An Investigation for Population Maintenance Mechanism in a Miniature Garden: Genetic Connectivity or Independence of Small Islet Populations of the Ryukyu Five-Lined Skink

Kazuki Kurita, Tsutomu Hikida, and Mamoru Toda

From the Department of Zoology, Graduate School of Science, Kyoto University, Sakyo, Kyoto, 606-8502, Japan (Kurita and Hikida) and Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan (Toda).

Address correspondence to Kazuki Kurita at the address above, or e-mail: kurita@zoo.zool.kyoto-u.ac.jp.

Abstract

The Ryukyu five-lined skink (Plestiodon marginatus) is an island lizard that is even found in tiny islets with less than half a hectare of habitat area. We hypothesized that the island populations are maintained under frequent gene flow among the islands or independent of each other. To test our hypotheses, we investigated genetic structure of 21 populations from 11 land-bridge islands that were connected during the latest glacial age, and 4 isolated islands. Analyses using mitochondrial cytochrome b gene sequence (n = 67) and 10 microsatellite loci (n = 235) revealed moderate to high levels of genetic differentiation, existence of many private alleles/haplotypes in most islands, little contemporary migration, a positive correlation between genetic variability and island area, and a negative correlation between relatedness and island area. These evidences suggest a strong effect of independent genetic drift as opposed to gene flow, favoring the isolation hypothesis even in tiny islet populations. Isolation-by-distance effect was demonstrated and it became more prominent when the 4 isolated islands were excluded, suggesting that the pattern is a remnant of the land-bridge age. In a few island populations, however, the possibility of occasional overwater dispersals was partially supported and therefore could not be ruled out.

Subject areas: Population structure and phylogeography

Key words: genetic structure, islands, microsatellite, mitochondrial DNA, Plestiodon marginatus

Small populations are prone to extinction because of low levels of genetic variation to adapt to environmental changes (Frankham et al. 2002). For instance, nonvolant terrestrial animals found on small islands have a higher risk of extinction because the surrounding sea prevents them from migrating between islands and they can rarely gain novel genetic variation, except for mutation within their own population. Indeed, Frankham (1997) noted that the majority of recorded extinctions after 1600 are of insular forms. Lizards are one of the nonvolant terrestrial animals that also inhabit fairly small islands (e.g., Perry et al. 1998; Pérez-Mellado et al. 2008). Although many attempts have been made to clarify the genetic characteristics of island lizards from historical biogeographic or conservation viewpoints (e.g., Malone et al. 2003; Tzika et al. 2008; Terrasa et al. 2009; Runemark et al. 2012; Rodríguez et al. 2013), studies focusing on population maintenance mechanisms are relatively rare.

The Ryukyu five-lined skink (Plestiodon marginatus) is an island lizard endemic to the Okinawa Group and a few islands in the Amami and Tokara Groups of the Ryukyu Archipelago, Japan, a continental island chain lying between the main islands of Japan and Taiwan. The occurrence of this endemic skink is considered as a consequence of vicariant divergence involved in the geographic isolation of the Ryukyu Archipelago from the continent (Kurita and Hikida 2014). This species is able to inhabit numerous regions including even tiny islets such as Komakajiam Island (0.02 km²) and Ejinajima Island (0.01 km²; Iwanaga et al. 2008; Kurita and Kadota 2010), where the
virtual habitat areas covered by vegetation are no more than half of a football pitch. This has raised questions about how *P. marginatus* has maintained itself in such a small islet.

To explain the maintenance mechanism of insular populations of *P. marginatus* in the Ryukyus, we present 2 hypotheses focusing on the 2 major aspects in the population genetics, gene flow and genetic drift. One possibility, based on the gene flow, is that the islet populations may have been maintained by rescue effects (rescue hypothesis; Iwana et al. 2008). The rescue effect is the basic feature of metapopulation theory, in which immigration of conspecifics compensates for severe population decline or loss of genetic variability in small populations, preventing extinction (Lomolino et al. 2006). This may be the case for *P. marginatus* because some empirical evidences showed that this skink has undergone long-distance overwater dispersal (Honda et al. 2008; Kurita and Hikida 2014). In addition, this skink is abundant in open habitats near the coast of many islands (Toyama 1979; Kurita and Kadota 2010) and the Ryukyu Archipelago has experienced tsunamis and typhoons. These circumstances suggest that the skink has occasionally been washed away offshore, enhancing the opportunity of overwater dispersals among nearby islands.

The other possibility is that the islet populations of this skink have essentially persisted as an isolated population since the islands separated (isolation hypothesis). Most of small islands in the Ryukyus were connected to larger islands and each other during the last glacial period at the end of the Pleistocene (approximately 18,000 years ago; Japan Association for Quaternary Research 1987). Thus, gene flow among the island populations has been interrupted after this period, and there have been no overwater dispersals, resulting in their survivals in situ over a period of thousands of years.

Here, we investigate genetic structure of island populations of this species on the basis of variations in mitochondrial DNA (mtDNA) and microsatellite markers to assess the above 2 hypotheses. The rescue hypothesis predicts that gene flow rather than genetic drift would vastly affect neutral genetic variation between island populations, resulting in the lower and homogenized genetic differentiation because of frequent gene exchanges among island populations. In particular, degree of genetic differentiation would increase with geographic distance among populations under the circumstance where gene flow is most likely to occur between neighboring populations (Hutchison and Templeton 1999). On the other hand, the isolation hypothesis predicts that genetic drift would overwhelm gene flow in affecting the neutral genetic variation, resulting in higher genetic differentiation among island populations despite their geographic proximities (Hutchison and Templeton 1999) and higher genetic variability in larger island (Jordan and Snell 2008).

We assumed individuals from an island to represent a single population except for those of Okinawajima Island, which is extremely large in area (1192 km²). Within Okinawajima Island, we collected the lizards from 7 separate localities (Oku, Yona, Ada, Bise, Nago, Naha, and Nashiro) and treated them as separate populations. We classified these 15 islands into 2 groups on the basis of the late Pleistocene geohistory (approximately 18,000 years ago; Japan Association for Quaternary Research 1987), where each coastline is assumed to correspond with current 100-m depth contour derived from sea level drop during continental glaciation: 1) the isolated islands which have been persisted as independent islands even during the latest glacial maxima (LGM; i.e., Okinoerabujima, Yoronjima, Izenajima, Agunijima, and Kumejima Islands) and 2) the land-bridge islands which were connected to the Okinawajima Island when the sea level was dropped (the remaining 10 islands). We used 67 specimens (3-7 individuals per locality or populations) representing all 15 islands for the mtDNA analysis and all (235) specimens for the microsatellite analysis. These specimens are deposited in the Zoological Collection of the Kyoto University Museum as vouchers. Genomic DNA was extracted from liver tissue, which was taken from each specimen and stored at −80 °C or in 99% ethanol, following the methods provided by Okamoto et al. (2006) with slight modifications.

### Materials and Methods

#### Study Sites and Samples Examined and DNA Extraction

A total of 235 individuals collected from 15 islands of various sizes in the Okinawa and Amami Groups were used in this study (Figure 1; Table 1; Supplementary Table S1 online).
of MrBayes 3.2.1 (Altekar et al. 2004; Ronquist et al. 2012) with the model (K80+I, HKY85+I, HKY85+G for the first, second, and third codon positions, respectively) selected using KAKUSAN4 under AICc. Two independent runs of 4 Markov chains were performed for 3,000,000 generations sampling a tree every 100 generations, producing 30,001 trees. After checking adequacy of the parameter estimates and convergence using Tracer 1.5 (Rambaut and Drummond 2007) for each run, we discarded the first 10,001 trees as burn-in, leaving 20,000 trees and calculated a consensus topology and Bayesian posterior probabilities. We regarded tree topologies with nodal support in the bootstrap values of 70% or greater as sufficiently resolved for ML analysis (Hillis and Bull 1993) and in the Bayesian posterior probability values of 95% or greater as significant support for BI analysis (Leaché and Reeder 2002; Huelsenbeck and Rannala 2004).

Microsatellite Genotyping and Analysis

We determined the genotypes of 235 lizards for 10 nuclear microsatellite loci. Of these 10 loci, 7 (Pki16-7, Pki17-1, Pki17-3, Pki17-5, Pki25-8, PlemaL53, and PlemaT68) and 2 (Eufa1 and Eufa15) were taken from Kurita et al. (2013) and Howes et al. (2004), respectively. The remaining one (PlemaL12) was newly designed from a sequence isolated from Kurita et al.’s (2013) genomic library (GenBank accession number: AB856581). For the former 7 loci, we performed polymerase chain reaction (PCR) amplifications following the method provided by Kurita et al. (2013) and classified their alleles into bins using MsatAllele 1.03 (Alberto 2009). In the latter 3 loci, PCR amplification was performed using the M13(-21) fluorescent-labeling protocol (Schuelke 2000) with the same primer pairs of Howes et al. (2004) for Eufa1 and Eufa15, and forward (5’-TCCCCACCTTAGCCATGAAA-3’) and reverse (5’-GATGCAACCACAATGCAGTC-3’) primers for PlemaL12. PCR was carried out in 10-μL reaction mixtures containing 7.0–145.8 ng of DNA template, 10× Ex Taq buffer, 0.2 mM of each dNTP, 0.025 μM of 5’-end-labeled locus-specific forward primer, 0.1 μM of locus-specific reverse primer, 0.1 μM of 5’-end-labeled primer with an Applied Biosystems dye (FAM, HEX, or NED), and 0.2 U of TaKaRa Ex Taq polymerase (TaKaRa BIO, Otsu, Japan). PCR amplifications was performed with GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA) or MyCycler (Bio-Rad Laboratories, Hercules, CA) under the PCR condition of Schuelke (2000) except for the number of cycles (35

Figure 1. Maps of (a) East Asia and (b) the islands of the Okinawa Group and the southern part of the Amami Group showing sampling sites of Plestiodon marginatus examined in this study. Numbers in black squares (5–11) indicate localities in Okinawajima Island. Numbers denoted by open squares indicate isolated islands (see text for the definition). All sites’ numbers correspond with those of Table 1 and Supplementary Table S1 online.
Table 1  Geographic and genetic characteristics of 21 populations of *Ple thrust* *marginitus* from the Okinawa and the southern Amami Groups examined in this study

<table>
<thead>
<tr>
<th>Population</th>
<th>Island group</th>
<th>Area (km²)</th>
<th>N</th>
<th>A</th>
<th>Ar1</th>
<th>Ar2</th>
<th>E</th>
<th>pAr1</th>
<th>pAr2</th>
<th>H_O</th>
<th>H_E</th>
<th>F_S</th>
<th>M</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Okinoerabujima</td>
<td>I</td>
<td>93.62</td>
<td>9</td>
<td>5.2</td>
<td>3.4</td>
<td>3.9</td>
<td>5</td>
<td>0.56</td>
<td>0.66</td>
<td>0.49</td>
<td>0.51</td>
<td>0.04</td>
<td>0.38</td>
<td>0.42</td>
</tr>
<tr>
<td>2. Yoronjima</td>
<td>I</td>
<td>20.46</td>
<td>17</td>
<td>9.3</td>
<td>4.1</td>
<td>4.7</td>
<td>15</td>
<td>0.82</td>
<td>1.08</td>
<td>0.55</td>
<td>0.57</td>
<td>0.03</td>
<td>0.39</td>
<td>0.31</td>
</tr>
<tr>
<td>3. Izennajima</td>
<td>I</td>
<td>14.02</td>
<td>11</td>
<td>4.9</td>
<td>3.3</td>
<td>3.7</td>
<td>11</td>
<td>0.76</td>
<td>0.85</td>
<td>0.47</td>
<td>0.52</td>
<td>0.09</td>
<td>0.26</td>
<td>0.52</td>
</tr>
<tr>
<td>4. Iejima</td>
<td>L</td>
<td>22.66</td>
<td>7</td>
<td>4.9</td>
<td>3.7</td>
<td>4.2</td>
<td>4</td>
<td>0.43</td>
<td>0.72</td>
<td>0.57</td>
<td>0.57</td>
<td>0.01</td>
<td>0.30</td>
<td>0.33</td>
</tr>
<tr>
<td>Okinawajima</td>
<td>L</td>
<td>1192</td>
<td>91</td>
<td>11.4</td>
<td>4.4</td>
<td>9</td>
<td>0.36</td>
<td>0.43</td>
<td>0.59</td>
<td>0.28</td>
<td>0.44</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Oku</td>
<td>—</td>
<td>16</td>
<td>4.2</td>
<td>2.6</td>
<td>0</td>
<td>2</td>
<td>0.16</td>
<td></td>
<td>0.40</td>
<td>0.41</td>
<td>0.03</td>
<td>0.31</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>6. Yona</td>
<td>—</td>
<td>27</td>
<td>4.1</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>0.19</td>
<td></td>
<td>0.43</td>
<td>0.42</td>
<td>0.02</td>
<td>0.43</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>7. Ada</td>
<td>—</td>
<td>13</td>
<td>4.4</td>
<td>2.9</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
<td></td>
<td>0.39</td>
<td>0.40</td>
<td>0.01</td>
<td>0.35</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>8. Bise</td>
<td>—</td>
<td>6</td>
<td>3.6</td>
<td>3.0</td>
<td>0</td>
<td>4</td>
<td>0.44</td>
<td></td>
<td>0.47</td>
<td>0.47</td>
<td>0.01</td>
<td>0.13</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>9. Nago</td>
<td>—</td>
<td>16</td>
<td>3.9</td>
<td>3.0</td>
<td>0</td>
<td>1</td>
<td>0.17</td>
<td></td>
<td>0.48</td>
<td>0.51</td>
<td>0.06</td>
<td>0.31</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>10. Naha</td>
<td>—</td>
<td>9</td>
<td>4.8</td>
<td>3.3</td>
<td>0</td>
<td>0</td>
<td>0.13</td>
<td></td>
<td>0.50</td>
<td>0.53</td>
<td>0.07</td>
<td>0.27</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>11. Nashiro</td>
<td>—</td>
<td>1</td>
<td>1.7</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td></td>
<td>0.30</td>
<td>0.30</td>
<td>0.00</td>
<td>0.21</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>12. Adagashima</td>
<td>L</td>
<td>0.08</td>
<td>7</td>
<td>3.0</td>
<td>2.4</td>
<td>2.6</td>
<td>0</td>
<td>0.003</td>
<td>0.01</td>
<td>0.33</td>
<td>0.33</td>
<td>0.02</td>
<td>0.29</td>
<td>0.64</td>
</tr>
<tr>
<td>13. Yagajishima</td>
<td>L</td>
<td>7.76</td>
<td>6</td>
<td>3.0</td>
<td>2.6</td>
<td>2.8</td>
<td>1</td>
<td>0.22</td>
<td>0.31</td>
<td>0.50</td>
<td>0.46</td>
<td>0.09</td>
<td>0.29</td>
<td>0.50</td>
</tr>
<tr>
<td>14. Ieikijima</td>
<td>L</td>
<td>1.75</td>
<td>14</td>
<td>4.7</td>
<td>2.9</td>
<td>3.1</td>
<td>3</td>
<td>0.30</td>
<td>0.38</td>
<td>0.46</td>
<td>0.48</td>
<td>0.05</td>
<td>0.30</td>
<td>0.43</td>
</tr>
<tr>
<td>15. Kudakajima</td>
<td>L</td>
<td>1.37</td>
<td>9</td>
<td>4.1</td>
<td>2.9</td>
<td>3.3</td>
<td>3</td>
<td>0.31</td>
<td>0.36</td>
<td>0.48</td>
<td>0.44</td>
<td>0.08</td>
<td>0.19</td>
<td>0.58</td>
</tr>
<tr>
<td>16. Komakajima</td>
<td>L</td>
<td>0.02</td>
<td>9</td>
<td>2.3</td>
<td>2.0</td>
<td>2.1</td>
<td>0</td>
<td>0.0001</td>
<td>0.07</td>
<td>0.36</td>
<td>0.30</td>
<td>0.20</td>
<td>0.14</td>
<td>0.76</td>
</tr>
<tr>
<td>17. Senagajima</td>
<td>L</td>
<td>0.21</td>
<td>5</td>
<td>2.8</td>
<td>2.6</td>
<td>2.8</td>
<td>0</td>
<td>0.16</td>
<td>0.17</td>
<td>0.36</td>
<td>0.43</td>
<td>0.19</td>
<td>0.14</td>
<td>0.51</td>
</tr>
<tr>
<td>18. Ejinajima</td>
<td>L</td>
<td>0.01</td>
<td>11</td>
<td>2.2</td>
<td>1.9</td>
<td>2.0</td>
<td>0</td>
<td>0.04</td>
<td>0.04</td>
<td>0.27</td>
<td>0.28</td>
<td>0.03</td>
<td>0.16</td>
<td>0.74</td>
</tr>
<tr>
<td>19. Tokashikijima</td>
<td>L</td>
<td>15.29</td>
<td>13</td>
<td>5.1</td>
<td>3.3</td>
<td>3.6</td>
<td>9</td>
<td>0.70</td>
<td>0.84</td>
<td>0.49</td>
<td>0.52</td>
<td>0.06</td>
<td>0.22</td>
<td>0.40</td>
</tr>
<tr>
<td>20. Agunajima</td>
<td>L</td>
<td>7.62</td>
<td>20</td>
<td>6.3</td>
<td>3.4</td>
<td>3.8</td>
<td>1</td>
<td>0.21</td>
<td>0.26</td>
<td>0.50</td>
<td>0.51</td>
<td>0.02</td>
<td>0.33</td>
<td>0.42</td>
</tr>
<tr>
<td>21. Kumejima</td>
<td>L</td>
<td>58.38</td>
<td>6</td>
<td>3.9</td>
<td>3.2</td>
<td>3.5</td>
<td>4</td>
<td>0.39</td>
<td>0.43</td>
<td>0.52</td>
<td>0.51</td>
<td>0.01</td>
<td>0.34</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Population numbers correspond with those of Figure 1. The values of “Okinawajima” were calculated for the pooled sample of all populations (5–11) in Okinawajima Island. N, number of samples; A, mean number of alleles; Ar1, allelic richness expected for 4 individuals (i.e., the sample size of the Nashiro population); Ar2, allelic richness expected for 5 individuals (i.e., the sample size of the Senagajima population); E, number of alleles private to the population; pAr1, private allelic richness expected for 4 individuals; pAr2, private allelic richness expected for 5 individuals; H_o, observed heterozygosities; H_e, unbiased expected heterozygosities; F_IS fixation indexes; M, bottleneck statistics of Garza and Williamson (2001); r, coefficient of relatedness; I, isolated islands; L, land-bridge islands (see text in detail). *P < 0.05.

cycles) and annealing temperature (62 °C) at the first cycle. One microliter of PCR products were mixed with 0.3 μL of size standard GeneScan-500 ROX (Applied Biosystems) and 10 μL of Hi-Di formamide, and then this mixture was electrophoresed on an Applied Biosystems 3100 Genetic Analyzer (Applied Biosystems) or Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems). The size of the fragments was analyzed and classified into bins using GeneMapper software 4.0 (Applied Biosystems) and the Peak Scanner Software 1.0 (Applied Biosystems). We performed the allele binning after correction of the size differences observed between outputs of the 2 kinds of sequencers employed for a series of same samples. In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary microsatellite data with Dryad.

The deviations from Hardy–Weinberg equilibrium (HWE) for each locus in each population were tested using GENEPOP 4.1.4 (Rousset 2008) on the basis of an exact test (Guo and Thompson 1992). Linkage disequilibrium for each locus pair in each population was also tested by GENEPOP using a log-likelihood ratio test. The presence of scoring errors, large allele dropout, and null alleles were checked using Micro-Checker 2.2.3 (Van Oosterhout et al. 2004). Mean number of alleles (A), the number of alleles private to the population (E), observed heterozygosity (H_o), and unbiased expected heterozygosity (H_e; Nei and Roychoudhury 1974) were calculated using GenAlEx 6.41 (Peakall and Smouse 2006) in order to evaluate the genetic diversity within the population. To allow for sample size variation, allelic richness (Ar) and private allelic richness (pAr) expected for the smallest sample size across populations were computed using ADZE 1.0 (Szpiech et al. 2008). Inbreeding coefficient (F_IS) was calculated and the significance of these values was tested using FSTAT 2.9.3.2 (Goudet 1995). Significance level to test of deviation from HWE, linkage disequilibrium, and F_IS was adjusted using the sequential Bonferroni procedure to take into account multiple comparisons (Rice 1989). We computed the bottleneck statistic (M; Garza and Williamson 2001) modified by Excoffier et al. (2005) using Arlequin 3.5.1.2 (Excoffier and Lischer 2010) to detect population bottlenecks. In this calculation, we used 4 perfect repeat loci (Pki17-3, Pki17-5, Pki25-8, and PlemaT68) following Garza and Williamson’s (2001) recommendation. We also estimated the coefficient of relatedness (r; Queller and Goodnight 1989) within populations using GenAlEx.
In order to estimate population differentiation, \( F_{ST} \) (Weir and Cockerham 1984) over all loci was calculated and its significance was tested by permuting alleles among populations with 10,000 randomizations using FSTAT. Pairwise \( F_{ST} \) between populations were also calculated and tested using the same program. The \( P \) values obtained for the significance test of pairwise \( F_{ST} \) were adjusted using the sequential Bonferroni procedure (Rice 1989). We assessed the statistical power of our microsatellite data set and sample size for detecting genetic differentiation using POWSIM 4.1 (Ryman and Palm 2006). Simulation was carried out with a constant condition (1000 dememorizations, 100 batches, 1000 iterations per batches, effective population size \([N_e] = 2000\), and 200 runs) except for number of generations (\( t \)). Principle coordinate analysis (PCoA) was performed on the basis of the pairwise \( F_{ST} \) values using GenAlEx to visualize their relationships. In addition to these analyses, we used STRUCTURE 2.3.4 (Pritchard et al. 2000) to assign individuals to genetically subdivided clusters and infer the number of such clusters (\( K \)) without predefined populations. We assumed an admixture model with correlated allele frequencies and performed 10 independent runs for each value of \( K \) (ranging from 1 to 21) with \( 1 \times 10^5 \) Markov chain Monte Carlo (MCMC) iteration after burn-in period of \( 2 \times 10^5 \) iteration. The value of \( K \) was accepted with the highest average “log probability of data,” and that of the membership coefficients for each individual (\( Q \)) was selected with the highest “log probability of data” of 10 runs in the accepted \( K \) value and visualized by DISTURCT (Rosenberg 2004).

We performed Mantel tests (Mantel 1967) to investigate isolation by distance among populations for the 3 datasets as follows: 1) all 21 populations from the 15 islands, 2) 16 populations from the 10 land-bridge islands, and 3) 7 local populations within Okinawajima Island. Geographic distances between sampling sites or between islands was measured as minimum distance at the closest point. In this analysis, genetic distances (\( F_{ST} \)) were used as the response variable and geographic distances (km) were used as the explanatory variable. It has been pointed out that tests for isolation-by-distance effect are biased by the presence of a hierarchical structure (Meirmans 2012). Therefore, we carried out a partial Mantel test (Smouse et al. 1986) to quantify the correlation between \( F_{ST} \) and geographic distance in consideration of population structure. We generated a categorical matrix of population assignment to the same (= 0) or different (= 1) group on the basis of the results of mtDNA and microsatellite data (see results). The Mantel and partial Mantel tests were performed using ecodist 1.2.7 package (Goslee and Urban 2007) in R. Pearson correlation coefficients (\( r \)) were calculated, and the statistical significance was assessed with 10,000 permutations. For comparison among the \( r \) at different dataset, 95% confidence intervals were estimated using 1000 bootstrap iterations that resampled 90% of the data.

We used BayesAss 3 (Wilson and Rannala 2003) to estimate recent migration among populations, in which we assumed <5 generations (i.e., approximately <10–20 years), given a generation time of 2–4 years for this group of the skink (Hikida 1981; Hasegawa 1994). We ran the program with \( 1 \times 10^8 \) MCMC iterations and sampled every 2000 steps. The delta value for migration rates, allele frequencies, and inbreeding coefficients were set to 0.60, 0.60, and 0.70, respectively, to ensure that acceptance rates for proposed changes to these parameters fell between 20% and 60% as indicated in the software manual (Rannala 2007). The first \( 4 \times 10^7 \) iterations were discarded as a burn-in after the check of the convergence for each parameter using Tracer.

**Results**

**mtDNA Variation**

We detected 31 haplotypes from 67 individuals of *P. marginalis* in the Okinawa and southern Amami Groups. Most haplotypes (26/31) were endemic to single locality except for haplotypes N7 (shared by Izenajima Island [site 3 in Figure 1] and Bise [site 8]), N9 (shared by Iejima Island [site 4], Bise, and Nago [site 9]), N10 (shared by Oku [site 5], Ada [site 7], and Adagashima Island [site 12]), N13 (shared by Ada and Adagashima Island), and S7 (shared by Kudakajima [site 15] and Komakajima [site 16] Islands). The sequence divergence between populations, which is expressed as mean uncorrected \( p \)-distance, ranged from 0.049 to 0.019, while that within populations ranged from 0.019 to 0.006.

ML (In \( L = -2458.06 \)) and BI (In \( L = -2532.57 \)) analyses yielded almost identical topologies. We thus provide the ML tree in Figure 2. The phylogenetic analyses showed the monophyly of *P. marginalis* from the Okinawa and southern Amami Groups (90%/1.0 = ML bootstrap value/Bayesian posterior probability). The ingroup samples were divided into 2 clades, Clade N (100%/1.0) and Clade S (83%/0.64). Clade N consisted of the haplotypes from the southern Amami Group and the northeastern part of the Okinawa Group including the northern part of Okinawajima Island. This clade further bifurcated into 2 subclades, N-I (98%/1.0) and N-II (86%/0.85). Subclade N-I comprised the haplotypes from 2 islands isolated during LGM: Okinoearabujima (site 1) and Yoronjima (site 2) Islands. Subclade N-II comprised the haplotypes from 5 localities (Oku, Yona [site 6], Ada, Bise, and Nago) in Okinawajima Island, its nearby islands (Iejima, Adagashima, and Yagajishima [site 13] Islands), and an isolated islands, Izenajima Island. Clade S consisted of the haplotypes from southeastern and southwestern parts of the Okinawa Group including the southern part of Okinawajima Island. Clade S was further divided into 2 subclades, S-I (82%/0.64) and S-II (74%/0.99). Subclade S-I consisted of the haplotypes from 2 localities (Naha [site 10] and Nashiro [site 11]) in Okinawajima Island, and 5 nearby islands (Ikeijima [site 14], Kudakajima, Komakajima, Senagajima [site 17], Ejinajima [site 18] Islands), and Tokashikijima [site 19] Islands. Subclade S-II consisted of the haplotypes from 2 islands that were isolated during LGM: Agunijima (site 20) and Kumejima (site 21) Islands.

**Microsatellite Variation**

We detected a total of 204 alleles across the 10 loci in the sample of 235 individuals from the 21 populations of...
The number of observed alleles in each locus ranged from 3 (at Pki16-7) to 47 (at Pki17-1). The deviation from HWE was not detected in 21 populations at 10 loci except for Pki17-5 in Tokashikijima Island and Pki25-8 in Ikeijima Island. The linkage disequilibrium was not detected between any pairs of loci in each population. Signs of scoring errors and large allele dropouts were not suggested at all loci in each population. Evidence of null alleles was suggested at Eufa15 in Adagashima Island, and Eufa1 and Pki17-5 in Tokashikijima Island, but all loci were retained for further analyses because these trends were not seen in any other populations.

**Genetic Diversity and Demographic History**

Genetic variability statistics of 21 populations are shown in Table 1. Mean number of alleles (\(\bar{A}\)) ranged from 1.7 (Nashiro in Okinawajima Island) to 9.3 (Yoronjima Island). Allelic richness expected for 4 individuals (Ar1) ranged from 0.0001 (Komakajima Island) to 0.82 (Yoronjima Island). \(H_O\) and \(H_E\) ranged from 0.27 (Ejinajima Island) to 0.57 (Yoronjima Island), and 0.28 (Ejinajima Island) to 0.57 (Yoronjima and Iejima Islands), respectively. \(F_{IS}\) ranged from −0.20 (Komakajima Island) to 0.19 (Senagajima Island), and it was not significantly different from zero in all populations. When all 7 local samples from Okinawajima Island were pooled, \(F_{IS}\) for the whole Okinawajima Island (0.28) was significantly larger than zero, indicating apparent population subdivisions. \(M\) ranged from 0.13 (Bise in Okinawajima Island) to 0.43 (Yona in Okinawajima Island). \(r\) ranged from 0.31 (Yoronjima Island) to 0.76 (Komakajima Island).

There were positive correlations between island area and \(Ar2\) (\(F_{1,13} = 42.81, R^2 = 0.77, P < 0.001\)), \(pAr2\) (\(F_{1,13} = 9.12, R^2 = 0.41, P = 0.000\)), or \(H_E\) (\(F_{1,13} = 86.51, R^2 = 0.87, P < 0.001\)), indicating that populations in bigger islands possess larger genetic variations and higher genetic uniqueness. On the other hand, there was a negative correlation between island area and \(r\) (\(F_{1,13} = 54.02, R^2 = 0.81, P < 0.001\)), indicating that populations in smaller islands exhibit high
relatedness. There was positive correlation between island area and $M$ ($F_{1,13} = 25.38, R^2 = 0.66, P < 0.001$), indicating that population in smaller islands are more likely to have experienced a serious population decline because the $M$ statistic would decrease with increasing severity and duration of population shrinking (Garza and Williamson 2001).

**Population Structure**

$F_{ST}$ value for all populations examined over all loci was 0.34 and statistically significant ($P < 0.0001$). The pairwise $F_{ST}$ values ranged from 0.07 (between Okinoerabujima and Yoronjima Islands) to 0.60 (between Adagashima and Ejinajima Islands), all of which were significantly greater than zero (Supplementary Table S2 online). The statistical power analysis using POWSIM showed that our microsatellite dataset have sufficient statistical power to detect $F_{ST}$ values $> 0.01$ with a probability of 100%. The plot of the pairwise $F_{ST}$ values in a PCoA showed separation between northern and southern populations, which roughly corresponded to the Clade N and Clade S of mtDNA phylogeny (Figure 3). However, a few populations belonging to Clade N (Izenajima [site 3 in Figure 1] and Iejima [site 4] Islands, and Bise in Okinawajima Island [site 8]) were close to the populations belonging to Clade S in the mtDNA tree. High genetic differentiation between populations was also indicated in the result of the STRUCTURE analysis (Figure 4). Eighteen genetic clusters were indicated as the most likely number of genetic clusters, and each corresponded to population in most case. However, some nearby populations (Iejima Island and Bise, Ada [site 7] in Okinawajima Island and Adagashima Island [site 12], and Kumejima [site 15] and Komakajima [site 16] Islands) belonged to the same genetic clusters.

The Mantel test showed significant positive correlation between $F_{ST}$ and geographic distance in the all 3 datasets (Figure 5 and Table 2). Such a significant correlation was also detected in the partial Mantel test that takes into account population structure for all populations (dataset 1: $r = 0.13, P = 0.0451$) and populations exclusive of isolated islands (dataset 2: $r = 0.36, P = 0.0003$), but not significant for populations within Okinawajima Island (dataset 3: $r = 0.39, P = 0.0651$). With respect to the former 2 cases, the correlation coefficient of dataset 2 was significantly larger than that of dataset 1.

**Estimating Gene Flow**

BayesAss analysis showed that 95% credible intervals of estimated recent migration overlapped with zero for almost all pairs of populations, indicating little or no recent migration among populations (Supplementary Table S3 online). Exceptionally, moderate recent migrations from Yoronjima Island to Okinoerabujima Island and from Komakajima Island to Kudakajima Island were indicated (mean migration rate: 0.11 in both), but the lower 95% credible limits of migration rates fell below 0.10.

**Discussion**

Our phylogenetic analyses clearly showed that there are 2 deeply divergent mitochondrial lineages in *P. marginatus* (Clades N and S in Figure 2; mean uncorrected $p$-distance between them was 0.044) and this grouping was largely supported by the microsatellite analysis (Figure 3). From their geographic pattern and degree of divergence, these 2 lineages are considered to correspond to those revealed by a previous study (Kurita and Hikida 2014). Kurita and Hikida (2014) estimated divergence time between the 2 lineages at some time during the Pliocene to the Early Pleistocene. Therefore, this divergence is too old to attribute to the island separations after the last glacial period in the Late Pleistocene (i.e., approximately 18,000 years ago), which we assumed as one possible cause of the current geographic genetic pattern. Nonetheless, it is worth noting that the populations of small islands were closely related to geographically nearby populations, suggesting long-distance overwater dispersals, if any, are not frequent enough to collapse the north–south geographical genetic structure.

Maintenance of islet populations by frequent overwater dispersals (i.e., rescue hypothesis) is less likely even among nearby islands within the same clade. The rescue hypothesis predicts frequent migration among island populations.

**Figure 3.** Principle coordinate analysis based on pairwise $F_{ST}$ values among the 21 populations in *Platysolon marginatus*. Numbers correspond with those of Figure 1 and Table 1, and codes correspond with Clade N (closed diamonds) and Clade S (open diamonds) in the mitochondrial DNA phylogeny of Figure 2.
However, results of the BayesAss analysis suggest very low levels of contemporary migration (Supplementary Table S3 online). In addition, significant pairwise $F_{ST}$ values among all populations (Supplementary Table S2 online) further suggest little gene flow in the past. This is also supported by the existence of many mtDNA haplotypes that are endemic to the single island with moderate to high frequencies. This provides evidence against high levels of gene flow (Slatkin 1985). One aspect that might be supportive to relatively frequent migration among island populations is the presence of the isolation-by-distance effect across islands. However, this seems to reflect gene flow among the populations when the islands were connected by land bridges rather than the concurrent dispersals across the sea, because correlation between $F_{ST}$ and geographic distance was stronger for populations excluding islands isolated during LGM (i.e., populations connected by land bridges) than for overall populations. Existence of the higher number of private alleles in the isolated islands (e.g., Okinoerabujima, Yoronjima, and Izenajiam Islands; Table 1) further suggests that the sea is an effective barrier to prevent migration in *P. marginatus*. We thus refute the hypothesis that the small island populations of *P. marginatus* are maintained by frequent gene flow across the sea.

To explain the existence of this skink in the tiny islets, we may consider another possibility that the skink only recently arrived in the islets by accidental overseas dispersal, where no skinks existed or there had become extinct. This source-sink population hypothesis, however, is also unlikely at least for part of the islet populations examined. For instance, 2 islet populations, Senagajima (0.21 km$^2$) and Ėjinajiam (0.01 km$^2$),
possessed endemic haplotypes of mtDNA. Moreover, their genetic compositions are apparently differentiated from any other populations, and large $F_{ST}$ values were obtained even with the respective nearby populations (i.e., Nashiro, Naha, Tokashikijima Island, and Senajima or Ejinaikima Islands; $F_{ST}$ between Senagajima and they were 0.18–0.42 and Ejinaikima and they were 0.32–0.45). These evidences suggest that these island populations have persisted there as isolated populations since the island separated at LGM. Considering that there are remarkable genetic differentiations among most island populations examined and that all but small islets possess private allele(s), we suppose that the populations of P. marginatus in many islands have maintained themselves as isolated populations since island separation. Such putatively long persistence of the lizard populations even in the small islets in the Ryukyus is surprising because these populations are expected to have occasionally experienced severe tsunami and typhoons. These cataclysmic environmental changes may enhance the opportunity for overseas dispersal on one hand; they may lead to remarkable population decline or extinction of islet populations on the other (Schoener and Schoener 1983).

If relatively long persistence as an isolated population on an islet is actually the case, we can further predict that those populations would retain only low levels of genetic variation because of the strong effect of genetic drift (Frankham et al. 2002). This is seen in our results. As shown in Table 1, genetic diversity was lower and the degree of inbreeding was higher in small islands. This result is well explained by assuming that island area is a good indicator of population size. Moreover, the small island populations are expected to have experienced more severe population bottleneck than those of larger islands because environmental deterioration by cataclysm would have been more serious in the former. This prediction is also supported by our results that there was significant correlation between the $M$ and island area (Table 1). Nonetheless, our data further showed that P. marginatus even in the larger islands also experienced a certain degree of bottleneck because all $M$ values we obtained were collectively lower than those of apparently declining populations or island populations of various mammals species presented by Garza and Williamson (2001: 0.6–0.7), but were comparable with the values obtained for island populations of Aegaean wall lizard (Podarcis erhardii, Hurston et al. 2009). Hurston et al. (2009) argued that wall lizard populations have experienced severe population fragmentation as a result of island separation caused by the rise in sea level after the LGM. We can apply this explanation to P. marginatus.

The above arguments do not necessarily mean that all of the island populations examined have persisted in their corresponding islands since island separations. For example, Adagashima and Komakajima populations may have originated from recent colonization or recolonization, or have received gene flow from nearby populations via overseas dispersal. This is because they shared haplotypes with populations on opposite banks (Oke and Ada in Okinawajima Island for the former island, and Kudakajima Island for the latter island). Actually, the Adagashima population shared all alleles at 8 out of the 10 examined loci with Ada population in the microsatellite data as well.

Another case of possible recent gene flow is the Izenajima population. Albeit all mitochondrial haplotypes detected from other non-land–bridge islands (i.e., Okinoerabujima, Yoronjima, Agunijima, and Kumejima Islands) are endemic to their respective islands, the Izenajima population shared a haplotype (N7) with Bise of Okinawajima Island. This haplotype belonged to one inner clade within Subclade N-II along with another haplotype (N6) from Izenajima and a few other haplotypes from the peninsula of Okinawajima Island including sites 8 and 9 and its nearby islands (sites 4 and 13; Figure 2). The Izenajima populations also possessed another endemic haplotype (N8), which belongs to the other inner clade that are mostly allopatrically distributed in the northeastern part of Okinawajima Island (sites 5, 6, 7, and 9) and a nearby islet (site 12). Coexistence of the haplotypes of these allopatric inner clades in the Izenajima population thus suggests secondary contact via recent overwater dispersal to Izenajima Island.

In conclusion, our results favor the isolation hypothesis as plausible explanation for the population maintenance mechanism of P. marginatus in most islands, including even tiny islets, of the Okinawa and Amami Groups. This emphasizes that genetic drift is important factor for the evolution and diversification of island lizards, as has been suggested for some other island lizard (Jordan and Snell, 2008; Hurston et al. 2009; Runemark et al. 2010). Nevertheless, the influence of the occasional overwater dispersals cannot be rejected for the maintenance or formation of a few island populations. Although our results did not show the evidence for gene flows frequent enough to increase genetic variation of island populations, they might partially contribute to resurrect island populations after extinction.

**Supplementary Material**

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

**Funding**

Grant-in-Aid for the Global COE Program “Formation of a Strategic Base for Biodiversity and Evolutionary Research: From Genome To Ecosystem” (A06) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan; the Grants for Excellent Graduate Schools program of MEXT, Japan; the Grant from the Japan Society for the Promotion of Science (JSPS KAKENHI grant number 22570094 to M.T.).

**Acknowledgments**

We are grateful to M. Inoue-Murayama for permitting us to use the analytical equipment and providing significant comments; E. Inoue, S. Yamamoto, T. Hamabata, and A. Mori for providing many valuable comments and useful suggestions; K. Kato and E. Kawaguchi for their technical support; Y. Kadota and T. Sasaki for help collecting specimens; and 3 anonymous reviewers for many useful suggestions.
References


Received December 25, 2013; First decision July 17, 2014; Accepted July 18, 2014

Corresponding Editor: Adalgisa Caccone