L. catesbeianus*	
12S 180 Fow	*12S rrn*	CCACACCYHCAAGGGHAYTCAGCAGT	●	●	●	●	Kurabayashi and Sumida 2009
12S Cend Fow	*12S rrn*	AAGTGTACTGGAAAGTGC		●		●	
16S 630 Fow	*16S rrn*	CTAGTAACAAGAAATTGC	●				
ATP8 Ce Fow	*atp8*	CCCTCAGAAAATCTTAAG	●		●		
Babihol bfCSBFow	CR	TATCACTTGTATGTTGC	●				Kurabayashi et al. 2010
Babihol CR bfRepFow	CR	ACATTGATGCCCCATATG	●				Kurabayashi et al. 2010
Babihol CR Fow	CR	CAAGTGAGTCCAACTGGA	●				Kurabayashi et al. 2010
Babihol CytbCeFow	*cob*	CTACATTAGGCCTCTTAGA	●		●		Kurabayashi et al. 2010
Babihol His Fow	*trnH*	TAAAACTCTAGATTGTGA	●		●		Kurabayashi et al. 2010
Babihol ND4 400Fow	*nad4*	TACTCGATGAGGAGCTCA	●		●		
Bhol_CR_EndFow	CR	AGATACCTATGGGACCAG	●				
Bok Cytb Cend Fow	*cob*	CCGCTCTCTTACATTTCG		●			
Bok ND4 270 Fow	*nad4*	CGCCTACATTGCCAATAG		●			
BoLc Cytb Ce Fow	*cob*	GGWCAACCAGTAGAAGACCC		●		●	
BoLc ND5 430 Fow	*nad5*	GTCCTTCWTATTAATTGG				●	
Bsub CR 850 Fow	*CR*	CGGACCTTCCCTTGCTTG			●		
Bsub CR befrep Fow	CR	GATCATGTTACATTGATG			●		
Bsub CR CSB Fow	CR	TTATCACTTGTATGTTGC			●		
Bsub CR RendFow	CR	CACATTACCGATAAGTAC			●		
Bsub_Cb_Ce_Fow	*cob*	ACTCCTACATTAGGCCTCTTGGA			●		
CO1 500 Fow	*cox1*	ATCCGTACTAATTACTGC	●		●		
CO1 Cend Fow	*cox1*	TATCTGAGAAGCTTTTGC	●		●		
CO2NeFow	*cox2*	CAAGAYGCRRYHTCHCCNATYATAGAAGA		●		●	
CO3 Nend Fow	*cox3*	GCMCACCAAGCWCAYGCHTWYCAYATRGT	●	●	●	●	Kurabayashi and Sumida 2009
CR af CSB Fow	CR	ACAATGCAATTGATATAGCC		●			
CR af rep Fow	CR	GTGAGTCCAACTGGATCTTC		●		●	
CR bef OH Fow	CR	ATACAAGCTCAGRCCACT		●		●	
Cytb Fow 1-1	*cob*	ACMGGHYTMTTYYTRGCHATRCAYTA	●	●	●	●	Kurabayashi and Sumida 2009
Cytb 840 Fow	*cob*	TGCCTACGCTATCCTCCG			●		
F51	*16S rrn*	CCCGCCTGTTTACCAAAAACAT	●	●	●	●	Sumida et al. 2002
Frog ND5 Fow1	*nad5*	TTYATHGGHTGRGARGGVGTNGG	●	●	●	●	Sano_et al. 2004
Lcat CO3 Cend Fow	*cox3*	GATCTACTGATGAGGCTC				●	
Lcat ND1 Cend Fow	*nad1*	TCTCCCACTTACACTTGC				●	
Lcat ND4 200 Fow	*nad4*	CTGGTTATTCCCTTTAAC				●	
ND1 100 Fow	*nad1*	TATGCAACACCGTAAAGG	●		●		
ND1 420 Fow	*nad1*	CGRGCHGTHGCHCAAACNATYTCHTAYGA	●	●	●	●	Kurabayashi and Sumida 2009
ND2 230 Fow	*nad2*	CCCACGAGCTATTGAAGC	●		●		
ND2 Cend Fow	*nad2*	CTACATTACTACCCTGAC			●		
ND3 120 Fow	*nad3*	AAACTNTCMCCNTAYGARTGYGGHTTTGAYCC	●	●		●	

Table 1. Continued.

ND3 Nend Fow	*nad3*	TATTTTGGCTACCATCAG			●		
ND5 1400 Fow	*nad5*	ATCATCCTCAGCTCCGCC	●		●		
ND5 700 Fow	*nad5*	TCHGCHCAATTYRGNYTHCACCCMTGRCT	●	●	●	●	Kurabayashi and Sumida 2009
ND5 Cend Fow	*nad5*	CTMACACTWGTTGCTACYGC		●		●	
Ranids Val 16S Fow	*16S rrn*	ATYTTGAGCCYHWAAYCTAGCC	●	●	●		Kurabayashi et al. 2010
Trp Fow	*trnW*	YHARACYAARRGCCTTCAAAGC	●	●		●	Kurabayashi and Sumida 2009
12S 600 Rev	*12S rrn*	CTGATAAGCGAGGAATGG	●		●		
16S 240 Rev	*16S rrn*	CCAACTTTATTTATGCTC	●		●		
AsnFow	*trnN*	AAGCTCKCTGGAWWGAGYGTTTAGCTGTTAA	●	●	●	●	Kurabayashi and Sumida 2009
ATP6 120 Rev	*atp6*	TTGGGCAGTTAAGAGGCG	●		●		
Babihol pseudoHis Rev	*ps-trnH*	TATAGAATTACACCTAGT	●				Kurabayashi et al. 2010
Babina ND4L Ce Rev	*nad4L*	GGAGAGTCCTAAWCCAGC	●		●		
Babina_Cytb_870Rev	*cob*	GCCTCCAAGTTTGTTTGG	●		●		
Bok ND6 Cend Fow	*nad6*	GGCTTTGGAGGTTGTGCG		●			
Boki CR Cend Rev	CR	ACACTACGGGTATATAC	●				
BoLc ND5 100 Rev	*nad5*	TTTTACTGCATTTTTYGC		●		●	
BoLc Thr Rev	*trnT*	CARTCTYCGGYTTACAAGACC		●		●	
Bsub CR Nend Rev	CR	GTTACGTATAGGATAGCC			●		
Bsub CR pHRev	CR	GGGTAGTCATTAGAATAC			●		
CO1 1000 Rev	*cox1*	ATARTGGAAGTGGGCAAC		●		●	
CO1 1400 Rev	*cox1*	ATGGAAATGTGCAACTAC	●		●		
CO1 520 Rev	*cox1*	GAAAATTATTACAAATGC	●		●		
CO3 630Rev	*cox3*	GCCAATAATAACATGTAG			●		
CR af rep Rev	CR	GAAGATCCAGTTGGACTCAC		●		●	
CR bef OH Rev	CR	AGTGGYCTGAGCTTGTAT		●		●	
Cyt b 230 Rev	*cob*	CAGCCRTWRTTWACRTCTCGRCARATRTG	●	●	●	●	Kurabayashi and Sumida 2009
Cytb 200 Rev	*cob*	TGATGCKCCRTTGGCATG		●		●	
Frog CO2 300Rev	*cox2*	AYTCRTARCTYCARTATCAYTGRTG		●		●	
Frog CO3 150Rev	*cox3*	CCTTCWCGRAYNAYRTCTCGYCAYCAYTG	●	●	●	●	
Frog ND1 660Rev	*nad1*	ACRTTRAANCCNGANACHAGTTCWGAYTC	●	●	●	●	
Frog ND5 Rev-1	*nad5*	CCYATTTTKCGRATRTCYTGYTC	●	●	●	●	
ND2 center Rev	*nad2*	TTCTGGTATTCAGAAGTG	●	●	●		
ND4 800 Rev	*nad4*	TGCGGTTGCTAAGATCCC	●		●		
ND5 720 Rev	*nad5*	GCDGADACWGGKGTDGGRCCYTCYAT	●	●	●		Kurabayashi and Sumida 2009
ND5 Cend Rev	*nad5*	TTTAATYAGGCCGGTTTG	●		●		
ND5NeRev	*nad5*	AATAAAAATGATCGACAC			●		
ND6 200 Fow	*nad6*	GCCATATCCTGAAGCTTG	●	●	●		
R16 M1	*12S rrn*	GGGTATCTAATCCCAGTTTG	●	●	●	●	Sano_et al. 2004
R51	*16S rrn*	GGTCTGAACTCAGATCACGTA	●	●	●	●	Sumida et al. 2002

Table 2. Sequence comparison of *trns* and their pseudogenes

Names of domains	Sequence (5'-3')
B. holsti trnH	GTAAATATAGTTTAGTAAAACTCTAGATTGTGATTCTAGCAACAAAGGTTAAACTCCTTTTATTTACC
B. holsti ps-*trnH*	..G..C......A.CA......---AGG...A......T--.........T..AC......G..C..-
B. subaspera trnH	GTAAATATAGTTTAGTAAAACTCTAGATTGTGATTCTAGCAACAAAGGTTAAACTCCTTTTATTTACC
B. subaspera ps-*trnH*	..G..C......A.CA....--.....-C..A......T--.T.......T..AC......G..C..T
B. okinavana trnH	GTGAATATAGTTTAACAAAAACCCTAGATTGTGATTCTAGCAACAAAGGTTAAATCCCTTTTATTCACC
B. okinavana ps-*trnH*	-C..G...--------...T.-....T.G...-.---..TGG.T.TGATG....--...C...ACT.G-
B. holsti trnE	TATTTCTGCCAGGACTTTAACCTGAACCTACAACTTGAAAAGCTATCGTTGTAATTCAACTACAAAAAC
B. holsti ps-*trnE*	.G......TT....T...C....-.C..AG..G..CA..-----....C.AC.--...G..T..G....
B. subaspera trnE	TATTTCTGCCAGGACTTTAACCTGAACCTATAACTTGAAAAGCTATTGTTGTTAT-TCAACTACAAAAAC
B. subaspera ps-*trnE*	.G......TT....T...C...--...TC.GC.GC.C....-------.C.C..CA.....CT..G....

"ps" represents pseudogene. Dots indicate sequence identity with compared sequence, and dashes indicate alignment gaps.

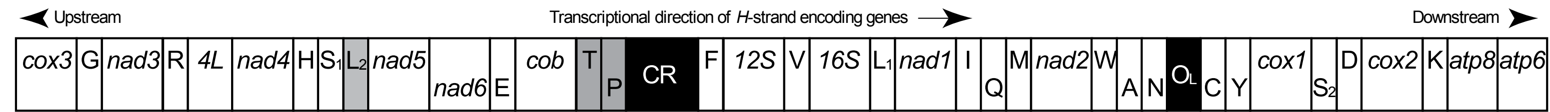
Table 3 Characterization of 8 microsatellite loci and cross-species amplification isolated from *Babina subaspera*

Loci	Accession no.	Repeat motif	Primer sequence (5'-3')	T_a (°C)	Size ranges (bp)	*B. subaspera* (N=10)				*B. holsti* (N=10)			
						N_a	H_O	H_E	PIC	N_a	H_O	H_E	PIC
Bas2091[a]		$(GT)_{23}$	F: [FAM]CTGGAGATGGTTAGGGCTCA R: TACTGCGCTGGGGTACTTCT	53	140-152					4	0.500	0.655	0.592
Bas2037[a]		$(CA)_{21}$	F: [HEX]TGTCGATGGTATGGTATCTGGA R: GGGTGTCACGAAGTACACGA	53	217-361					20	1.000	0.950	0.948
Bas2044[a]		$(GT)_{18}$	F: [NED]GGGTGGGCAAACTCAATATG R: AAAATCGCCCACTCTGTCC	53	175-185					4	0.700	0.615	0.544
Bas2027[a]		$(CA)_{26}$	F: [PET]GCGACAGAAACCTTCTCAGC R: CAAGTGCCCTTTGCTCTTTC	53	208-235					8	0.700	0.860	0.844
Bas1033[a]		$(GT)_{19}$	F: [FAM]TCCCACAGCAAAGAGAAACA R: GGCTAAGCATGTTTTCCAGTAA	53	226-260					12	1.000	0.860	0.849
Bas1036[a]		$(CA)_6GA(CA)_{21}$	F:[HEX] TTTCCCCATTGAGATCTGCT R: TCCCCTGTCATTGTGTTCCT	53	199-209					4	0.500	0.480	0.450
Bas2011[a]		$(GT)_{20}$	F: [NED]CAGGGAGAAGAGCCAGATTG R: ACTGAGTCACCCGTCCGTAG	57	199-262					14	0.900	0.915	0.909
Bas2051[a]		$(GT)_{19}$	F: [PET]GTGAGCCAGGTCTTCTCGTC R: CCATAGTGTGAGAGCCGTTG	53	209-234					8	0.800	0.860	0.844
Bas1047	AB817964	$(GT)_{18}G_6$	F: TGGAAGCACCAAGGACTGG R: TGGACTTGTAGTGCAAAGTGG	58	184-202	5	0.500	0.755	0.713	4	0.600	0.570	0.492
Bas2056	AB817969	$(GT)_{20}$	F: GAGATTGTGTTGCCGGGAC R: CACCATTCCACGAGTATGCG	58	334-461	10	1.000	0.860	0.846	14	**0.600**	0.915	0.909
Bas2003	AB817966	$(CA)_{18}$	F: CTGGAATGAATGGGCAGCG R: GAGGACCGAGGAATTCGGG	58	304-355	6	0.600	0.680	0.642	12	0.800	0.895	0.886
Bas2007	AB817967	$A_{11}(CA)_{48}C(GA)_5$	F: CCTCCCTCTGCTAGCTTGG R: CTCTGCTTGCATGGGCTTC	58	384-467	12	0.800	0.905	0.897	12	0.900	0.880	0.869
Bas2002	AB817965	$(GT)_{24}ATGT(AT)_3$	F: ACAAGCTAGTTGGACAACCG R: GACCGCTGCGCAAATACAG	58	205-238	6	0.700	0.745	0.709	-	-	-	-
Bas2067	AB817971	$(TA)_3(CA)_{15}$	F: AAAGAGGTGGCACTGGGAG R: TGTCTGCCTTGCATAATGTATCAC	58	286-298	5	0.600	0.620	0.571	-	-	-	-
Bas2054	AB817968	$(TA)_3CATA(CA)_{22}$	F: TAAGCAATTCATGCAGCGG R: CAGTGGCAACCTGTGAAGC	56	329-351	7	0.600	0.810	0.773	-	-	-	-
Bas2064	AB817970	$(GT)_{22}A(AT)_5$	F: GGGAGGTCGAGACAAATGC R: ACTCCGATCACACGACTGC	58	343-365	7	0.900	0.755	0.719	-	-	-	-

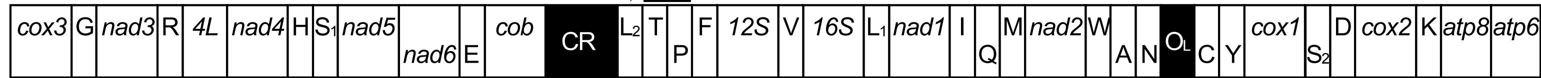
T_a anealing temperature, N_a numberof alleles, H_O observed heterozygosity (in bold numbers, if values are significantly deviated from HWE), N_E expected heterozygosity, PIC polymorphic information content

[a] indicates previously developed microsatellite primers by Iwai et al. 2011; - indicates no polymorphism

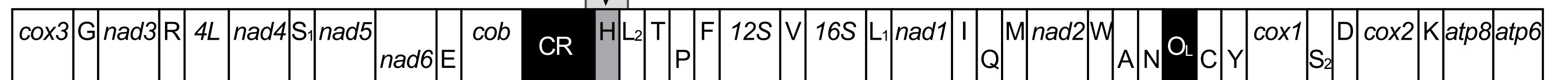
Typical vertebrate-type arrangement



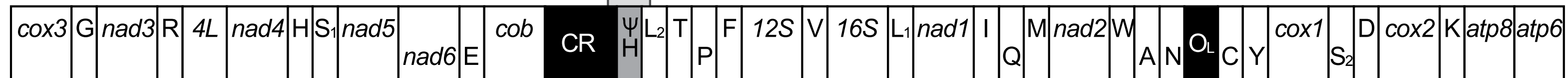
Neobatrachian-type arrangement (*Lithobates catesbeianus*)



Odorrana tormota



Babina okinavana



Babina holsti and *B. subaspera*

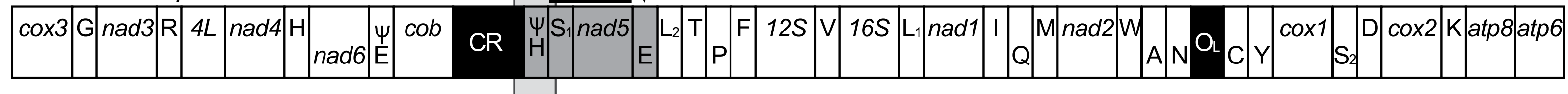


Fig. 1. Comparison of mt gene arrangements among ranid taxa. The transcriptional direction of *H-strand* encoding genes and the upstream and downstream notations used in this paper are shown by a closed arrow and closed arrowheads, respectively. The *H*- and *L*-strand encoded genes are denoted at the top and bottom of each gene box, respectively. The sizes of the boxes do not reflect actual gene length. Closed arrows show the rearranged genes and infer the evolutionary directions of the rearrangements (see the text). Gray boxes represent the rearranged genes from the neobatrachian-type positions. The derived *trnH* position shared by *Odorrana tormota* and *Babina* species is also shown. Transfer RNA genes (*trns*) are designated by single-letter amino acid codes. L1, L2, S1, and S2 indicate *trns* for Leu (UUR), Leu (CUN), Ser (AGY), and Ser (UCN), respectively. Trn boxes with "Ψ" indicate the pseudogenes. The control region is abbreviated as CR. O_L indicates the region of the light-strand replication origin including a typical stem-loop structure. Other genes are abbreviated as follows: *12S* and *16S*, 12S and 16S ribosomal RNA; *cox1-3*, cytochrome c oxidase subunits 1-3; *cob*, cytochrome b; *nad1-6* and *4L*, NADH dehydrogenase subunits 1-6 and 4L.

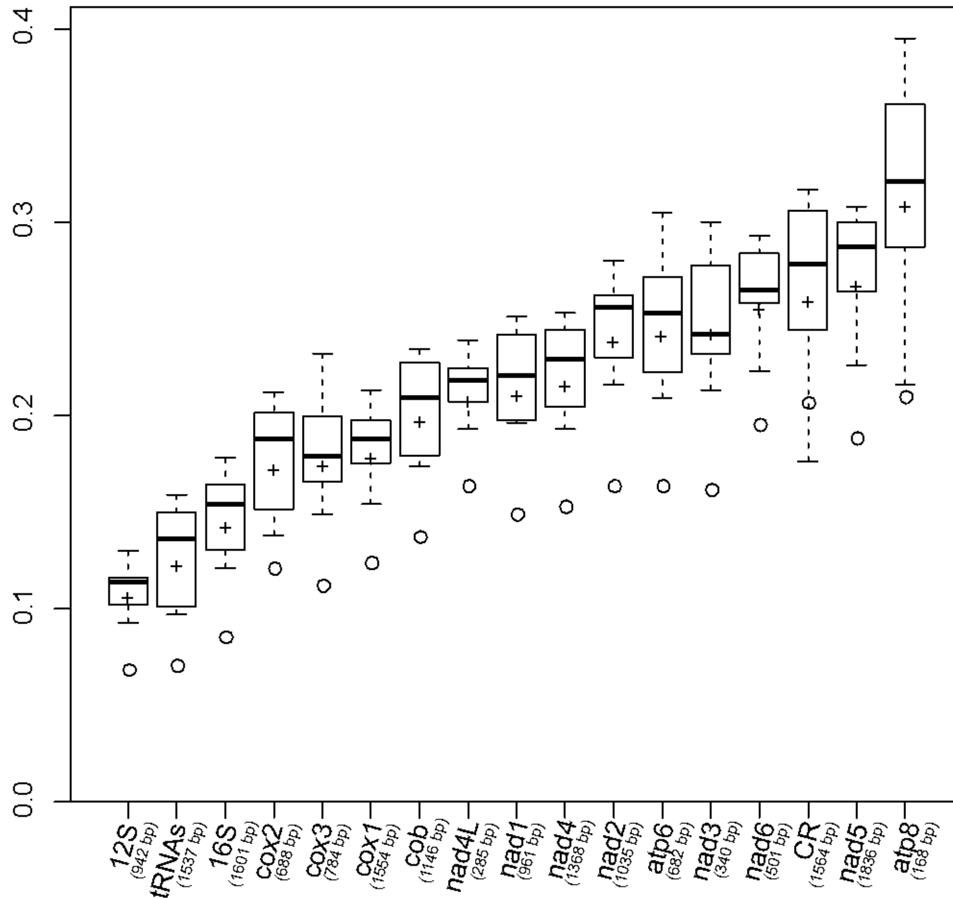


Fig. 2. Box plot of the nucleotide divergence of each mt gene and the CR in ranid frogs
Each box plot shows the currently available nucleotide divergence of each mt gene or CR among 6 ranid frogs (3 *Babina*, 2 *Odorrana*, and 1 *Lithobates* species) (see Fig. 3). Abbreviations of gene names are provided. Box plots have a line representing median divergence, and the surrounding box contains the middle 50% (25–75%) of the data, with whiskers showing the 1.5 inter-quartile range. Pluses indicate average nucleotide divergence of the mt genes (and the CR). The genes and the CR are arranged from the smallest average divergence to the largest average divergence. Open circles indicate differences of *Babina* (*B. holsti* vs. *B. okinavana* vs. *B. subaspera*).

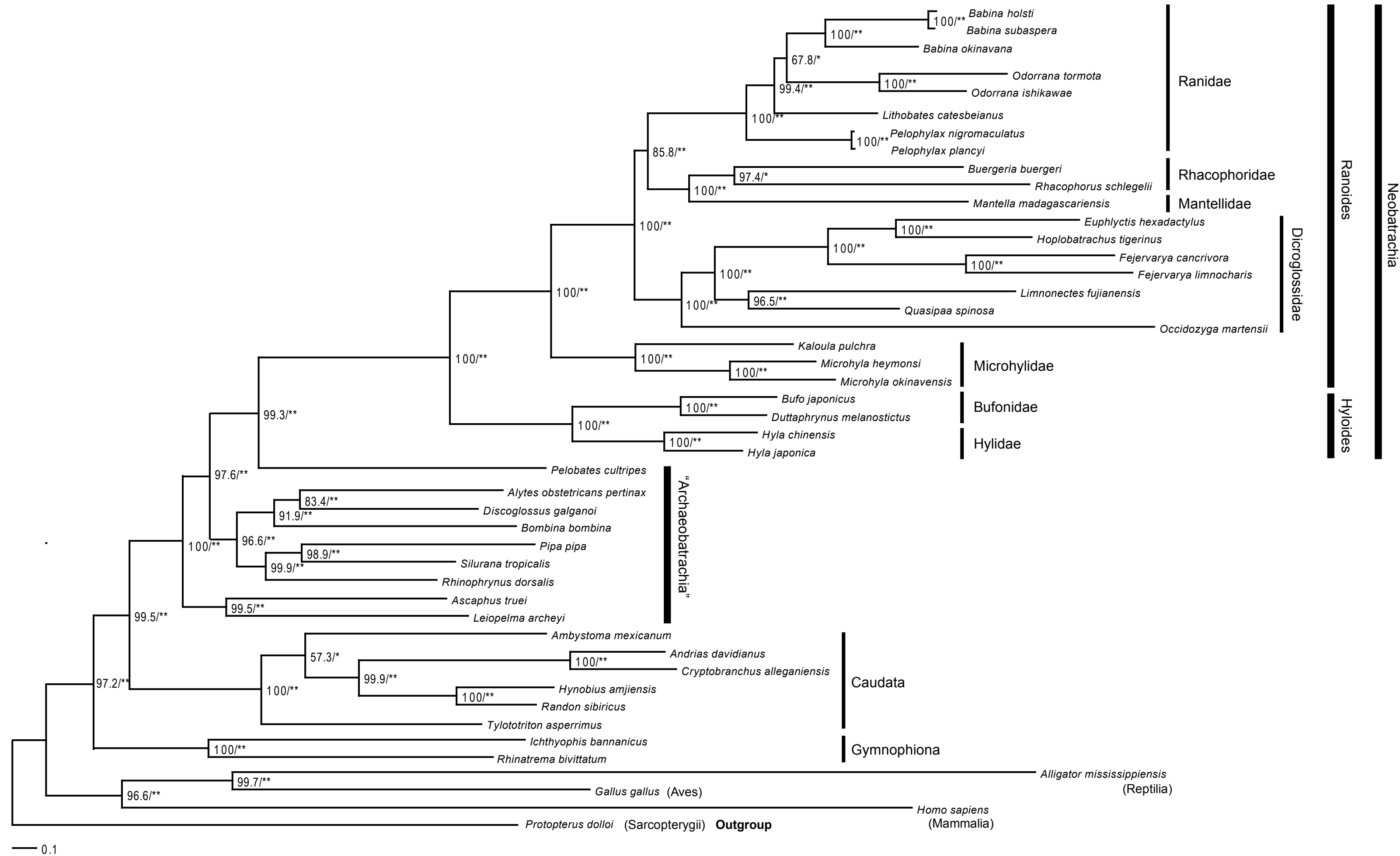
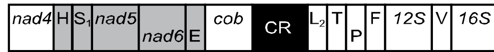
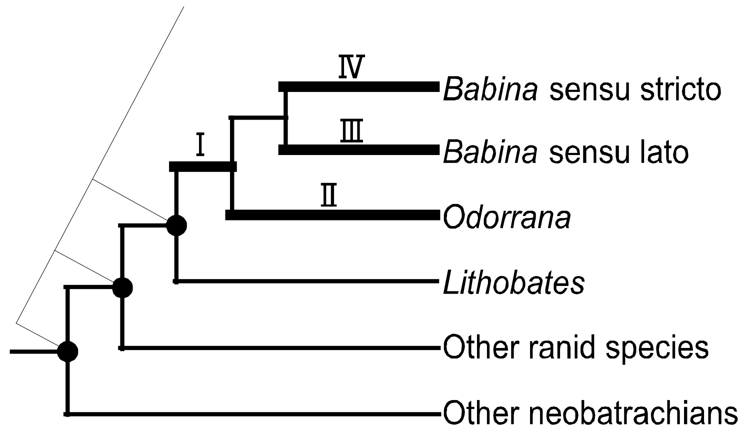
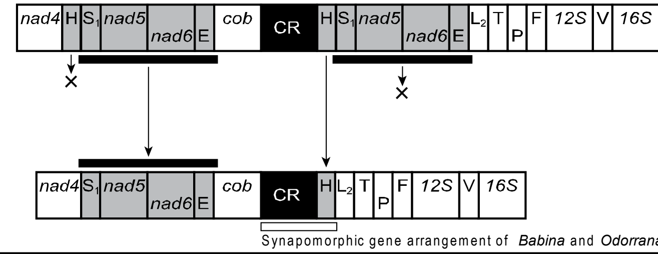


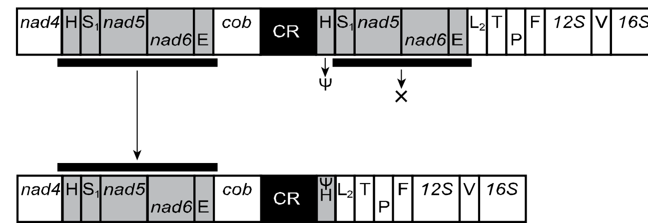
Fig. 3. Anuran phylogeny inferred from mt genomic data

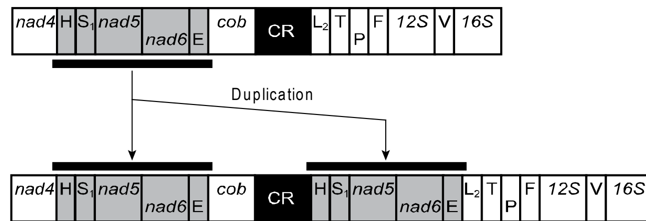
The BI tree is shown here. The ML tree (-log likelihood = 270376.650) has the same tree topology. Bootstrap support (BP) and Bayesian posterior probability (BPP, ** >99 and ** >95) of each node are denoted. Excluding *B. holsti*, *B. okinavana*, *B. subaspera*, and *L. catesbeianus*, the nucleotide sequence data used were taken from the previous studies: *Alligator mississippiensis*, Y13113; *Alytes obstetricans pertinax*, AY585337 ; *Ambystoma mexicanum*, AY659991; *Odorrana tormota*, NC_009423; *Andrias davidianus*, NC_004926; *Ascaphus truei*, AJ871087; *Bombina bombina*, AY458591; *Buergeria buergeri*, AB127977; *Bufo japonicus*, AB303363; *Cryptobranchus alleganiensis*, GQ368662; *Discoglossus galganoi*, AY585339; *Duttaphrynus melanostictus*, AY458592; *Euphlyctis hexadactylus*, NC_014584; *Fejervarya cancrivora*, EU652694; *Fejervarya limnocharis*, AY158705; *Gallus gallus*, X52392; *Homo sapiens*, AF347015; *Hoplobatrachus tigerinus*, NC_014581; *Hyla chinensis*, AY458593; *Hyla japonica*, AB303949; *Hynobius amjiensis*, DQ333808; *Ichthyophis bannanicus*, AY458594; *Kaloula pulchra*, AY458595; *Leiopelma archeyi*, NC_014691 ; *Limnonectes fujianensis*, AY974191; *Mantella madagascariensis*, AB212225; *Microhyla heymonsi*, AY458596; *Microhyla okinavensis*, AB303950; *Occidozyga martensii*, NC_014685; *Odorrana ishikawae*, AB511282; *Quasipaa spinosa*, FJ432700 ; *Pelobates cultripes*, AJ871086; *Pelophylax plancyi*, NC_009264; *Pelophylax nigromaculatus*, AB043889; *Pipa pipa*, GQ244477; *Protopterus dolloi*, L42813; *Ranodon sibiricus*, AJ419960; *Rhacophorus schlegelii*, AB202078; *Rhinatrema bivittatum*, AY456252; *Rhinophrynus dorsalis*, NC_015620; *Silurana tropicalis*, AY789013; *Tylototriton asperrimus*, EU880340.











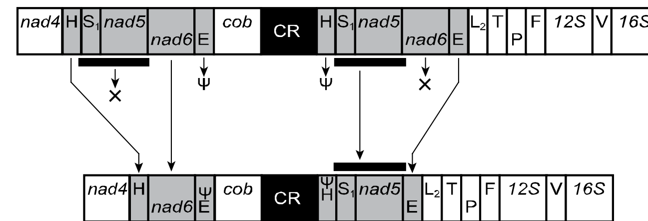


Fig. 4. Mitochondrial gene rearrangement pathway of *Babina* and related taxa
Possible rearrangement lineages and putative duplication or deletion events in each lineage are illustrated. M L and BI tree topologies (Fig. 3) are used here. “X” and ”Ψ” under the gene boxes indicate “deleted gene” and “pseudo gene” in extant species, respectivel y. Gene names and the other abbreviations are the same as those in Fig. 1.

公表論文

(1) Mitochondrial genomes of Japanese *Babina* frogs (Ranidae, Anura): unique gene arrangements and the phylogenetic position of genus *Babina*.

Ryosuke Kakehashi, Atsushi Kurabayashi, Shohei Oumi, Seiki Katsuren, Masaki Hoso, and Masayuki Sumida

Genes & Genetic Systems 88(1): 59-67 (2013)

(2) Development and characterization of new microsatellite loci in the Otton frog (*Babina subaspera*) and cross-amplification in a congeneric species, Holst's frog (*B. holsti*).

Ryosuke Kakehashi, Takeshi Igawa, Noriko Iwai, Etsuko Shoda-Kagaya, and Masayuki Sumida

Conservation Genetics Resources 5: 1071-1073 (2013)

参考論文

(1) Complete mitochondrial genome of *Amolops mantzorum* (Anura: Ranidae).

Xiang Shan, Yun Xia, Ryosuke Kakehashi, Atsushi Kurabayashi, Fang-Dong Zou, and Xia-Mao Zeng

Mitochondrial DNA 1736: 1-3 (2014)

(2) Improved Transport of the Model Amphibian, *Xenopus tropicalis*, and Its Viable Temperature for Transport.

Atsushi Kurabayashi, Ryosuke Kakehashi, Ichiro Tazawa, Yoshikazu Haramoto, Tomomi Oshima, Yuzuru Ito, and Masayuki Sumida

Current Herpetology 33(1): 75-87 (2014)