Molecular phylogenetic relationship of toads distributed in the Far East and Europe inferred from the nucleotide sequences of mitochondrial DNA genes

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ABSTRACT

The toads of the *Bufo bufo* species group are widely distributed in the Eurasian continent and Japanese Archipelago. In this study we analyzed the mtDNA gene sequences of this species group and estimated the divergence time to clarify the evolutionary relationships and biogeography of toads distributed in the Far East and Europe. The phylogenetic tree indicated that this group produced *Bufo bufo* in Europe, whereas it produced *B. japonicus* in the Far East. *B. japonicus* was divided into three major clades corresponding to a group consisting of *B. j. gargarizans* in China, *B. j. bankorensis* in Taiwan, and *B. j. miyakonis* on Miyako Isl. and eastern and western groups of Japanese *B. j. japonicus* subspecies group. The eastern and western groups were divided into several subclades which tended to reflect the region-specific geographic distribution of all localities except *B. j. japonicus* from Hakodate. The estimated branching times of these clades suggest that geological events may have influenced the divergence of the toads distributed in the Far East and Europe.

Keywords: Molecular phylogeny; Mitochondrial genes; Bufonidae; *Bufo*; Molecular clock

1. Introduction

The toads of *Bufo bufo* species group, including the *B. j. japonicus* subspecies group, are widely distributed from the Eurasian continent to the Japanese Archipelago (Inger, 1972; Matsui, 1984). As such, they might be expected, like other frogs, to provide a useful example for studying speciation and/or evolutionary processes caused by paleogeographic events relevant to Eastern Eurasian continent and Japanese island arc. It may also become possible to infer a hypothesis on the evolution of other lineages of non-volant terrestrial vertebrates in this region in the course of its complex geological history.

Based on the reproductive isolation mechanisms elucidated by crossing experiments, Kawamura et al. (1980, 1982) classified the toads from Japan, China and Taiwan as the subspecies group *Bufo japonicus*. According to their reports, this group comprises seven subspecies: *Bufo j. japonicus*, *B. j. montanus*, and *B. j. torrenticola* from the Japan mainland; *B. j. yakushimensis* from Yaku Isl., Japan; *B. j. miyakonis* from Miyako Isl., Japan; *B. j. gargarizans* from China and Taiwan; and *B. j. bankorensis* from Taiwan. Based on an analysis of the numerous morphometric features of the Japanese toads, however, Matsui (1984) concluded that the Japanese toads are divided into three species: *Bufo japonicus* (*B. j. japonicus* and *B. j. formosus* synonymized with *B. japonicus japonicus* in present study), *B. gargarizans miyakonis* (synonymized with *B. j. miyakonis* in present study), *B. torrenticola* (synonymized with *B. j. torrenticola* in this study). In a study on the genetic relationships among *Bufo* species and subspecies distributed in the Palearctic and Oriental regions, Nishioka et al. (1990) found that the *B.*

j. japonicus subspecies group is divided into two groups: the first, consisting of the three subspecies *B. j. miyakonis* in Miyako Isl., Japan, *B. j. gargarizans* in China and Taiwan, and *B. j. bankorensis* in Taiwan; the second consisting of four subspecies in the mainland and on Yaku Isl. in Japan. In an examination of the genetic relationships among *B. j. japonicus* populations distributed in the Japanese mainland by allozyme analysis, Kawamura et al. (1990) found that these populations are divided into two major groups, the western and eastern groups. This grouping corresponds to *B. j. japonicus* and *B. j. formosus* designated by Matsui (1984). However, the molecular phylogenetic relationships and the process of the geographic distribution of the *Bufo japonicus* subspecies group remain to be clarified due to the limited number of polymorphic loci and the lack of informative markers for divergence time.

Mitochondrial DNA can be a powerful molecular marker for reconstructing evolutionary lineages in animals (Avise, 1994; Kocher and Stepien, 1997). Many recent phylogenetic studies have also applied mitochondrial DNA markers to infer the histories of animals with respect to geography, geology, and paleoclimatology (Macey et al., 1998; Mulcahy and Mendelson, 2000). We thus used mtDNA gene sequence data in phylogenetic analysis to elucidate the evolutionary relationships within the *B. japonicus* subspecies group. Finally, we estimated the divergence times of *Bufo* taxa based on the mtDNA data and discussed possible geologic events believed to explain the current distributions of these toads.

2. Materials and methods

2.1. Specimens

We analysed 33 *Bufo* individuals in total consisting of 30 *B. japonicus* from different populations, one *B. bufo*, one *B. viridis* and one *B. americanus.* The collecting stations were shown in Table 1 and Fig. 1. Taxonomic status were followed as previously published (Kawamura et al., 1990; Nishioka et al., 1990), and accordingly, *B. japonicus* was divided into six subspecies (see Table 1).

2.2. DNA sequencing

Total DNA was extracted from clipped toe, liver or muscle samples of each toad specimen using the Qiagen DNEasy tissue extraction kit according to the manufacturer's instructions. The total DNA was used for amplifying DNA fragments by polymerase chain reaction (PCR) with a set of primers for cytbF1/bufo and R51 (Appendix A). PCR mixtures were prepared with an LA-Taq Kit (TaKaRa) according to the manufacturer's protocol. The resulting fragments were amplified by 35 shuttle PCR cycles consisting of denaturation for 20 sec at 98˚C followed by annealing and extension for 5 min at 60 ˚C. The amplified DNA fragments were approx. 6 kbp in length and corresponded to nucleotide positions 16869–17802 and 1-5149 of the complete mitochondrial DNA sequence of the frog species *Rana nigromaculata* (Sumida et al., 2001, GenBank Accession No. AB043889) (contains Cyt *b*, D-loop, tRNA-Leu(CUN), tRNA-Thr, tRNA-Pro, tRNA-Phe, 12S rRNA and 16S rRNA genes in *Bufo japonicus* (our unpublished data)). The PCR fragments were purified with PEG

precipitation (Kraft et al., 1988) then partial portions of the Cyt *b*, 12S rRNA, and 16S rRNA genes and four entire tRNA (Leu(CUN), Thr, Pro, and Phe) genes were directly sequenced with each primer (Appendix A) using a cycle sequencing kit (DYEnamic ET dye terminator, Amersham) and automated DNA sequencer (ABI 373A, Applied Biosystems). The resultant sequences were deposited in the EMBL/GenBank/DDBJ database (Table 1).

2.3. Phylogenetic analyses

The resultant Cyt *b*, tRNAs, 12S rRNA, and 16S rRNA gene sequences from the 33 *Bufo* specimens were aligned using the ClustalW program (Thompson et al., 1994). Initially, we obtained alignment data for each gene region then eliminated any ambiguous and gap sites by observation. To survey the phylogenetic utilities of the different gene regions, we used these data for further phylogenetic analyses. Furthermore, we made a combined data consisting of all four alignment data to construct total evidence tree. Before combining four data sets, we conducted an ILD test for all possible combinations using the partition homogeneity test (parsimony method of Farris et al., 1995) implemented in PAUP* 4.0b10 (Swofford, 2002) to check whether all of the sequences were suitable for combination. The alignment data used in this study are available at: http://home.hiroshima-u.ac.jp/amphibia/sumida/bufoaln.

Maximum likelihood (ML), maximum parsimony (MP), and neighbor joining (NJ) analyses for all five data sets were carried out using PAUP*. In all cases, *B. americanus* was used as the outgroup. The ML and NJ analysis was conducted using substitution

models and parameters estimated by Modeltest version 3.06 (Posada and Crandall, 1998). The proposed model for each data set was denoted in Table 2. The reliabilities of the resultant trees were evaluated by Bootstrap statistics (BP). BP values were calculated by 1000 replications in the MP and NJ optimality criterion and by 100 replications in the ML analysis.

2.4. Estimating the date of divergence

The divergence times of the Japanese *Bufo* taxa were estimated from all usable alignments sites (2073 sites) based on the Bayesian relaxed molecular clock method using MultiDivTime version 9/25/03 (Thorne and Kishino, 2002). First, we separately estimated the model parameters for each alignment data set (Cyt *b*, four tRNAs, 12S, and 16S) using the baseml program in the PAML package (Yang, 1997). The resultant parameters were used to estimate the branch lengths with the estbranches program in the MultiDivTime package (Thorne and Kishino, 2002). Following this, we performed MCMC analysis to approximate the posterior distribution rates and divergence times based on the topology of the combined analysis (Fig. 2A) using multidivtime in the MultiDivTime package. In this analysis, an internal node between the Asian clade and *B. bufo* was saved as a fixed reference point for comparison of the relative divergence ages; the branching date, 10 Ma, was previously reported by Macey et al. (1998). A paleogeographical event, aridization of Central Asia caused by uplifting of the Himalayas was also reported to have occurred 10 Ma (Harrison et al., 1992).

3. Results

3.1. Nucleotide sequences

Nucleotide sequences were determined for partial portions of the Cyt *b*, 12S rRNA, and 16S rRNA genes and four complete tRNA (Leu(CUN), Thr, Pro, Phe) genes from 33 *Bufo* taxa. Four sets of alignment data were obtained with the resultant sequences. The alignment data is summarized in Table 1. We checked for saturated substitutions in each data set by plotting transition/transversion ratio. Although the Cyt *b* sequence seemed to show a weak saturated substitution pattern, the four tRNA, 12S, and 16S sequences showed no saturation.

Figure 3 shows the mean nucleotide divergences (with uncorrected P values) of the sequenced genes at each taxonomic level. The magnitudes of divergence were correlated to the taxonomic level for all genes examined.

The partition homogeneity test (Farris et al., 1995) revealed that all four alignment data sets were suitable for combination (homogeneity not rejected, $P = 0.93$). Thus, we obtained a combined alignment data containing all sequenced portions (see Table 2) and used this in addition with the four individual alignment data sets in subsequent phylogenetic analyses.

3.2. Phylogenetic analyses

The results of phylogenetic analyses based on each data set are summarized in Table 3. Similar phylogenetic trees were reconstructed for all data sets used. The BP values varied considerably across data sets; however, the combined data tended to provide

higher bootstrap values than the individual data (Table 3). Therefore, we mainly describe the phylogenetic relationships based on the combined data.

Figure 2A shows a single ML tree (-ln*L* = 7707.14526) obtained from the combined data. *Bufo bufo*, the continental and Taiwanese toads (*B. j. gargarizans*, and *B. j. bankorensis*), and all of the Japanese toads (*B. j. japonicus, B. j. montanus, B. j. torrenticola, B. j. yakushimensis,* and *B. j. miyakonis*) in this tree formed a clear monophyletic group (clade 1, BP: 100). Within the *Bufo bufo* species group (corresponding to clade 1 in Fig. 2A), *B. bufo* was placed in the basal position and the other taxa were divided into three major groups (clades 4, 5, and 11) supported by high BP values (> 95%) (see Table 3). One of these three groups, clade 11 consisting of *B. j. miyakonis*, *B. j. bankorensis*, and *B. j. gargarizans,* was initially included as a sister group of the other Japanese *Bufo* taxa. The localities of the initially divided taxa (Miyako Isl., Taiwan, and China) are separate from the Japan mainland. The *Bufo* taxa living on the Japan mainland (*B. j. japonicus, B. j. montanus, B. j. yakushimensis, B. j. torrenticola*) formed a clear monophyletic group, clade 3, which was subdivided into two major groups with high BP values (> 96%), clades 4 and 5. Clade 5 corresponded to the eastern Japan group and consisted of *B. j. japonicus* and *B. j. montanus*, while clade 4 corresponded to the western Japan group and consisted of *B. j. japonicus, B. j. torrenticola,* and *B. j. yakushimensis.*

These eastern and western clades were each further divided into three subclades supported by high BP values (>90%). The eastern group (clade 5 in Fig. 2A) was divided into three subclades corresponding to three areas of eastern Japan: clade 8,

central (Kanto and Tokai districts), clade 9, northern (Tohoku district); and clade 10, northeastern (Kanto and Tohoku districts, excluding Hakodate). Similarly, the western group (clade 4) was divided into three subclades corresponding to areas of western Japan: clade 15, central (Kinki, Chugoku, and Shikoku districts); clade 16, comprising two populations of *B. j. torrenticola*; and clade 17, southern (Kyushu district, including Yaku Island). These phylogenetic relationships closely matched the geographic distribution pattern in all but one case; namely, *B. j. japonicus* from Hakodate. Although the Hakodate is situated in the northernmost area of the *B. j. japonicus* distribution, *B. j. japonicus* from Hakodate was included in the eastern group (clade 10) rather than the northern group (clade 9).

3.3. Estimating the date of divergence

Divergence ages of the Asian *Bufo* taxa were estimated by Bayesian molecular dating. In this analysis, we used the divergence time between *B. bufo* and the Asian clade (Macey et al., 1998) as a fixed reference divergence point (10 Ma of clade 1 in Fig. 2B). The resultant ultrametric tree based on the ML topology and estimated divergence time of the *Bufo* taxa are shown in Fig. 2B. The estimated date of divergence was well matched to the proposed geological date.

4. Discussion

4.1. Phylogenetic relationships and taxonomic implications

Our phylogeny indicated that the *B. japonicus* taxa examined here have a closer

common ancestor with European *B. bufo* than *B. viridis*. This seems to support the hypothesis recognizing these species as members of the *Bufo bufo* species group based on morphology (Inger, 1972). Our result also matches the results from karyology (Matui, 1980) and allozyme analysis (Nishioka et al., 1990).

All our analyses strongly support the monophyly of *B. j. japonicus* subspecies group, corresponding to the results of previous crossing experiments (Kawamura et al., 1980, 1982). In these previous analyses, all hybrids between the *B. j. japonicus* subspecies group were fertile, while male hybrids between *B. bufo* and the *B. j. japonicus* subspecies group were sterile.

The resultant trees showed that the *B. j. japonicus* subspecies group was divided into three major clades. In the initial divergence, the ancestors of the clade consisting of *B. j. gargarizans, B. j. bankorensis,* and *B. j. miyakonis* from the China, Taiwan and Miyako Isl. diverged from another clade consisting of *B. j. japonicus* from the Japan mainland. This was followed by further divergence into eastern and western groups. These divergences were in agreement with three clusters detected by allozyme analyses (Nishioka et al., 1990; Kawamura et al., 1990). Morphometric analyses also agree with these relationships. Matsui (1984) divided Japanese common toads (*B. j. japonicus* in this study) into two subspecies (*B. j. formosus* and *B. j. japonicus*) based on their morphology; these two subspecies roughly correspond to the eastern and western groups in our study. Within these eastern and western groups, our results identified subclusters that tended to reflect a region-specific geographic distribution similar to those found by allozyme analyses (Nishioka et al., 1990; Kawamura et al., 1990).

Although Hakodate is located near Tohoku (Fig. 1), the Hakodate population was exceptionally grouped with several Kanto populations rather than the Tohoku populations (clade 10 in Fig. 2A). Furthermore, pairwise sequence divergence between the Hakodate and Kashiwa (one population from Kanto) populations was the lowest (Cyt *b*: 0.0230, tRNA: 0.0000, 12S: 0.0049, 16S: 0.0039, and combined data: 0.0019) of all the pairwise divergences identified (from 0.0019 to 0.1410 between *B. bufo* and *B. americanus*, mean = 0.0569). Noting the same disjunct distribution in a separate study using allozyme data, Kawamura et al. (1990) postulated that some *B. j. japonicus* toads were artificially introduced into Hokkaido from the Kanto district surrounding Tokyo Bay. Furthermore, Matsui (1984) suggested that the morphometric value of the Hakodate population completely overlapped those of the Yokohama population near Kashiwa. Although the small genetic divergence between the Hakodate and Kashiwa populations could be explained by such as the presence of ancestral polymorphism and/or incidental similar nucleotide sequences due to quite low genetic divergences among eastern *B. japonicus* populations, our findings and previous knowledge support the artificial introduction scenario.

Matsui (1976) designated the Japanese stream toad (*B. j. torrenticola* here) a new species (*B. torrenticola*) based on morphological and ecological observations; however our present analysis and previous allozyme analysis (Kawamura et al., 1990) reveal that the Japanese stream toad forms one of the subgroups within the western group of *B. j. japonicus*. However, the detailed position of *B. j. torrenticola* in the western group was not resolved by the present study. Both Kishino-Hasegawa (1989) and

Shimodaira-Hasegawa (1999) tests did not reject the topologies (*B. j. torrenticola* + all western *B. j. japonicus*) and (*B. j. torrenticola* + western *B. j. japonicus* from Arashi to Nichinan) at $P < 0.05$. Thus, further studies are required to clarify the position of *B. j. torrenticola* in the western *B. j. japonicus* subgroup.

4.2. Divergence time and biogeography

The *Bufo* taxa used in our study were broadly divided into seven clades. These groupings basically agreed with previous allozyme analysis and morphological studies; studies in which the authors were unable to discuss the biogeographical hypotheses for the *B. j. japonicus* subspecies group due to a lack of molecular clocks. However, in the present study, no fossil records were available to identify the branching times between Japanese and Asian continental *Bufo* taxa. Accordingly, we used a molecular timescale calibrated with geographic evidence presented by Macey at al. (1998) who indicated that the European and Asian *Bufo* species split occurred 10 Ma. The divergence between the *B. japonicus* subspecies and European common toad *B. bufo* was estimated to have taken place with uplifting of the Transhimalaya and Tibetan Plateau, which effectively dried out Central Asia by blocking the Indian monsoons (Harrison et al., 1992) (Fig. 4B). A similar branching pattern between European and Asian taxa in this era was also reported for the pond frog (*Rana*) species group (Sumida et al. 2000) with an estimated divergence at around 10 Ma.

As shown in Fig. 2B, the following divergence times were obtained from Bayesian molecular dating: (1) 6.8 ± 0.9 Ma between the Chinese, Taiwanese and Miyako Isl. and

Japanese clades (clade 2 in Fig. 2B), (2) 5.7 ± 1.0 Ma between the eastern and western groups on the Japan mainland (clade 3 in Fig. 2B), (3) 4.4 ± 1.0 Ma for the several western subgroups (clade 4 in Fig. 2B), and (4) 3.6 ± 1.0 Ma for the several eastern subgroups (clade 5 in Fig. 2B). On the basis of these estimations, we inferred the biogeographic events that caused the main branchings of the *Bufo* groups. Initial branching between eastern continental and Japanese toads was estimated to have occurred in the Late Miocene (Fig. 2B, clade 2). Takehana et al. (2003) estimated branching of the freshwater fish *Oryzias latipes* at almost the same date. This branching took place during the same era when the Philippine Sea plate (PHS) resumed its subduction (Kamata, 1999; Kamata and Kodama, 1994; Itoh and Nagasaki, 1996). The PHS subduction caused two distinct geologic events: the formation of a large volcano-tectonic depression in the center of Kyushu district and back-arc spreading of the Ryukyu Arc (Fig. 4C). The stock of Japanese *Bufo* species dispersed from the continent through the land bridge from the Korean Peninsula to the Japanese Archipelago (Hikida et al., 1989). The geographic events that occurred in the Kyushu district and Ryukyu Arc might have broken this land bridge and played a critical role in isolating the ancient Japanese *Bufo* species from continental species.

Our findings suggested that *B. j. miyakonis* was divided from the continental *B. j. gargarizans* and Taiwanese *B. j. bankorensis* in the Pleistocene era at approx. 0.9 Ma and 1.3 Ma, respectively. The oldest fossil record of *B. j. miyakonis* from the Late Pleistocene (< 1 Ma) deposit on Miyako Island (Nokariya and Hasegawa, 1985) seems to agree with the result. Furthermore, no native populations or fossil records of the

genus *Bufo* have ever been found in the other Ryukyu Islands (Ota, 2003). Our results and the *Bufo* distribution pattern seem to support the hypothesis that the disjunction between Miyako Island and the other Ryukyu Islands (approx. 6 Ma, Late Miocene) preceded the fragmentation of Miyako Island and the Eurasia continent/Taiwan (approx. 1 Ma, Pleistocene) (Ota, 1998). A similar distribution pattern and divergence have also been reported for the lizard *Takydromus toyamai* (Ota et al., 2002) and snakes *Amphiesma concelarum* and *Calamaria pfefferi* (Ota, 1998).

The Japanese *Bufo* species diverged into two major groups (eastern and western) in the Early Pliocene (5.7 Ma) after separation from the Asia continental species (clade 3 in Fig. 2B). Similar geographical divergence patterns have also been observed in other frogs such as *Rana rugosa* (Nishioka et al., 1993) and *Buergeria buergeri* (Atsumi et al., 1998; Sumida et al., 2004). These findings suggest that divergence among these frog species might have been brought about by the same geographic event. An event corresponding to expansion of this ancient basin might have caused divergence between the western and eastern Japan in the Early Pliocene (Fig. 4D). For example, many basins expanded in an intra-arc depressional zone called the Setouchi Geologic Province and in the back arc area of a mainland known as the Green Tuff region (Itoigawa, 1991; Yoshida, 1992; Kuwahara, 1985). These basins might have segmented the distribution range of the ancestral Japanese *Bufo* species.

There are several possible phylogenetic positions for *B. j. torrenticola* as mentioned above; however, all possible topologies support the grouping of *B. j. torrenticola* and western *B. j. japonicus*. Thus, the morphological appearance of this subspecies seems to

have developed after divergence from eastern *B. j. japonicus* (< 5.7 Ma). The distribution range of this subspecies is limited to a mountainous area near the border of the two major groups, western and eastern. As mentioned above, the ancient basin expanded to these areas during the Early Pliocene followed by a period of uplifting and volcanic activity, which transformed the neighboring landmass in the Pliocene (Takeuchi, 1999a, b). The mountains, basins, and other geographic features in this area might have restricted dispersal of *B. j. torrenticola* from this mountainous area, thereby isolating this subspecies and setting the stage for morphological differentiation.

In conclusion, we showed that the branching dates estimated from mtDNA data are closely related to the geographic history of the Eurasian continent and Japanese Archipelago. We point out, however, that the fossil records and specimens used to identify the branching time in this study were somewhat limited. Consequently, Eurasia and the Japanese Archipelago might be attractive areas for combining phylogenetic and biogeographic studies. Further molecular analyses and fossil records are necessary to provide deeper insight into the evolutionary history of the *B. j. japonicus* subspecies group and *B. bufo* species group.

Acknowledgments

The authors are especially indebted to the late Emeritus Professor T. Kawamura, Hiroshima University, for his invaluable support throughout this work. We are grateful to Professor Chih-Ye Chang, Institute of Zoology, Academica Sinica, Mr. C. S. Wang and Mr. P. S. Lin, National Taiwan University, Dr. E. Crespo, Museu e Laboratorio, Zoologico e Antropologica, Faculdade de Ciencias-Universidade, Portugal, Professor T. Otsu, Yamagata University, Dr. N. Shinozaki, Amphibian Laboratory of Nikko, Professor M. Tadano, Gifu University, Professor S. Ishikubo, Kagoshima University, Professor M. Kuramoto, Fukuoka Educational University, Professor M. Matsui, Kyoto University, Mr. R. Shimoyama, Nagano Prefecture, Mr. M. Sakuyama, Hakodate City, Mr. S. Okada, Tottori University, and Mr. Y. Yuasa, Hyogo Prefecture for collecting and providing valuable specimens. We are also grateful to Dr. H. Yamasaki, Hiroshima University, and Professor H. Ota, University of the Ryukyus, for helpful comments and suggestions on geology and biogeography. This work was supported by Grants-in-Aid for Scientific Research to M. Sumida (No. 13839012) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Figure legends.

Fig. 1. Map showing the collecting stations of the toads used in the present study.

Fig. 2. (A) Maximum Likelihood tree (-ln *L*= 7707.14526) of 33 *Bufo* taxa obtained based on 2,075bp of the mitochondrial DNA genes with the TrN+I+G substitution model. The numbers of each clade correspond to those in the text and Table 3. The Bootstrap supports are given in order for ML/MP/NJ. (B) ML tree topology of the combined dataset converted to an ultrametric tree by estimating the relative divergence dates of the *Bufo* species used in this study. As a fixed reference point, we used the split between European and Asian *Bufo* species (*B. bufo* and the *B. japonicus* subspecies group) (indicated by an arrow).

Fig. 3. Comparisons of the average sequence divergences of the mitochondrial DNA genes among three different taxonomic levels.

Fig. 4. Summary of the paleogeography in eastern Asia. (A) Map of Eastern Asia at

present showing areas represented in the other paleogeographic maps. (B) Paleogeographic map of Southern Asia (Middle Miocene, 10 Ma; modified from Harrison et al., 1992). The solid line indicates the Indian Plate and open arrows indicate movements of the Indian Plate and Indian subcontinent. (C) Paleogeographic map of Eastern Eurasia and the Japanese Archipelago (Late Miocene, 6 Ma; modified from Maruyama et al., 1997 and Kamata and Kodama, 1999). The solid line indicates plates in this area and open arrows indicate movements of the Philippine Sea Plate. Dotted lines and solid arrows indicate the area where land dividing events are expected. (D) Paleogeographic map of the central Japan mainland (Early Pliocene, 5 Ma; modified from Itoigawa, 1991 and Yoshida 1992). Dotted circles indicate the ancient basin. The dotted line and solid arrows indicate the direction of basin expansion.

Fig. 1.

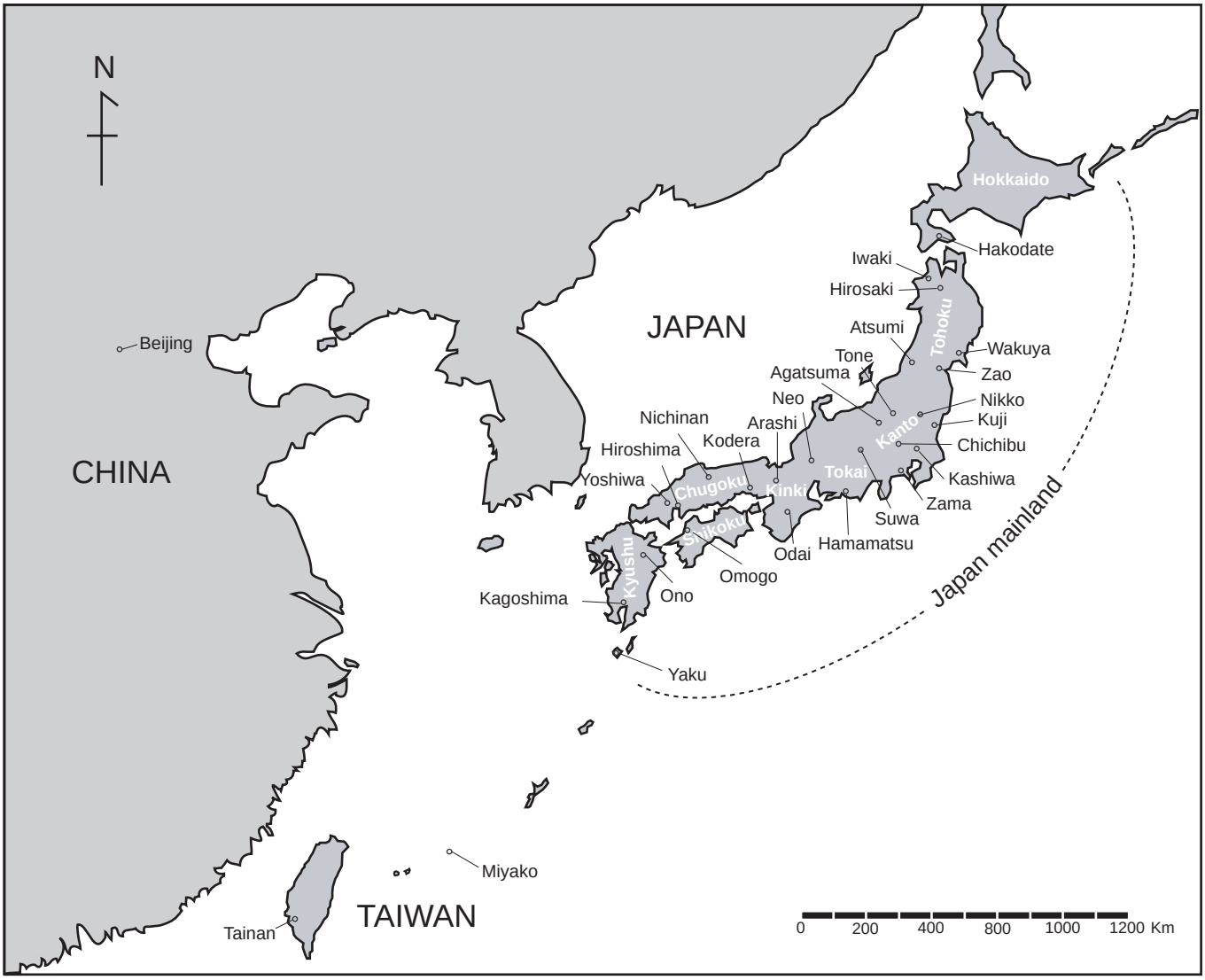
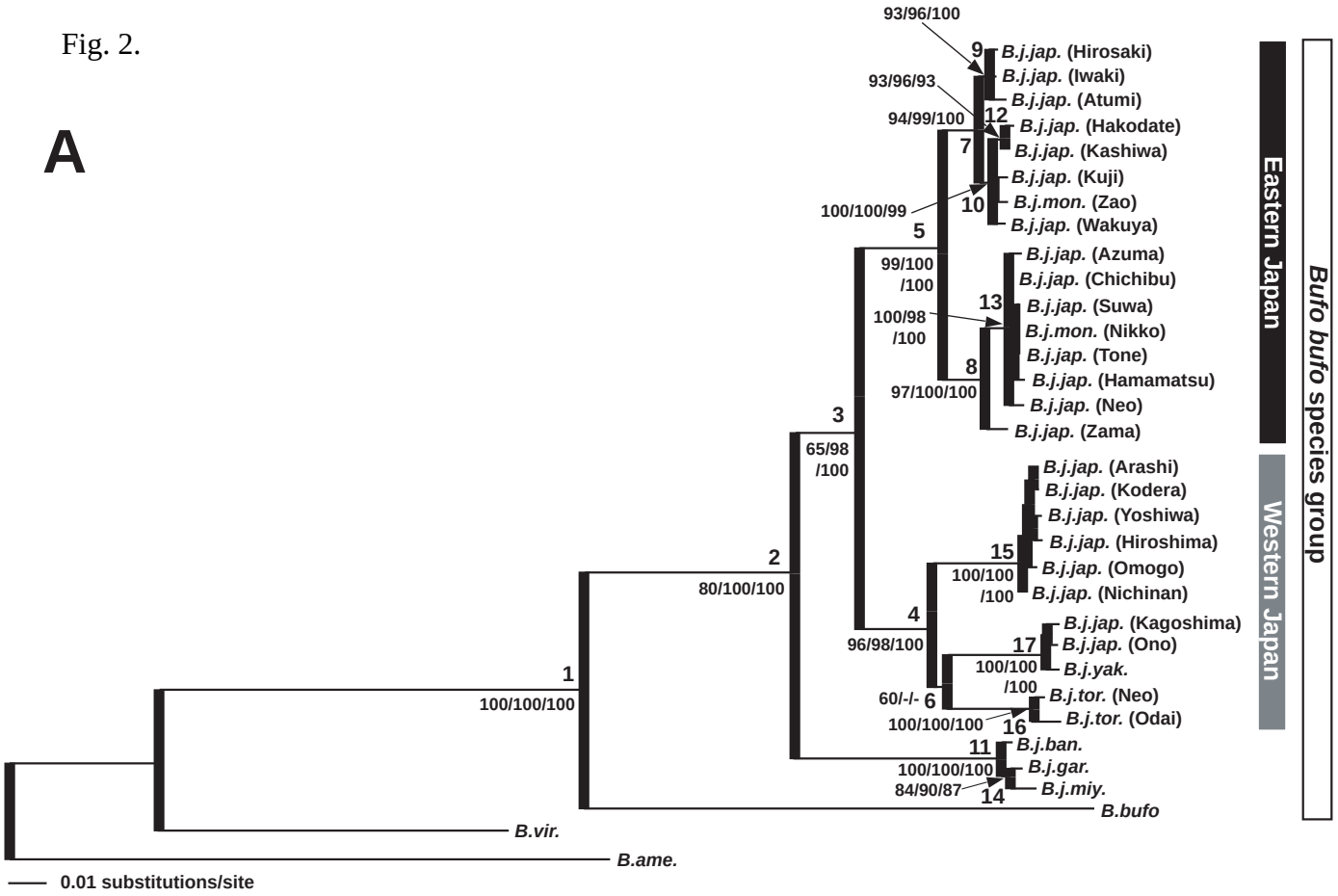

N
Hokkaido
Hakodate
Iwaki
Hirosaki
JAPAN
Tohoku
Atsumi
Wakuya
Tone
Zao
Agatsuma
Neo
Nikko
Kanto
Nichinan
Arashi
Kuji
Kodera
Chichibu
Hiroshima
Tokai
Kashiwa
Yoshiwa
Chugoku
Kinki
Zama
Suwa
Shikoku
Hamamatsu
Odai
Kyushu
Omogo
Ono
Kagoshima
Yaku
Japan mainland
Beijing
CHINA
Miyako
TAIWAN
Tainan
0 200 400 800 1000 1200 Km

Fig. 2.


A
93/96/100
9
B.j.jap. (Hirosaki)
B.j.jap. (Iwaki)
93/96/93
B.j.jap. (Atumi)
94/99/100
12
B.j.jap. (Hakodate)
7
B.j.jap. (Kashiwa)
B.j.jap. (Kuji)
100/100/99
10
B.j.mon. (Zao)
B.j.jap. (Wakuya)
5
99/100
/100
B.j.jap. (Azuma)
B.j.jap. (Chichibu)
13
100/98
/100
B.j.jap. (Suwa)
B.j.mon. (Nikko)
B.j.jap. (Tone)
8
97/100/100
B.j.jap. (Hamamatsu)
B.j.jap. (Neo)
3
65/98
/100
B.j.jap. (Zama)
Eastern Japan
B.j.jap. (Arashi)
B.j.jap. (Kodera)
B.j.jap. (Yoshiwa)
15
B.j.jap. (Hiroshima)
2
100/100
/100
B.j.jap. (Omogo)
80/100/100
B.j.jap. (Nichinan)
4
B.j.jap. (Kagoshima)
96/98/100
17
B.j.jap. (Ono)
100/100
B.j.yak.
1
/100
60/-/-
6
B.j.tor. (Neo)
100/100/100
16
B.j.tor. (Odai)
Western Japan
11
B.j.ban.
100/100/100
B.j.gar.
84/90/87
14
B.j.miy.
B.bufo
Bufo bufo species group
B.vir.
B.ame.
0.01 substitutions/site

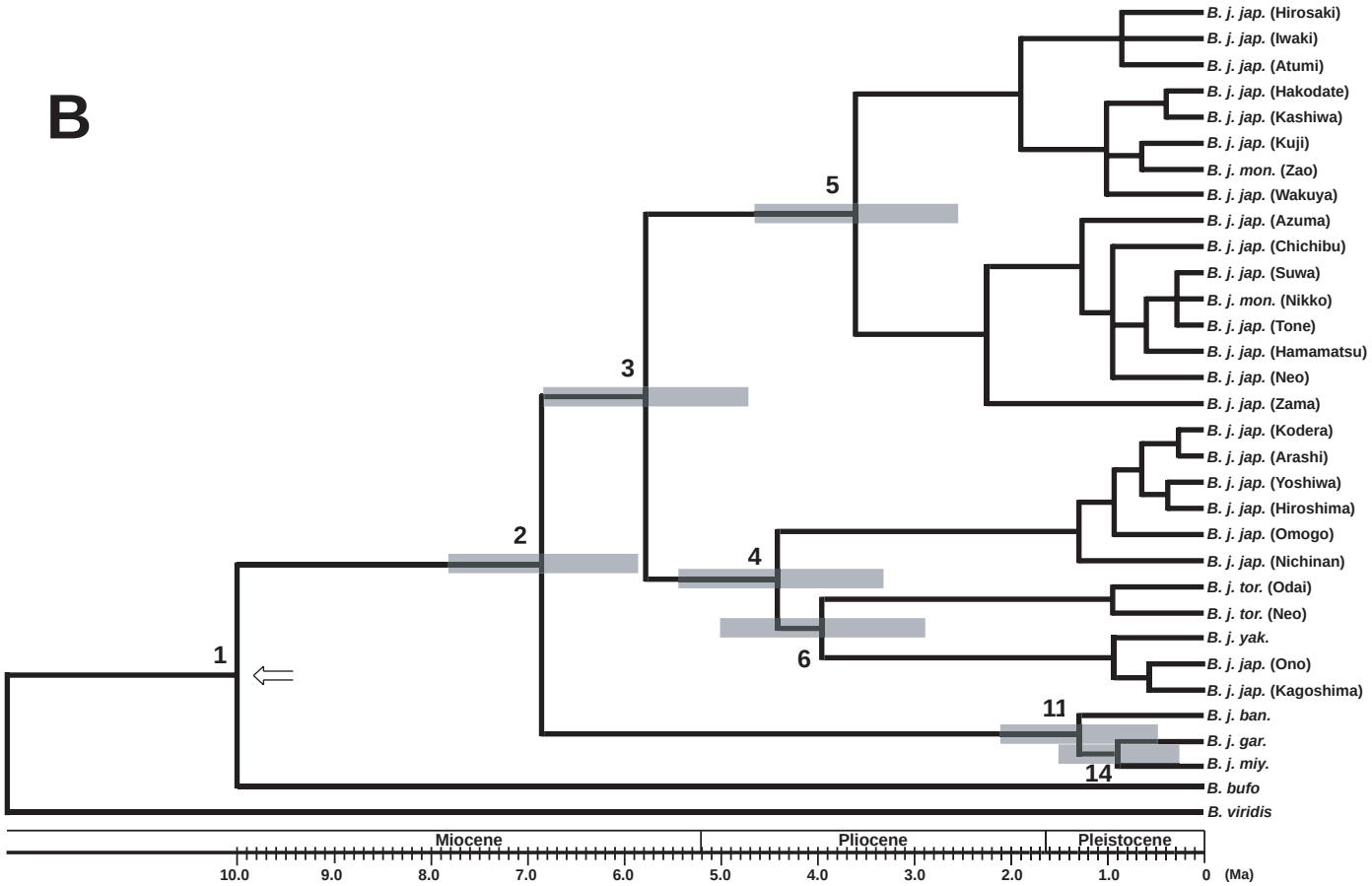

B
B. j. jap. (Hirosaki)
B. j. jap. (Iwaki)
B. j. jap. (Atumi)
B. j. jap. (Hakodate)
B. j. jap. (Kashiwa)
B. j. jap. (Kuji)
B. j. mon. (Zao)
5
B. j. jap. (Wakuya)
B. j. jap. (Azuma)
B. j. jap. (Chichibu)
B. j. jap. (Suwa)
B. j. mon. (Nikko)
B. j. jap. (Tone)
B. j. jap. (Hamamatsu)
3
B. j. jap. (Neo)
B. j. jap. (Zama)
B. j. jap. (Kodera)
B. j. jap. (Arashi)
B. j. jap. (Yoshiwa)
B. j. jap. (Hiroshima)
2
B. j. jap. (Omogo)
B. j. jap. (Nichinan)
4
B. j. tor. (Odai)
B. j. tor. (Neo)
1
6
B. j. yak.
B. j. jap. (Ono)
B. j. jap. (Kagoshima)
11
B. j. ban.
B. j. gar.
B. j. miy.
14
B. bufo
B. viridis
Miocene
Pliocene
Pleistocene
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
(Ma)

Fig. 3.

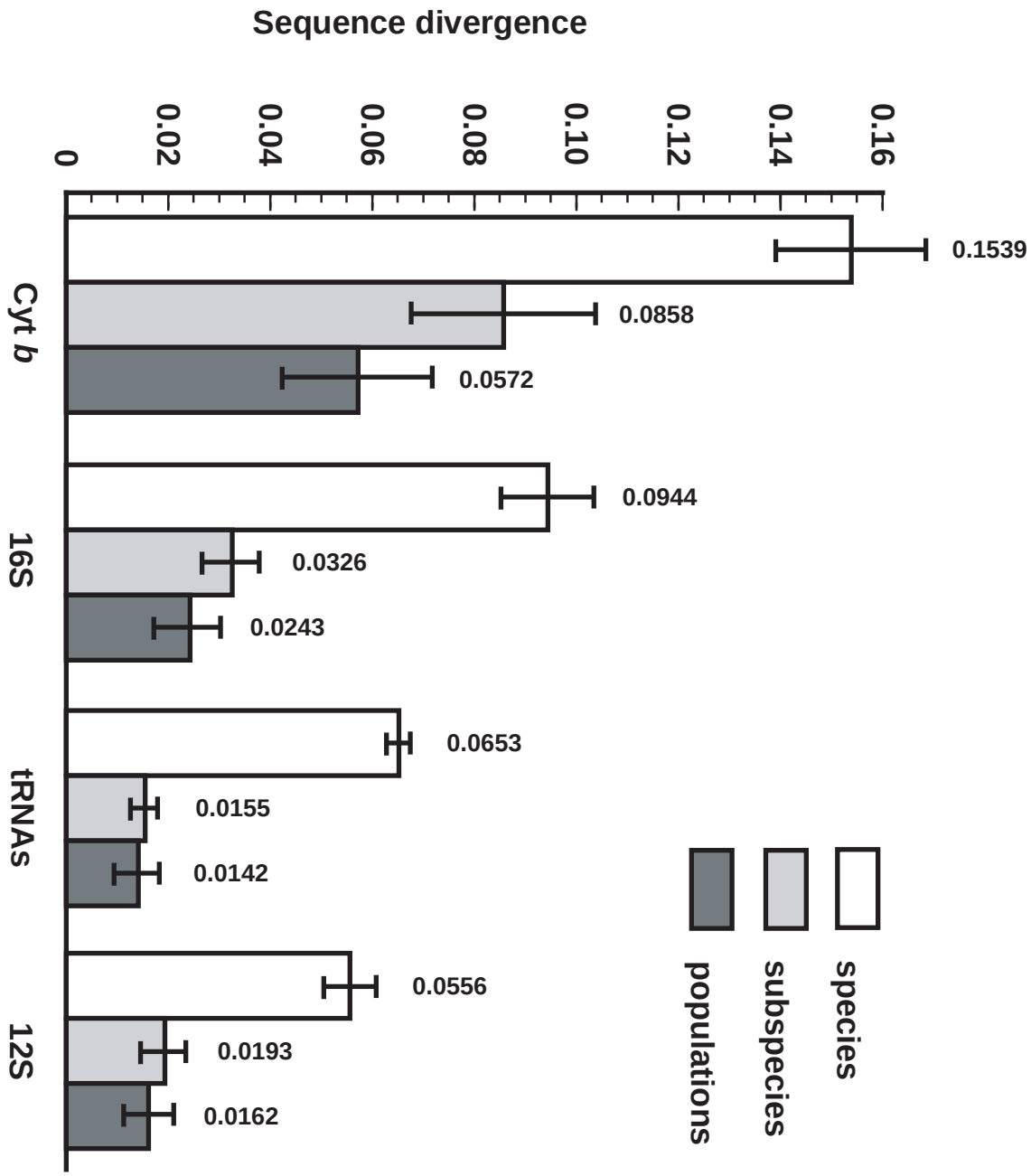

Sequence divergence
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
Cyt b
16S
tRNAs
12S
0.1539
0.0858
0.0572
0.0944
0.0326
0.0243
0.0653
0.0155
0.0142
0.0556
0.0193
0.0162
species
subspecies
populations

Fig. 4.

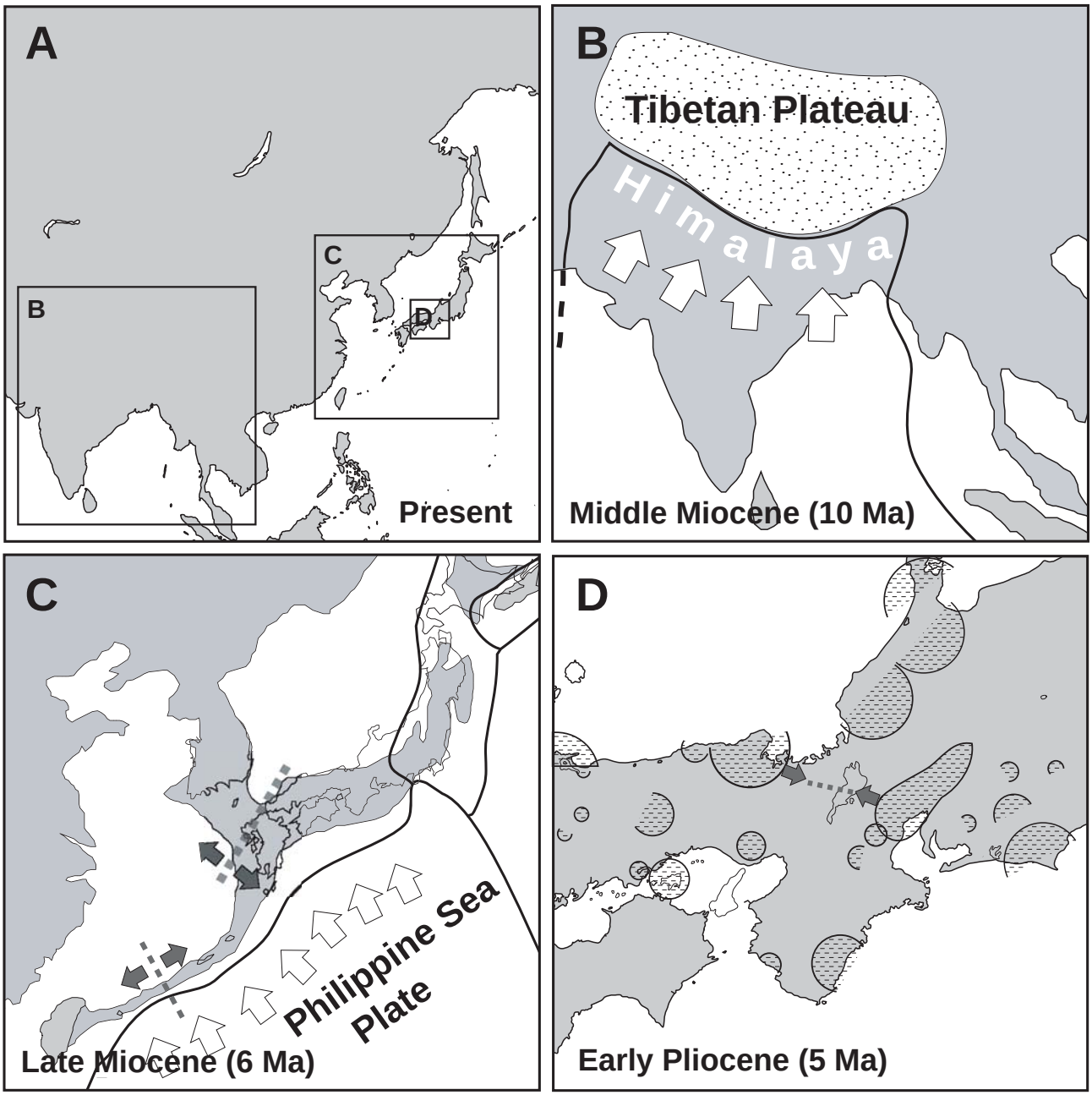

A
B
C
D
Present
B
Tibetan Plateau
Himalaya
Middle Miocene (10 Ma)
C
Philippine Sea Plate
Late Miocene (6 Ma)
D
Early Pliocene (5 Ma)

Table 1. Specimens used and haplotypes of nucleotide sequences of the mitochondrial DNA genes

Species	Collecting station		Abbreviation	Accession Nos.		
	Country	Locality, Prefecture		Cyt *b*	tRNAs and 12S rRNA	16S rRNA
1. *Bufo japonicus japonicus*	Japan	Hakodate, Hokkaido	B.j.jap. (Hakodate)	AB159232	AB159448	AB159561
2. *Bufo japonicus japonicus*	Japan	Hirosaki, Aomori	B.j.jap. (Hirosaki)	AB159233	AB159449	AB159562
3. *Bufo japonicus japonicus*	Japan	Mt. Iwaki, Aomori	B.j.jap. (Iwaki)	AB159234	AB159450	AB159563
4. *Bufo japonicus japonicus*	Japan	Wakuya, Miyagi	B.j.jap. (Wakuya)	AB159235	AB159451	AB159564
5. *Bufo japonicus japonicus*	Japan	Atsumi, Yamagata	B.j.jap. (Atsumi)	AB159236	AB159452	AB159565
6. *Bufo japonicus montanus*	Japan	Zao, Yamagata	B.j.mon. (Zao)	AB159237	AB159453	AB159566
7. *Bufo japonicus montanus*	Japan	Nikko, Tochigi	B.j.mon. (Nikko)	AB159238	AB159454	AB159567
8. *Bufo japonicus japonicus*	Japan	Kuji, Ibaraki	B.j.jap. (Kuji)	AB159239	AB159455	AB159568
9. *Bufo japonicus japonicus*	Japan	Kashiwa, Chiba	B.j.jap. (Kashiwa)	AB159240	AB159456	AB159569
10. *Bufo japonicus japonicus*	Japan	Agatsuma, Gunma	B.j.jap. (Azuma)	AB159241	AB159457	AB159570
11. *Bufo japonicus japonicus*	Japan	Tone, Gunma	B.j.jap. (Tone)	AB159242	AB159458	AB159571
12. *Bufo japonicus japonicus*	Japan	Chichibu, Saitama	B.j.jap. (Chichibu)	AB159243	AB159459	AB159572
13. *Bufo japonicus japonicus*	Japan	Zama, Kanagawa	B.j.jap. (Zama)	AB159244	AB159460	AB159573
14. *Bufo japonicus japonicus*	Japan	Suwa, Nagano	B.j.jap. (Suwa)	AB159245	AB159461	AB159574
15. *Bufo japonicus japonicus*	Japan	Hamamatsu, Shizuoka	B.j.jap. (Hamamatsu)	AB159246	AB159462	AB159575
16. *Bufo japonicus japonicus*	Japan	Neo, Gifu	B.j.jap. (Neo)	AB159247	AB159463	AB159576
17. *Bufo japonicus torrenticola*	Japan	Neo, Gifu	B.j.tor. (Neo)	AB159248	AB159464	AB159577
18. *Bufo japonicus torrenticola*	Japan	Odai, Nara	B.j.tor. (Odai)	AB159249	AB159465	AB159578
19. *Bufo japonicus japonicus*	Japan	Arashiyama, Kyoto	B.j.jap. (Arashi)	AB159250	AB159466	AB159579
20. *Bufo japonicus japonicus*	Japan	Kodera, Hyogo	B.j.jap. (Kodera)	AB159251	AB159467	AB159580
21. *Bufo japonicus japonicus*	Japan	Nichinan, Tottori	B.j.jap. (Nichinan)	AB159252	AB159468	AB159581
22. *Bufo japonicus japonicus*	Japan	Yoshiwa, Hiroshima	B.j.jap. (Yoshiwa)	AB159253	AB159469	AB159582
23. *Bufo japonicus japonicus*	Japan	Hiroshima, Hiroshima	B.j.jap. (Hiroshima)	AB159254	AB159470	AB159583
24. *Bufo japonicus japonicus*	Japan	Omogo, Ehime	B.j.jap. (Omogo)	AB159255	AB159471	AB159584
25. *Bufo japonicus japonicus*	Japan	Ono, Oita	B.j.jap. (Ono)	AB159256	AB159472	AB159585
26. *Bufo japonicus japonicus*	Japan	Kagoshima, Kagoshima	B.j.jap. (Kagoshima)	AB159257	AB159473	AB159586
27. *Bufo japonicus yakushimensis*	Japan	Yaku Isl, Kagoshima	B.j.yak.	AB159258	AB159474	AB159587
28. *Bufo japonicus miyakonis*	Japan	Miyako Isl, Okinawa	B.j.miy.	AB159259	AB159475	AB159588
29. *Bufo japonicus bankorensis*	Taiwan	Kuantzuling, Tainan	B.j.ban.	AB159260	AB159476	AB159589
30. *Bufo japonicus gargarizans*	China	Beijing	B.j.gar.	AB159261	AB159477	AB159590
31. *Bufo bufo*	Portugal	Minho	B.bufo	AB159262	AB159478	AB159591
32. *Bufo viridis* (outgroup)	Turkey		B.vir.	AB159263	AB159479	AB159592
33. *Bufo americanus* (outgroup)	USA	Ann Arbor, Michigan	B.ame.	AB159264	AB159480	AB159593

Table 2. Summary of alignment data and evolutionary models estimated with Modeltest (Posada and Crandall, 1998)

Alignment data	Total sites	Variable sites	Parsimoniously informative sites	Model
Cytochrome *b*	689	318	236	TrN+I+G
tRNAs	279	38	23	TrN+I
12S rRNA	411	57	28	TVM+I+G
16S rRNA	519	106	63	TrN+I+G
Combined data	2073	478	169	TrN+I+G

Table 3. Summary of phylogenetic analyses of the mitochondrial DNA gene fragments

Node	Description	All combined data			Cytochrome *b*			tRNAs			12S rRNA			16S rRNA		
		ML	MP	NJ	ML	MP	NJ	ML	MP	NJ	ML	MP	NJ	ML	MP	NJ
1	*B. viridis* most basal	+(100)	+(100)	+(100)	+(93)	+(90)	+(99)	+(87)	+(87)	+(88)	+(68)	+(84)	+(89)	+(93)	+(99)	+(99)
2	*B. bufo* most basal in *B. bufo* species group	+(80)	+(100)	+(100)	+(58)	+(88)	+(90)	+(76)	+(88)	+(96)	+(60)	+(83)	+(90)	+	+(90)	+(91)
3	*B. j. gar. B. j. miy. B. j. ban.* most basal in *B. j. japonicus* group	+(65)	+(96)	+(99)	+	+(93)	+(99)	+	-	+(51)	-	-	-	+	-	+(91)
4	*B. j. torrenticola* in a clade with *B. j. japonicus* West Japan group	+(96)	+(98)	+(100)	+(95)	+(94)	+(100)	-	-	-	+(64)	+(54)	+(70)	+	-	+
5	*B. j. japonicus* East Japan group	+(99)	+(100)	+(100)	+(77)	+(98)	+(100)	+(62)	+(63)	+(67)	+(73)	+(73)	+(76)	-	-	+(82)
5a	*B. j. torrenticola* in a clade with *B. j. japonicus* East Japan group	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
5b	*B. j. gar. B. j. miy. B. j. ban.* in a clade with *B. j. japonicus* East japan group.	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
6	*B. j. japonicus* Kyusyu district sister to *B. j. torrenticola*	+(60)	+	-	-	-	-	-	-	-	+	+	+	-	-	+
6a	*B. j. japonicus* Chugoki Kinki Shikoku district sister to *B. j. torrenticola*	-	-	+	+	-	+(56)	-	-	-	-	-	-	-	-	-
6b	*B. j. torrenticola* most basal in *B. j. japonicus* West Japan	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
7	*B. j. japonicus* Kanto Tohoku Hokkaido district	+(94)	+(99)	+(100)	+(89)	+(84)	+(100)	+	-	+(55)	-	-	-	+(90)	+(92)	+(98)
8	*B. j. japonicus* Chubu Tokai Kanto district	+(97)	+(100)	+(100)	+(83)	+(96)	+(100)	-	+(57)	+	+(53)	-	+(54)	+(78)	+(82)	+(84)
9	*B. j. japonicus* Tohoku district	+(93)	+(96)	+(100)	+(99)	+(97)	+(99)	-	-	-	-	-	-	-	-	-
10	*B. j. japonicus* Kanto Hokkaido district	+(100)	+(100)	+(99)	+(100)	+(100)	+(100)	-	-	-	-	-	-	-	-	-
11	*B. j. miy. B. j. gar. B. j. ban.* in a clade	+(100)	+(100)	+(100)	+(100)	+(100)	+(100)	-	-	-	+(87)	+(86)	+(95)	+(73)	+(99)	+(100)
12	*B. j. jap.*(Hakodate) sister to *B. j. jap.*(Kashiwa)	+(95)	+(96)	+(93)	+(76)	+(82)	+(75)	-	-	-	+	-	+(59)	+	+(52)	+(63)
13	*B. j. jap.*(Zama) most basal in a *B. j. jap.* Kanto district	+(100)	+(98)	+(100)	+(91)	+(98)	+(98)	-	-	-	+(76)	+(84)	+(90)	+(73)	+(83)	+(86)
14	*B. j. miy.* sister to *B. j. gar.*	+(84)	+(90)	+(87)	+(79)	+(73)	+(87)	-	-	-	-	-	+	+(61)	+(62)	+(68)
15	*B. j. japonicus* Kinki Chugoku Shikoku district in a clade	+(100)	+(100)	+(100)	+(100)	+(100)	+(100)	+(79)	+(90)	-	+(83)	+(88)	+(88)	+(68)	+(92)	+(96)
16	*B. j. torrenticola* in a clade	+(100)	+(100)	+(100)	+(100)	+(100)	+(100)	-	-	-	+(88)	+(93)	+(97)	+(99)	+(99)	+(100)
17	*B. j. japonicus* Kyushu district in a clade	+(100)	+(100)	+(100)	+(100)	+(100)	+(100)	+(61)	+(71)	-	+(87)	+(91)	+(98)	+(62)	+(90)	+(99)

Note. +, a certain topology was supported in analysis; -, the topology was not supported. Numbers in parentheses are bootstrap support values (ML, MP, and NJ) (only given if >50%). Numbering of nodes corresponds to Fig. 2; numbers followed by a or b are alternative topologies not favored by the combined analysis. Combined and separate ML analyses recovered single topology with highest –logML. The –logML were 7707.14526, 5081.08200, 650.15971, 1029.49256, and 1641.63165, for combined data, Cyt *b*, tRNAs, 12S rRNA, and 16S rRNA, respectively. As for MP analyses, combined data reconstructed 110 maximum parsimonious trees (length = 1,360, CI = 0.6593, and RI = 0.8218). Separate data made following MP trees: Cyt *b*, the consensus tree of twelve trees (length = 664 steps CI = 0.6057 RI = 0.8067); tRNAs, the consensus tree of fifty two trees (length = 57 steps CI = 0.7018 RI = 0.8317); 12S, the consensus tree of eighty trees (length = 84 steps CI = 0.7976 RI = 0.8859); 16S, the consensus tree of 179 trees (length = 187 steps CI = 0.6684 RI = 0.8019).

Appendix A. Primers used for amplifying and sequencing fragments of the mitochondrial DNA genes in this study

Name	Sequence 5’-3’	Primer Position	Sequence position	References
12SR1/bufo	CGGATACTTGCATGTGTATA	H2750	tRNA-Leu (CUN)	Present study
			tRNA-Thr	
			tRNA-Pro	
			tRNA-Phe	
FS01	AACGCTAAGATGAACCCTAAAAGTTCT	L2675	5’ portion of 12S rRNA	Sumida *et al.* (1998)
R16	ATAGTGGGGTATCTAATCCCAGTTTGTTTT	H3117	5’ portion of 12S rRNA	Sumida *et al.* (1998)
F51	CCCGCCTGTTTACCAAAAACAT	L4553	3’ portion of 16S rRNA	Sumida *et al.* (2002)
R51	GGTCTGAACTCAGATCACGTA	H5128	3’ portion of 16S rRNA	Sumida *et al.* (2002)
cytbF1/bufo	ATCTGCCGAGATGTAAACAACGG	L16869	5’ portion of Cytochrome *b*	Present study
cytbF2/bufo	AACCTTCTCTCCGCCGCCC	L17106	5’ portion of Cytochrome *b*	Present study
cytbF3/bufo	CATTTATYATTGCAGGCGCC	L17221	5’ portion of Cytochrome *b*	Present study
cytbF3a/bufo	CRTTTATTATTGCAGGCGC	L17221	5’ portion of Cytochrome *b*	Present study
cytbF4/bufo	TACGCCATTCTTCGSTCAATCCC	L17496	3’ portion of Cytochrome *b*	Present study

Note. Primer position corresponds to the sites in the *Rana nigromaculata* sequence (Sumida et al., 2001) and is preceded by the amplification direction indicated as H (heavy strand) or L (light strand). Sequence position indicates the genes sequenced by using each primer.