Influence of geology and human activity on the genetic structure and demography of the Oriental fire-bellied toad (*Bombina orientalis*) ☆

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ABSTRACT

The Oriental fire-bellied toad (*Bombina orientalis*) is a commonly used study organism, but knowledge of its evolutionary history is incomplete. We analyze sequence data from four genetic markers (mtDNA genes encoding cytochrome c oxidase subunit I, cytochrome b, and 12S–16S rRNA; nuDNA gene encoding recombination activating gene 2) from 188 individuals across its range in Northeast Asia to elucidate phylogeographic patterns and to identify the historic events that shaped its evolutionary history. Although morphologically similar across its range, *B. orientalis* exhibits phylogeographic structure, which we infer was shaped by geologic, climatic, and anthropogenic events. Phylogenetic and divergence-dating analyses recover four genetically distinct groups of *B. orientalis*: Lineage 1—Shandong Province and Beijing (China); Lineage 2—Bukhan Mountain (Korea); Lineage 3—Russia, Northeast China, and northern South Korea; and Lineage 4—South Korea. Lineage 2 was previously unknown. Additionally, we discover an area of secondary contact on the Korean Peninsula, and infer a single dispersal event as the origin of the insular Jeju population. Skyline plots estimate different population histories for the four lineages: Lineages 1 and 2 experienced population decreases, Lineage 3 remained stable, while Lineage 4 experienced a sharp increase during the Holocene. The timing of the population expansion of Lineage 4 coincides with the advent of rice cultivation, which may have facilitated the increase in population size by providing additional breeding habitat.

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1. Introduction

The Oriental fire-bellied toad (*Bombina orientalis*) is easy to rear and to manipulate (Kawamura et al., 1972), making it a useful study organism in the sciences. Using *B. orientalis*, ecological research addressed the impact of maternal investment on offspring (Kaplan, 1992; Kaplan and King, 1997; Kaplan and Phillips, 2006), biochemical studies discovered useful compounds from skin secretions (e.g. bombinin- and bombesin-like peptides, bradykinins; Erspamer, 1988; Gibson et al., 2001; Chen et al., 2002), and ecotoxicological work quantified the negative effect of insecticides and

other chemicals on frog survival and development (Kang et al., 2008; Park et al., 2010). Yet to be resolved is the evolutionary history of *B. orientalis*, which may provide additional insight into these previous studies.

We studied *B. orientalis* with two goals—to elucidate the genetic relationships of populations across its range and to identify the events that shaped its evolutionary history. *Bombina orientalis* is widespread across Northeast Asia, and a previous study showed individuals from Shandong Province and Beijing to be genetically distinct from those in Northeast China, Russia, and Korea (Zheng et al., 2009). Additionally, Beijing samples were genetically verified as being artificially introduced from Shandong populations (Zheng et al., 2009). This previous work included only one sample from the Korean Peninsula, leaving us with an incomplete picture of the evolutionary history of the species. Here we extend the findings

of Zheng et al. (2009) by including comprehensive sampling in Korea.

Northeast Asia has undergone complex tectonic activity (Yi et al., 2003; Metcalfe, 2006; Shi, 2006) and dramatic climatic change (An et al., 2001; Lambeck and Chappell, 2001; Chen et al., 2003). Pleistocene glaciation is accepted as a major factor that influenced the diversification of plants and animals in Northeast Asia (Serizawa et al., 2002; Aizawa et al., 2007; Lee et al., 2008; Zhang et al., 2008; Bai et al., 2010; Ding et al., 2011), including *B. orientalis* (Zheng et al., 2009). We evaluate this hypothesis of Pleistocene diversification of *B. orientalis* by performing divergence dating and demographic history analyses.

By sampling across its range, we uncover the phylogeographic pattern of *B. orientalis*. We identify genetically distinct groups in Korea and elucidate the origin of the Jeju Island population. Also, we estimate the diversification of *B. orientalis* to be during the Miocene, casting doubt on hypotheses of a Pleistocene glaciation effect. We offer new hypotheses on the events that shaped the evolutionary history of *B. orientalis*.

2. Materials and methods

2.1. Sampling

We collected data from 188 *B. orientalis* specimens from across its range (Fig. 1; Table S1). Of these samples, 161 were newly sequenced in this study and 27 retrieved from GenBank. Tissue samples (liver or toe clips) were preserved in 95% ethanol. *Bombina bombina* and *B. variegata* were chosen as outgroups for phylogenetic analyses based on the results of a previous study (Pabijan et al., 2013).

2.2. Laboratory protocols

Genomic DNA was extracted using a standard salt extraction protocol (Sambrook and Russell, 2001). We built upon a previous

B. orientalis dataset (Zheng et al., 2009), sequencing three mitochondrial DNA (mtDNA) fragments (encoding cytochrome c oxidase subunit I [*COI*], cytochrome b [*cytb*], and 12S rRNA and 16S rRNA [*12S–16S*]) and one nuclear gene (encoding recombination activating gene 2 [*RAG2*]). The primers COI-1/COI-2 were used to amplify *COI* (Zheng et al., 2009), cytb-1/cytb-2 to amplify *cytb* (Zheng et al., 2009), 12S-1/16S-2 to amplify *12S–16S* (Kocher et al., 1989; Palumbi, 1996), and rag2-1/rag2-4 to amplify *RAG2* (Zheng et al., 2009). PCR products were purified and sequenced in both directions using the PCR primers at the National Instrument Center for Environmental Management (Seoul National University) using an ABI3730XL machine (Applied Biosystems, Foster City, CA, USA). Sequences were assembled and edited using Geneious v.5.3.6 (Biomatters, Auckland, New Zealand). Multiple sequence alignments were performed using MUSCLE (Edgar, 2004) and manually checked. Additionally, *COI*, *cytb*, and *RAG2* alignments were checked for erroneous stop codons by translating nucleotides to amino acid. Haplotypes of *RAG2* were reconstructed using PHASE (Stephens et al., 2001), with the aid of SeqPhase (Flot, 2010).

2.3. Phylogenetic analyses

Phylogenetic analyses were performed on two datasets: concatenated mtDNA and *RAG2*. All phylogenetic analyses performed on the mtDNA dataset were partitioned by gene. Maximum Likelihood (ML) analyses were performed in RAxML v.8.0.2 (Stamatakis, 2014), while Bayesian Inference (BI) analyses were performed in MrBayes v.3.2 (Ronquist et al., 2012). ML analyses were performed using the combined rapid bootstrap and search for the best-scoring ML tree analysis. The GTR + G model of nucleotide substitution was used for the ML tree search and 1000 bootstrap replicates. For BI analyses, models of nucleotide substitution were chosen within MrBayes using the reversible jumping model choice (nst = mixed) with both rate variation and invariable sites (rates = invgamma). Two BI searches with random starting trees were run and
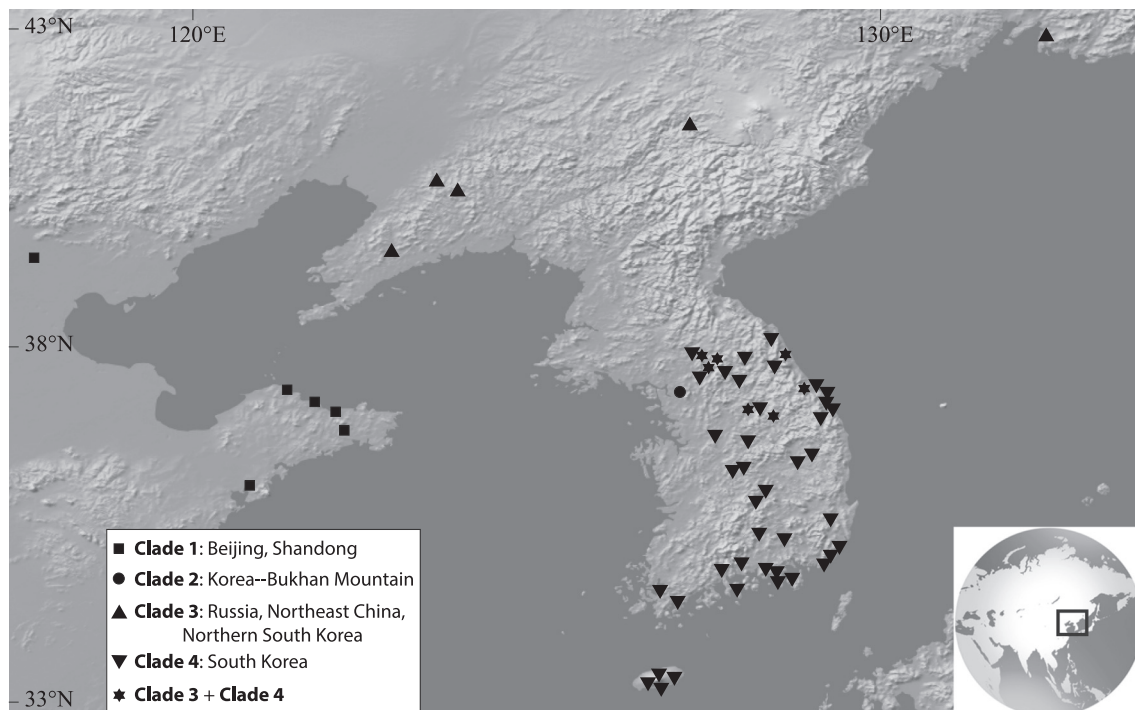
Fig. 1. Map of *Bombina orientalis* samples used in this study. Different shapes denote the four mitochondrial haplotype clades inferred to represent population lineages. The star shape indicates localities where individuals from Clade 3 and Clade 4 were found living syntopically.

compared using four Markov Chain Monte Carlo (MCMC) chains, 10 million generations, and sampling over 1000 generations. Stationarity was evaluated by plotting the log-likelihood scores in Tracer v.1.5 (Rambaut et al., 2013), and data from the first 2 million generations were discarded before building a consensus tree.

For mtDNA, uncorrected *p*-distances were calculated for the major groups using MEGA v.6.06 (Tamura et al., 2013). Calculations were made with partial deletion of missing data and a site coverage cutoff of 95%.

Approximate unbiased topology tests (Shimodaira, 2002) were used to examine whether the ML tree inferred from mtDNA was statistically significant compared to trees with different relationships among the four major haplotype clades. Tests compared four topologies: (1) the ML topology with Clade 2 (Bukhan Mountain) being the sister taxon to a clade comprising all other samples, (2) Clade 2 as the sister group to Clade 1 (China only), (3) Clade 2 as the sister group to Clade 3 (Russia, China, Korea), and (4) Clade 2 as the sister group to Clade 4 (Korea only). We ran constrained RAxML analyses and calculated the per-site log likelihoods. These per-site log likelihoods were then input into the program CONSEL (Shimodaira and Hasegawa, 2001).

2.4. Divergence dating analyses

For the mtDNA dataset, divergence dates were estimated using BEAST v.1.8.0 (Drummond et al., 2012). An uncorrelated lognormal relaxed clock model was implemented with MCMC chains run for 100 million generations and sampled every 10,000 generations. A GTR + I + G substitution model was used with a Yule process tree prior. Two independent analyses were run and combined using LogCombiner v.1.8. Stationarity and effective sample sizes were evaluated in Tracer v.1.5 (Rambaut et al., 2013). Tree data were summarized in TreeAnnotator v.1.8.0 after removing data from the first 10 million generations as burn-in. We estimated the rate of evolution of each mtDNA marker by unlinking substitution models.

We set priors for two calibration points based on geologic and fossil data following a previous study of *Bombina* (Pabijan et al., 2013; Table S2). Parham et al. (2012) outline the data needed for justifying fossil calibrations, but as all data are not available, we use these analyses as a heuristic. The first calibration point was set for the divergence between Asian (*B. orientalis*) and European (*B. bombina* and *B. variegata*) *Bombina* based on range of possible dates for the dispersal from Asia to Europe: the oldest *Bombina* fossil from Europe (~13 Ma [mega-annum]; Miynarski et al., 1982; Böhme and Ilg, 2003) and the closing of the Turgai Strait (~30 Ma; Sanmartin et al., 2001). For the oldest *Bombina* fossil from Europe, Pabijan et al. (2013) tested two alternative hypotheses (13 Ma vs. 20 Ma), but we chose 13 Ma since these results of Pabijan et al. (2013) were consistent with mid-Miocene climate fragmenting *Bombina* species. We implemented a normal distribution with a mean of 22.5 and SD of 5.7 (13.12–33.67 Ma). The second calibration point was set for the divergence between *B. variegata* and *B. bombina* based on the oldest *B. bombina* fossil from Poland (~4 Ma; Sanchiz and Miynarski, 1979), *B.* cf. *bombina* from Austria (~9 Ma; Tempfer, 2005; Böhme and Ilg, 2003), and *B.* cf. *variegata* from the Czech Republic (~9 Ma; Hodrova, 1987; Böhme and Ilg, 2003). We implemented a normal distribution with a mean of 7.2 and SD of 2.1 (3.74–10.65 Ma).

2.5. Estimating demographic history

The demographic history of *B. orientalis* was estimated from the mtDNA dataset using BEAST v.1.8.0 (Drummond et al., 2012). Separate analyses were run for the four groups recovered in the phylogenetic analyses. Priors for the root height of each group were set

based on the results from divergence-dating analyses. A GTR + I + G substitution model was used with a "Coalescent: Bayesian Skyline" tree prior. MCMC chains were run for 500 million generations, sampling every 10,000 generations. The amount of burn-in removed varied between analyses, and was determined using Tracer v.1.5.

3. Results

3.1. Phylogenetic analyses

Phylogenetic trees from ML and BI analyses were similar, differing only in relationships of the terminal branches and support
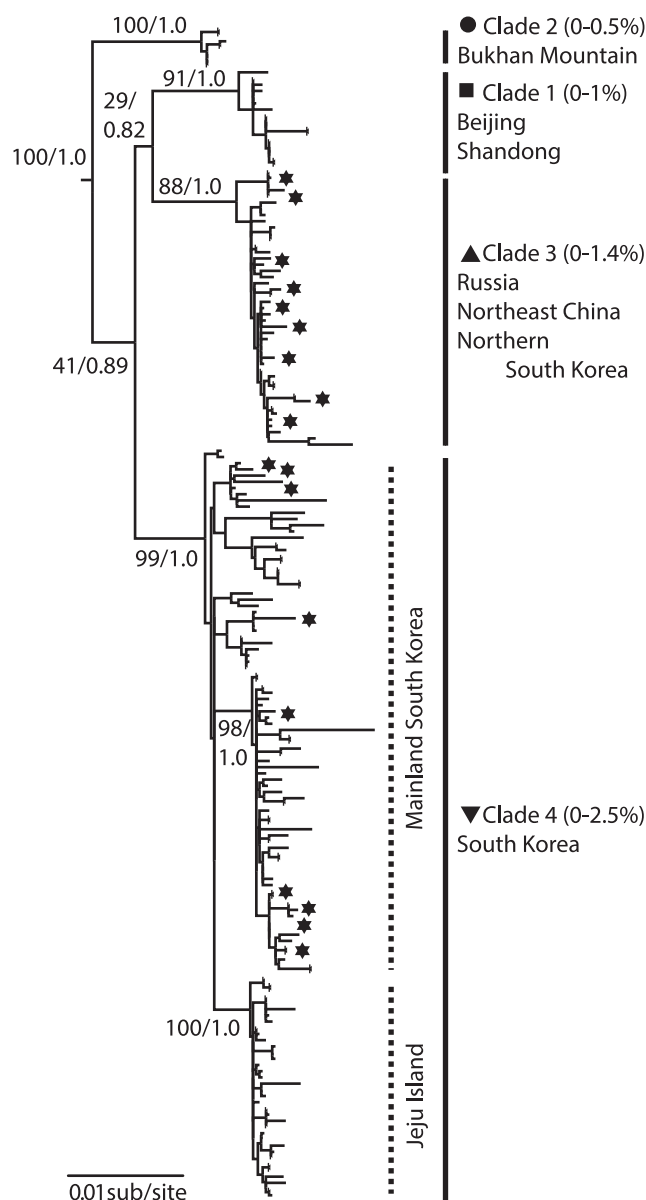
Fig. 2. Maximum likelihood (ML) phylogeny of *Bombina orientalis* inferred from the concatenated mitochondrial DNA dataset. Support values for major clades are labeled on the branches (ML bootstrap/Bayesian posterior probability). Uncorrected *p*-distance values among haplotypes within clades 1–4 are next to the clade labels. *P*-distances between clades tended to be higher (2.4–4.2%). The four major clades are denoted with the same shapes used in Fig. 1. Stars on terminal branches indicate sites where individuals from Clade 3 and Clade 4 were found living syntopically. *Bombina bombina* and *B. variegata* were used as outgroups for analyses (not shown).

values for clades. Analyses of the mtDNA were well resolved and recovered four major clades, herein designated as Clade 1, Clade 2, Clade 3, and Clade 4 (Fig. 2). Each clade was strongly supported by ML and BI analyses (ML bootstrap = 88–100, BI posterior probability = 1.0), but relationships among clades were unclear due to low support values. Clade 1 contains specimens from Shandong Province and Beijing, China (Figs. 1 and 2), while Clade 2 includes specimens from a single site in South Korea—Bukhan Mountain. Clade 3 contains samples from the Russian Far East, Northeast China, and northern South Korea. Lastly, Clade 4 covers samples from South Korea. Within Clade 4 is a strongly-supported clade of Jeju Island samples. Seven sites in northern South Korea (37.4–38°N) had individuals from both Clades 3 and 4 (denoted by the star shape in Figs. 1 and 2). *RAG2* had low phylogenetic signal and the phylogenetic trees were poorly resolved, with many polytomies present (Fig. S1). New sequences were submitted to GenBank (KR869168–KR869627; Table S1), and the dataset used for phylogenetic analyses submitted to TreeBASE (http://purl.org/-phylo/treebase/phylows/study/TB2:S17697).

The maximum uncorrected *p*-distances of mtDNA within the major haplotype clades (Clade 1: 1%, Clade 2: 0.5%, Clade 3: 1.4%, Clade 4: 2.5%) were generally lower than among clades (2.4–4.2%). Uncorrected *p*-distances among haplotypes obtained from Jeju Island were <1%. The mean uncorrected *p*-distances for each mtDNA marker are listed in Table S3.

Results from the topology tests comparing alternative topologies for the placement of Clade 2 (Bukhan Mountain) were not significant (all *p*-values > 0.1). These results indicate that non-ML topologies were statistically equal to the ML tree and therefore possible.

3.2. Divergence dating analyses

After removing the burn-in, the majority divergence dating analysis parameters had effective sample sizes greater than 200, indicating the MCMC chain reached stationarity. All divergence-date estimates had broad confidence intervals. The same four major haplotype clades were recovered as with phylogenetic analyses, but the relationship between these groups differed. Comparison of the divergence-date estimates to previous studies is in Table S4. The estimate for the origin of *B. orientalis* was 14.8 Ma (6.5–23.52 Ma). For the four major haplotype clades, the divergence dates varied (Table 1). The estimate for the divergence among the haplotypes within the Jeju clade was 4.32 Ma (1.64–7.61 Ma) (Table 1). Groups corresponding to the four major haplotype clades are considered distinct evolutionary lineages and called Lineages 1–4 in the discussion.

Inferred rates of evolution (substitutions/site/Ma) of the three mtDNA partitions are as follows: *COI*—0.00632, *cytb*—0.00415, *12S–16S*—0.00113. Comparisons of rates to previous studies (Evans et al., 2003; Mueller, 2006) are found in Table S5.
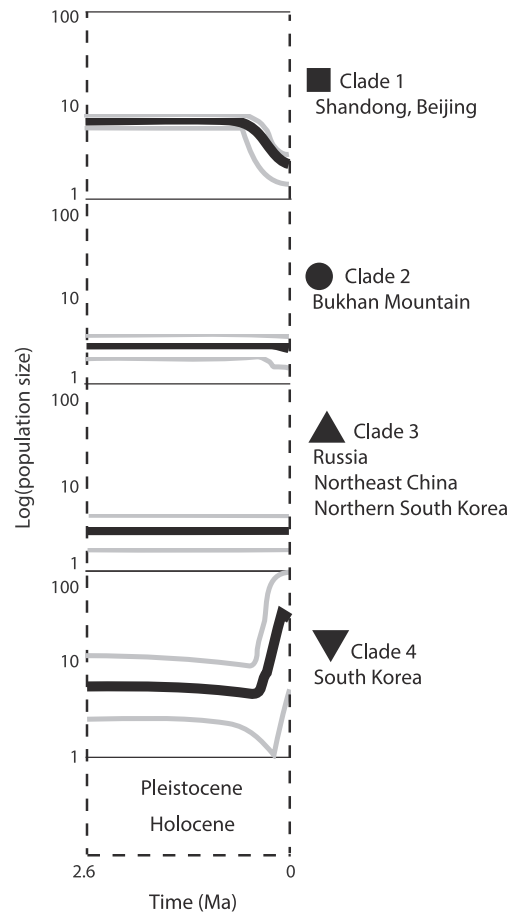
Fig. 3. Skyline plots inferring the demographic history of the four major *Bombina orientalis* lineages recovered in our study. The *y*-axis represents the log-transformed population size (effective population size × generation time). Graphs have been truncated to only show the Pleistocene and Holocene.

3.3. Demographic history

The Bayesian skyline graphs inferred the demographic histories of the four *B. orientalis* lineages (Fig. 3). The demographic patterns of the four lineages differed during the Pleistocene–Holocene: Lineage 1 and 2 showed population decreases, Lineage 3 showed a stable population size, and Lineage 4 showed a population expansion.

4. Discussion

4.1. Genetic structure of B. orientalis

Bombina orientalis occurs across Northeast Asia in Northeast China, the Russian Far East, and Korea. A previous phylogeographic

Table 1
Divergence date estimates of the major groups recovered in this study. Ma = mega-annum.

Group	Description	Mean (95% confidence interval)
All	*B. orientalis, B. bombina, B. variegata*	17.17 Ma (8.56–26.15 Ma)
B. orientalis	Age of *B. orientalis*	14.80 Ma (6.50–23.52 Ma)
Clade 1	Beijing, Shandong	5.01 Ma (1.72–9.29 Ma)
Clade 2	Bukhan Mountain	3.12 Ma (0.61–6.58 Ma)
Clade 3	Russia, Northeast China, Northern South Korea	6.31 Ma (2.29–10.79 Ma)
Clade 4	South Korea	9.20 Ma (3.77–15.33 Ma)
Jeju	Jeju Island population	4.32 Ma (1.64–7.61 Ma)

study of *B. orientalis* uncovered two genetically distinct groups: (1) Shandong Province and Beijing, China and (2) Northeast China, Russia, and Korea (Zheng et al., 2009). In our study, we increased sampling throughout Korea and uncovered additional genetic structure in *B. orientalis*. Since these results are based largely on the mtDNA dataset (low variation and genetic structure in *RAG2*), these results should be considered preliminary.

Four major mtDNA lineages were recovered: (1) Shandong Province and Beijing (China), (2) Bukhan Mountain (South Korea), (3) Russia, Northeast China, and northern South Korea, and (4) South Korea (Figs. 1 and 2). The first group from Zheng et al. (2009) in Shandong and Beijing corresponds to Lineage 1 of our study, while their second group is divided into two genetically distinct lineages in our study (Lineages 3 and 4). We discovered another genetically distinct lineage (Lineage 2) restricted to a single region in South Korea (Bukhan Mountain). These four lineages are all strongly supported (ML bootstrap = 88–100, BI posterior probability = 1.0), but based on support values (Fig. 2) and topology tests, the relationship among them is unclear. Morphologically, there were no obvious differences among the lineages. As our study provided increased sampling and new insight in Korea, we focus our discussion on the three lineages in Korea.

4.2. Lineage 2—Bukhan Mountain

We uncovered a genetically distinct group with a distribution restricted to Bukhan Mountain (Figs. 1 and 2). Seven individuals were collected across 2012 and 2013 field seasons from two localities ~3 km apart. Genetically, all samples were highly similar (*p*-distance < 0.5%) and formed a strongly supported haplotype clade distinct from other populations (ML bootstrap = 100, BI posterior probability = 1.0). The closest collecting locality was ~45 km to the northwest in Myoungji Mountain (data retrieved from Gen-Bank); this individual was a member of Lineage 4 (Fig. 2). Based on ML and BI analyses, Lineage 2 was inferred to be the sister group to a clade comprising all other *B. orientalis*, but this relationship was not strongly supported, and topology tests could not exclude alternative positions in the phylogeny. To determine the extent of Lineage 2's distribution and whether there are areas of sympatry with other lineages, areas around Bukhan Mountain and Seoul should be surveyed further.

4.3. Lineages 3 and 4—Russia, China, Korea

Excluding Lineage 2, two major groups of *B. orientalis* occur across the Korean Peninsula. Lineage 3 is widespread in Northeast China, Russia Far East, and the Korean Peninsula, while Lineage 4 is restricted to Korea (Fig. 1). The uncorrected *p*-distances between individuals in these two lineages ranged between 2.4% and 3.8%. At seven locations across northern South Korea, where more than five samples were collected (Pyunggang Botanical Garden, Gapyeong, Chuncheon, Chiak Mountain, Taebaek Mountain, Gangneung, Yeoncheon), individuals from Lineages 3 and 4 lived syntopically (indicated by stars in Figs. 1 and 2). *RAG2* had low variability, and these data did not provide evidence on whether the two groups hybridized—shared alleles could be a result of either hybridization or shared ancestral polymorphisms.

The genetic distinctness and geographic distributions of the two lineages lead us to hypothesize that the range overlap resulted from allopatry, divergence, and then secondary contact. The area of sympatry spans approximately 67 km between 37.4 and 38°N. However, since we do not have samples from North Korea, we do not know the northern extent of the sympatric area. *Bombina orientalis* in North Korea probably belongs to Lineage 3, as individuals in this lineage surround North Korea, but the range of Lineage 4 possibly extends northward. Sequencing additional nuclear markers

will help to clarify the relationship between Lineages 3 and 4, and whether they hybridize in the region of sympatry.

4.4. Jeju Island colonization

Lineage 4 consists of a group from Jeju Island, nested in a paraphyletic mainland South Korea group (Fig. 2). This pattern is typical of a single colonization event from continent to island (Bellemain and Ricklefs, 2008). Based on our heuristic, we estimated the age of the Jeju population to be 1.64–7.61 Ma (95% confidence interval). The sister group to the Jeju group has members spread throughout the Korean Peninsula, so we could not determine the source population.

Jeju is a volcanic island that sits atop the 100 m-deep continental shelf of the Korean Peninsula. Jeju formed primarily through volcanic activity (Sohn and Park, 2004). The geological history of Jeju is still under debate. Surface rock layers of Jeju are primarily lavas, which overlay fossiliferous, volcaniclastic sediment (Koh, 1997). The age estimates of Jeju based on different methods are similar: 0.5–1.88 Ma based on ^{40}Ar/^{39}Ar dating (Koh et al., 2013), 0.85–1.72 Ma based on nannofossils (Yi et al., 1998), and 0.78–1.95 Ma based on paleomagnetic and biostratigraphic data (Oh et al., 2000). Our estimate for the age of the Jeju *B. orientalis* population (1.64–7.61 Ma) has a broad confidence interval that overlaps geologic estimates of the age of Jeju Island in the 2 Ma range. The broad confidence interval of our estimate is likely due to the uncertainty in our calibration points, but it raises the possibility that Jeju Island is older than previously believed. Regardless of the timing, these data imply that the *B. orientalis* population on Jeju Island is the result of a single colonization event with no detectable gene flow with the Korean Peninsula, despite intervals of connectedness during lower sea levels of the Quaternary (Butenko et al., 1985; Voris, 2000).

4.5. Miocene diversification

The phylogeographic patterns and diversification of many plants and animals of Northeast Asia can be explained by glacial cycles and refugia during the Quaternary (Serizawa et al., 2002; Aizawa et al., 2007; Lee et al., 2008; Zhang et al., 2008; Bai et al., 2010; Ding et al., 2011). However, our analyses estimate that *B. orientalis* diverged during the Miocene (Table 1), a result that differs from previous studies that estimated a Pleistocene–Pliocene diversification (Zheng et al., 2009; Pabijan et al., 2013). As estimates for age of the tree root and divergence between *B. bombina* and *B. variegata* were consistent across studies (Table S4), we believe that past studies erroneously inferred younger estimates for *B. orientalis* due to sampling and analytical approaches. For sampling, we expected our dates to be older compared to previous studies because we sampled deeper lineages (Zheng et al. did not have samples from Clade 2 and Pabijan et al. did not have samples from Clades 1 and 2). Additionally for analytical approaches, Zheng et al. (2009) set a prior on the rate of evolution (0.011 substitutions/site/ Ma), artificially constraining the analyses. We compared the rates of mtDNA evolution inferred in our study to previous studies and found *B. orientalis* to be evolving at a slightly slower rate than other amphibians (Table S4). As there are no studies investigating mtDNA evolution rate across frog diversity, it is unclear whether *B. orientalis* is especially slow, or falls within the normal rate range.

Our results indicate that *Bombina orientalis* diverged in the Miocene rather than the Pleistocene. The question then becomes: what events during the Miocene caused diversification? Although the broad confidence intervals on the divergence divergence-date estimates do not allow us to identify the specific events leading toward diversification, these results help us understand the broad patterns in *B. orientalis* evolution. Climate probably was not a

major factor, as reconstruction of Northeast Asian vegetation suggests stable, warm climate through the Miocene (Kong, 1996, 2000; Liu and Leopold, 1994). The flora was dominated by thermophilous, evergreen, broadleaf trees (*Castanopsis, Cycloblanaopsis, Cinnamomum, Ilex, Myrica*) extending around the northern Pacific Rim from Northeast China eastward across the Bering land bridge into North America (Kong, 1996, 2000; Liu and Leopold, 1994). Geologically, the Miocene was a time of major events in Northeast Asia such as uplift, faulting, and volcanic activity forming the major mountain ranges along the Korean Peninsula (Wang et al., 1989; Park and Son, 2005, 2008; Wan, 2012), opening of the East Sea/Sea of Japan (Otofuji et al., 1985, 2003), and low sea level exposing a dry Yellow Sea basin (Kim, 1987; Park, 2001; Yi et al., 2003). Based on divergence-dating estimates, these events during the Miocene coincided with and probably influenced the diversification of *B. orientalis*. Additional analyses clarifying the relationship among the four clades and estimating the timing of diversification events more precisely will allow us to understand the exact geological events driving divergence.

4.6. Demographic history and human activity

Estimates of demography imply different histories for each of the four lineages (Fig. 3). Lineages 1 and 2 show population decreases during the Pleistocene–Holocene. These are the two lineages with relatively restricted distributions, with Lineage 1 found in Shandong Province, China (Beijing is an introduced population; Zheng et al., 2009) and Lineage 2 found in Bukhan Mountain, Korea (Fig. 1). It is possible that these populations were previously widespread, but experienced a population size and distribution contraction influenced by the four to five Pleistocene glacial cycles in Northeast Asia (Liu, 1988).

Lineage 3, since its appearance during the late Miocene, maintained a stable population size. Lastly, Lineage 4 had a stable population for most of its history, followed by a sharp population expansion during the Holocene. We suggest that human activity contributed to the population expansion of Lineage 4. Previous studies reveal that agricultural ponds provide breeding habitats for amphibians (Beebee, 1997; Knutson et al., 2004), and population sizes may fluctuate with the availability of these man-made water bodies (Beebee, 1997; Curado et al., 2011). Individuals of *B. orientalis* aggregate and breed in shallow water bodies in the springtime (Kuzman et al., 2004), just as paddies are flooded for rice cultivation. Rice paddies provide stable water bodies in which *B. orientalis* breed, and population densities in rice paddies are often much higher than in natural habitats (pers. obs.). Currently, rice paddies occupy approximately 10% of Korea's total area (934 km^2; KOSTAT, 2015). The cultivation of rice (*Oryza sativa japonica*) in Asia is believed to have begun 8000–13,900 years ago in southern China (Zhao, 1998; Jiang and Liu, 2006), and arrived in Korea between 4000 BC (Im, 1990; Park and Lee, 1995) and 300 BC (Ahn, 2010). As the timing of population expansion is similar to development of rice cultivation, we hypothesize that the increased availability of rice paddies during the Holocene contributed to the population size increase of *B. orientalis*.

5. Conclusion

Bombina orientalis is a genetically diverse species with four distinct lineages across Northeast Asia. In the middle of the Korean Peninsula, two lineages overlap, with genetically distinct individuals living syntopically. Individuals from Jeju Island were monophyletic for mitochondrial haplotypes and nested within a paraphyletic mainland grouping, indicating that the population arose from a single colonization event. Divergence-dating analyses

imply that this colonization occurred soon after the formation of Jeju Island. Contrary to previous views that Pleistocene glacial cycles caused population substructure in *B. orientalis*, our analyses suggest a Miocene diversification. As climate was relatively stable in the Miocene, diversification was probably driven by geologic events. However, the Pleistocene was an important time for *B. orientalis*; Pleistocene glacial cycles may have caused population decreases in some lineages, while rice cultivation provided stable breeding habitats for a population expansion in another lineage.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.12.019.

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