Effects of Isolation by Continental Islands in the Seto Inland Sea, Japan, on Genetic Diversity of the Large Japanese Field Mouse, Apodemus speciosus (Rodentia: Muridae), Inferred from the Mitochondrial Dloop Region

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To study the effects of post-glacial isolation by islands on population genetic diversity and differentiation of the large Japanese field mouse, *Apodemus speciosus*, we examined partial nucleotide sequences of the mitochondrial *Dloop* region (ca. 300 bp) in 231 individuals collected from islands in the Seto Inland Sea and adjacent regions on Honshu and Shikoku Islands in the western part of the Japanese archipelago. Molecular phylogenetic and network analyses showed that haplotypes in each island tended to form monophyletic groups, while those in Honshu and Shikoku (the major Japanese islands) showed scattered relationships and were connected with island haplotypes. These observations suggest that a set of Honshu and Shikoku haplotypes became the ancestral lineages of the island population. No gene flow was detected among island populations, indicating that independent evolution occurred on each island, without the influence of human activities, since the establishment of the islands in the Holocene. Population genetic diversities on each island were lower than those on Honshu and Shikoku. Comparison between genetic diversity and island area size showed positive correlations and supported the suggestion that genetic drift is a major factor that shaped the current haplotype constitution of the islands in the Seto Inland Sea.

Key words: island biogeography, Seto Inland Sea, field mice, mitochondrial DNA, genetic diversity

INTRODUCTION

Since the era of Darwin and Wallace in the 19th century, islands have been examined extensively as natural experimental laboratories to support macrobiological research (Losos and Ricklefs, 2009; Kueffer et al., 2014; Warren et al., 2015). In particular, multiple islands provide independent replicates to be tested and could therefore represent model systems for exploring the ecological and evolutionary processes, which are often difficult to study experimentally (Warren et al., 2015). MacArthur and Wilson (1967) laid the groundwork for the research field of island biogeography and proposed various hypotheses, such as the species-area relationship, that remain current issues in island biogeography. Although islands have a fruitful research history, there

has been a tendency to focus on limited numbers of species on some oceanic islands with relatively old histories (e.g., Darwin's finches on the Galapagos Islands). Novel island theory could also be obtained from continental islands, such as those in the Japanese archipelago, which provide a younger time scale for isolation.

Extinction is one area of research that makes islands valuable as model systems. Islands have seen a number of extinction events of terrestrial vertebrates, and this vulnerability is suspected to be partly due to the low degree of genetic diversity in small island populations (Frankham, 1996, 2003; Frankham et al., 2010). Low genetic diversity is predicted to lead to extinction through inbreeding and/or by limiting immunological and evolutionary capabilities to cope with changing environments (Hughes and Stachowicz, 2004; Frankham et al., 2010). In fact, threatened species tend to have lower levels of genetic diversity (Frankham, 1996; Spielman et al., 2004), and the genetic diversity of small island populations has been reported to be lower than

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those of populations on nearby larger land masses (e.g., White and Searle, 2007; Sato et al., 2009; Kinoshita et al., 2015). However, the associations between limited genetic variation and island areas are not yet fully understood, especially with respect to mammals (White and Searle, 2007; see also Frankham, 1996). Unraveling this relationship would provide insight into the link between genetic diversity and population size in wild populations, which is a major issue in population genetics (Hague and Routman, 2016). Moreover, the aforementioned hypothesis that island populations have lower genetic diversity has not always been supported, particularly for plant species (e.g., Yamada and Maki, 2012; McGlaughlin et al., 2014; García-Verdugo et al., 2015). It is therefore important to investigate the effects of isolation by islands of variable area on genetic characteristics of the island populations to provide insights into extinction mechanisms, which should be clarified in the framework of conservation biology.

To investigate the genetic characteristics of island populations, we focused on islands in the Seto Inland Sea (called *Setonaikai* in Japanese) located in the southwestern region of the Japanese archipelago. The Seto Inland Sea (450 km from east to west and 15−55 km from north to south) is surrounded by three major islands of Japan (Honshu, Shikoku, and Kyushu; Fig. 1A) and harbors 727 islands in total (defined as islands > 0.1 km in circumference) with diverse areas (Japan Coast Guard). Among these islands, we conducted a field survey on Geiyo Islands (Fig. 1B). As the average depth of the sea is ca. 30 m and 98% of the sea beds are located at < 70 m depth (Japan Coast Guard), most are likely to have been created during the Pleistocene, where a drop of ca. 120 ± 7 m in sea level was recorded in the last glacial maximum (Oba and Irino, 2012). Recent paleobiogeographic and geological studies suggested that the Seto Inland Sea was established in the

Holocene by ca. 8 kya (kilo years ago) due to the Jomon (or Frandrian) marine transgression that started ca. 11 kya (Masuda et al., 2000; Shioya et al., 2007; Yasuhara, 2008), suggesting that these islands may have been formed by 8 kya at the latest. Therefore, islands in the Seto Inland Sea would provide test areas to determine the effects of isolation by islands after the last glacial period. We expected that traces of the effects of island divisions by marine transgression in the Seto Inland Sea would be reflected in the
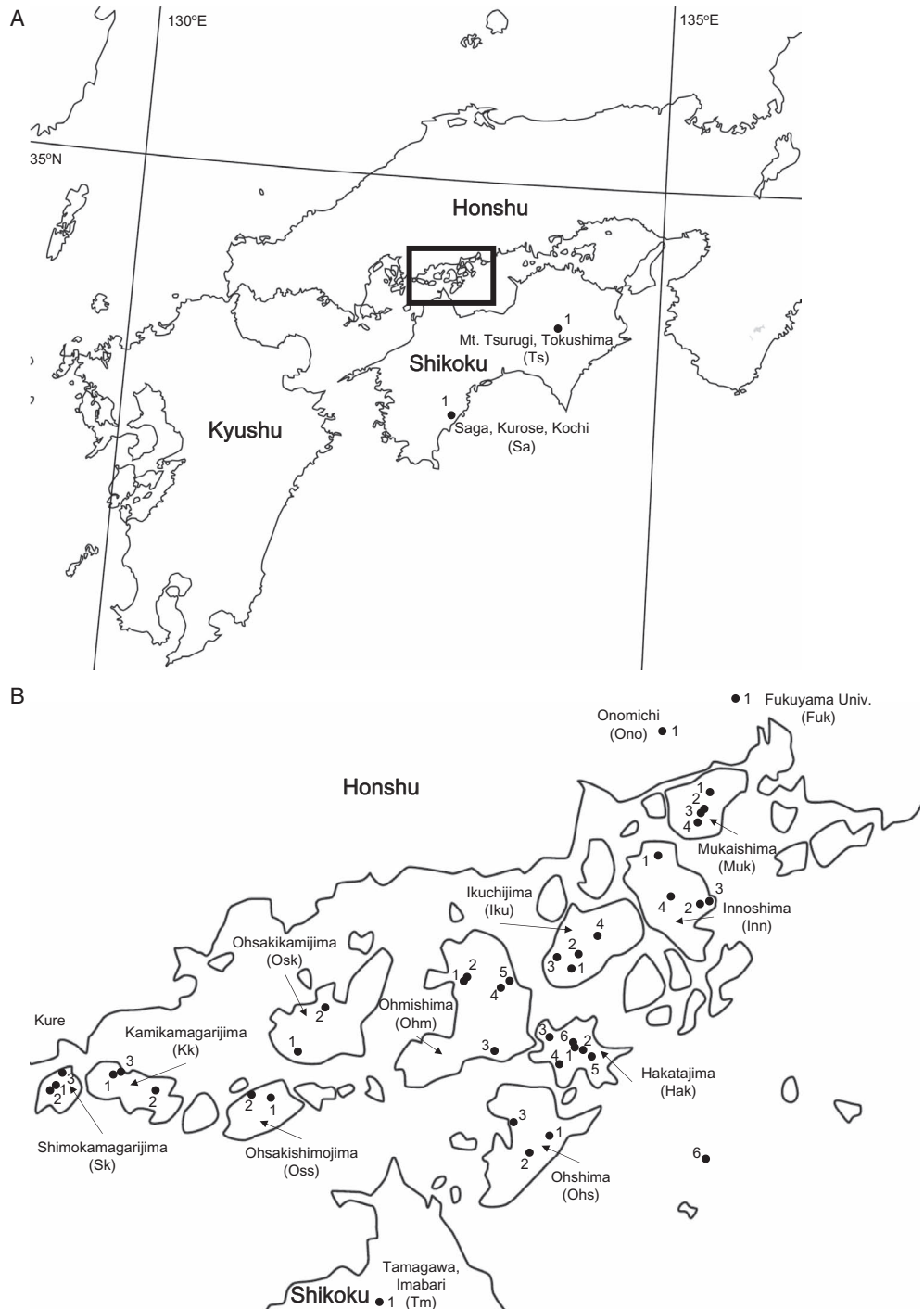

Fig. 1. Sampling locality. Maps of **(A)** the western Japanese islands and **(B)** Geiyo Islands in the Seto Inland Sea. The numbers and dots on each island indicate sampling points.

genomes of the organisms inhabiting the islands.

The large Japanese field mouse, *Apodemus speciosus* (Temmink, 1844), is an indigenous and the most commonly observed rodent in Japan (Nakata et al., 2009), and is one of the oldest Japanese mammals (Sato, 2016). *Apodemus speciosus* is considered a typical rodent adapted to forest environments and utilizing forest resources, such as acorns (Saitoh et al., 2008; Shimada, 2015), but is also observed in artificially modified habitats near human activity (Takada, 1983; Takada et al., 2012). This species also lives in forest ecosystems in most of the islands in the Seto Inland Sea except for small islands < 13 km^2 in area (Kawaguchi, 2003; see also Kaneko, 1992). Thus, this species is well-suited to the investigation of the features of population genetic diversity on islands in the Seto Inland Sea. Takada et al. (2014) examined the mandible morphometric variation of *A. speciosus* on islands in the Seto Inland Sea, and showed that animals from each island have divergent morphologies compared to those from Honshu and Shikoku. However, there have been no genetic studies of these populations. Previous genetic studies examined the phylogeography and differentiation among populations on a large geographic scale (Suzuki et al., 2004; Tomozawa and Suzuki, 2008; Tomozawa et al., 2014; Suzuki et al., 2015), focusing on the main and peripheral islands of the Japanese archipelago and long evolutionary history of a few million to hundreds of thousands of years. On the other hand, Sato et al. (2014) examined the effects of forest fragmentation on the genetic diversity of this species over a smaller area (university campus) and shorter time scale (a few decades). This study provides data regarding the trends in genetic diversity in populations considered to have been isolated on small islands for ca. 8000–11,000 years, and therefore fills the gaps between previous studies.

In this study, we examined partial sequences of the mitochondrial *Dloop* region in 231 individuals of *A. speciosus* collected from multiple islands in the Seto Inland Sea and adjacent main Japanese islands (Honshu and Shikoku), and explored the island effect on genetic diversity. Hereafter, unless otherwise noted, we used the term "islands" to refer to those in the Seto Inland Sea and regarded samples from Honshu and Shikoku as "non-island" samples, as Honshu and Shikoku are much larger than the islands in the Seto Inland Sea and therefore have relatively non-island trends within the framework of this study (see below).

MATERIALS AND METHODS

Specimens, sampling locations, and nucleotide sequences

The voucher specimens examined are summarized in Supplementary Table S1 online. We conducted the field survey during the period 2004–2015 on 10 islands (36 locations) in the Seto Inland Sea (Geiyo Islands), one location on Honshu island (Onomichi), and one location on Shikoku island (Tamagawa, Imabari) in the Japanese archipelago (Fig. 1B), and collected 204 individuals of *A. speciosus* with Sharman live-traps baited with oatmeal with permission from Hiroshima and Ehime Prefectures (see Supplementary Table S1 online). We also examined six previously collected samples from two locations on Shikoku Island (Saga, Kochi, and Mt. Tsurugi, Tokushima; Tomozawa and Suzuki, 2014). As one of the aims of this study was to test the hypothesis that island populations have lower genetic diversity, only one site was explored in each region of Honshu and Shikoku and multiple sites on islands in the

Seto Inland Sea to set the condition that island populations can more easily have higher genetic diversity. If the results indicated that island populations have lower genetic diversity even under these severe conditions, this would strongly support the hypothesis of lower genetic diversity of island populations.

We obtained permission for all animal experiments from the Animal Ethics Committee at Fukuyama University and performed the experiments in accordance with the guidelines provided by Fukuyama University and the Mammal Society of Japan.

Approximately 300 bp of nucleotide sequences of the mitochondrial DNA, including the tRNA-Pro (26 bp) gene and displacement loop region (*Dloop*; 273 or 274 bp), were determined for all *A. speciosus* samples, as described previously (Sato et al., 2014). Hereafter, we refer to these sequences as *Dloop*. The determined sequences have been deposited in the International DDBJ/ENA/GenBank DNA database with accession numbers LC153575–LC153613 (Table 1). In addition to the sequences obtained in this study, we downloaded and examined previously determined sequences from the DNA database (Hap1–Hap12 from the Fukuyama University campus [Sato et al., 2014]; Table 1). Including data of 21 samples collected from southern region in Fukuyama University campus (Sato et al., 2014), we used a total of 231 *Dloop* sequences for the analyses in this study.

DNA extraction, polymerase chain reaction (PCR) amplification, and Sanger sequencing

We used the conventional phenol-chloroform method (Sambrook and Russell, 2001) to isolate the genomic DNA from liver or muscle tissues preserved in ca. 100% ethanol. Amplification was performed by PCR using an automated thermal cycler (Life Touch thermal cycler; Bioer Technology, Hangzhou, China). An AmpliTaq Gold 360 Master Mix kit (Applied Biosystems, Foster City, CA, USA) was used for PCR amplification. Aliquots of 0.1–0.2 μg of template DNA were added to a total volume of 20 μL of PCR mixture. The primers were M15997 and H16401 originally designed by Hirota et al. (2004) and similarly adopted by Sato et al. (2014). The PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. We also conducted PCR with a negative control, and confirmed no amplification on agarose gel electrophoresis. Sequencing of the PCR products was carried out using a Big-Dye Terminator Cycle Sequencing kit v3.1 (Thermo Fisher Scientific, Tokyo, Japan), followed by analyses on an ABI3130 genetic analyzer (Thermo Fisher Scientific).

Phylogeny, network, and genetic diversity

A phylogenetic tree was constructed by the neighbor-joining (NJ; Saitou and Nei, 1987) method with the uncorrected *p*-distance in MEGA version 6 software (Tamura et al., 2013). Supports for each relationship in the phylogeny were evaluated by nonparametric bootstrap proportion (BP; 10000 replicates; Felsenstein, 1985). The median-joining network was drawn using the program PopART version 1.7 (Leigh and Bryant, 2015). Genetic diversities in each local population were evaluated by haplotype diversity (*h*) and nucleotide diversity (*π*), implemented in the program DnaSP version 5 (Librado and Rozas, 2009). In calculating the genetic diversity in the population from Fukuyama University campus, we used only 21 individuals from the southern region of this sampling location as this region is unlikely to have been affected by forest fragmentation (Sato et al., 2014).

RESULTS

Obtained haplotypes and sequence variations

We found 40 haplotypes in a partial sequence of the *Dloop* region (ca. 300 bp) among 210 individuals newly

Table 1. Haplotype distribution among sampling locations and genetic diversity indices.

Haplotypes	Sampling locations[a]															Accession No.
	Fuk	Ono	Muk	Inn	Iku	Ohm	Hak	Ohs	Osk	Oss	Kk	Sk	Tm	Sa	Ts	
Hap1	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819741
Hap2	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819742
Hap3	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819743
Hap4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819744
Hap5[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819745
Hap6	3	1	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819746
Hap7	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819747
Hap8	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819748
Hap9[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819749
Hap10[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819750
Hap11	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819751
Hap12[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AB819752
Ono1	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	LC153605
Ono2	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	LC153606
Ono3	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	LC153607
Muk1	–	–	28	–	–	–	–	–	–	–	–	–	–	–	–	LC153575
Inn1	–	–	–	13	–	–	–	–	–	–	–	–	–	–	–	LC153576
Inn2	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	LC153577
Inn3	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	LC153578
Iku1	–	–	–	–	23	–	–	–	–	–	–	–	–	–	–	LC153579
Ohm1	–	–	–	–	–	10	–	–	–	–	–	–	–	–	–	LC153580
Ohm2	–	–	–	–	–	6	–	–	–	–	–	–	–	–	–	LC153581
Ohm3	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	LC153582
Ohm4	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	LC153583
Hak1	–	–	–	–	–	–	10	–	–	–	–	–	–	–	–	LC153584
Hak2	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	LC153585
Hak3	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	LC153586
Ohs1	–	–	–	–	–	–	–	24	–	–	–	–	–	–	–	LC153587
Ohs2	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	LC153588
Ohs3	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	LC153589
Ohs4	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	LC153590
Osk1	–	–	–	–	–	–	–	–	4	–	–	–	–	–	–	LC153598
Osk2	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	LC153599
Oss1	–	–	–	–	–	–	–	–	–	15	–	–	–	–	–	LC153600
Kk1	–	–	–	–	–	–	–	–	–	–	17	–	–	–	–	LC153601
Sk1	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–	LC153602
Sk2	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	LC153603
Sk3	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	LC153604
Tm1	–	–	–	–	–	–	–	–	–	–	–	–	9	–	–	LC153591
Tm2	–	–	–	–	–	–	–	–	–	–	–	–	6	–	–	LC153592
Tm3	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	LC153593
Tm4	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	LC153594
Tm5	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	LC153595
Tm6	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	LC153596
Tm7	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	LC153597
Sa1	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	LC153613
Ts1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	LC153608
Ts2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	LC153609
Ts3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	LC153610
Ts4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	LC153611
Ts5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	LC153612
N[c]	21	5	28	16	23	20	16	28	5	15	17	9	22	1	5	
Nh[d]	8 (38)	4 (80)	1 (3.6)	3 (18.8)	1 (4.3)	4 (20.0)	3 (18.8)	4 (14.3)	2 (6.7)	1 (6.7)	1 (5.9)	3 (33.3)	7 (31.8)	1 (100)	5 (100)	
h[e]	0.833	0.9	0	0.342	0	0.668	0.575	0.267	0.4	0	0	0.667	0.766	–	1	
π[f]	0.01459	0.02207	0	0.00245	0	0.00474	0.00217	0.00094	0.00134	0	0	0.00966	0.01606	–	0.0194	
Area size[g]	–	–	22.31	35.03	31.21	64.58	20.93	41.87	38.27	17.37	18.79	7.96	–	–	–	

[a]: Fuk (Fukuyama University, South), Ono (Kurihara, Onomichi), Muk (Mukaishima), Inn (Innnoshima), Iku (Ikuchijima), Ohm (Ohmishima), Hak (Hakatajima), Ohs (Ohshima), Osk (Ohsakikamijima), Oss (Ohsakishimojima), Kk (Kamikamagarijima), Sk (Shimokamagarijima), Tm (Tamagawa, Imabari, Ehime), Sa (Saga, Kurose, Kochi), Ts (Mt. Tsurugi, Tokushima)

[b]: These haplotypes were detected around Fukuyama University, but not observed in the South location (see Sato et al., 2014) and were listed here only to show that these haplotypes were not detected from islands.

[c]: Number of individuals

[d]: Number of haplotypes (percentage of the number of haplotypes per the number of individuals)

[e]: Haplotype diversity

[f]: Nucleotide diversity

[g]: km^2

examined in this study, of which 39 were novel haplotypes and one from specimen YT2005-1-Ap5 (Kurihara, Onomichi) was Hap6 that was detected previously in the Fukuyama University campus (Sato et al., 2014; Table 1; see Supplementary Table S1 online). All except this haplotype were region-specific, with no haplotypes shared by different island populations (Table 1). In the aligned haplotype-by-sequence matrix, also including previously determined sequences (Hap1–12 from [Sato et al., 2014]; Table 1), there were 46 variable sites and a 1-bp size variation among 51 haplotypes.
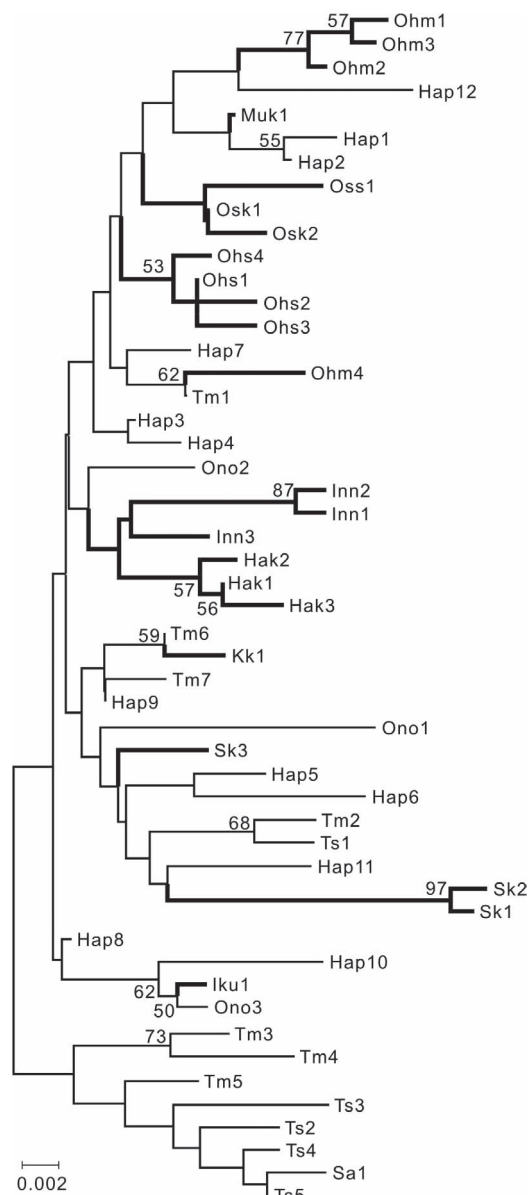
Fig. 2. Phylogenetic relationships among obtained haplotypes. Neighbor-joining tree among identified haplotypes of the large Japanese field mouse (*Apodemus speciosus*) constructed with the uncorrected *p*-distance matrix. The numbers on branches are bootstrap proportions (10,000 replicates). Values < 50% are not shown. The thick branches show lineages of islands in the Seto Inland Sea.

Phylogenetic and network relationships among haplotypes

Phylogenetic relationships inferred from the NJ analysis were not well resolved (bootstrap values mostly < 50%; Fig. 2). However, three major trends could be extracted from the observed phylogenetic structures. First, island haplotypes tended to have a single origin (Fig. 2). Specifically, haplotypes from Innoshima (Inn1, Inn2, and Inn3), Hakatajima (Hak1, Hak2, and Hak3), Ohshima (Ohs1, Ohs2, Ohs3, and Ohs4), and Ohsakikamijima (Osk1 and Osk2) islands each formed a clade (Fig. 2). Haplotypes from Mukaishima (Mk1), Ikuchijima (Iku1), Ohsakishimojima (Oss1), and Kamikamagarijima (Kk1) islands were monotypic on each island, also suggesting single origins (Table 1). On the other hand, diphyletic lineages were observed for haplotypes on Ohmishima (Ohm1-Ohm2-Ohm3 and Ohm4) and Shimokamagarijima (Sk1-Sk2 and Sk3) islands, but one of diphyletic lineages was dominant on each island (19/20 and 7/9 individuals shows Ohm1-Ohm2-Ohm3 and Sk1-Sk2 haplotypes in Ohmishima and Shimokamagarijima islands, respectively). Second, in contrast to the island haplotypes, those from Honshu (Fukuyama University campus and Onomichi) and Shikoku (Ehime, Kochi, and Tokushima) mainland areas did not tend to show monophyly. Rather, they were scattered throughout the phylogeny (Fig. 2). It should be noted that the extents of dispersion of haplotypes from Honshu and Shikoku differed. Haplotypes from Honshu (Hap and Ono) were more scattered and were shown to be closely related to many island haplotypes (Hap1-Hap2 and Muk1, Hap11 and Sk1-Sk2, Hap12 and Ohm1-Ohm2-Ohm3, Ono2 and Inn1-Inn2-Inn3-Hak1-Hak2-Hak3, and Ono3 and Iku1). In contrast, although also scattered, most of the Shikoku haplotypes, including those from diverse areas (Ehime, Kochi, and Tokushima), clustered in one clade (eight of 13 haplotypes detected on Shikoku; Tm3-Tm4-Tm5-Ts2-Ts3-Ts4-Ts5-Sa1; Fig. 2). Meanwhile, similar to the Honshu haplotype, two Shikoku haplotypes were connected to the island haplotypes (Tm1 and Ohm4 and Tm6 and Kk1; Fig. 2). Third, both phylogenetic (Fig. 2) and median-joining network (Fig. 3) analyses showed almost no geographic association among island haplotypes except for the close relationship between haplotypes in Ohsakikamijima (Osk) and the neighboring island Ohsakishimojima (Oss).

Genetic diversity

We evaluated the genetic diversity of each population, excluding those with relatively few samples < 10 individuals (Ono, Osk, Sk, Sa, and Ts). Overall, genetic diversity of the *A. speciosus* populations on the islands of the Seto Inland Sea was lower than the mainland regions on Honshu and Shikoku. The proportion of a dominant haplotype in each island population exceeded 50% (ranging from 50% on Ohmishima [Ohm] to 100% on Mukaishima [Muk], Ikuchijima [Iku], Ohsakishimojima [Oss], and Kamikamagarijima [Kk]; Table 1), while those in each Honshu and Shikoku population did not reach 50% (33% at Fukuyama University [Fuk] to 41% in Tamagawa, Imabari [Tm]; Table 1). The percentages of the number of haplotypes per number of individuals were also lower in the island populations, where the values were 3.6–20.0% for the island populations and 31.8–38.0% for the Honshu and Shikoku populations (Table 1). In agree-

ment with these trends, haplotype and nucleotide diversities (h and π, respectively) were also lower in the island populations (Honshu and Shikoku, $h = 0.766–0.833$, $\pi = 0.01459–0.01606$; Islands in the Seto Inland Sea, $h = 0–0.668$, $\pi = 0–0.00474$; Table 1).

Similarly, removing less well-sampled island populations from the analyses (Osk and Sk), we compared the genetic diversity indices (h and π) and each island area according to the Geospatial Information Authority of Japan (http://www.gsi.go.jp/KOKUJYOHO/MENCHO/201510/shima.pdf [accessed on 12 April 2016]), where the areas of Muk, Inn, Iku, Ohm, Hak, Ohs, Oss, and Kk are recorded as 22.31, 35.03, 31.21, 64.58, 20.93, 41.87, 17.37, and 18.79 km^2, respectively (Table 1). The results of regression analyses revealed marginally non-significant and significant positive correlations between haplotype and nucleotide diversities and island area, respectively (Fig. 4; h, $P = 0.082$; π, $P < 0.05$). The marginal non-significance for the former comparison was due to the relatively higher genetic diversity of the population on Hakatajima (Hak) island (an outlier), which has a smaller area (Fig. 4). Excluding the Hak population from the analysis, we obtained significant positive relationships between haplotype diversity and area ($y = 0.0147x – 0.3023$, $R^2 = 0.8821$, $P < 0.05$; data not shown). It

should be noted here that we captured mice from six localities on Hak island, showing the most extensive sampling points in this study, and probably leading to sampling bias. It is also probable that the Hak island may have specific characteristics to produce genetic variations within islands (e.g., artificial factors such as roads; Sato et al. [2014]). Furthermore, as the Ohm population has diphyletic lineages albeit with very low support on the phylogeny, it may be problematic to include both lineages in the calculation of the nucleotide diversity (π) for fare comparisons to the other island populations that have each monophyletic lineage. Excluding the Ohm4 haplotype from the analyses resulted in positive correlation although not statistically supported ($y = 4E–05x – 0.0004$, $R^2 = 0.3461$, $P = 0.125$; data not shown). This tendency of the positive relationship should therefore be treated as tentative and with some caution because the number and the origin of samples in each island population may affect the genetic diversity. We nevertheless provide this possible correlation in this study to provide a hypothesis for further testing with more samples and robust phylogenetic analyses in future.

DISCUSSION

Establishments of island and Shikoku haplotypes of *A. speciosus*

The phylogenetic relationships obtained in this study showed that haplotypes on Honshu and Shikoku, especially the former, were widely scattered among island haplotypes on the phylogeny (Fig. 2), suggesting that islands in the Seto Inland Sea had been connected to the Honshu and Shikoku islands in the past, and a set of haplotypes originating from these major islands produced ancestral haplotypes of island populations in the Seto Inland Sea. This inference is consistent with the geological theory for the sea level drop in the late Pleistocene, as explained in the Introduction. Although not strongly supported, the extents of scattering of haplotypes on the phylogeny were different between Honshu and Shikoku haplotypes, with the former being more dispersed and the latter tending to cluster into one group (Fig. 2). These observations suggest earlier differentiation of the Shikoku haplotypes from the others under conditions where there was still gene flow between the islands in the Seto Inland Sea and Honshu.
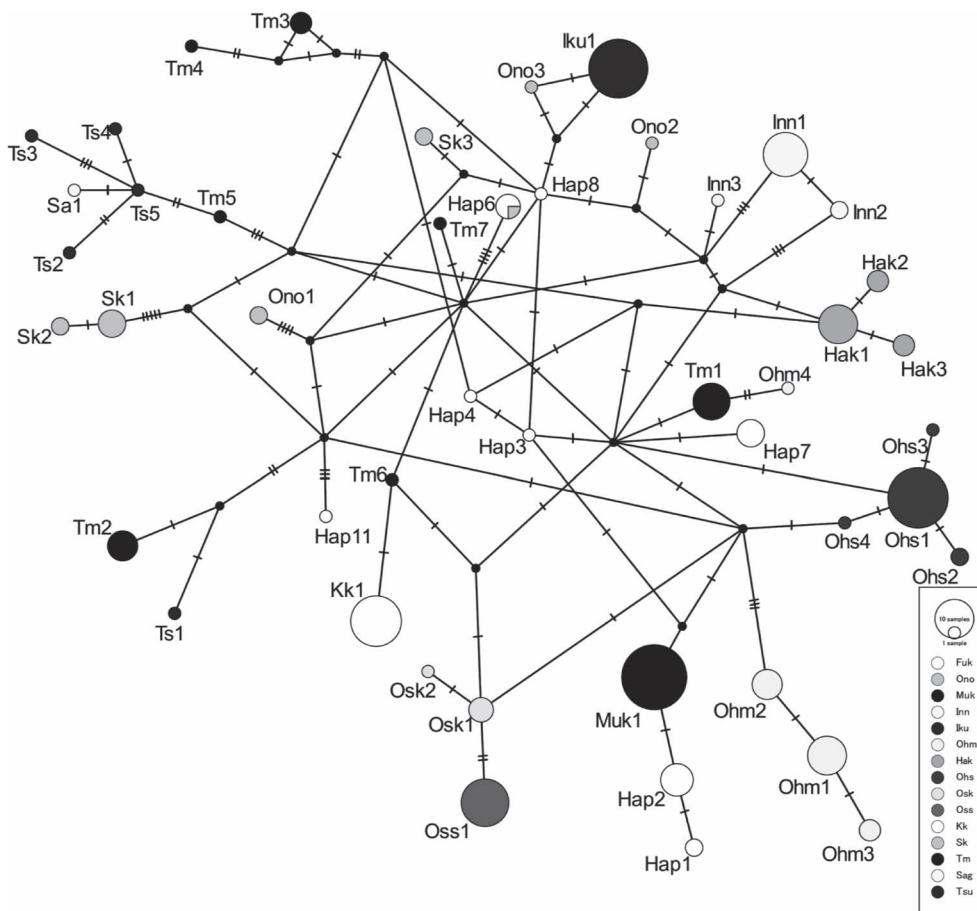

Fig. 3. Network relationships among obtained haplotypes. Median-joining network among identified haplotypes of the large Japanese field mouse (*A. speciosus*). Slashes on branches between nodes indicated mutations. Each grayscale shade shows different sampling locations and circle size reflects the number of individuals for each haplotype, as indicated in the legend at the bottom right. The smallest black nodes indicate unobserved haplotypes (median vectors).
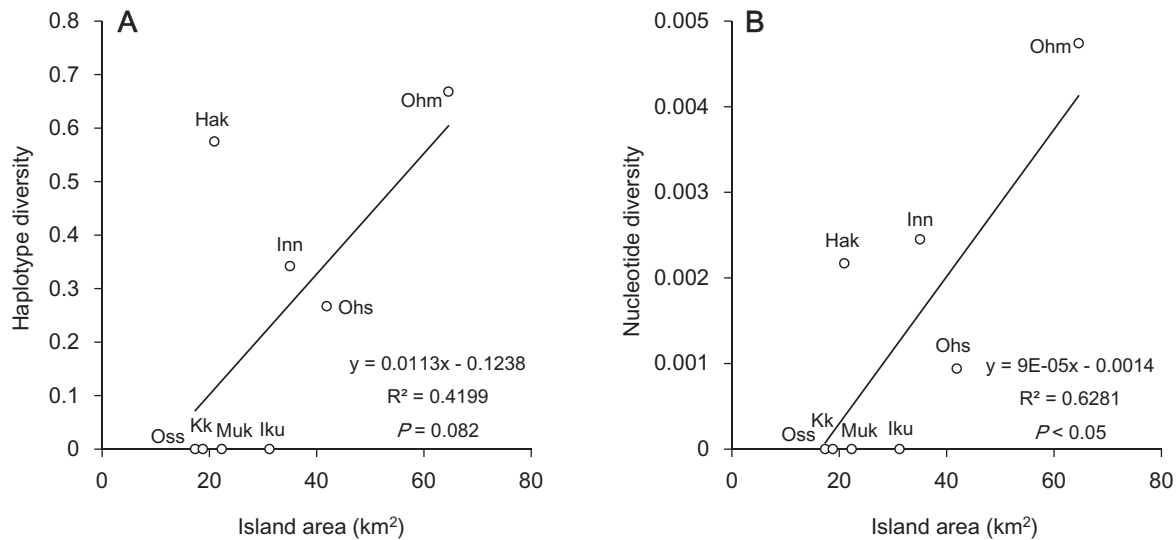
Fig. 4. Genetic diversity-Area relationships. Comparisons between population genetic diversities of the large Japanese field mouse (*A. speciosus*) on islands in the Seto Inland Sea and the area of each island: **(A)** haplotype diversity (*h*) and island area size and **(B)** nucleotide diversity (π) and island area size. The results of regression analyses (equation, R^2, and *P*-value) are shown in the lower right corner of each graph. The abbreviations of island names are shown near each plot.

Consistent with this suggestion, it is known that one large ancient river system existed between Ohshima (Ohs) and Shikoku in the late Pleistocene (Kuwashiro, 1959; see also Kondo et al., 2009 for clearer map), as also supported by the presence of a deep strait between these islands (Kurushima Strait; Japan Coast Guard). The pattern of the marine transgression process in the Holocene inferred from the ostracod paleobiogeography also supports the earlier division of Shikoku from Ohshima (Yasuhara et al., 2008). It is therefore likely that, around this region, the earliest divergence of the *A. speciosus* lineages occurred between Ohshima and Shikoku by the ancient river. On the other hand, it was demonstrated that most haplotypes detected from Kochi (Saga on southwestern Shikoku) and Tokushima (Mt. Tsurugi on eastern Shikoku) prefectures were derived from one of the Tm haplotypes (Tm5) in Ehime prefecture (Tamagawa, Imabari). These observations suggest that diverse distributional expansion within Shikoku may have originated from a limited stock of haplotypes derived from the northwestern part of Shikoku after the geological division between Ohshima and Shikoku. More sampling from the entire island of Shikoku and other islands in the Seto Inland Sea that could provide other routes from Honshu is necessary to test the hypothesis that the recent expansion of *A. speciosus* of Shikoku originated from the northwestern part.

Time scale for the establishment of island populations and rate of mitochondrial DNA evolution

The island haplotypes obtained in this study were specific to each island. This may be because in situ independent mutations have accumulated in the ancestral haplotypes of the island populations, such that island haplotypes differed from those of Honshu, Shikoku, and the other islands, and therefore there are no common haplotypes among islands in the Seto Inland Sea (Table 1). These results suggest that evolutionary changes occurred independently on each island without gene flow among islands.

Focusing on these independent mutations, we calculated the evolutionary rate of the mitochondrial DNA (mtDNA) and dated the geological separations among islands using the intra- and inter-island genetic divergences determined in this study. Recently, Suzuki et al. (2015) calculated the evolutionary rate of mtDNA gene sequences of two Japanese *Apodemus* species (*A. argenteus* and *A. speciosus*) and suggested that a higher evolutionary rate than traditionally thought should be used for dating evolutionary events at the younger time scale of < 100,000 years. In that study, the appropriate evolutionary rate for the control region (including *Dloop*) was estimated to be 17–24%/site/lineage/million years for this scale. Here, the intra-island genetic divergences were calculated based on the phylogenetic tree (Fig. 2) for Inn1-Inn2-Inn3, Ohm1-Ohm2-Ohm3, Hak1-Hak2-Hak3, Ohs1-Ohs2-Ohs3-Ohs4, Osk1-Osk2, and Sk1-Sk2, and ranged from 0.3 to 1.7% (0.56% on average). On the other hand, the inter-island genetic divergence (Muk1 vs. Hap1-Hap2, Inn1-Inn2-Inn3 vs. Hak1-Hak2-Hak3, Iku1 vs. Ono3, Ohm1-Ohm2-Ohm3 vs. Hap12, Ohm4 vs. Tm1, Osk1-Osk2 vs. Oss, Kk1 vs. Tm6, and Sk1-Sk2 vs. Hap11) varied from 0.3 to 2.7% (1.33% on average). We supposed that the geological division among islands occurred almost simultaneously in the Seto Inland Sea (8–11 kya; Masuda et al., 2000; Shioya et al., 2007; Yasuhara, 2008) and used the average genetic divergences among island lineages as calculated above. If we assume that differences among haplotypes within an island can reflect mutations that occurred after the establishment of the island, and use 8–11 kya for the divergences among haplotypes within the island, the evolutionary rate was calculated to be 25–35%/site/lineage/million years, which is only slightly higher than that reported by Suzuki et al. (2015; 17–24%/site/lineage/million years). Nevertheless, this result is consistent with the time dependency of the evolutionary rate, where higher evolutionary rate was observed in younger lineages (Ho et al., 2005). If we applied a rate of 25–35%/site/lineage/million years for

inter-island genetic divergence (1.33% on average), these events were estimated to have taken place at 19–26 kya, just before the last glacial maximum (ca. 18 kya) when the Seto Inland Sea dried up, except for deep rivers. Divergence of the ancestral haplotypes of the island population may have already been produced by some ancient geographical features probably remaining on the sea floor although not identified (e.g., ancient river systems). However, it should be noted that the island populations were influenced by pressures that would have led to low genetic diversity (see also the next section). Therefore, the number of mutations used for the time estimation here may only represent the minimum of all mutations that have occurred to date. Therefore, it is possible that the evolutionary rate may be more rapid than that obtained in this study.

It should be discussed here from another perspective that we did not detect gene flows among island populations (Table 1). This indicates that the bridges constructed among islands (Shimanami Kaidou Highway from Onomichi to Imabari through Muk-Inn-Iku-Ohm-Hak-Ohs islands and Akinada Tobishima Kaidou Highway from Kure to Oss through Sk-Kk islands) did not affect the genetic constitution of *A. speciosus* on these islands. While repeated anthropogenic translocations from Europe to Africa were known for forest-living species *A. sylvaticus* (Lalis et al., 2016), *A. speciosus* inhabiting islands in the Seto Inland Sea did not show a trend for such commensality to human activities. The Seto Inland Sea has been a prosperous transportation route for ships since the mid-Yayoi period (ca. 2000 years ago), and there have been many historical port cities around the region (Kitagawa et al., 2007). The area was also famous for pirates and ancient navies (e.g., Murakami-Suigun) that could have moved extensively around the islands in the Seto Inland Sea in the Middle Ages (14th–16th centuries; Kitagawa et al., 2007). Furthermore, *A. speciosus* on islands in the Seto Inland Sea can be found not only in forests but also near farmland (Takada et al., 2012). Nevertheless, anthropogenic activity has seldom affected the gene flow of this species, indicating that it would have been difficult for this species to cross over the open areas between forests and island coast or pass the long bridges with no cover and little food. Therefore, islands in the Seto Inland Sea could be a model system for clarifying evolutionary forces that maintain the genetic diversity of field mice because we can avoid the effects of gene flow that could otherwise contribute to the population size and genetic properties of the population in the wild (Hague and Routman, 2016). However, we also should note that the population on Shimokamagarijima (Sk) island showed relatively higher genetic diversity, although the sample size and area were small (Table 1). This may be suggestive of the presence of continued gene flows from Honshu. Kawaguchi (2003) suggested migrations of *A. speciosus* from Honshu to this island via the artificial bridge, since it is unusual for small islands < 13 km^2 in area to maintain this species. On the other hand, Tomozawa et al. (2014; *A. speciosus* on Izu Islands) and White and Searle (2007; common shrew, *Sorex araneus*, on Scottish islands) assumed the occurrence of over-water dispersal onto small islands from the main source islands to explain the higher genetic diversity of the island populations. Further sampling of *A. speciosus* from Sk island and the nearby Honshu

region (Kure) is needed to test these alternative hypotheses (gene flow by over-bridge or over-water dispersal or merely sampling bias).

A simple evolutionary role of genetic drift on the island populations

This study also showed that all of the island populations had lower levels of genetic diversity than Honshu and Shikoku populations (Table 1). These results are analogous to the numbers of species on a fragmented island, where multiple species exist on islands at the time of fragmentation and then the number of species subsequently diminishes (Warren et al., 2015). The present results suggest that fragmentation worked similarly on multiple haplotypes of the island populations and then led to reductions in genetic diversity on each island. The phylogeny (Fig. 2) and the median-joining network (Fig. 3) analyses revealed almost no geographical relationships among island populations, implying that haplotypes on each island have been fixed stochastically and independently after nearly simultaneous fragmentation of the islands. Therefore, it is likely that genetic drift has worked on each small isolated island. Although additional sampling efforts are needed, the tendency of a positive relationship between genetic diversity and island area (a possible representative of the population size; Fig. 4) also suggests that smaller population size could have affected the lower genetic diversity, supporting the strong effect of genetic drift in formation of the current haplotype constitutions on islands.

Wang et al. (2014) analyzed the genetic diversity of pond frog populations on similar continental islands (Zhoushan Archipelago) in China with microsatellite markers, and concluded that small population size and long isolation time could have influenced the lower genetic diversity. On the other hand, our study showed that islands that have been isolated for less time relatively near Honshu had the lowest genetic diversity (Table 1; Mukaishima [Muk], Ikuchijima [Iku], Osakishimojima [Oss], and Kamikamagarijima [Kk]), suggesting that the isolation time and the distance from the mainland may not have had a strong influence on the genetic diversity in this case. Wang et al. (2014) also noted that the island area was a poor surrogate (or predictor) of the population size because population density and habitat structures may differ between islands. Recent studies on plant species further supported this suggestion (García-Verdugo et al., 2015). A number of complicated factors could be responsible for the genetic features of island populations. For a plant species on Honshu and Izu islands, over-sea gene flows were inferred to have occurred frequently and maintained high levels of genetic diversity in the island populations (Yamada and Maki, 2012). Similarly, as noted above, terrestrial *A. speciosus* was shown to have dispersed onto the Izu islands from Honshu, suggesting the occurrence of over-water colonization (Tomozawa et al., 2014). In contrast to these observations, the possible positive relationship between island area and genetic diversity, as implied in this study, suggests that confounding effects, such as differences in times of origin and gene flow, can be disregarded in the present case, and it is likely that the island area could be a surrogate for the population size of the field mouse populations in the islands of the Seto Inland Sea. Therefore, in

future studies, the islands in the Seto Inland Sea could be used as a simple model system in which to understand the effects of population size (inferred from island size) on the genetic diversity, excluding the influences of isolation times and gene flows, which could affect the population genetic characteristics.

CONCLUSIONS

Populations of the large Japanese field mouse, *A. speciosus*, inhabiting islands in the Seto Inland Sea located in the western Japanese archipelago, were demonstrated to be genetically differentiated from each other without gene flows and showed lower levels of genetic diversity than those on the major Japanese islands (Honshu and Shikoku). These results suggest that each island population has experienced stochastic effects of genetic drift. This was supported by the positive relationship between genetic diversity and island area (a surrogate for population size), although more sampling would be necessary. As the establishment of each island could be considered to have occurred nearly simultaneously, *A. speciosus* on islands in the Seto Inland Sea could be a model system for future studies to investigate the ecological and evolutionary processes without confounding effects, such as gene flows or differences in isolation times.

COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

JJS designed the research project and obtained fund for this study. JJS, YT, RT, KG, YY, YT, YU, TT, and YY conducted field works to collect tissue samples of *A. speciosus*. YT, RT, KG, and YY did experiments for DNA sequences. JJS performed the phylogenetic and network analyses and wrote the paper.

SUPPLEMENTARY MATERIALS

A supplementary material for this article is available online (URL: http://www.bioone.org/doi/suppl/10.2108/zs160113).
Supplementary Table S1. Voucher information and Dloop haplotypes.

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