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# Phylogeography of the Miyako Toad, *Bufo gargarizans miyakonis* (Anura: Bufonidae), Inferred from Mitochondrial Sequence Data

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**Abstract:** The Miyako toad, *Bufo gargarizans miyakonis*, is a subspecies endemic to the Miyako Islands of the Ryukyu Archipelago. The distribution of *B. g. miyakonis* is curious since no toads are found on other islands in the remaining part of the Ryukyu Archipelago. In this study, we conducted phylogenetic estimation and population genetic analyses using sequence data of the mitochondrial control region to clarify the phylogenetic position of *B. g. miyakonis* in the *B. gargarizans* species group and its population history. Results of phylogenetic analyses suggested that *B. g. miyakonis* was closely related to eastern populations of *B. gargarizans* and that there is incomplete lineage sorting among these populations. The divergence time between *B. g. miyakonis* and its closest lineages was estimated to be 0.54–0.75 Ma, the Middle Pleistocene, which was a little younger than the estimated value provided by the previous study. It is probable that the migration of the ancestor of *B. g. miyakonis* was strongly affected by the Middle Pleistocene glacial cycle. Population genetics analyses suggested that this subspecies experienced a rapid population size expansion, and that there is moderate genetic differentiation of sub-populations. Although the *B. g. miyakonis* population has a relatively high-level genetic diversity, it is also unique, having a significant number of accumulated mutations following rapid demographic expansion.

Key words: *Bufo gargarizans miyakonis*; *Bufo gargarizans* species group; Miyako Islands; Phylogeny; Population Genetics

## INTRODUCTION

The *Bufo gargarizans* species group is widely distributed in eastern Asia, including China, Taiwan, Russia, Korea, and Japan (Frost, 2021). This group includes the mainland Asiatic toad, *B. gargarizans* sensu lato

(which includes *B. tibetanus*, *B. andrewsi*, and *B. minshanicus*), as well as the Taiwanese Bankor toad, *B. bankorensis* (Macey et al., 1998; Fu et al., 2005; Zhan and Fu, 2011), but taxonomy of the group is debatable because of its genetic complexity (Othman et al., 2022). Although widely distributed, previous molecular phylogenetic studies have revealed a relatively low level of genetic differences among populations from central to eastern Asia including islands close to the mainland (Fu et

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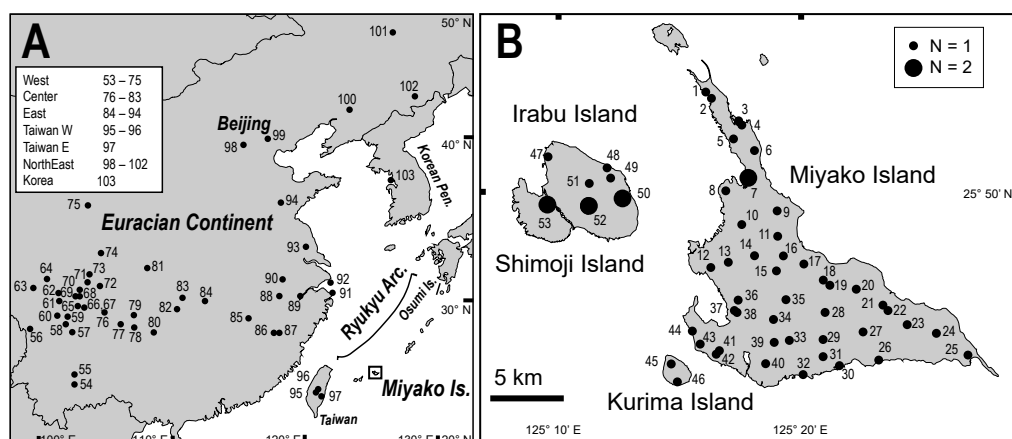


FIG. 1. A: Map of the Eastern area of the Eurasian continent and adjacent regions showing the sampling locations of specimens of the *Bufo gargarizans* species group (Liu et al., 2000; Fu et al., 2005; Cao et al., 2006; Hu et al., 2007; Yu et al., 2014; Tong et al., 2017; Borzée et al., 2017). Location names denoted by dots with numerals are further described in Appendix II. B: Map of the Miyako islands showing the sampling locations of the specimens of *Bufo gargarizans miyakonis* used. Location names denoted by dots with numerals are further described in Appendix I.

al., 2005; Zhan and Fu, 2011; Othman et al., 2022). These findings suggest that the common ancestor of these populations rapidly dispersed from southwest China to northeast China (Hu et al., 2007; Zhan and Fu, 2011; Othman et al., 2022). Previous authors argued that the genetic structure of this species group has been formed by repeated range expansions and secondary admixture among the populations during the Pleistocene glaciation cycle and anthropogenic introductions (Zhan and Fu, 2011; Lee et al., 2021; Othman et al., 2022).

The Miyako toad, *B. gargarizans miyakonis*, is a subspecies endemic to the Miyako Islands of the Ryukyu Archipelago, Japan (Matsui and Maeda, 2018). The Ryukyu Archipelago contains many islands between the Osumi Islands and Taiwan, but the Miyako islands are only the island group where a toad occurs (Fig. 1A) (Ota, 2003; Matsui and Maeda, 2018). Because of this curious distribution pattern of the toad in the Ryukyu Archipelago, Inger (1947) regarded *B. g. miyakonis* as introduced by humans from continental China. Subsequently, Late Pleistocene fossils of bufonids were excavated from the Pinza-Abu cave on Miyako Island

(Nokariya and Hasegawa, 1985). *Bufo g. miyakonis* is therefore now considered as indigenous endemic subspecies (Ota, 1998, 2003; Matsui and Maeda, 2018).

The Miyako Islands in the Ryukyu Archipelago are flat and small (Miyakojima City, 2012). Despite this, there are many unique endemic animals in these islands (Ota and Takahashi, 2008). The Late Pleistocene fossil fauna known from Miyako Island contains unique species and it also includes many species of Palearctic animals (Hasegawa, 1985a, 2012). The unique fauna of the Miyako Islands has therefore attracted considerable attention (Ota and Takahashi, 2008; Ota, 2012), but the origins of the endemic taxa and population history of many species has been controversial (Shokita et al., 2006).

The origin and population history of *B. g. miyakonis* remain unknown. A previous morphological analysis suggested that *B. g. miyakonis* was closely related to a continental population around Shanghai (Matsui, 1984). Several subsequent molecular studies have confirmed that *B. g. miyakonis* is a member of the *B. gargarizans* species group (allozyme:

Nishioka et al., 1990; mitochondrial (mt) DNA: Igawa et al., 2006, Hase et al., 2012, Fukutani et al., 2022: in these studies, a few samples of the *B. gargarizans* species group, including *B. g. miyakonis*, were analyzed as out groups of *B. japonicus* species group). Igawa et al. (2006) estimated that the divergence time between the Miyako population and its close relative, the Beijing lineage, was approximately 0.9 Ma. However, this analysis used only three samples of the *B. gargarizans* species group. Considering that the genetic structure of the *B. gargarizans* species group is complex (see above), it is necessary to more accurately estimate the relationship between *B. g. miyakonis* and other populations of *B. gargarizans* species group.

In this study, we investigated the phylogenetic position of *B. g. miyakonis* within the *B. gargarizans* species based on many samples of *B. g. miyakonis* and many samples of the *B. gargarizans* species group using DNA sequences of the mitochondrial control region. The sequence data for many samples of the *B. gargarizans* species group are available and previous studies (e.g., Liu et al., 2000) showed that the mitochondrial control region is suitable to resolve fine scale phylogeny in this group. We first analyzed sequence data of 341 bp, because this, relatively short region of mtDNA was available for samples from the greatest number of collection sites. We used these data to infer the phylogenetic position of the *B. gargarizans miyakonis* and to estimate the divergence time between *B. g. miyakonis* and *B. g. gargarizans*. Second, we conducted population genetic analyses using data of a 615 bp sequence from the mitochondrial control region to assess the genetic diversity and demographic history of *B. g. miyakonis*. We then considered the population history of *B. g. miyakonis* based on these results.

## MATERIALS AND METHODS

### Sample collection

57 individuals of *B. g. miyakonis* were collected from 53 locations throughout the

Miyako Islands (Fig. 1B, Appendix I). All animals were euthanized using CO<sub>2</sub> (Suzuki and Kurosawa, 2005) and were then stored at -20°C.

### Laboratory procedures

Total DNA was extracted from skeletal muscles using DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). Sequences from the mitochondrial control region of *B. g. miyakonis* were amplified by polymerase chain reaction (PCR). PCR was performed using ExTaq Kit (TaKaRa Bio., Kusatsu, Japan) using the following primers: CytbA-L (5'-GAA TYG GRG GWC AAC CAG TAG AAG ACC C-3') and ControlB-H (5'-GTC CAT TGG AGG TTA AGA TCT ACC A-3') (Goebel et al., 1999). PCR amplification was performed on a MiniAmp Thermal Cycler (Applied Biosystems, Massachusetts, USA) using the following protocol: predenaturation at 95°C for 5 min.; 29–35 cycles of denaturation at 95°C for 30 s, annealing at 65.5–67.5°C for 30 s, extension at 72°C for 90 s; and final extension at 72°C for 5 min. The PCR products were cleaned up by polyethylene glycol precipitation before determination of sequence identity by cycle sequencing. Cycle sequence reactions used a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and the PCR primers listed above along with the following protocol: 95°C predenaturation for 1 min; 30 cycles of denaturation at 95°C for 10 s, annealing at 65.5–67.5°C for 5 s, and extension at 60°C for 4 min. Reaction products were then cleaned up by ethanol precipitation. The Sequences were read on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). All fragments were sequenced both in the forward and reverse senses and were assembled using GeneStudio v.2.2.0.0 (Genestudio Inc., Suwanee, USA).

### Inference of the genealogy of the *Bufo gargarizans* species group

To infer the phylogenetic position of *B. g. miyakonis* within the *B. gargarizans* species group, we used sequences from the mitochon-

drial control region. Although the obtained sequences reached a maximum of 348 bp, we used a 341 bp sequence that was shared by all sequences of the *B. gargarizans* species group. The sequences of *B. g. miyakonis* were aligned by ClustalW, as implemented in MEGA X (Kumar et al., 2018), with sequences from continental populations of *B. gargarizans* (Fu et al., 2005; Cao et al., 2006; Hu et al., 2007; Borzée et al., 2017; Tong et al., 2017) and Taiwanese populations of *B. bankorensis* (Liu et al., 2000; Yu et al., 2014) obtained from GenBank (NCBI; Fig. 1A, Appendix II). Similar to the previous studies, the common toad *B. bufo* (GenBank Accession No: MN122891), which had diverged with *B. gargarizans* species group in the Late Miocene (Zhan and Fu, 2011; Othman et al., 2022), was chosen as an outgroup. In total 120 haplotypes were analyzed (57 sequences from *B. g. miyakonis* and 63 sequences from members of the *B. gargarizans* species group collected at 50 locations: Fig. 1A, Appendix II). The continental populations of *B. gargarizans* were treated as four geographic groups as follows: western China (W), central China (C), eastern China (E), and northeastern China (NE), following Fu et al. (2005) and Hu et al. (2007). The previous studies suggested that the groups from eastern Taiwan (TE), western Taiwan (TW), and Korean peninsula (K) were all monophyletic (Yu et al., 2014; Borzée et al., 2017), so only one haplotype from each of these groups was used for our analyses.

We then performed phylogenetic analyses by both maximum likelihood (ML) and Bayesian inference (BI) approaches. The most appropriate substitution models were determined using MEGA X based on the corrected Akaike Information Criterion (Akaike, 1974; Kumar et al., 2018) for ML and Bayesian Information Criterion (Schwarz, 1978) for BI. For the ML tree, the HKY+G+I (Hasegawa et al., 1985) model was chosen. MEGA X was used to construct the ML tree. The reliability of the ML tree was assessed by calculating bootstrap probability (BP) (Felsenstein, 1985) with 1,000 replications; all other settings were set to default. To

estimate divergence time as well as to infer phylogeny, the BI tree was constructed using BEAST v.2.6.6 (Bouckaert et al., 2019) based on the HKY+G+I model using a coalescent constant model and a relaxed log-normal molecular clock (Drummond et al., 2006). As calibration points, the divergence time of the *B. gargarizans* species group and *B. bufo* was set as  $14.5 \pm 1$  Ma, and the time to the most common recent ancestor (TMCRA) of the *B. gargarizans* species group was set as  $9.99 \pm 1$  Ma (Othman et al., 2022). This tree was initiated with random starting trees, consisting of Markov chain Monte Carlo (MCMC) chains that ran for 50 million generations, with sampling every 1,000 generations. The first 20% of generations were excluded as burn-in. Convergence of the chains to a stationary distribution and the effective sample size (ESS) were assessed visually using Tracer v.1.7.2. (Rambaut et al., 2018). Furthermore, to infer the phylogenetic relationships among haplotypes, median-joining network tree was constructed using NETWORK v.10.2.0.0. (Bandelt et al., 1999; Fluxus Technology Ltd., 2021).

#### Genetic diversity and population structure

We analyzed the genetic diversity and population structure of *B. g. miyakonis* samples, using a 615 bp sequence shared by all samples of this subspecies, including the extended region of the  $\leq 348$  bp sequence described above. Haplotype diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) were calculated using Arlequin v3.5.2.2 (Excoffier and Lischer, 2010). Populations of *B. g. miyakonis* were then divided into two sub-populations: the Miyako Island–Kurima Island (MY–KR) and Irabu Island–Shimoji Island (IR–SM) groups. To investigate the genetic structure of these groups, we conducted an analysis of molecular variance (AMOVA; Excoffier et al., 1992) with a permutation test of 1,000 random replications using Arlequin v.3.5.2.2.

#### Demographic history

To investigate the population demographic history of *B. g. miyakonis*, the mismatch distri-

TABLE 1. Genetic variation and statistics of *Bufo gargarizans miyakonis* (615 bp). In the IR–SM population, the number of samples was small, so the mismatch distribution analysis did not converge. n: sample size, hn: number of haplotypes, h: haplotype diversity value,  $\pi$ : nucleotide diversity value, SD: standard deviation, SSD: sum of square deviations, Rag: Harpending's Raggedness index. Asterisks indicate levels of significance: double asterisks (\*\*) for  $P \leq 0.01$ .

Group	n	hn	$h \pm SD$	$\pi \pm SD$	Tajima's $D$	Fu's $F_s$	SSD	Rag
MY–KR	47	19	0.8871 $\pm$ 0.0339	0.005177 $\pm$ 0.003025	–1.17474	–8.43017**	0.01027978	0.03944338
IR–SM	10	3	0.6000 $\pm$ 0.1305	0.002493 $\pm$ 0.001840	–1.14612	1.35813	N/A	N/A
All	57	19	0.8972 $\pm$ 0.0232	0.004984 $\pm$ 0.002919	–1.13053	–7.5377**	0.00729951	0.03136915

bution (Rogers and Harpending, 1992), Tajima's  $D$  (Tajima, 1989) and Fu's  $F_s$  (Fu, 1997) were computed using Arlequin v3.5.2.2. The statistical significance of these tests was then tested using parametric bootstrapping with 1,000 replicates. In addition, an extended Bayesian skyline plot (EBSP) analysis (Drummond et al., 2005) was used to infer the demographic history. The EBSP was constructed based on an HKY model using a coalescent extended Bayesian skyline model and a relaxed log-normal clock consisting of MCMC chains run for 100 million generations, with sampling every 1,000 generations. The first 20% of generations were excluded as burn-in, and convergence of the chains to a stationary distribution and ESS were assessed visually using Tracer v1.7.2. The evolution rate of the Miyako population was set to 1.69% per Ma, as inferred from the previously mentioned divergence time estimation. Because reproductive generations overlap in bufonids (e.g., Okuno, 1986), we did not estimate the effective population size in a strict sense. Bayesian skyline plots were produced using plotEBSP.R, an R package, implemented in R v4.2.0 (R Core Team, 2021).

## RESULTS

### Sequence characteristics

Of the 57 *B. g. miyakonis* individuals studied, we identified 12 haplotypes using a 347 bp sequence of the mitochondrial control region (shared by all sequences of *B. g. miyakonis* in the first dataset for phylogenetic inference of

the *B. gargarizans* species group) and 19 haplotypes using the 615 bp sequence (used for population analyses) (Table 1; Appendix I; GenBank Accession No.: LC683658–LC683676). Haplotypes, MYK01, 05, 07, 10, recognized in the 347 bp data were divided into more than one haplotypes in the 615 bp dataset.

### Phylogenetic position of *Bufo gargarizans miyakonis*

Our phylogenetic trees were consistent with previous studies (e.g., Igawa et al., 2006, Zhan and Fu, 2011; Fig. 2A). The result of phylogenetic inference showed that *B. g. miyakonis* was included in the clade that consisted of many eastern populations of *B. gargarizans* (Fig. 2A). In contrast, the TE population, which was most geographically proximate, was relatively far from *B. g. miyakonis* in the tree. The monophyly of *B. g. miyakonis* was not supported (Fig. 2B). The monophyly of haplotypes, MYK01–10 (hereafter, haplogroup MYK), was supported by high BP and posterior probability (PP), but the remaining two haplotypes, MYK11 and MYK12, were located outside of the haplogroup MYK Clade along with a few the eastern populations of *B. gargarizans* (Fig. 2; E1 from Locality No. 90 and No. 93, and E2 from Locality No. 93, in Fig. 1A). In the network, *B. g. miyakonis* was not distinct from the continental *B. gargarizans* group. MYK11, MYK12 were placed between the other haplotypes from the Miyako Islands and those from the continental China (Fig. 2C).



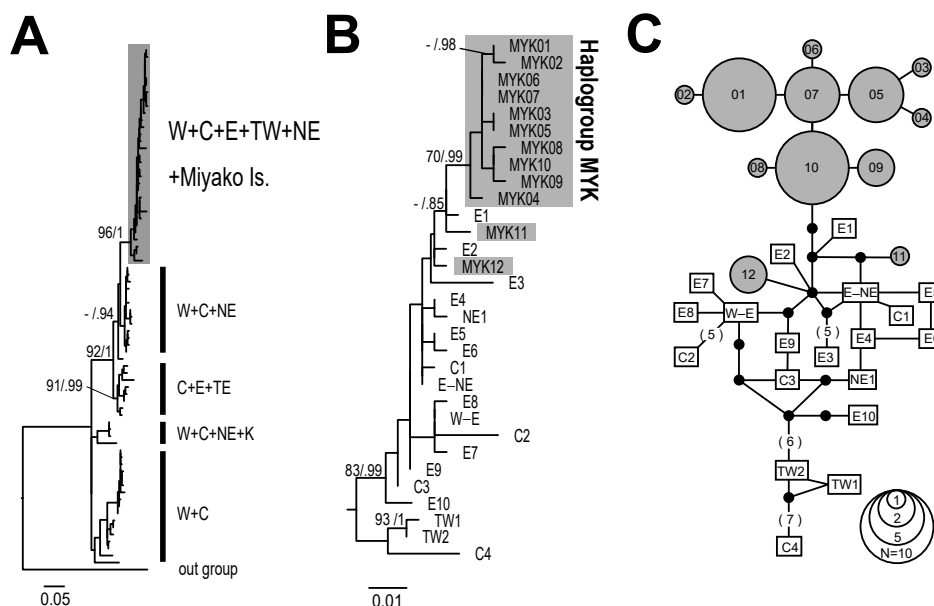


FIG. 2. Genetic trees of the *Bufo gargarizans* species group reconstructed from the mt control region (341 bp). Bootstrap probability (BP) for maximum likelihood (ML) and posterior probability (PP) values for Bayesian inference (BI) models are given in order at each node, although only when  $BP \geq 70\%$  and  $PP \geq 0.80$ . A: ML trees of the *B. gargarizans* species group. The clade that includes *B. g. miyakonis* is indicated in gray. B: ML trees of the clade that includes *B. g. miyakonis*. Haplotypes of *B. g. miyakonis* are colored in gray. See the main text for information on how *B. g. miyakonis* samples were grouped. C: Median-Joining network trees of the clade that includes *B. g. miyakonis*. Gray circles indicate haplotypes of *B. g. miyakonis*, white rectangles indicate haplotypes of *B. gargarizans* or *B. bankorensis*, and black dots indicate missing haplotypes, respectively. When nucleotide substitution between haplotypes has occurred more than once, the number of times is shown as a number in parentheses.

### Divergence time

The average evolution rate of the mitochondrial control region of *B. gargarizans* species group was estimated to be  $1.37\% \pm 0.27\%$  (ESS=5,188) per Ma. The divergence time between haplogroup MYK and other haplotypes was 0.75 Ma (95% HPD=0.289–1.02 Ma) in the Middle Pleistocene (Fig. 3), and the evolution rate of haplogroup MYK was 1.69% per Ma. In addition, the divergence times of MYK11 from E1 and MYK12 from E2 were estimated to be 0.62 Ma (95% HPD=0.072–0.749 Ma) and 0.54 Ma (95% HPD=0.022–0.736 Ma), respectively (Fig. 3).

### Genetic diversity and population structure

For the 615 bp *B. g. miyakonis* population

dataset, our estimate of  $h$  was  $0.8972 \pm 0.0232$ , and our estimate of  $\pi$  was  $0.004984 \pm 0.002919$  (Table 2). When comparing the genetic diversity of the sub-populations, IR–SM samples showed approximately half the diversity (as measured by  $\pi$ ) as MY–KR samples (Table 1).

Our AMOVA results revealed that there was significant genetic differentiation between sub-populations (Table 2).

### Demographic history

For the whole *B. g. miyakonis* population, Tajima's  $D$  was negative ( $-1.13053$ ) although not statistically significant ( $p=0.11$ ). Moreover, Fu's  $F_s$  was also negative, and it was statistically significant (Table 1). Tajima's  $D$  was also not significant for both sub-populations. Fu's

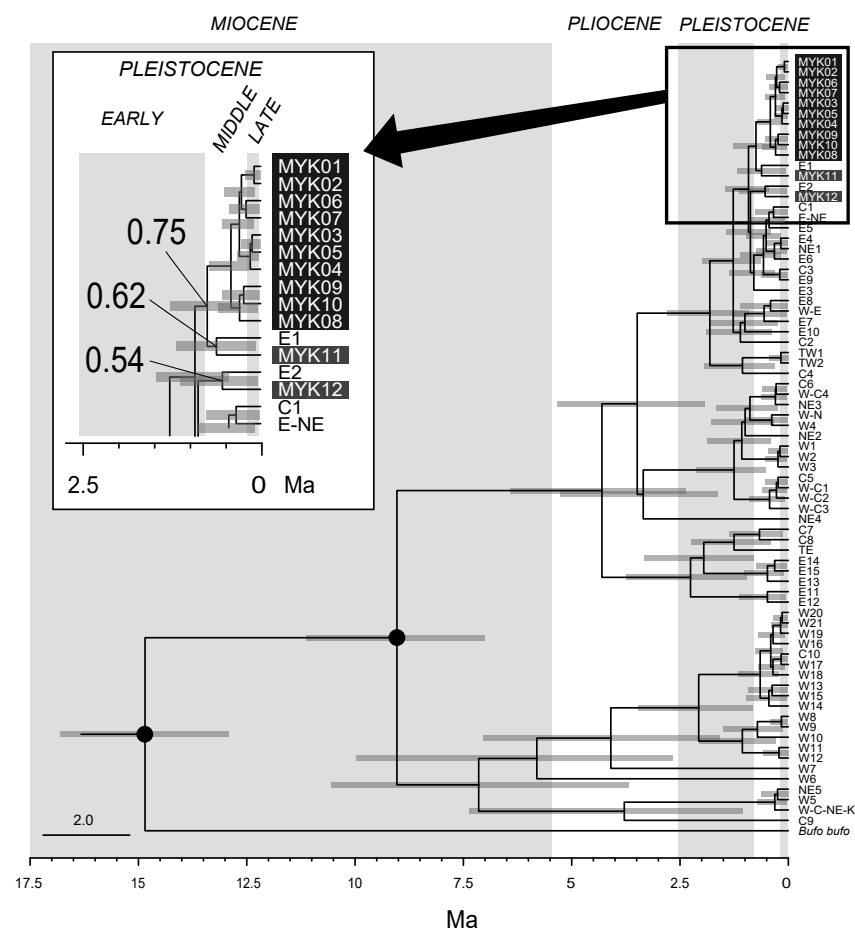


FIG. 3. Bayesian divergence time estimates of the mt control region (341 bp) of the *Bufo gargarizans* species group. The haplotypes of *B. g. miyakonis* (the haplogroup MYK and MYK11–12) are colored in dark gray and gray, respectively. The black dot nodes indicate calibration points based on Othman et al. (2022). Gray bars on nodes indicate 95% credible intervals.

TABLE 2. AMOVA results. Asterisks indicate levels of significance: double asterisks (\*\*) for  $P \leq 0.01$ .

	Sum of squares	Variation components	Variation (%)	$\Phi_{ST}$
Among population	5.691	0.25671	14.98015	0.1498015**
Within population	80.134	1.45698	85.01985	
Total	85.825	1.7137		

$F_s$  was negative and significant only for the MY–KR group. The mismatch distribution of the population was a unimodal curve, with a peak around 3–4 ( $\tau=3.129$ ,  $\theta_0=0.000$ ,  $\theta_1=17.188$ , average pairwise difference=3.065).

The sum of square variance (SSD) and Harpending’s Raggedness index (Rag) were also not significant, suggesting the rapid expansion of this population (Table 1). The mismatch distribution of the MY–KR group



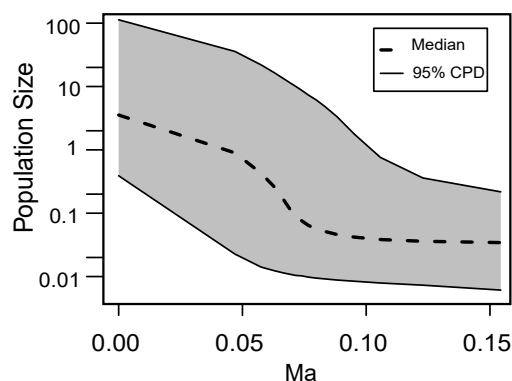


FIG. 4. The EBSP of haplogroup MYK. The vertical axis and the horizontal axis are the logarithmic relative population size and the absolute time before the present (Ma), respectively.

was generally similar to that of the population as a whole. The distribution for the IR–SM group was not computed because of insufficient sample size. The EBSP of haplogroup MYK also indicated a rapid population expansion since 0.1 Ma ca (Fig. 4).

## DISCUSSION

### *Origin and formation history of Bufo gargarizans miyakonis*

Phylogenetic inferences suggested that the origin of *B. g. miyakonis* were from the eastern continental populations. In contrast, the Taiwanese groups, that are geographically closest to the Miyako Islands populations, were not closely related to the Miyako populations genetically. These phylogenetic relationships were consistent with the results of biochemical (Nishioka et al., 1990) and morphological (Matsui, 1984) analyses. Our results also suggested that *B. g. miyakonis* is not monophyletic in the mtDNA phylogeny. The non-monophyly of *B. g. miyakonis* might be attributable to an incomplete lineage sorting. In this species group, some cases of what appear to be incomplete lineage sorting have been reported (Yu et al., 2014; Othman et al., 2022). To clarify the relationship between *B. gargarizans* species group and *B. g. miyakonis*, more markers such

as nuDNA loci should be used in future studies.

The divergence time between haplogroup MYK and the continental haplotypes was estimated to be 0.75 Ma, sometime in the Middle Pleistocene. The divergence times of MYK11 and MYK 12 from respective continental counterparts were estimated to be 0.54 and 0.62 Ma approximately, respectively. These estimates were slightly younger than 0.9 Ma, the estimate given by Igawa et al. (2006) who used the Beijing population as a representative of *B. gargarizans*. Since our dataset included closer lineages (E1 and E2) to Miyako populations, which collected in sites closer to the Miyako Islands than Beijing (Locality No. 90 and No. 93 in Fig. 1A), our estimates were slightly younger.

The distribution of close populations suggests that the ancestral *B. g. miyakonis* population migrated over a land bridge connecting eastern Eurasia and the Miyako Islands. A land bridge between continental Eurasia and the Miyako Islands has been suggested by fossil evidence, including many Palearctic species, from the Pinza-Abu cave on Miyako Island (Hasegawa, 1985a; Oshiro and Nohara, 2000). Almost all of these Palearctic species such as *Capreolus miyakoensis* and *Rattus miyakoensis* are considered to be closely related to other species present in northeastern Eurasia (Hasegawa, 1985a, 1985b; Zhen and Hasegawa, 1985; Kawaguchi et al., 2009). In the Early Pleistocene, the Miyako Islands were last connected to neighboring islands (Kizaki and Oshiro, 1977, 1980; Ota, 1998; Kimura, 2003). At 0.12–0.2 Ma in the Middle Pleistocene, the area between the continent and the Miyako Islands was likely shallow and there was a temporal land bridge at the ebb (Kimura, 1990; Kimura et al., 2001). Kimura et al. (2001) suggested a probability that some of the above Palearctic species might have migrated to the Miyako Islands across this temporal land bridge during the Middle Pleistocene. Considering that the toads depend on fresh water, it is unlikely that *B. g. miyakonis* could migrate through a shallow sea like mammal species

(e.g., *C. miyakoensis*) could. However, our results suggest that the migration route of *B. g. miyakonis* was similar to other Palearctic species. We considered that this land bridge had been a corridor for the ancestor of *B. g. miyakonis*. Although recent phylogenetic analyses have suggested that several frog species inhabiting the Ryukyu Archipelago dispersed overseas (Komaki et al., 2017; Yu et al., 2020), the oversea dispersal of *B. g. miyakonis* is unlikely to happen, because its expected migration route is perpendicular to the present-day and past Kuroshio Current (Gallagher et al., 2015).

Stratigraphic studies have suggested that the Miyako Islands submerged (completely or partly) during the Early–Middle Pleistocene, 0.41–0.95 Ma (Iryu et al., 2006). Since this estimate is more recent than the divergence time between *B. g. miyakonis* and the continental *B. gargarizans* group, 0.54–0.75 Ma, the migration event of *B. g. miyakonis* may have occurred later than the genetic divergence. It is consistent with the inference that the ancestral population of *B. g. miyakonis* had migrated to the Miyako Islands at 0.12–0.2 Ma, when the land bridge had connected the continent and the Miyako Islands (see above; Kimura et al., 2001). Furthermore, the scale of crustal movements during the Late Pleistocene and the uniqueness of the fossils found on individual islands of the Ryukyu Archipelago both provide reasons to be skeptical of the existence of a Late Pleistocene land bridge in the Ryukyu Archipelago (Hasegawa, 2012; Furukawa and Fujitani, 2014; Yokoyama et al., 2018). Our results suggested that the ancestral population of *B. g. miyakonis* migrated to the Miyako Islands at the Middle Pleistocene, consistent with the absence of a land bridge in the Late Pleistocene. The curious distribution of the *B. g. miyakonis* may be affected by the formation history of the Miyako Islands. Future research should focus on seeking geographic or stratigraphic evidence for this land bridge.

The migration history of *B. g. miyakonis* is notably different from that inferred for other species from the Miyako Islands (land snails: Kameda et al., 2007; freshwater crab: Segawa,

2011; Asian keelback snake: Kaito and Toda, 2016; skinks: Kurita and Toda, 2017; narrow-mouthed frog: Tominaga et al., 2019). Moreover, on the Miyako Islands, almost all animal species that originated from northeastern Eurasia are considered extinct (Hasegawa, 2012), and there are few modern species that have their closest relatives on the continent like *B. g. miyakonis* (e.g., Miyako grass lizard: Lin et al., 2002; Ota et al., 2002). Therefore, our results may be important for understanding the formation process of present-day terrestrial fauna of the Miyako Islands.

#### Population genetics

Our population genetic analyses suggested rapid demographic expansion of *B. g. miyakonis*. For the *B. g. miyakonis* population, we measured a high value for  $h$  ( $0.8972 \pm 0.0232$ ) and a low value for  $\pi$  ( $0.004984 \pm 0.002919$ ) relative to other members of the *B. gargarizans* species group. Hu et al. (2007) gave  $h$  and  $\pi$  values for the continental populations of the *B. gargarizans* species group: the mean  $h$  was  $0.947 \pm 0.006$  with a range from  $0.000$ – $0.855 \pm 0.085$ , and the mean  $\pi$  was  $0.04739 \pm 0.00215$  with a range from  $0.0000$ – $0.04438 \pm 0.00524$  (Hu et al., 2007). Inferred from the relationship between a relatively high  $h$  and a low  $\pi$  values (Grant and Bowen, 1998; Avise, 2000), it was suggested that *B. g. miyakonis* was initially genetically uniform and subsequently accumulated mutations due to a rapid expansion of the population size. Our mismatch distribution analysis and Fu's  $F_s$  test results also supported this demographic trend. The EBSP also suggested a rapid expansion from 0.1 Ma until the present. Although our estimate suggests that this expansion started before the last glacial maximum (LGM), it might also have been caused by increases in land area following glacial regression. Because of the high diversity of fossil fauna and the existence of large mammals in the Late Pleistocene, the environment of the Miyako Islands is thought to have been significantly larger in both land area and carrying capacity before the LGM relative to the present (Ota and Takahashi, 2008). After the

LGM, the land area and carrying capacity decreased, and large animals became extinct (Ota and Takahashi, 2008). However, it was estimated that the expansion of *B. g. miyakonis* population was maintained after the LGM.

We also found evidence of moderate genetic differentiation of *B. g. miyakonis* subpopulations. We found many unique haplotypes in the MY–KR sample set, which had a relatively large sample size. On the other hand, we found no unique haplotype from the IR–SM dataset, partially because of the sample size was smaller. Nevertheless, we found evidence of differentiation among these subpopulations. Interestingly, no such genetic differentiation has been reported for other species present on the Miyako Islands (smooth skinks: Koizumi et al., 2014; anchialine atyid shrimp: Weese et al., 2012, 2013). All collection sites used in this study were part of a continuous landmass at the LGM (Japan Association for Quaternary Research, 1987). After the LGM, geographic isolation due to the transgression of the sea level likely contributed to the genetic differentiation between subpopulations.

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## APPENDIX I

List of specimen information of *Bufo gargarizans miyakonis*. Area was referred to by the subpopulation name in AMOVA. n: sample size.

Locality	n	Area	latitude (N)	longitude (E)	Haplotype (615 bp)	GenBank accession numbers
1. Miyako Island, Okinawa, Japan	1	MY-KR	24.90	125.27	MYK03	LC683661
2. Miyako Island, Okinawa, Japan	1	MY-KR	24.89	125.27	MYK01a	LC683658
3. Miyako Island, Okinawa, Japan	1	MY-KR	24.88	125.29	MYK01a	LC683658
4. Miyako Island, Okinawa, Japan	1	MY-KR	24.88	125.29	MYK10a	LC683672
5. Miyako Island, Okinawa, Japan	1	MY-KR	24.87	125.29	MYK01a	LC683658
6. Miyako Island, Okinawa, Japan	1	MY-KR	24.86	125.30	MYK10b	LC683673
7. Miyako Island, Okinawa, Japan	2	MY-KR	24.84	125.30	MYK05b, c	LC683664, LC683665
8. Miyako Island, Okinawa, Japan	1	MY-KR	24.83	125.28	MYK07b	LC683669
9. Miyako Island, Okinawa, Japan	1	MY-KR	24.82	125.32	MYK10c	LC683674
10. Miyako Island, Okinawa, Japan	1	MY-KR	24.81	125.29	MYK09	LC683671
11. Miyako Island, Okinawa, Japan	1	MY-KR	24.81	125.32	MYK10b	LC683673
12. Miyako Island, Okinawa, Japan	1	MY-KR	24.79	125.27	MYK07a	LC683668
13. Miyako Island, Okinawa, Japan	1	MY-KR	24.79	125.28	MYK10b	LC683673
14. Miyako Island, Okinawa, Japan	1	MY-KR	24.79	125.30	MYK01b	LC683658
15. Miyako Island, Okinawa, Japan	1	MY-KR	24.78	125.32	MYK01a	LC683658
16. Miyako Island, Okinawa, Japan	1	MY-KR	24.79	125.32	MYK10b	LC683673
17. Miyako Island, Okinawa, Japan	1	MY-KR	24.79	125.34	MYK08	LC683670
18. Miyako Island, Okinawa, Japan	1	MY-KR	24.78	125.35	MYK07a	LC683668
19. Miyako Island, Okinawa, Japan	1	MY-KR	24.78	125.35	MYK12	LC683676
20. Miyako Island, Okinawa, Japan	1	MY-KR	24.77	125.37	MYK01a	LC683658
21. Miyako Island, Okinawa, Japan	1	MY-KR	24.76	125.39	MYK05a	LC683663
22. Miyako Island, Okinawa, Japan	1	MY-KR	24.76	125.39	MYK01a	LC683658
23. Miyako Island, Okinawa, Japan	1	MY-KR	24.75	125.41	MYK01a	LC683658
24. Miyako Island, Okinawa, Japan	1	MY-KR	24.75	125.43	MYK01a	LC683658
25. Miyako Island, Okinawa, Japan	1	MY-KR	24.73	125.45	MYK07a	LC683668
26. Miyako Island, Okinawa, Japan	1	MY-KR	24.73	125.39	MYK09	LC683671
27. Miyako Island, Okinawa, Japan	1	MY-KR	24.75	125.38	MYK02	LC683660
28. Miyako Island, Okinawa, Japan	1	MY-KR	24.76	125.35	MYK01a	LC683658
29. Miyako Island, Okinawa, Japan	1	MY-KR	24.74	125.35	MYK04	LC683662
30. Miyako Island, Okinawa, Japan	1	MY-KR	24.73	125.36	MYK01a	LC683658
31. Miyako Island, Okinawa, Japan	1	MY-KR	24.73	125.35	MYK09	LC683671
32. Miyako Island, Okinawa, Japan	1	MY-KR	24.72	125.34	MYK05d	LC683666
33. Miyako Island, Okinawa, Japan	1	MY-KR	24.74	125.33	MYK01a	LC683658
34. Miyako Island, Okinawa, Japan	1	MY-KR	24.75	125.31	MYK01a	LC683658
35. Miyako Island, Okinawa, Japan	1	MY-KR	24.77	125.32	MYK11	LC683675
36. Miyako Island, Okinawa, Japan	1	MY-KR	24.77	125.29	MYK12	LC683676
37. Miyako Island, Okinawa, Japan	1	MY-KR	24.76	125.29	MYK05d	LC683666

## APPENDIX I

(continued)

Locality	n	Area	latitude (N)	longitude (E)	Haplotype (615 bp)	GenBank accession numbers
38. Miyako Island, Okinawa, Japan	1	MY–KR	24.76	125.29	MYK01a	LC683658
39. Miyako Island, Okinawa, Japan	1	MY–KR	24.74	125.32	MYK09	LC683671
40. Miyako Island, Okinawa, Japan	1	MY–KR	24.73	125.31	MYK05d	LC683666
41. Miyako Island, Okinawa, Japan	1	MY–KR	24.73	125.28	MYK07a	LC683668
42. Miyako Island, Okinawa, Japan	1	MY–KR	24.73	125.28	MYK12	LC683676
43. Miyako Island, Okinawa, Japan	1	MY–KR	24.74	125.26	MYK10b	LC683673
44. Miyako Island, Okinawa, Japan	1	MY–KR	24.75	125.26	MYK01a	LC683658
45. Kurima Island, Okinawa, Japan	1	MY–KR	24.73	125.25	MYK06	LC683667
46. Kurima Island, Okinawa, Japan	1	MY–KR	24.72	125.25	MYK05d	LC683666
47. Irabu Island, Okinawa, Japan	1	IR–SM	24.86	125.16	MYK10b	LC683673
48. Irabu Island, Okinawa, Japan	1	IR–SM	24.85	125.20	MYK10a	LC683672
49. Irabu Island, Okinawa, Japan	1	IR–SM	24.84	125.20	MYK10a	LC683672
50. Irabu Island, Okinawa, Japan	2	IR–SM	24.83	125.21	MYK10a	LC683672
51. Irabu Island, Okinawa, Japan	1	IR–SM	24.84	125.19	MYK10a	LC683672
52. Irabu Island, Okinawa, Japan	2	IR–SM	24.83	125.19	MYK10a, 12	LC683672, LC683676
53. Shimoji Island, Okinawa, Japan	2	IR–SM	24.83	125.16	MYK10b	LC683673

## APPENDIX II

List of the collection sites of the *Bufo gargarizans* species group coincides with the collection point numbers used in Fig 1A. Area of *B. gargarizans* species was referred to by the regional division used in previous studies (Fu et al., 2005; Hu et al., 2007).

Locality	Area	Latitude (N)	Longitude (E)	Haplotype (341 bp)	GenBank accession numbers	References
54. Kunming, Yunnan, China	W	24.27	102.73	W6, W8	AF190230, AY924312	Liu et al. 2000; Fu et al. 2005
55. Jiangchuan, Yunnan, China	W	25.00	102.70	W-E, W10	DQ288705, DQ288720	Hu et al. 2007
56. Derong, Sichuan, China	W	28.12	99.20	W8, W9	AY924311, AY924313	Fu et al. 2005
57. Xichang, Sichuan, China	W	27.87	102.52	W11	AY924317, AY924318	Fu et al. 2005
58. Mianning, Yunnan, China	W	28.50	102.10	W11	AY924315, AY924316	Fu et al. 2005
59. Yele, Sichuan, China	W	28.93	102.19	W12	AY924314	Fu et al. 2005
60. Jiulong, Sichuan, China	W	29.00	101.50	W21	AY924339	Fu et al. 2005
61. Xinduqiao, Sichuan, China	W	30.03	101.47	W1	AY924368, AY924369	Fu et al. 2005
62. Daofu, Sichuan, China	W	30.49	101.47	W1, W2	AY9243780, AY924371	Fu et al. 2005
63. Baiyu, Sichuan, China	W	30.81	99.59	W1, W3	AY924372, AY924373	Fu et al. 2005
64. Luhou, Sichuan, China	W	31.40	100.62	W1	AY924368, AY924369	Fu et al. 2005
65. Hongya, Sichuan, China	W	29.65	102.95	W-C1	AY924328, AY924354	Fu et al. 2005
66. E' mei, Sichuan, China	W	29.60	103.40	W-C1	DQ288715	Hu et al. 2007
67. Mt Omei, Sichuan, China	W	29.58	103.28	W-C1, W16, W19	AY924320, AY924320, AY924324, AY924325, AY924355, AY924356, AY924357	Fu et al. 2005
68. Qionglai, Sichuan, China	W	30.25	103.09	W16, W17, W18	AY924329, AY924330, AY924331, AY924332	Fu et al. 2005
69. Baoxing, Sichuan, China	W	30.30	102.80	W16, W19, W21	AY924319, AY924322, AY924323	Fu et al. 2005
70. Dayi, Sichuan, China	W	30.63	103.17	W15, W20	AY924326, AY924327	Fu et al. 2005
71. Pengxian, Sichuan, China	W	31.23	103.75	W13	AY924361	Fu et al. 2005
72. Zhongjiang, Sichuan, China	W	31.00	104.60	W-C1, W-C2	AY924337, AY924352, AY924353, AY924360	Fu et al. 2005
73. Maoxian, Sichuan, China	W	31.70	103.88	W14, W16	AY924333, AY924334, AY924335, AY924336	Fu et al. 2005
74. Wengxian, Gansu, China	W	33.06	104.69	W7	AY924340, AY924341, AY924342	Fu et al. 2005
75. Lanzhou, Gansu, China	W	36.03	103.73	W-C-NE-K, W-NE, W-C3, W-C4, W4, W5	DQ288711, DQ288712, DQ288713, DQ288714, DQ288717, DQ288719	Hu et al. 2007
76. Fushun, Liaoning, China	C	29.20	105.00	W-C1, C5	AY924358, AY924363	Fu et al. 2005
77. Xishui, Gansu, China	C	28.33	106.20	W-NE	DQ288709	Hu et al. 2007
78. Suiyang, Guizhou, China	C	28.23	107.16	W-C2	AY924359	Fu et al. 2005
79. Nanchuan, Chongqing, China	C	29.07	107.19	W-C1	AY924351	Fu et al. 2005
80. Jiangkou, Guizhou, China	C	27.90	108.72	C9	AY924343	Fu et al. 2005
81. Wangyuan, Sichuan, China	C	32.06	108.17	W-C3, C10	AY924338, AY924362	Fu et al. 2005
82. Shimen, Hunan, China	C	29.30	110.40	C2, C3, C4	DQ288704, DQ288706, DQ288708	Hu et al. 2007
83. Zhangjiajie, Hunan, China	C	30.10	110.80	C7, C8	AY924346, AY924347	Fu et al. 2005

## APPENDIX II

(continued)

Locality	Area	Latitude (N)	Longitude (E)	Haplotype (341 bp)	GenBank accession numbers	References
84. Shishou, Hubei, China	C	29.82	112.55	W–C–NE–K, W–C4, C1	DQ288696, DQ288711, DQ288717	Hu et al. 2007
85. Xinjian, Jiangxi, China	E	28.70	115.80	E–NE, E15	DQ288693, DQ288695	Hu et al. 2007
86. Guadun, Fujian, China	E	27.73	117.66	E11, E14	AY190234, AY924350	Liu et al. 2000; Fu et al. 2005
87. Wuyishan, Fujian, China	E	27.70	118.00	E12, E13	DQ288692, DQ694694	Hu et al. 2007
88. Huangshan, Anhui, China	E	30.20	118.10	E13	DQ288692	Hu et al. 2007
89. Lin'an, Zhejiang, China	E	30.20	119.70	E13	AY924348, AY924349	Fu et al. 2005
90. Wuhu, Anhui, China	E	31.33	118.38	E1, E4, E7, E13	DQ275350, DQ288692, DQ288699, DQ288701	Cao et al. 2006; Hu et al. 2007
91. Dinghai, Zhejiang, China	E	30.64	122.06	E8	KY806353	Tong et al. 2017
92. Shennongsi, Zenjiang, China	E	30.14	122.04	W–E	KY806314	Tong et al. 2017
93. Yancheng, Jiangsu, China	E	33.38	120.13	E1, E2, E5, E10	DQ288697, DQ288700, DQ288701, DQ288707	Hu et al. 2007
94. Yiyuan, Shandong, China	E	36.18	118.17	E3, E6, E9	DQ288698, DQ288702, DQ288703	Hu et al. 2007
95. Dongpu, Taiwan	T	23.55	120.91	TW1	KF692222	Yu et al. 2014
96. Taiwan	T	No detail location data		TW2	AF190231	Liu et al. 2000
97. Shanfong, Taiwan	T	23.32	121.23	TE	KF692208	Yu et al. 2014
98. Baihuashan, Beijing, China	NE	39.78	115.40	NE1, NE3	AY924364, AY924365	Fu et al. 2005
99. Jixian, Beijing, China	NE	40.10	117.20	E–NE	AY924367	Fu et al. 2005
100. Shenyang, Liaoning, China	NE	41.80	123.38	W–NE, NE2, NE4	DQ288709, DQ288710, DQ288716	Hu et al. 2007
101. Harbin, Heilongjiang, China	NE	45.75	126.63	W–C–NE–K, NE5	DQ288717, DQ288718	Hu et al. 2007
102. Antu, Jilin, China	NE	42.55	128.30	W–C–NE–K	AY924344, AY924345	Fu et al. 2005
103. Ganghwa, Korea	K	37.72	126.40	W–C–NW–K	KY295994	Borzee et al. 2017