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Phylogeny and Differentiation of Wide-ranging Ryukyu Kajika Frog *Buergeria japonica* (Amphibia: Rhacophoridae): Geographic Genetic Pattern Not Simply Explained by Vicariance Through Strait Formation

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To investigate geographic genetic structures and taxonomic relationships among isolated populations of *Buergeria japonica,* occurring very widely in various habitats of the Ryukyu Archipelago and Taiwan, we conducted phylogenetic and demographic analyses among individuals from various localities, representing their entire distributional ranges. *Buergeria japonica* is genetically greatly differentiated and comprises three major clades (the Southern Taiwan [ST] clade, the Northern Taiwan + Southern Ryukyu [NT/SR] clade, and the Central + Northern Ryukyu [CR/NR] clade), each of which seems to represent independent species. The first divergence in the species is estimated to have occurred in the middle to late Miocene in areas of current Taiwan, then eastern periphery of the Asian continent. Split of the ST and the remaining clades, and subsequent divergence between the NT/SR and the CR/NR clades in the latter, indicate consecutive south to north vicariant diversifications. However, these vicariances are not always associated with formation of significant barriers such as deep straits. Less but still prominently diverged subclades (the Amami + Tokara [AM/TK] and the Okinawa [ON] subclades) in the CR/NR clade were recognized in spite of the absence of an intervening deep strait. Contrariwise, individuals from Amami and Tokara Groups formed the AM/TK subclade in spite of the presence of the intervening Tokara Gap (a long-standing deep tectonic strait). Furthermore, in the AM/TK subclade, low but definite genetic divergence was found between the Northern Amami + Tokara (NAM/TK) lineage and the Southern Amami (SAM) lineage. Estimated divergence time and gene flow rate within the NAM/TK lineage indicate that this species reached northern Tokara from the south by overseas dispersal over the Tokara Gap long after its formation, but not by more recent artificial transportation. This overseas dispersal would have been facilitated by its more frequent occurrence around coastal habitats than other frogs.

Key words: insular amphibia, phylogeography, Ryukyu, Taiwan, mitochondrial DNA

INTRODUCTION

Genetic differentiation of amphibian species of the Ryukyu Archipelago, located between Japan mainland and Taiwan, has been vigorously studied as these animals, with low dispersal capability over saltwater (e.g., Inger and Voris, 2001), are ideal materials for examining the effects of past strait and land-bridge formations on their geographic genetic structures. Thus, past studies revealed that their geographic pattern and degree of genetic differentiations differ more or less among amphibian species endemic to this archipelago (*Echinotriton andersoni*, *Cynops ensicauda*, *Rana ulma-R. kobai*, the *Odorrana narina* complex, *Odorrana ishikawae-O. splendida*, *Babina subaspera-B. holsti*, and *Microhyla okinavensis*) but that they invariably deeply related to the geohistory of this region (Honda et al., 2012; Tominaga et al., 2010, 2014; Matsui, 2011; Matsui et al., 2005a, b).

Faunistically, the Ryukyu Archipelago is classified into three regions: the Northern (Osumi and Northern Tokara Groups), the Central (Southern Tokara, Amami and Okinawa Groups), and the Southern Ryukyus (Miyako and Yaeyama Groups; Ota, 1998, 2000) (Fig. 1). The Northern Ryukyus, largely predominated by the Palearctic elements, is isolated from the Central Ryukyus by the Tokara Gap (tectonic Strait), which was supposedly formed between the Pleistocene and

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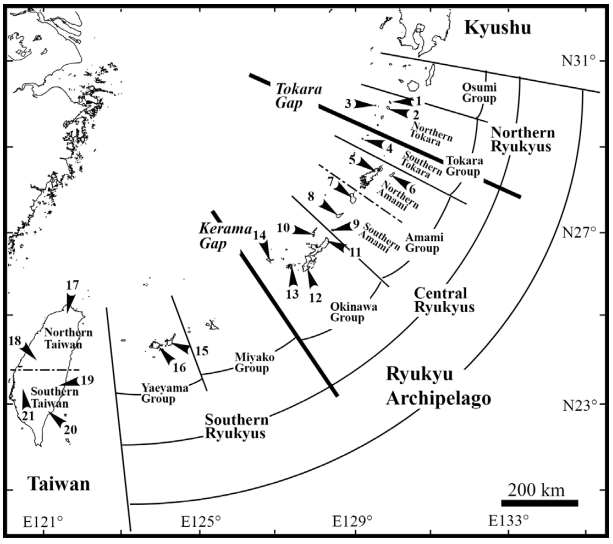

Fig. 1. Map of the Ryukyu Archipelago showing distributional ranges of *Buergeria japonica* and sampling locations in this study.

the late Miocene (1.55–8 MYA: Kizaki and Ohshiro, 1980; Kimura, 2003; Osozawa et al., 2012). The Central Ryukyus, characterized by numerical dominance of many relict species, is isolated by the Kerama Gap from the Southern Ryukyus, where the fauna resembles that of Taiwan and the continental China. Most amphibian species of this archipelago are restricted to each of these three regions, and only two frog species (*Buergeria japonica* and *Microhyla okinavensis* [Maeda and Matsui, 1999; Matsui et al., 2005a]) are distributed in all three regions across the intervening straits. Furthermore, *B. japonica* also occurs on Taiwan, where some species confined to the Southern Ryukyus in the Japanese territory, are otherwise found.

Such unusually wide distribution of *B. japonica* from the Northern Ryukyus to Taiwan, across the deep Tokara and Kerama Gaps (Maeda and Matsui, 1999), poses the question of whether it is really a single species, especially after *M. okinavensis* populations, formerly erroneously considered conspecific with morphologically resembling Taiwanese, continental, and tropical Asian populations, proved to be endemic to the Ryukyu Archipelago (Matsui et al., 2005a). Another notable characteristic of this *B. japonica* is its very wide habitat. It inhabits various environments from small mountain stream to artificial ditch around urban area and even small river mouth around sandy beach (Maeda and Matsui, 1999). This last habitat is particularly unique among Japanese and Taiwanese frogs, and its high adaptation and tactics to coastal habitats have been demonstrated (Haramura, 2004, 2007a, b, 2008, 2011).

For both clarifying geographic genetic structures across its wide range, and correctly understanding taxonomic relationships among isolated populations of *B. japonica*, detailed study of genetic variation is inevitable. Limited information on genetic variation in this species has been available (Nishioka et al., 1987; Nishizawa et al., 2011). Only recently Komaki et al. (2014) developed microsatellite markers of this species that would be useful for future study of phylogeography and demography of *B. japonica*. However, to date, overall genetic differentiation among populations of *B. japonica* has never been studied and their divergence times remain vaguely estimated.

The aims of this study are twofold. One is to clarify phylogenetic relationships and degree of genetic differentiations among local populations so as to assess their taxonomic relationships. The other is to clarify why this species is distributed in a much wider area than other amphibian species of the Ryukyu–Taiwan region. To address the latter question, three alternate hypotheses were tested. The first is overseas dispersal hypothesis, the second is a hypothesis that postulates an original wide distribution around current range with some dispersal through the landmass and subsequent isolation by strait (gap) in old ages, and the third is the mixture of the first and the second hypotheses. In the first case, we would predict smaller genetic variation throughout their range, while we would conversely predict almost invariably large genetic differentiations among populations in the second case. If both larger and smaller genetic differentiations among isolated populations were observed, the third hypothesis would be the most plausible.

MATERIALS AND METHODS

Sampling

Tissues were obtained from a total of 134 individuals of *B. japonica* from 15 islands (16 populations) of the Ryukyu Archipelago and five populations of Taiwan, representing whole of the current distributional range of this species (Fig. 1; Table 1). Individuals from Toshima village, except for that from Gajajima Island collected in July 1991(before the species was listed as a protected species), were collected under permissions to AT and HO from the local government.

Sequencing

Ethanol-preserved tissues were homogenized in 0.6 mL of STE buffer containing 10 mM Tris/HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA (pH 8.0). In total, 60 μL of 10% SDS solution and 6 μL of Proteinase K (0.1 mg/mL) were added to the homogenate solutions and digested proteins for 12 h at 36°C. The solution was treated with phenol and chloroform/isoamyl alcohol, and DNA was precipitated with ethanol. DNA precipitates were dried and dissolved in 1 mL of TE [10 mM Tris/HCl, 1 mM EDTA (pH 8.0)], and 1 μL was subjected to polymerase chain reaction (PCR).

For PCR amplification of the cytochrome *b* (cyt *b*) gene, the primers BueCBF1 (Lin et al., 2012) (5′-TTTCTGCCAGGRTTYTAACCTAGACC-3′) and cytb_R2_Bue5 (5′-CAGTTGGCCAATTAGAATAAATGGGTCTTC-3′: newly designed for cyt *b* of this frog) were used. For PCR amplification of 16S rRNA region, the primers 16SL2021 (5′-CCTACCGAGCTTAGTAATAGCTGGTT-3′) by Tominaga et al. (2006) and Hedges16H1 (5′-CTCCGGTCTGAACTCAGATCACGTAGG-3′) by Hedges and Maxson (1993) were used. The reaction conditions were initial heating at 94°C for 4 min; 35 cycles of 94°C (30 s), 50°C (30 s), and 72°C (1.5 min); and a final extension at 72°C for 7 min. The amplified DNA fragments were purified using polyethylene glycol (PEG, 13%). Cycle-sequencing reactions were performed using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using four primers described above and four additional intervening primers: cytb_F2_Bue2 (5′-GCTACACTRACCCGMTTYTTTACATT-3′) and cytb_R1_Bue (5′-TTTATGCCTGTRGGRTTRGARGATCC-3′) for cyt *b* and 16Sh2715 (5′-AAGCTCCATAGGGTCTTCTCGTG-3′) by Tominaga et al. (2006) and 16SL2510 (5′-CCGACTGTTTACCAAAAACAT-3′) by Fu (2000) for 16S rRNA. Following this, sequencing was performed on ABI 3100 or ABI 3130 automatic sequencers. We sequenced all 134 individuals of *B. japonica* for 1032 bp of partial cyt *b* gene, and 55 representative individuals for

Table 1. Population name, number of individuals, number of haplotypes, and haplotype compositions based on cytochrome *b*.

Population number	Population name	Number of indivduals	Number of haplotypes	Haplotype
1	Kuchinoshima Island	12	2	h01, h02
2	Nakanoshima Island	5	1	h02(5)
3	Gajajima Island	1	1	h03
4	Takarajima Island	10	2	h04(9), h05
5	Amamioshima Island	9	4	h04(6), h06, h07, h08
6	Kikaijima Island	9	5	h09, h10, h11(2), h12, h13(4)
7	Tokunoshima Island	10	5	h14(3), h15(2), h16, h17(3), h20
8	Okinoerabujima Island	7	3	h18(5), h19, h23
9	Yoronjima Island	10	3	h18(8), h21, h22
10	Izenajima Island	4	2	h35(3), h36
11	Okinawajima (Kunigami)	12	5	h24(2), h26, h27, h30(7), h31
12	Okinawajima (Nanjo)	7	4	h25, h28(3), h29, h34(2)
13	Tokashikijima Island	3	2	h32, h33(2)
14	Kumejima Island	2	1	h37(2)
15	Ishigakijima Island	3	3	h38, h39, h46
16	Iriomotejima Island	15	8	h40, h41, h42, h43, h44, ,h45, h46(6), h47
17	Taipei	3	3	h48, h49, h50
18	Puli	1	1	h51
19	Ruisui	4	4	h55, h56, h59, h60
20	Zhiben	5	5	h52, h53, h54, h57, h58
21	Guanziling	2	2	h61, h62

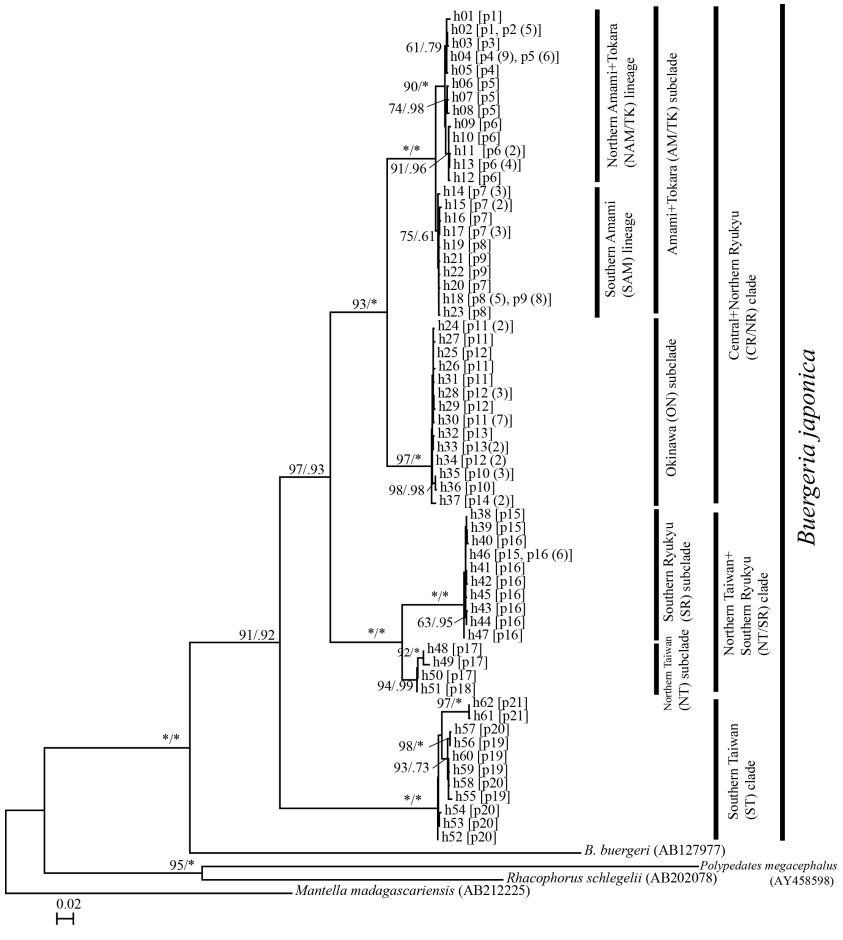

Fig. 2. Maximum likelihood phylogram of 1032 bp of the mitochondrial cytochrome *b* gene for haplotypes of *Buergeria japonica* and its related species. Numbers preceded by "h" and "p" indicate haplotype number and population number, respectively. Numbers in parentheses after population number indicate number of individuals, included. Nodal numbers represent ML bootstrap supports/ Bayesian posterior probability. Asterisks indicate 100% bootstrap support values or 1.00 Bayesian posterior probabilities.

1004–1006 bp of partial 16S rRNA. New sequences of cyt *b* and 16S rRNA obtained in this study were deposited in GenBank (Accession number: AB998751–AB998867). Alignment of data from all individuals was performed using the Clustal option in the BioEdit software (Hall, 1999).

Phylogenetic analyses

Buergeria is the monotypic genus in the subfamily Buergeriinae unlike all other genera comprising Rhacophorinae (Frost et al., 2006). We thus chose four outgroup taxa, including a buergeriine member (*B. buergeri* [AB127977]), two rhacophorine genera (*Polypedates megacephalus* [AY458598] and *Rhacophorus schlegelii* [AB202078]), and a manteliid (*Mantella madagascariensis* [AB212225]), which represents the family sister to Rhacophoridae, and made the following two datasets. Dataset I: 1032 bp of partial cyt *b* for all 62 haplotypes detected from 134 individuals of ingroup and four outgroup taxa; Dataset II: combined 2065 bp dataset of cyt *b* and 16S rRNA for 55 ingroup individuals and four outgroup taxa. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian inference (BI) methods. The optimum substitution models for each partition were selected by Kakusan4 (Tanabe, 2011) based on the Akaike information criterion. For ML analysis, HKY (Hasegawa et al., 1985) + G was selected for the Dataset I and J2 + G was selected for both cyt *b* and 16S rRNA for the Dataset II. The ML tree was searched using TREEFINDER ver. Oct. 2008 (Jobb et al., 2004; Jobb, 2008) and Phylogears2 (Tanabe, 2008) through 100 trials of the likelihood ratchet method (Vos, 2003). For Bayesian analyses, HKY + G (Hasegawa et al., 1985) was selected as the best substitution model for the Dataset I, and GTR + G was selected for both cyt *b* and 16S rRNA genes. Bayesian analysis was conducted by MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001), and two independent runs of four Markov chains were conducted for 10 million generations. The first three million generations were discarded as burn-in. Convergence of parameters was checked using Tracer ver. 1.5 (Rambaut and Drummond, 2009). For ML analysis, nonparametric bootstrap (bs)

analysis (Felsenstein, 1985) with 1000 replicates was used. Branches with bs values of 70% or higher were regarded as sufficiently resolved (Huelsenbeck and Hillis, 1993). For Bayesian analysis, posterior probabilities (bpp) were used as an indicator of node credibility, and those of 95% or higher were considered significant (Leaché and Reeder, 2002).

Calculation of genetic distance and estimation of divergence time

The mean genetic *p*-distance for pairwise combinations of haplotypes was calculated using MEGA, version 4 (Tamura et al., 2007). To estimate divergence times, the Bayesian analyses using BEAST ver. 1.7.5 (Drummond et al., 2012) were conducted. For each calibration, 10 million generations of run (of which the first three million were discarded as burn-in) were conducted under a non-autocorrelated log-normal relaxed clock model. Tracer ver. 1.5 (Rambaut and Drummond, 2009) was used to check the parameter distributions and effective sample size. Because calibration points properly applicable for these data were not available, we applied the following two evolutionary rates from published studies of other frogs:

Calibration I: Divergence rates of cyt *b* in frogs have been usually observed in the range of 1.0–1.5% per million years (e.g., 1.41%/MY [Jang-Liaw et al., 2008]; 1.14%/MY [Fouquet et al., 2009]; 1.0%MY [Vences et al., 2013]), excepting an unusually high rate (3.6%/MY) reported by Babik et al. (2004). The divergence rate of 1.41% (0.705% per lineage)/MY, which was estimated for the cyt *b* region of *Sylvirana latouchii* (Fouquet et al., 2009), was applied for cytb *b* under the HKY model in this study because both *Sylvirana* and *Buergeria* belong to the superfamily Ranoidea and their adults are small- to medium-sized (Fei et al., 2012) which implies they share similar life cycles. We set the prior distribution of the substitution rate to a normal distribution with a mean of 0.00705 substitutions/site/million years and standard deviation of 0.0015 substitutions/site/million years.

Calibration II: Because substitution rate for 16S rRNA is ca. 0.7 fold of that of cyt *b* in anurans (Kakehashi et al., 2013), we applied the evolutionary rate of 1.00% (0.50% per lineage) per MY for 16S rRNA data under the HKY model. We set the prior distribution of the substitution rate to a normal distribution with a mean of 0.005 substitutions/site/million years and standard deviation of 0.0015 substitutions/site/million years.

Estimation of historical demography

To examine the possibility of overseas dispersal across the Tokara Gap, the historical demography, especially the pattern of gene flow and divergence times within the Amami + Tokara (AM/TK) subclade (See result section), was examined using coalescent analysis with the Bayesian IM model. We analyzed the mitochondrial cyt *b* data using the program IMa2 (Hey, 2010), and estimated the effective population size, *Ne*, population migration rate, *2NeM*, and population divergence time, *T*. We applied 0.705% per MY per lineage following Jang-Liaw et al. (2008). The geometric mean of these values, approximately $7.28^{*}\ 10^{-6}$ mutations per year per locus, was used as the mutation rate (μ) to scale each demographic parameter. Based on several test runs, the upper bounds for the parameters were set at $\theta = 20 - 50$, $t = 20 - 30$, and $m = 10 - 25$, and two million steps (sampling frequency one tree per 20 steps) of calculations were performed for 40 heated chains after ten million burn-in steps. We conducted three independent runs, and finally combined the results using the L-mode option of IMa2. Since *B. japonica* typically starts to breed at the age of one year old (Nakasone and Tominaga, unpublished), we adopted this value as the generation time of this species. The trendline plots and effective sample sizes were monitored to ensure good mixing and convergence of parameters.

RESULTS

Phylogenetic relationships

ML analysis based on the Dataset I generated a topology with lnL = –6,237.572. The mean lnL score of Bayesian analyses based on the Dataset I for all trees sampled at stationarity was –6,358.042. Based on Dataset II, ML analysis generated a topology with lnL = –10,598.744, and the mean lnL score of Bayesian analyses for all trees sampled at stationarity was –10,705.633.

i) All phylogenetic analyses yielded essentially identical topologies (Figs. 2, 3). Monophyly of *B. japonica* was strongly supported in all trees with support in MLbs and

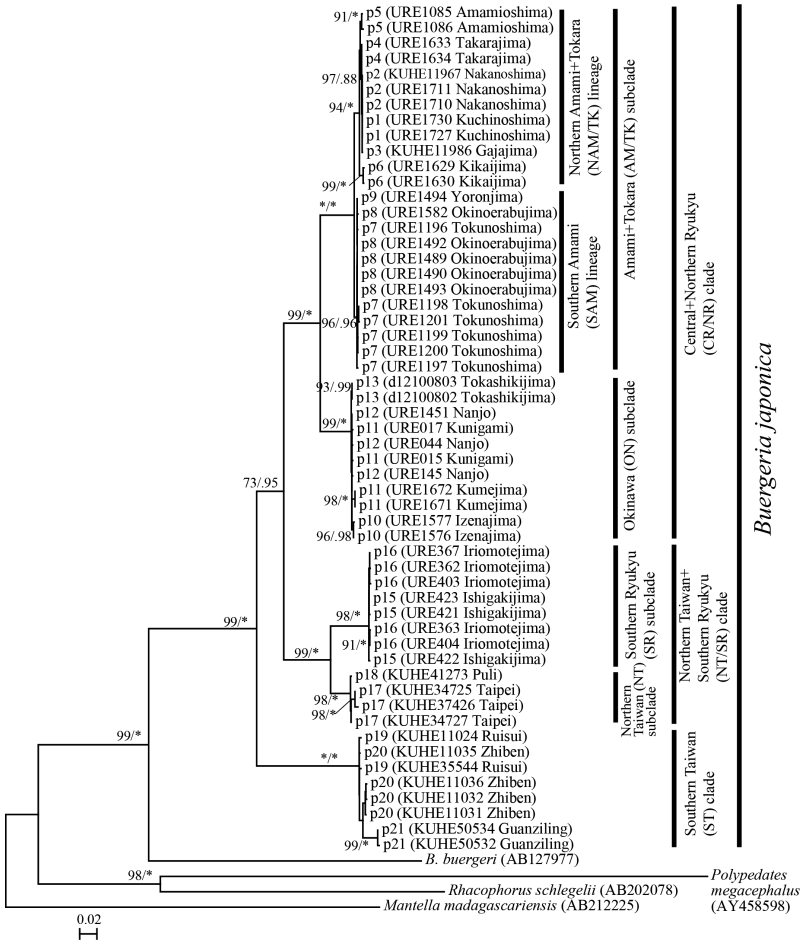

Fig. 3. Maximum likelihood phylogram of 2065 bp of the mitochondrial cytochrome *b* + 16S ribosomal RNA gene of *Buergeria japonica* and its related species. Nodal numbers represent ML bootstrap supports/ Bayesian posterior probability. Asterisks indicate 100% bootstrap support values or 1.00 Bayesian posterior probabilities. Numbers preceded by "p" indicate population number. Numbers in parentheses preceded by "URE" and "KUHE" with sampling localities indicate voucher specimens numbers.

bpp, respectively, of 91% and 0.92 for the dataset I, and 99% and 1.00 for dataset II.

ii) Within the ingroup, individuals from Southern Taiwan formed a clade (the ST clade: 100%, 1.00, and 100%, 1.00) and were separated from the clade consisting of all remaining individuals from the Ryukyu Archipelago and Northern Taiwan (97%, 0.93, and 73%, 0.95).

iii) In the latter group, individuals from Northern Taiwan and the Southern Ryukyus formed a clade (the NT/SR clade: 100%, 1.00 and 99%, 1.00) and were separated from the other clade of individuals from the Central and the Northern Ryukyus (the CR/NR clade: 93%, 1.00, 99%, 1.00).

iv) Within the NT/SR clade, individuals of each subclade were separated with high supported values (100%, 1.00 and 98%, 1.00 for the Southern Ryukyus [SR] subclade, and 94%, 0.99 and 98%, 1.00 for the northern Taiwan [NT] subclade).

v) In the CR/NR clade, individuals from the Amami and Tokara Groups were separated from those from the Okinawa Group (100%, 1.00 and 100%, 1.00 for the Amami + Tokara [AM/TK] subclade, 97%, 1.00 and 99%, 1.00 for the Okinawa [ON] subclade).

vi) In the AM/TK subclade, individuals from Tokara Group, Amamioshima, and Kikaijima Islands formed the Northern Amami + Tokara [NAM/TK] lineage (90%, 1.00 and 94%, 1.00), and were separated from the Southern Amami [SAM] lineage, which included individuals from Tokunoshima, Okinoerabujima, and Yoronjima Islands (75%, 0.61 and 96%, 0.96).

Genetic differentiation and divergence time among populations

The means (± 1 SE) of the uncorrected *p*-distances among clades or subclades are shown in Table 2. The distances obtained in the two gene fragments differed greatly. For example, distance was 12.8 ± 0.9–17.3 ± 1.0% in cyt *b*, but 5.2 ± 0.6–6.9 ± 0.8% in 16S rRNA among the three major clades. The distances between the AM/TK and the ON subclades (8.8 ± 0.8% in cyt *b* and 3.2 ± 0.5% in16S rRNA) were similar to those observed between the SR and the NT subclades (7.7 ± 0.7% in cyt *b* and 3.3 ± 0.5% in 16S rRNA).

The divergence times obtained by the two calibrations differed slightly (Table 3). The ST clade diverged from the other lineages at 14.66 (95% CI: 12.40–16.98) MYA in the Calibration I (cyt *b*), but at 10.59 (7.08–16.75) MYA in the Calibration II (16S rRNA).

In the Calibration I, the split was estimated at 10.63 (8.30–12.57) MYA between the CR/NR and the NT/SR clades, and at 4.98 (2.73–6.73) between the SR and the NT subclades. The AM/TK and the ON subclades were estimated to have diverged at 6.07 (3.81–7.75) MYA. The estimated times of divergence within each clade, subclade, or haplogroup varied from 0.41 (0.17–0.67) MYA in the SR subclade to 1.60 (1.03–2.16) MYA in the AM/TK subclade.

Corresponding values in MYA estimated in the Calibration II were smaller ranging from 3.21 (1.77–4.97) to 10.59 (7.08–16.75) between clades or subclades. Whereas within subclade or lineage, values ranged from 0.44 (0.06–0.92) to 1.19 (0.64–1.90), that were nearly equal to those obtained in the Calibration I.

In the demographic analysis using IMa2, we define the following three operational haplogroups: the Northern Tokara [NTK] haplogroup consisting of individuals from Kuchinoshima, Nakanoshima, and Gajajima islands that are north of the Tokara Gap; the Northern Amami + Southern Tokara [NAM/STK] haplogroup including individuals from Takarajima, Amamioshima, and Kikaijima Islands, which are south of the Tokara Gap; and the Southern Amami [SAM] haplogroup (= SAM lineage) including individuals from Tokunoshima, Okinoerabujima, and Yoronjima Islands, which are regionally allied in Amami Group but phylogenetically largely isolated from the northern Amami (see Figs. 2 and 3).

The population divergence time obtained between the NTK and the NAM/STK haplogroups was ca. 0.55 (0.13–2.92) MYA (Table 4), which is similar to the value (0.75

Table 2. Uncorrected *p*-distance (mean ± SE in %) among populations. Above diagonal: *p*-distance based on 16S rRNA, below diagonal: *p*-distance based on cytochrome *b*.

	NAM/TK lineage	SAM lineage	ON subclade	SR subclade	NT subclade	ST clade
NAM/TK lineage		0.7 ± 0.2	3.1 ± 0.5	5.6 ± 0.7	5.5 ± 0.7	6.8 ± 0.8
SAM lineage	1.8 ± 0.3		3.2 ± 0.5	5.7 ± 0.7	5.6 ± 0.7	6.9 ± 0.8
ON subclade	9.0 ± 0.8	8.6 ± 0.8		5.7 ± 0.7	5.2 ± 0.6	6.6 ± 0.7
SR subclade	14.6 ± 1.0	14.2 ± 1.0	14.3 ± 1.0		3.3 ± 0.5	6.1 ± 0.7
NT subclade	13.3 ± 0.9	13.1 ± 1.0	12.8 ± 0.9	7.7 ± 0.7		6.6 ± 0.6
ST clade	17.2 ± 0.9	17.3 ± 1.0	16.1 ± 0.9	16.6 ± 1.0	15.8 ± 1.0	

Table 3. Mean divergence time (95% CI lower-upper) estimated by BEAST analyses for between clades/subclades/lineages/populations and within clade/subclade/lineage.

	Calibration I (cyt *b*)	Calibration II (16S rRNA)
Within the NAM/TK lineage	0.75 (0.45–1.05)	0.97 (0.46–1.59)
Wtihin the SAM lineage	0.55 (0.25–0.90)	0.59 (0.24–1.02)
Within the AM/TK subclade	1.60 (1.03–2.16)	1.19 (0.64–1.90)
Within the the ON subclade	0.71 (0.36–1.12)	0.72 (0.25–1.31)
Between the AM/TK and the ON subclades	6.07 (3.81–7.75)	3.21 (1.77–4.97)
Within the SR subclade	0.41 (0.17–0.67)	0.62 (0.20–1.16)
Within the NT subclade	0.90 (0.46–1.41)	0.44 (0.06–0.92)
Between the SR and the NT subclades	4.98 (2.73–6.73)	3.34 (1.85–5.04)
Between the CR/NR and the NT/SR clades	10.63 (8.30–12.57)	6.40 (4.36–8.82)
Within the ST clade	2.79 (1.85–3.86)	0.76 (0.26–1.41)
Between the ST clade vs other clades	14.66 (12.40–16.98)	10.59 (7.08–16.75)
Between *Buergeria japonica* and *B. buergeri*	21.16 (18.43–23.80)	13.20 (9.54–18.53)
Between *Rhacophorus* and *Polypedates*	17.2 (12.31–21.15)	19.21 (16.28–21.97)
Between *Buergeria* and *Rhacophorus* + *Polypedates*	27.15 (24.22–30.07)	24.31 (19.36–30.14)

Table 4. Demographic parameters estimated in the IM analysis. *Ne*, effective population size (million individuals); 2*NeM*, effective population migration rate (number of gene copies/generation), for which 2*NeM*1→2 (2*NeM*2→1) indicates gene flow from group 1 to 2 (2 to 1) forwards in time; T, population divergence time (MYA). Values supported by the highest probability are shown as HiPt, and HPD95 indicate the 95% highest posterior density interval. Parameter with an asterisk indicates the value with statistical support in LLR test.

	N1	*N2*	*2NeM1→2*	*2NeM2→1*	*T*
(1) NTK vs. (2) NAM/STK					
HiPt	0.004	0.81	0.11	0.61*	0.55
HPD95	(0.001–0.154)	(0.32–2.09)	(0.00–36.51)	(0.00–4.20)	(0.13–2.92)
(1) NTK vs. (2) SAM					
HiPt	0.004	0.48	0.04	0.02	–
HPD95	(0.001–0.154)	(0.20–1.06)	(0.00–4.39)	(0.00–1.90)	–
(1) NAM/STK vs. (2) SAM					
HiPt	0.81	0.48	0.03	0.04	–
HPD95	(0.32–2.09)	(0.20–1.06)	(0.00–2.77)	(0.00–3.68)	–

[0.45–1.05] MYA) obtained for the divergence time within the NAM/TK lineage in BEAST analysis. The reliable divergence times between the NTK and the SAM haplogroups and between the NAM/STK and SAM haplogroups could not be estimated by IM analysis because of poor degrees of their convergences. The estimates of effective population size (*Ne*) varied among the haplogroups although their CI overlapped largely. Notably, the estimated *Ne* in NTK (0.004 [0.001–0.154] million individuals) is much smaller than in others (0.81 [0.32–2.09] million individuals in NAM/STK; 0.48 [0.20–1.06] million individuals in SAM). For the gene flow, only one direction of population migration rate from the NAM/STK haplogroup to the NTK haplogroup (2*NeM* = 0.61 [0.00–4.20]) was shown to be significantly larger than zero ($p < 0.05$ in log-likelihood ratio [LLR] test).

DISCUSSION

Phylogenetic relationship, genetic differentiations, and taxonomic implications

Three major clades (the CR/NR, the NT/SR, and the ST clades) were recognized in *B. japonica* studied. Genetic distances obtained among these three clades (12.8–17.3% in cyt *b* and 5.2–6.9% in 16S rRNA) are regarded as quite large compared with reported values among various congeneric anuran species [e.g., 4.7–5.3% in 16S rRNA between two sibling species of *Rana* (Matsui et al., 2011), 3.0–18.1% in 16S rRNA among species of *Kurixalus* (Nguyen et al., 2014)] . Therefore, each of the three major clades appears to represent a taxonomically independent entity. In order to determine their taxonomic status, detailed morphological comparisons are needed.

Distribution patterns and divergence history

Of the three major clades detected, two are distributed in Taiwan with the deepest divergence from each other. This indicates that the first divergence of *B. japonica* occurred in this area. The age of the first divergence is estimated at the late Miocene (14.66 [12.40–16.98] MYA in the Calibration I and 10.59 [7.08–16.75] MYA in the Calibration II), when the current Taiwan was a part of the eastern coast of the continent (Kimura, 2003). Thus, the ancestral stock of this species is thought to have first diverged at the periphery of the continent.

The individuals from the Southern Ryukyus are closer to those from Northern Taiwan than from the Central Ryukyus. This pattern supports Ota (2000), who pointed out a close similarity of amphibian fauna between Taiwan and the Southern Ryukyus.

Divergence time between the CR/NR and the NT/SR clades is estimated as10.63 (8.30–12.57) MYA in the Calibration I and 6.40 (4.36–8.82) MYA in the Calibration II. The estimated age of the formation of the Kerama Gap, which separates the Central and the Southern Ryukyus, varies from the late Miocene (Ota, 1998; Kimura, 2003) to the early Pleistocene (1.55 MYA: Osozawa et al., 2012), but our data more favor for the former estimation. Our result also does not discord with the hypothesis proposed by Ota (1998), who insisted on the isolation of the Central Ryukyus from the other landmass since the Pliocene.

Within the CR/NR clade, two subclades were recognized. Presence of two distinct groups (Amami and Okinawa) in the Central Ryukyus is a common phenomenon observed in most amphibian species occurring in this region (*Cynops ensicauda* [Hayashi and Matsui, 1988; Tominaga et al., 2010]; *Echinotriton andersoni* [Honda et al., 2012]; the *Odorrana narina* complex and the *O. ishikawae* species group [Matsui et al., 2005b]; *Microhyla okinavensis* [Matsui et al., 2005a]; the *Babina* species group [Tominaga et al., 2014]). Divergence time between the AM/TK and the ON subclades is estimated as 6.07 (3.81–7.75) MYA in the Calibration I and 3.21 (1.77–4.97) MYA in the Calibration II. The estimated times of divergence between the Amami and Okinawa populations in several amphibians falls within the range from the late Miocene to the early Pleistocene (1.7 [1.1–2.4] MYA for two species of the *Odorrana narina* complex [Matsui et al., 2005b]; 2.3 [1.5–3.2] MYA for two species of the *O. ishikawae* species group [Matsui et al., 2005b]; 2.4–8.3 MYA for two species of *Babina* [Tominaga et al., 2014]; 3.3–6.8 MYA for *Cynops ensicauda* [Tominaga et al., 2010, 2013]; and 3.1–18.0 MYA for *Echinotriton andersoni* [Honda et al., 2012; Kurabayashi et al., 2012]). Most of these estimations are much older than the formations of straits in the Pleistocene (0.12–1.3 MYA; Kizaki and Oshiro, 1980; Ota, 1998; Kimura, 2003), which have separated the Amami and the Okinawa Groups, and should have induced divergence of the terrestrial animals of the Central Ryukyus into two island group elements. This suggests that, most amphibian species had already diverged on the paleo-Central Ryukyu landmass before the formation of the straits, which currently separate the two regions (Amami and Okinawa).

Even so, however, geographic distribution pattern of these two subclades in *B. japonica* differs from those of other species. Matsui et al. (2005a), for example, demonstrated that the boundary of Amami and Okinawa lineages of *M. okinavensis* is located on the area between

Tokunoshima and Yoronjima Islands. On the other hand, the boundary of the two lineages (the AM/TK and the ON subclades) of *B. japonica* is located on the area between Yoronjima and Okinawajima Islands. This discordance might be attributable to differential patterns of secondary range expansions after initial separation in *M. okinavensis* and *B. japonica*.

We made three alternative hypotheses; namely, overseas dispersal; an original wide distribution with dispersal through the landmass and subsequent isolation by strait (gap) in old ages: or a mixture of the first and the second hypotheses. The considerations for divergence times described above favor our second and third hypotheses, and indicate that *B. japonica* had been originally distributed in the range from current Taiwan to the Ryukyu Archipelago before separation of the two regions by the straits.

Gene flow across the Tokara Gap

The divergence time within the NAM/TK lineage is estimated as 0.75 (0.45–1.05) MYA in the Calibration I and 0.97 (0.46–1.59) in the Calibration II by BEAST analyses and 0.55 (0.13–2.92) MYA based on cyt *b* by IM analysis. The separation of the NTK (Kuchinoshima, Nakanoshima, and Gajajima Islands) and the STK (Takarajima Island) populations is supposed to have occurred more recently because their genetic differentiations (*p*-distance: 0.27±0.11% in cyt *b*, 0.12±0.08% in 16SrRNA) are much smaller than those between TK + Amamioshima and Kikaijima populations (= maximum *p*-distance combinations within NAM/TK lineage; 0.88 ± 0.23% in cyt *b*, 0.76 ± 0.24% in 16S rRNA), although the time between the NTK and the STK populations could not be estimated by BEAST and IM analysis probably because of an insufficient amount of information in the data set. Individuals from the NTK have minor, but unique genetic characteristics. Moreover, the gene flow from the STK to the NTK after their isolation is also detected in the IM analysis (Table 4). The Tokara Gap is believed to have formed during the late Miocene to the Pleistocene time (1.55–8 MYA: Kizaki and Ohshiro, 1980; Kimura,1996; Osozawa et al., 2012), older than most of our estimates, although some ranges partially overlapped. Thus, the present result suggests that this frog extended its range to the northern Tokara not by artificial introduction that should have been possible after 0.035 MYA, when anthropological activities began (Tsutsui, 2009), but by the natural overseas dispersal across the strait, such as rafting.

Taking overall large genetic differentiation and old divergence of this species into considerations, the results of demographic analyses indicate that our third hypothesis is most plausible and that wide distribution of this species has been attained by both original wider distribution before the islands separation and overseas dispersal.

Buergeria japonica inhabits various environments including coastal area (Haramura, 2004) and lays eggs in the mouths of streams, no more than 100 m from sea (Maeda and Matsui, 1999), unlike other sympatric frog species. Although embryos of this species are not particularly strong in salinity tolerance (Haramura, 2007), its high abundance around seacoasts would facilitate the spreading of its range across the sea. To evaluate actual ability of overseas dispersal of this species, future studies of salinity tolerance throughout its life stage are clearly needed.

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