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Genetic Diversity and Differentiation of the Ryukyu Endemic Frog *Babina holsti* as Revealed by Mitochondrial DNA

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We surveyed the genetic diversity and genetic differentiation of an endangered frog, *Babina holsti*, endemic to Okinawajima and Tokashikijima Islands of the Ryukyus, to elucidate its divergence history and obtain basic data for its conservation. Genetic differentiation between the two island lineages is moderate (3.1% *p*-distance in the cyt *b* gene). This result suggests that the two island lineages have been isolated between the late Pliocene and the middle Pleistocene and have never migrated between the current northern part of Okinawajima and Tokashikijima Islands, which were once connected in the late Pleistocene glacial age. On Okinawajima Island, the southernmost sample was constituted by a unique haplotype, without considerable genetic distance from haplotypes detected from northern samples. This unique haplotype composition in the southernmost sample would have resulted from the restricted gene flow between the southernmost population and the other populations in Okinawajima Island. Furthermore, the absence of genetic diversity within the southernmost sample indicates that this population has recently experienced population size reduction, possibly by predation pressure from an introduced mongoose, which is more abundant in the southern part than in the northern part of the island. Lower genetic diversity in the Tokashikijima sample implies a small effective population size for mitochondrial DNA (mtDNA) in *B. holsti* on the island. Immediate conservation measures should be taken for the populations from the southernmost range in Okinawajima and Tokashikijima.

Key words: island amphibian, phylogeography, mongoose, Okinawa, mitochondrial DNA, ESU

INTRODUCTION

Island populations of nonvolant animals sometimes show higher genetic differentiation among populations from different islands (Matsui et al., 2005) and lower genetic diversity within each island than continental populations (Iguchi and Nishida, 2000). In particular, in animals such as amphibians with generally low dispersal and low migration ability beyond saltwater barriers (Duellman and Trueb, 1986; Inger and Voris, 2001), genetic variations would be strongly affected by vicariance (Ota, 2000; Tominaga et al., 2010). Because of this unique trait, amphibians offer a good opportunity to investigate historical biogeography of insular regions (Vences et al., 2003), while they raise problems of local extinction in island environments. Thus, investigation of the genetic diversity and differentiation of island amphibians provides important information for historical biogeography and conservation biology in insular regions.

Babina holsti is an endemic frog of the Ryukyu Archipelago with a very limited distribution, being restricted to the northern part of Okinawajima Island and the adjacent small islet Tokashikijima Island (Maeda and Matsui, 1999). These two islands were originally connected before the early Pleistocene (ca. 1.5 MYA), but began to separate from each other in the middle Pleistocene (ca. 1.3–0.85 MYA), and were reconnected by a land bridge in the late Pleistocene (ca. 0.20–0.01 MYA) (Kamiya, 1984; Kimura, 2003; Iryu et al., 2006). Thus, we can propose three alternative hypotheses about the formation of the current disjunct distribution of this frog. First, we hypothesize that this frog was present in the area of the current Okinawajima and Tokashikijima Islands prior to their first separation in the middle Pleistocene, and that the two isolated populations have survived on these two islands to date. Second, we can alternatively hypothesize that this frog occurred on one of the two islands and expanded its range to another in the late Pleistocene glacial age (ca. 0.20–0.01 MYA) when the land bridge connected the two islands. Thirdly, we also hypothesize that two lineages had been separated by some vicariant events in the middle Pleistocene and that their ranges secondarily overlapped via the land bridge in the late Pleistocene glacial age. From the first hypothesis, we can expect high genetic divergence between the two island samples, while from the second hypothesis, we can expect low genetic divergence between them. From the third hypothesis, we can expect

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genetically highly diverged lineages to coexist on one or both islands. In this study, we surveyed genetic differentiation among samples of this frog using mitochondrial DNA (mtDNA) sequences and estimated the divergence time between mtDNA lineages to clarify the divergence history of this species and the formation of its current disjunct distribution.

Babina holsti is thought to be a high risk of extinction and has been protected by law from illegal collection. On Okinawajima Island, the distributional range of this frog was estimated to have decreased in past decades (Toyama, 1995). This situation would lead to limited migrations among locally isolated populations (Hitchings and Beebee, 1997) and decreased genetic diversity within this species. However, details of geographic genetic variation and diversity in this species have not previously been surveyed. In this study, we clarified the current genetic geographic structure and elucidated the evolutionary significant units (ESUs, Ryder 1986) for efficient conservation of this endangered species.

MATERIALS AND METHODS

Sampling

Tissues from a total of 74 individuals of *B. holsti* were collected by toe (for metamorphs) or tail (for larvae) clipping methods from 10 sampling localities representing the current distributional range of *B. holsti* (Fig. 1; Table 1). A set of individuals from a sampling locality is called a sample. Each one individual of a sister species *B. subaspera* from Amamioshima and Kakeromajima Islands, respectively, was added to the phylogenetic analyses. These were collected before April 19, 2005, before the species was listed as a protected species. Two other species of the genus *Nidirana* (*N. okinavana* from Southern Ryukyu and *N. adenopleura* from Taiwan) were added as outgroup taxa. The genus *Nidirana* was originally designated as a subgenus of *Rana* by Dubois (1992) and was elevated to a generic rank by Chen et al. (2005). However, Frost et al. (2006), without actual comparisons, synonymized *Nidirana* with *Babina*. Although subsequent molecular studies proved monophyly of *Nidirana* and *Babina*, morphological and ecological synapomorphies listed from literature by Frost et al. (2006) are not convincing and require further studies. Therefore, in this study, we use the generic name *Nidirana* for species *okinavana* and *adenopleura*, following some previous authors (Matsui, 2007; Cuaynkern et al., 2010), although the use of *Babina* is now becoming popular simply for the sake of convenience in referencing database.

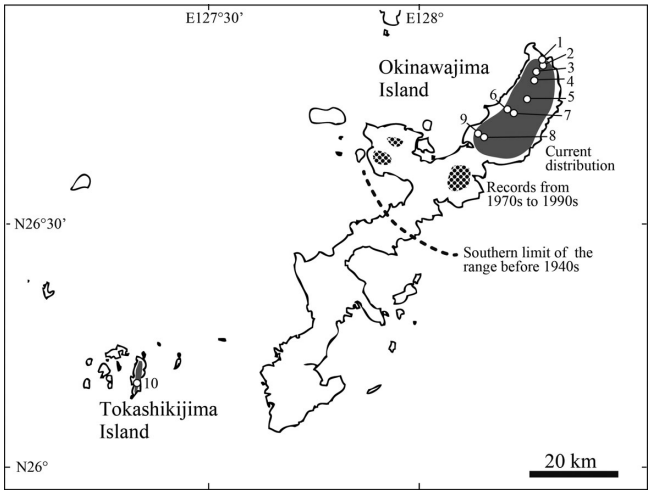

Fig. 1. Map of Okinawa showing current (gray) and past (halftone dot) distributional ranges of *Babina holsti* and sampling locations in this study.

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, the primers Cytb_F1_Rana (5′-ACAAACAWAATTCYGCWATCATRTGTTTCT-3′) and Cytb_R2_Rana (5′-CTTTMAGAAGYTTATTTTCTAGGAGGCC-3′), which were newly designed for the cyt *b* gene of frogs, were used. The reaction conditions were initial heating at 94°C for 4 min; 35 cycles of 94°C (30 s), 55°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 the primers described above and two newly designed primers: Cytb_F2_Rana (5′-TTCAGTSGAYAACGCCACCCTCACCCG-3′) and Cytb_R1_Rana (5′-TCCTYCACCAAACKGGMTCWTCYAACC-3′). Following this, sequencing was performed on ABI 3100 or ABI 3130 automatic sequencers. New sequences of cyt *b* that were obtained were deposited in GenBank (Accession number: AB826407–AB826435). Alignment of data from all individuals was performed using the Clustal option in the BioEdit software (Hall, 1999).

Phylogenetic analysis

Phylogenetic trees were constructed by maximum parsimony (MP), Bayesian inference (BI), and maximum likelihood (ML) meth-

Table 1. Sample number, name, number of individuals, number of haplotypes, genetic diversity indices, and haplotypes observed in each sample.

Sample No.	Sample Name	N of individuals	N of haplotypes	Haplotype diversity	Nucleotide diversity	H 01	H 02	H 03	H 04	H 05	H 06	H 07	H 08	H 09	H 10	H 11	H 12	H 13	H 14	H 15	H 16	H 17	H 18	H 19	H 20	H 21	H 22	H 23	H 24	H 25
1	Oku1	5	3	0.70 ± 0.22	0.0018 ± 0.0014											1			1				3							
2	Oku2	8	4	0.79 ± 0.11	0.0044 ± 0.0028	1	3					1																3		
3	Nishimei	12	7	0.89 ± 0.06	0.0054 ± 0.0031		1	3		1							1		2				1			3				
4	Benoki	13	8	0.94 ± 0.04	0.0052 ± 0.0030		2				2						1				1	2		1		2	2			
5	Okuni	4	3	0.83 ± 0.22	0.0026 ± 0.0021								1	1				2												
6	Okuma	1	1	–	–																				1					
7	Yonaha	8	4	0.79 ± 0.11	0.0019 ± 0.0014		3								1					1			3							
8	Nuha	10	1	0.00 ± 0.00	0.0000 ± 0.0000				10																					
9	Oganeku	1	1	–	–				1																					
10	Tokashiki	12	2	0.53 ± 0.08	0.0005 ± 0.0005																								7	5

ods. The optimum substitution models for each partition were selected by Kakusan4 (Tanabe, 2011) based on the Akaike information criterion. For ML analysis, TN93 (Tamura and Nei, 1993) +I, HKY85 +I, and GTR +I were selected as the optimal models for the first, second, and third codon positions of the cyt *b* gene, respectively. 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, K2P (Kimura, 1980) +I, HKY85 (Hasegawa et al., 1985), and GTR (Rodriguez et al., 1990) +I were selected as the best substitution model for the first, second, and third codon positions of the cyt *b* gene, respectively. Bayesian analysis was conducted using MrBayes software v3.1.2 (Huelsenbeck and Ronquist, 2001). Two independent runs of four Markov chains were conducted for 5 million generations in Bayesian analyses. The MP tree was constructed using PAUP* 4.0b10 (Swofford, 2002). MP phylogenies were estimated using the heuristic search algorithm for each tree-building methodology. Hundred random taxon addition replicates were used for all analyses to minimize the effect of entry sequence on the topology of the resulting cladogram. Analyses were conducted using accelerated character transformation (ACCTRAN) optimization and tree bisection–reconnection (TBR) branch swapping, with characters unordered and equally weighted. For ML and MP analyses, nonparametric bootstrap (bs) analysis (Felsenstein, 1985) with 1,000 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 95% or higher were considered significant (Leaché and Reeder, 2002).

Network 4.6 (Foster et al., 2007) was used to compute the median joining network (Bandelt et al., 1999) based on default settings.

Calculation of genetic distance and estimation of divergence time

Genetic distances were calculated using the mean genetic *p*-distance for pairwise combinations of haplotypes using MEGA, version 4 (Tamura et al., 2007). To estimate divergence times, the Bayesian method using BEAST ver. 1.6.2 (Drummond and Rambaut, 2007) was applied. Because no known calibration points exist, two different substitution rates were used as the substitution rates of cyt *b* evolution for genus *Rana* [1.0%/MY (Vences et al., 2013); 3.6%/MY (Babik et al., 2004)]. BEAST analyses were performed using the strict clock model under the HKY + G model of sequence evolution, and the topology obtained from ML analyses was used as a starting tree. Default prior distributions were used for all other parameters, and analyses were run for 50 million generations, sampling every 1000 generations. Suitable burn-in and convergence of parameters were determined using Tracer ver. 1.5 (Rambaut and Drummond, 2007), and the first 3 million generations were discarded as burn-in.

Genetic diversity and genetic differentiation

Haplotype diversity (*h*) and nucleotide diversity (π, based on pairwise differences) values were calculated in each sample consisting of more than two individuals using the ARLEQUIN3.1 software (Excoffier et al., 2005). The data set was tested for population subdivision by two different approaches using ARLEQUIN3.1 (Excoffier et al., 2005). First, the population pairwise fixation indices F_{ST} were calculated and their significances were tested by a nonparametric permutation approach with 1,000 permutations of haplotypes among sampling localities. Second, an exact test of population differentiation (Raymond and Rousset, 1995) was conducted for comparisons of haplotype frequencies among samples.

RESULTS

In total, 25 cyt *b* haplotypes were recognized in *B. holsti* and two were recognized in *B. subaspera*. For 29 haplotypes including the outgroup taxa, phylogenetic analyses were conducted using the 1,085 sites of the partial cyt *b* gene; 254 of these sites were variable and 56 were parsimony-informative. MP analysis yielded more than 10,000 equally most parsimonious trees [L = 1,124 steps, retention index = 0.889, and consistency index = 0.894]. ML analysis generated a topology with lnL = –2,667.63188. The mean lnL score of Bayesian analyses for all trees sampled at stationarity was –2,720.81. All three phylogenetic analyses yielded essentially identical topologies, and only an ML phylogeny is shown in Fig. 2. Monophyly of *B. holsti* and *B. subaspera* was strongly supported in all trees (92%, 1.00, and 100% support in MLbs, bpp, and MPbs, respectively). Within the two species of *Babina*, two haplotypes of *B. subaspera* are separated from 25 haplotypes of *B. holsti* with moderate support values for each species (88%, 0.97, and 100% for *B. subaspera*; 81%, 0.61, and 94% for *B. holsti*). *Babina holsti* was divided into two main lineages: one consisting of two haplotypes observed in the sample from Tokashikijima (95%, 1.00, and 100%) and the other consisting of 23 haplotypes found only in the Okinawajima samples (73%, 0.61, and 91%).

The mean ± standard deviation (SD) of the uncorrected *p*-distances was 5.0 ± 0.6% between *B. holsti* and *B. subaspera* and 3.1 ± 0.5% between the Tokashikijima and Okinawajima lineages. The maximum uncorrected *p*-distance among 23 haplotypes of the Okinawa lineage was

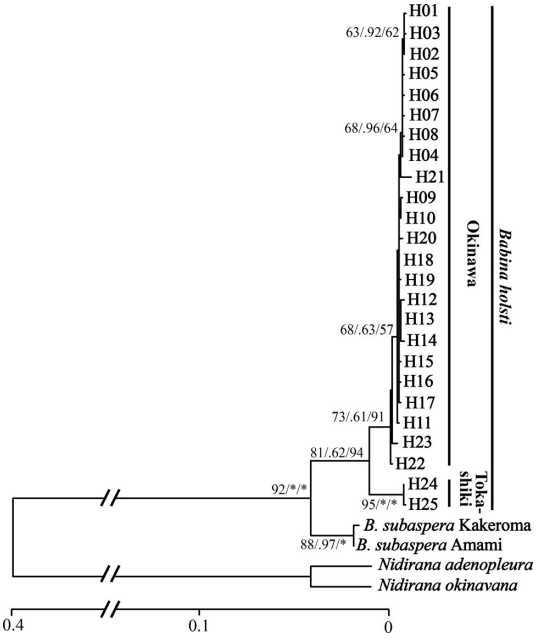

Fig. 2. Maximum likelihood phylogram of 1085 bp of the mitochondrial cytochrome *b* gene for haplotypes of *Babina holsti* and its related species. Numbers preceded by "H" indicate haplotype number. Nodal Numbers represent ML bootstrap supports/ Bayesian posterior probability/ MP bootstrap supports. Asterisks indicate 100% bootstrap support values or 1.00 Bayesian posterior probabilities.

1.1%. The mean divergence times with 95% credibility interval (CI) were 6.53 MYA (4.76–8.32 MYA) between *B. holsti* and *B. subaspera* and 3.4 MYA (2.38–4.60 MYA) between two lineages of *B. holsti* based on the 1.0%/MY substitution rate, while they were 1.81 MYA (1.33–2.32 MYA) and 0.96 MYA (0.65–1.28 MYA), respectively, based on 3.6%/MY substitution rate.

The genetic structure in each sample is summarized in Table 1. The overall nucleotide diversity ($\pi \pm$ SD) was 0.01120 ± 0.00154, and that for all individuals from Okinawajima was 0.00388 ± 0.00039. In many cases, two or more different haplotypes were found from each sample of Okinawajima Island and several haplotypes were shared by two or more samples (Fig. 3; Table 1). However, sample 8 from the southernmost limit of the range in Okinawajima had only one haplotype, and sample 10 from Tokashikijima had only two haplotypes. The π values in these two samples (mean: 0–0.000489) were lower than those in other samples (mean: 0.001843–0.005418) (Table 1).

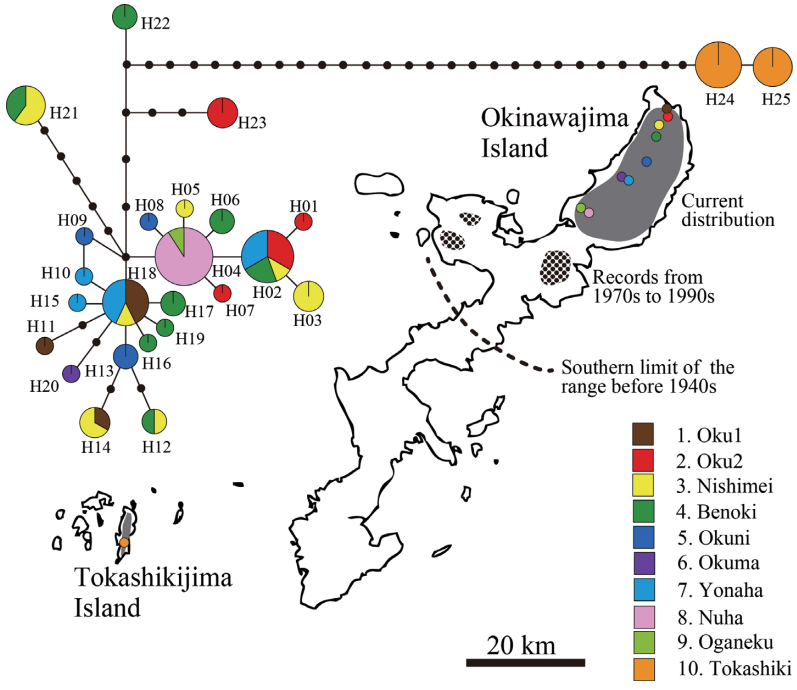

Fig. 3. Haplotype network tree based on median joining network method for the cytochrome *b* haplotypes of *Babina holsti*. The size of each circle represents haplotype frequency. Black circles indicate missing haplotypes. The color of each circle corresponds to sampling locality on the map.

The results of the test for sample differentiation are shown in Table 2. Both F_{ST} and the result of exact tests suggest significant differentiation in various combinations of samples. In particular, the southernmost sample in Okinawajima (sample 8) had a unique haplotype, which was shared by only the neighboring sample 9, and showed significant differentiations from the other samples of Okinawajima.

DISCUSSION

Genetic differentiation between island lineages

Our results indicate monophyly of each of the Tokashikijima and Okinawajima lineages of *B. holsti*, although the support for the latter lineage was insufficient in Bayesian analysis. The mean genetic distance between the two lineages from different islands is moderate (3.1%). Iwai and Shoda-Kagaya (2012) surveyed the population structure of *B. subaspera*, the sister species of *B. holsti*, using the mitochondrial cytochrome *c* oxidase subunit I (CO I) gene and revealed low genetic diversity (π = 0.00136 ± 0.0003). The maximum *p*-distance among seven haplotypes of *B. subaspera* is calculated as 0.4% (vs. 1.1% among 23 haplotypes of the Okinawajima lineage and 3.4% among 25 haplotypes of *B. holsti* in the cyt *b* gene). Because the substitution rate of the cyt *b* gene is closely comparable with that of the CO I gene (Kakehashi et al., 2013), it is possible that the degree of genetic diversity is much greater in *B. holsti* than in *B. subaspera*. The estimated divergence time between lineages from the two islands of *B. holsti* is also considerable (mean = 3.1 MYA in the late Pliocene based on 1.0% divergence/MY and 0.96 MYA in the middle Pleistocene based on 3.6% divergence/MY). Because the rate of 3.6% divergence/MY is much higher than the rates proposed for other vertebrates (Babik et al., 2004), the actual divergence time of the two island lineages seems to be around the late Pliocene. This result supports our first hypothesis and indicates that *B. holsti* was present in an area including both the current islands before the formation of the Pleistocene land bridge.

Several amphibian species are distributed on both these islands. Tominaga et al. (2010) revealed that the samples of *Cynops ensicauda* from the two islands shared several haplotypes, indicating that the species migrated between

Table 2. Pairwise fixation indices (F_{ST}; above diagonal) and *P* values for exact test (Raymond and Rousset, 1995; below diagonal) for samples of *Babina holsti*. Samples with N < 3 were omitted. *: differentiation is significant ($P < 0.05$).

	1: Oku1	2: Oku2	3: Nishimei	4: Benoki	5: Okuni	7: Yonaha	8: Nuha	10: Tokashiki
1: Oku1		0.3393*	0.1079	0.0675	0.0625	0.1329	0.7740*	0.9720*
2: Oku2	0.014 ± 0.001*		0.1629*	0.1233*	0.2063	0.2138*	0.3277*	0.9314*
3: Nishimei	0.231 ± 0.006	0.013 ± 0.001*		–0.0095	–0.0006	0.0509	0.2067*	0.9067*
4: Benoki	0.013 ± 0.001*	0.070 ± 0.003	0.147 ± 0.004		–0.0204	0.021	0.1908*	0.9056*
5: Okuni	0.085 ± 0.003	0.027 ± 0.002*	0.028 ± 0.003*	0.034 ± 0.002*		0.0331	0.6002*	0.9694*
7: Yonaha	0.393 ± 0.005	0.103 ± 0.004	0.049 ± 0.003*	0.063 ± 0.004	0.030 ± 0.001*		0.4796*	0.9656*
8: Nuha	0.000 ± 0.000*	0.000 ± 0.000*	0.000 ± 0.000*	0.000 ± 0.000*	0.001 ± 0.000*	0.000 ± 0.000*		0.9910*
10: Tokashiki	0.000 ± 0.000*	0.000 ± 0.000*	0.000 ± 0.000*	0.000 ± 0.000*	0.001 ± 0.000*	0.000 ± 0.000*	0.000 ± 0.000*	

Tokashikijima and Okinawajima in the last glacial age. In contrast, a high genetic differentiation between the Tokashikijima and Okinawajima lineages of *B. holsti* indicates that there was no migration between Okinawajima and Tokashikijima lineages of this species when the two islands were connected by a land bridge in the last glacial age, not favoring our second hypothesis. The fact that these two lineages are allopatrically distributed with each other and do not coexist on both islands indicates that the third hypothesis is not supported.

However, other considerations are necessary. Fossils of *B. holsti* have been detected from the layer approximately 32,000–20,000 YA from the southern part of Okinawajima (Hasegawa, 1980; Nakamura and Ota, 2009). This region is thought to have been submerged in the middle Pleistocene (ca. 0.85 MYA) and to have emerged in the early late Pleistocene (ca. 0.41 MYA) (Iryu et al., 2006). Thus, the population extinguished in the southern part of Okinawajima must have migrated to there from the northern part of this Island and/or Tokashikijima Island after 0.41 MYA. The possibility that the migration from Tokashikijima through land formation in the glacial ages is not precluded. Honda et al. (2012) surveyed the genetic differentiation of *Echinotriton andersoni* and showed that the samples from the southern part of Okinawajima are closer to Tokashikijima samples than to the northern Okinawajima samples. Similarly, in *Microhyla okinavensis*, individuals from southern Okinawajima are reported to be closer to individuals from Kumejima Island, which is located in the west off Okinawajima Island, than to individuals from northern Okinawajima (Matsui et al., 2005). These studies indicate that several amphibians in the southern part of Okinawajima have migrated between western small islets rather than northern Okinawajima in the late Pleistocene glacial age. Thus, the possibility remains that the extinct population of *B. holsti* in the southern part of Okinawajima is related to the present Tokashikijima population (represented by sample 11). To determine whether this is the case, detailed morphological comparison of fossil and extant specimens as well as the development of fossil DNA analysis is required.

Genetic diversity within samples

In the present study, two southern samples (samples 8 and 9) showed a low genetic diversity within Okinawajima. The southernmost sample (sample 8) contained only one haplotype, which is unique. The sample 8 showed significant differentiations from samples from northern range on the Okinawajima. These results indicate samples 8 and 9 belong to a same population (hereafter referred as the southern population), that were diverged from the northern population containing samples 1–7. A distributional survey of *B. holsti* conducted by Okinawa Prefecture (2013) revealed that the southern population (represented by samples 8 and 9) is largely isolated from the northern population (represented by samples 1–7) (Fig. 4). The existence of a unique haplotype in the southern samples without considerable genetic distance from haplotypes from the northern samples indicates that the gene flow between the southern and northern population on Okinawajima Island was originally restricted. Furthermore, the absence of genetic diversity within the southern samples indicates that the southern population has recently experienced population size reduction by the degradation of intervening habitats and possible predation by invasive predators, such as mongoose.

Because the exotic mammal mongoose (*Herpestes auropunctatus*) preys upon small vertebrates, including *B. holsti*, and is more abundant in the southern range than in the northern range of this frog in Okinawajima (Ministry of the Environment Government of Japan, 2012), it is likely that predation pressures by the mongoose have accelerated further decreasing genetic diversity in the southern population of the island, which would already have little chance of gene flow between the northern population.

Several alternative environmental variables such as human population density, forest cover, precipitation levels,

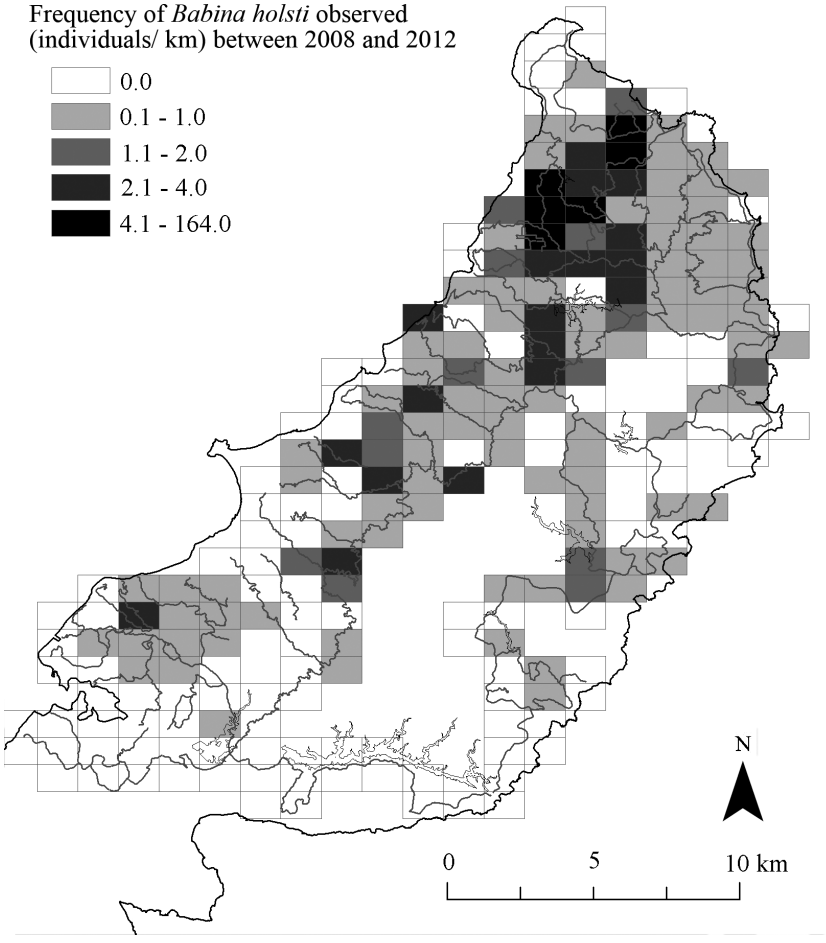

Fig. 4. Estimated distributional range and frequency of *Babina holsti* observed. Size of mesh is set as 1.15 km^2 (latitudinal width: 1249 m × longitudinal width: 923 m). Data modified from Okinawa Prefecture (2013).

breeding site availability, and road density may also affect the distribution and density of *B. holsti*. However, none of the alternative variables apear to differ significantly between the current southern and northern distributional areas of this frog on Okinawajima. For strict assessment of the effects of these variables, including the presence/absence of mongoose, on the distribution and density of *B. holsti*, statistical analyses with respect to the relationships between them are required.

These results also showed a lower genetic diversity in the Tokashikijima sample (sample 10). Although further investigations are required for detailed evaluation of the genetic diversity and demography of *B. holsti* on this island, as tissues could only be collected from one locality, our field observation indicates lower density of this species on this island than on Okinawajima. Furthermore, the range of this species on this island is much smaller than that on Okinawajima; thus, the current low genetic diversity in the sample may have resulted from a past or current small population size.

Conservation of *Babina holsti*

The distributional range of *B. holsti* in Okinawajima has become narrower in this half century because of habitat destruction and possibly by the effect of predation by invasive species. Seventy years ago, this frog was found in two or three times larger areas than the present range (Toyama, 1995). Distributional assessment conducted by Okinawa Prefecture (2013) and the Ministry of the Environment Government of Japan (2012) indicates that the current distribution of *B. holsti* is fragmented, and this fragmentation appears to have affected the current genetic geographic pattern. Because our data are based on limited samples and single genes, further analyses are required to elucidate the detailed genetic structure of this species. However, because *F*st values between the southernmost sample and more northern samples are relatively high, it is possible that this species has low migration ability where its habitat is fragmented. For conservation of the southern population, recovery of the suitable habitat and eradication of mongoose at the range separated from the northern populations are necessary to maintain the metapopulations.

A moderate (3.1% in *p*-distance) genetic differentiation between the two island lineages suggests that they are independent ESUs (Ryder, 1986) that should be treated as different conservation units. The Tokashikijima lineage showed distinct and uniform genetic properties, indicating a long, independent divergence history on the island, which has only a small population capacity. Thus, a more intensive study and conservation measures of this island population are urgently required.

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