

Population size and time since island isolation determine genetic diversity loss in insular frog populations

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Abstract

Understanding the factors that contribute to loss of genetic diversity in fragmented populations is crucial for conservation measurements. Land-bridge archipelagoes offer ideal model systems for identifying the long-term effects of these factors on genetic variations in wild populations. In this study, we used nine microsatellite markers to quantify genetic diversity and differentiation of 810 pond frogs (*Pelophylax nigromaculatus*) from 24 islands of the Zhoushan Archipelago and three sites on nearby mainland China and estimated the effects of the island area, population size, time since island isolation, distance to the mainland and distance to the nearest larger island on reduced genetic diversity of insular populations. The mainland populations displayed higher genetic diversity than insular populations. Genetic differentiations and no obvious gene flow were detected among the frog populations on the islands. Hierarchical partitioning analysis showed that only time since island isolation (square-root-transformed) and population size (log-transformed) significantly contributed to insular genetic diversity. These results suggest that decreased genetic diversity and genetic differentiations among insular populations may have been caused by random genetic drift following isolation by rising sea levels during the Holocene. The results provide strong evidence for a relationship between retained genetic diversity and population size and time since island isolation for pond frogs on the islands, consistent with the prediction of the neutral theory for finite populations. Our study highlights the importance of the size and estimated isolation time of populations in understanding the mechanisms of genetic diversity loss and differentiation in fragmented wild populations.

Keywords: genetic diversity, genetic drift, land-bridge island, microsatellite markers, *Pelophylax nigromaculatus*, population size, time since island isolation

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Introduction

Island systems provide ideal natural experiments for testing hypotheses about population genetics and evolution and the effects of fragmentation on genetic

diversity loss in wild populations (Frankham 1997; Grant 1998; Thorpe 2005; Losos & Ricklefs 2009). Islands are discrete entities that provide an opportunity to understand how random genetic drift from a finite population contributes to genetic diversity loss in wild populations. Because many islands are younger than continents, the evolutionary processes of insular populations have occurred in more recent times, which

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allows us to accurately assess the role of population history (e.g. time since island isolation) in genetic diversity. Furthermore, the geographical barriers of oceans restrict gene flow between islands and the mainland or among islands, helping us to infer the genetic consequences of isolation and population differentiation.

According to neutral theory (Kimura 1983), the proportion of initial genetic diversity (expected heterozygosity) retained for neutral loci after t generations in a diploid finite population of random mating can be described by the following formula (Wright 1931; Kimura 1983; Falconer & Mackay 1996):

$$H_t/H_0 = [1 - 1/(2N_e)]^t = 1 - F_t$$

where N_e is the effective population size, H_t and H_0 are the genetic diversity of generations (t) and (0), respectively, and F_t is the inbreeding coefficient of generation (t). Assuming that mutation rates are similar among populations, this formula indicates that retained genetic diversity in a population due to random genetic drift at a given time is positively correlated with the effective population size and negatively correlated with the number of generations (t) (Frankham 1996, 2012). Provided that the current population size (N) reflects the long-term N_e , the genetic diversity is positively associated with census population sizes. The loss in genetic variation in the population may be reduced or recovered if there is a population expansion from the finite population (Nei *et al.* 1975; Kimura 1983) or small amounts of immigration (Kimura 1983; Keller *et al.* 2001). Numerous studies have tested these predictions by comparing the genetic diversity of insular populations with mainland populations (Frankham 1997; Bromham & Woolfit 2004; Garcia-Verdugo *et al.* 2009; Mason *et al.* 2011; Yamada & Maki 2012). An overwhelming majority of insular populations studied have been shown to have less genetic variation than their mainland counterparts, with no gene flow or restricted gene flow observed between island and mainland populations or among island populations (White & Searle 2007; Velo-Anton *et al.* 2012). Studies have used the island area as a surrogate for island population size and revealed a positive relationship between genetic diversity and island area (Frankham 1996; White & Searle 2007). The results of these studies suggest that the population densities for a species are the same across different islands and the mainland. This assumption, however, is unlikely to be realistic. There is ample evidence that population densities (density compensation) are higher on islands than on the mainland and on small islands than on larger island, due to changes in competitors, predators and food availability between them (MacArthur & Wilson 1967; Adler & Levins 1994; Rodda & Dean-Bradley 2002; Wang *et al.* 2009; Li *et al.* 2011). A few

studies have investigated the effects of island population size and time since island isolation on genetic diversity loss (e.g. island population size: White & Searle 2007; island age: Hurston *et al.* 2009). Some studies have also employed the distance to the mainland or the nearest larger island as an indicator of the degree of geographical barrier or the dispersal ability of species between an island and the mainland or between an island and a larger island (Bottin *et al.* 2005). As far as we are aware, no studies have quantified the relative contributions of island area, island population size, time since island isolation and distance to the mainland or nearest larger island on insular genetic diversity and population differentiation.

Globally, most studies of genetic diversity on archipelagoes have focused mainly on America (Jordan & Snell 2008; Martinez-Solano & Lawson 2009; Barker *et al.* 2012; Bell *et al.* 2012), Australia (Hinten *et al.* 2003; Furlan *et al.* 2012), the Mediterranean Sea (Bittkau & Comes 2005; Hurston *et al.* 2009) and Europe (Velo-Anton *et al.* 2012). There has been relatively less focus on Asia, and studies of genetic diversity on archipelagoes in Asia have focused mainly on the Japanese Ryukyu Archipelago (Maki *et al.* 2003), the Indo-Malay Archipelago (Kochzius & Nuryanto 2008) and the Philippine Archipelago (Esselstyn & Brown 2009). Fewer genetic studies have been conducted on islands in China, although China has more than ten thousand islands, with climates ranging from tropical to cold temperate conditions (Committee 2013). The Zhoushan Archipelago is the largest archipelago in China. It is located in the East China Sea (29°31'–31°04'N, 121°30'–123°25'E) in the northeast of Zhejiang Province (Fig. 1). The archipelago currently consists of 1339 islands with a total land area of approximately 1371 km². This land-bridge archipelago was connected to the mainland during the glacial period, and all the islands were separated from the mainland 7000–9000 years ago by the rising sea level during the Holocene (Zhou 1987). As information on isobaths in the East China Sea and the sea level curve of this sea for the last 20 000 years are available (Zhao *et al.* 1979), the time since island isolation for each island of the archipelago can be determined easily. This provides a good opportunity to examine the effects of time since island isolation on genetic loss of natural populations, especially on a recent timescale (the last 10 000 years).

In this study, we report on the genetic diversity of pond frog (or black spotted frogs, *Pelophylax nigromaculatus* = *Rana nigromaculata*) populations on 24 islands of the Zhoushan Archipelago and three sites on the adjacent mainland in China using nine microsatellite markers, and we identified the factors affecting the genetic diversity on the islands. The pond frog is a large-sized

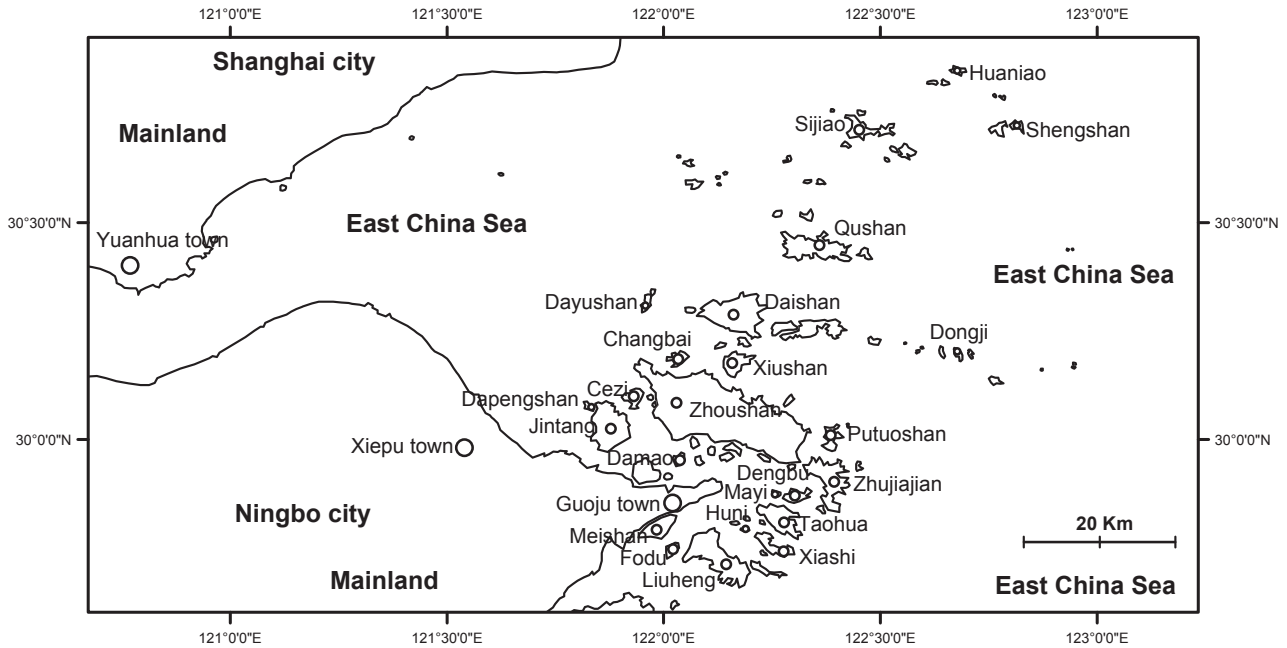


Fig. 1 Study areas for pond frog in the Zhoushan Archipelago and neighbouring mainland, China. Open circle indicates towns. Sampling covers all accessible habitats in an island or a site.

frog, widely distributed in most provinces of China, Japan, Russia and North and South Korea (Fei *et al.* 1999). The frog is found on most islands greater than 1 km² in size in the archipelago (Li *et al.* 1998) and is commonly found in crop fields (including rice fields and irrigated fields), ditches, rivers, ponds, reservoirs and surrounding areas (Fei *et al.* 1999; Wang *et al.* 2007). In the Zhoushan Archipelago, the pond frog is one of the most widespread and an abundant amphibian, which allows measurements to be made using large sample sizes (Huang *et al.* 1990). Like most amphibians (Balinsky 1981; Duellman & Trueb 1994), the pond frog is intolerant of seawater (Yang 2003) and is therefore an ideal species for examining the effects of seawater barriers on genetic diversity of insular amphibians.

We first compared the genetic diversity of pond frog populations among islands of the archipelago and sites on the mainland. We then investigated gene flow between populations among islands and mainland sites. Finally, we evaluated the relative importance of island area, island population size, time since island isolation and distance to the mainland or the nearest larger island on the genetic diversity of the insular populations.

Materials and methods

Study area

We conducted the study at three sites in the mainland (Guoju, Xiepu and Yuanhua) and 24 islands: Meishan,

Fodu, Liuheng, Huni, Xiashi, Mayi, Taohua, Dengbu, Zhujiajian, Putuoshan, Zhoushan, Damao, Cezi, Jintang, Dapengshan, Changbai, Xiushan, Dayushan, Daishan, Dongji, Qushan, Sijiao, Shengshan and Huaniao (Fig. 1). The islands of the Zhoushan Archipelago were originally part of the neighbouring mainland, but became separated from the mainland due to rising of the sea level. Over the past 20 000 years, the East China Sea level has changed during some periods, which has affected the timing of the Zhoushan Archipelago separation (Zhao *et al.* 1979). The sea level fell to between -150 and -160 m below its present level between 20 000 and 15 000 years before the present (BP). It then rose rapidly 15 000–6000 years BP. The sea level rose up to 2–4 m above its present level 6000–5000 years BP. This was the highest sea level position of the postglacial period. The sea level then fell to 1–2 m above its present level, with oscillations at 4700–4000 years BP, 3800–3000 years BP and 2500–1100 years BP. Since then, the sea has been stable at its present level.

The archipelago and the nearby mainland are similar in topography, climate, vegetation and fauna (Zhou 1987; Huang *et al.* 1990; Zhuge & Gu 1990). Both are located in the coastal hill–plain zone of Zhejiang Province, which is covered with hills (70% of the total area) and plains (30% of the total area). The highest peak on the mainland has an elevation of 657 m, compared to the 544-m elevation of the highest peak in the Zhoushan Archipelago. The climate is typical of a subtropical ocean monsoon zone and is highly seasonal, with hot

summers and cool winters. The annual rainfall is *c.* 1200–1400 mm. The natural vegetation of the area is dominated by subtropical evergreen broad leaf forest. Species richness on the islands is lower than on the mainland, with 10 amphibian species discovered in the Zhoushan Archipelago, compared with 17 species on the mainland (Huang *et al.* 1990; Li *et al.* 1998). At least 24 islands possess amphibian populations (Yuan 1965; Li *et al.* 1998). People inhabit 98 of the islands, and fishing is the primary occupation. Agriculture, including irrigated fields and rice fields, occupies about 20% of island areas.

Time since isolation estimation

The length of time that has passed since island isolation occurred can be estimated based on minimum ocean depths (the depth of the underwater saddles that connect adjacent islands or the mainland) and data on the rates of sea level rise since the last glacial maximum (Foufopoulos & Ives 1999). We reconstructed the historical pattern of island fragmentation for the Zhoushan Archipelago from the rates of change of the East China Sea level since the late Pleistocene in China (Guo 1979) and from detailed bathymetric maps used for navigational purposes in the East China Sea (NGDCNHZA 2012). We first determined the minimum depth contour around the islands from bathymetric maps (NGDCNHZA 2012). We then compared the islands' minimum isobaths values and the sea level change curve (Guo 1979). The length of time since the year when the sea level rose above the minimum isobaths value was defined as the time since island isolation (Foufopoulos & Ives 1999). The effects of geologic processes, such as crustal uplifting or coastal erosion, might affect the timing of island separation for some islands, but it should have little effect on the timing of the Zhoushan Archipelago island separation for two reasons. First, the geological conditions of the islands since isolation in the East China Sea have been relatively stable (Xu & Le 1988), and no crustal uplifting has occurred. Second, because the time since isolation of the islands in the Zhoushan Archipelago is very recent (<10 000 years), coastal erosion has most likely contributed little to the minimum isobaths values.

Data on population size of the frog, island area and distance to mainland or the nearest larger island

We estimated the frog population size on an island by multiplying the population density by the suitable habitat area on the island for the frog. Line transect methods were used (Heyer *et al.* 1994; Li *et al.* 2011) to investigate the density of the frog population during

the nonbreeding season between 2011 (for 13 islands and Guoju and Xiepu on the mainland), 2012 (for six islands and Yuanhua on mainland) and 2013 (for five islands) (Fig. 1). We set 12–20 line transects at each sampled site on the mainland or each island, with larger islands having more transects. The line transect sampling on an island was conducted to cover all accessible habitats for the frog. Each transect was 2 m wide and 50–200 m long and situated through dry field and grassland or along accessible edges of rice fields and shorelines of ponds and ditches. These transects were set in various locations in suitable habitats at a site or on an island. These transects were searched at night (19:00–21:30) with a 12-V DC lamp and a geographical positioning system (GPS), and all frogs encountered along each transect were counted. The search speed was approximately 1–2 km/h. Each site or island was surveyed for 3–6 nights. The frog density (individuals/m²) at a site or on an island was determined by summing the densities of frogs across transects and dividing by the number of all transects.

We estimated the area of suitable habitat for the frog on each island by quantifying suitable land cover for each island, including percentages of irrigated field, rice fields, forests, resident areas, factories, roads, ditches, rivers, ponds and reservoirs and salt pan (Jiang *et al.* 1991; Committee 1994, 2006, 2007). We estimated the suitable habitat area for pond frog on each island by multiplying the percentage of suitable habitats by the island area. Distances among the mainland and islands or from an island to the larger island were inferred from maps, and information on island areas was obtained from the work of Chen (Chen 1989).

Sample collections, DNA extraction and multilocus microsatellite genotyping

We collected pond frog tissue samples after conducting the density surveys. We randomly captured 30 frogs (15 adults of each sex) along transects on each site or island (Fig. 1). The frogs were caught by hand or dip net. The tips of second hind toes were clipped for DNA analysis, and then, the frogs were released to the places where they were captured. Each clipped sample was preserved in 95% ethanol for the laboratory analysis.

DNA was extracted following the published procedure (Bai *et al.* 2012a,b). Microsatellite loci are frequently used to detect the patterns of genetic diversity that were caused by the effects of selection or drift, or a combination of both in isolated populations (Hancock 1999; Hinten *et al.* 2003; Selkoe & Toonen 2006; White & Searle 2007; Hurston *et al.* 2009). We selected putatively neutral nine microsatellite markers (GenBank accessions nos: Rnh1-Rnh4, Rnh-6, Rnh9, Rnh10, Rnh12 and

Rnh-13) (Gong *et al.* 2010) to estimate genetic diversity, genetic differentiation and gene flow. All primers were labelled with 5'-fluorescein bases (HEX, FAM or TAMRA). PCRs were carried out as described by Gong *et al.* (2010), and PCR products were resolved on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). The microsatellites were scored using GENESCAN version 3.7 (Applied Biosystems) and GeneMarker version 1.71 (SoftGenetics).

Statistical analysis

We applied MICRO-CHECKER 2.2.3 to quantify null alleles and scoring errors resulting from factors such as stuttering or large allele dropout (Van Oosterhout *et al.* 2004). We tested for linkage disequilibrium and for significant deviations from Hardy–Weinberg equilibrium using GENEPOP version 4.0 (Raymond & Rousset 1995). Bonferroni corrections were used for tests involving multiple comparisons (Rice 1989). We quantified the mean number of alleles (N_a), the expected heterozygosity (H_e) and the observed heterozygosity (H_o) using GENETIX version 4.03 (Belkhir *et al.* 2004). We used Mann–Whitney test to examine the difference in genetic diversity of pond frog populations between the mainland and islands.

Genetic differentiation between the populations was calculated using pairwise F_{ST} values (Weir & Cockerham 1984) using FSTAT version 2.9.3.2 (Goudet 2002). The significance of differences in pairwise F_{ST} values was assessed by means of a 10 000-permutations test, and the P values were adjusted using the Bonferroni correction (Rice 1989).

We estimated migration rates using the program MIGRATE-N 3.2.7 (Beerli & Felsenstein 1999). The program MIGRATE-N uses coalescent theory and the Monte Carlo Markov Chain (MCMC) method to estimate pairwise gene flow. We analysed the microsatellite data using a Brownian approximation model and assumed a constant mutation rate for all loci. We conducted an initial run of our data using F_{ST} estimates and a UPGMA tree for the estimation of M ($M = m/\mu$, where m = the migration rate and μ = the mutation rate). We performed five independent runs using one long chain. For each run, we used a run of 1×10^7 recorded parameter genealogies, after discarding the first 1×10^5 genealogies as burn-in for each locus.

We used the program BOTTLENECK to test for recent population bottleneck events (Cornuet & Luikart 1996; Piry *et al.* 1999). This software could measure heterozygosity excess for each population to infer recent population bottlenecks. BOTTLENECK was run under two-phase mutation models: 10% of the infinite allele model and 90% of the stepwise-mutation model, using 100 000 replicates. The significance of heterozygosity excess for each of island population values was determined by one-tailed Wilcoxon tests ($P = 0.01$).

We estimated the relative importance of population size, island area, distance to the mainland, distance to the nearest larger island and island isolation time on the genetic diversity of insular populations. Insular population size, island area and distance to the mainland or the nearest larger island were log-transformed and island isolation time was square-root-transformed for normality (Vittinghoff *et al.* 2011). We first determined the relationship between the expected heterozygosity of an insular population and predictors such as population size, island area, distance to the mainland, distance to the nearest larger island and time since island isolation using single-variable regression. Because some predictors were collinear, we used hierarchical partitioning (HP) analysis (Chevan & Sutherland 1991; Mac Nally 2002) to evaluate the unique (unshared) and shared variance of each predictor of expected heterozygosity. HP analysis estimates the independent and shared contributions of each predictor to a dependent variable by considering all possible models (Mac Nally 2002). We calculated the statistical significance of each predictor's unique variance in the HP analysis as a pseudo- Z -score, using 1000 randomizations (Mac Nally 2002). These analyses were conducted using the 'hier.part' and 'rand.hp' functions in the R statistical software package (R Development Core Team 2008).

Results

Genetic diversity and differentiation on islands and the nearby mainland

MICRO-CHECKER did not detect the presence of null alleles or scoring errors. For each population and the entire sample, all microsatellite loci were in HWE ($P > 0.05$). After Bonferroni correction, we found no pattern of linkage disequilibrium among the loci and populations ($P > 0.05$). The loci were polymorphic within all populations.

The genetic diversity was relatively higher in all mainland populations than in island populations ($z = -2.778$ $P = 0.005$), and the island populations (Dongji, Shengshan, Damao and Huaniao) exhibited the lowest genetic diversity indexes (Table 1). The pairwise population F_{ST} differences ranged from 0.024 to 0.395 ($P < 0.001$ for all comparisons). These results indicated a significant genetic differentiation between the population pairs (Table S1, Supporting Information). Low-immigration-rate 95% CIs estimated from MIGRATE-N showed that differences among populations did not differ significantly from zero, suggesting a likely absence of gene flow between those populations (Table S2, Supporting Information). We did not find evidence for recent bottlenecks in any population (one-tailed Wilcoxon test, $P > 0.01$).

Table 1 Genetic diversity indicators of pond frogs and island features on the Zhoushan Archipelago, China

Location	H_e	H_o	N_a	Area (km ²)	DTM (km)	DTI (km)	Isolation (years)	MD ± SD (no. transects)	PZ (frogs)
Mainland									
Guoju	0.68 ± 0.04	0.70 ± 0.03	7.22 ± 4.63						
Yuanhua	0.70 ± 0.05	0.73 ± 0.03	6.89 ± 3.89						
Xiepu	0.73 ± 0.05	0.72 ± 0.03	8.78 ± 6.12						
Islands									
Zhoushan	0.60 ± 0.08	0.65 ± 0.03	5.78 ± 3.27	501.8	9.0	9.0	8500	300 ± 58 (20)	3 378 777
Daishan	0.63 ± 0.05	0.64 ± 0.03	4.89 ± 2.80	105.0	38.0	12.8	8500	655 ± 321 (20)	862 927
Liuheng	0.59 ± 0.06	0.59 ± 0.03	4.44 ± 1.51	92.9	7.3	23.5	8500	599 ± 79 (20)	1 156 076
Jintang	0.66 ± 0.06	0.66 ± 0.03	6.33 ± 4.12	76.6	4.0	6.2	8250	1310 ± 570 (20)	2 233 736
Zhujiajian	0.62 ± 0.04	0.62 ± 0.03	4.44 ± 1.51	60.9	19.3	1.2	7950	320 ± 30 (20)	293 036
Qushan	0.60 ± 0.05	0.65 ± 0.03	4.11 ± 1.62	59.8	55.2	12.2	8300	320 ± 90 (20)	200 193
Taohua	0.65 ± 0.05	0.67 ± 0.03	5.56 ± 3.43	40.1	9.4	11.5	8750	880 ± 50 (20)	399581
Meishan	0.64 ± 0.08	0.72 ± 0.03	5.67 ± 3.39	26.9	0.6	5.2	7000	210 ± 13 (20)	239 547
Xiushan	0.64 ± 0.07	0.71 ± 0.03	6.00 ± 3.81	22.9	27.1	3.2	8550	720 ± 131 (20)	221 377
Sijiao	0.67 ± 0.04	0.66 ± 0.03	5.56 ± 3.32	21.3	46.6	25.3	8300	1390 ± 630 (20)	73 184
Xiashi	0.64 ± 0.05	0.70 ± 0.03	4.44 ± 1.74	16.4	14.1	2.6	8150	1582 ± 218 (20)	145 239
Dengbu	0.60 ± 0.05	0.60 ± 0.03	4.78 ± 2.33	15.9	13.8	1.5	8650	1310 ± 140 (20)	165 148
Cezi	0.55 ± 0.08	0.59 ± 0.03	4.33 ± 2.45	14.2	13.3	2.4	8500	530 ± 310 (20)	142 429
Putuoshan	0.67 ± 0.05	0.75 ± 0.03	5.00 ± 2.45	11.8	24.7	3.2	8150	1105 ± 693 (20)	87 782
Changbai	0.61 ± 0.04	0.68 ± 0.03	3.44 ± 0.88	10.9	29.6	1.9	8250	740 ± 41 (20)	173 604
Fodu	0.59 ± 0.08	0.63 ± 0.03	4.67 ± 2.92	7.1	7.5	1.9	8150	1107 ± 217 (15)	129 875
Dayushan	0.59 ± 0.04	0.65 ± 0.03	4.44 ± 1.67	6.3	41.9	7.7	8500	2510 ± 21 (15)	104 584
Damao	0.47 ± 0.07	0.50 ± 0.03	2.67 ± 1.00	6.1	5.5	3.3	9050	440 ± 51 (15)	50 131
Shengshan	0.45 ± 0.06	0.52 ± 0.03	2.78 ± 0.83	4.2	82.0	26.2	9700	2383 ± 111 (12)	3570
Dapengshan	0.56 ± 0.04	0.61 ± 0.03	3.78 ± 1.30	3.9	12.2	0.6	7750	1950 ± 100 (12)	65 650
Huaniao	0.49 ± 0.08	0.52 ± 0.03	3.00 ± 1.23	3.6	66.4	19.0	9550	2408 ± 144 (12)	6989
Dongji	0.52 ± 0.02	0.57 ± 0.03	2.78 ± 0.67	2.6	62.7	25.2	9350	1742 ± 150 (12)	4176
Mayi	0.56 ± 0.06	0.61 ± 0.03	4.00 ± 1.80	2.3	10.4	1.7	8400	1308 ± 132 (12)	14 061
Huni	0.59 ± 0.08	0.66 ± 0.03	4.00 ± 2.29	1.4	10.1	2.6	8150	1825 ± 214 (12)	38 160

H_e , expected heterozygosity; H_o , observed heterozygosity; N_a , mean number of alleles; Area, island area; DTM, distance to the mainland; DTI, distance to the nearest larger island; Isolation, time since island isolation before the present; MD, mean densities of pond frogs (frogs/ha); PZ, population size.

Effects of island features on genetic diversity

The islands areas ranged from 1.39 km² for Huni Island to 501.78 km² for Zhoushan Island (Table 1). The distance to the mainland ranged from 0.6 to 82 km, and the distance to the nearest larger island ranged from 0.6 to 26.2 km (Table 1). The time since island isolation ranged from 7000 years for Meishan Island to 9700 years for Shengshan Island (Table 1).

The number of pond frogs per hectare ranged from 210 to 2510 on the islands (Table 1). There was a negative correlation between island area and population density ($r = -0.663$, $P < 0.001$). The estimated population sizes on the islands ranged from 3570 adult frogs on Shengshan Island to over 3 378 777 adult frogs on Zhoushan Island (Table 1).

A regression analysis showed that the genetic diversity of the frog populations on the islands was positively correlated with island area (Pearson's correlation,

$r = 0.523$, $P = 0.009$) and population size ($r = 0.671$, $P < 0.001$), but negatively correlated with time since island isolation ($r = -0.668$, $P < 0.001$). Genetic diversity was not correlated with distance to the mainland ($r = -0.281$, $P = 0.184$) or with the distance to the nearest larger island ($r = -0.294$, $P = 0.163$). However, there were correlations between some island features: population size on island increased with island area ($r = 0.872$, $P < 0.001$), but decreased with time since island isolation ($r = -0.483$, $P = 0.017$) and distance to mainland ($r = -0.454$, $P = 0.026$). Time since island isolation was positively correlated with distance to mainland ($r = 0.634$, $P = 0.001$) and distance to the nearest larger island ($r = 0.564$, $P = 0.004$) (Fig. 2). When two furthest islands were excluded from our data set, results were similar (Table S3, Supporting Information).

The HP analysis showed that time since island isolation (44%) and population size (29%) accounted for significant portions of the genetic variation in the frog

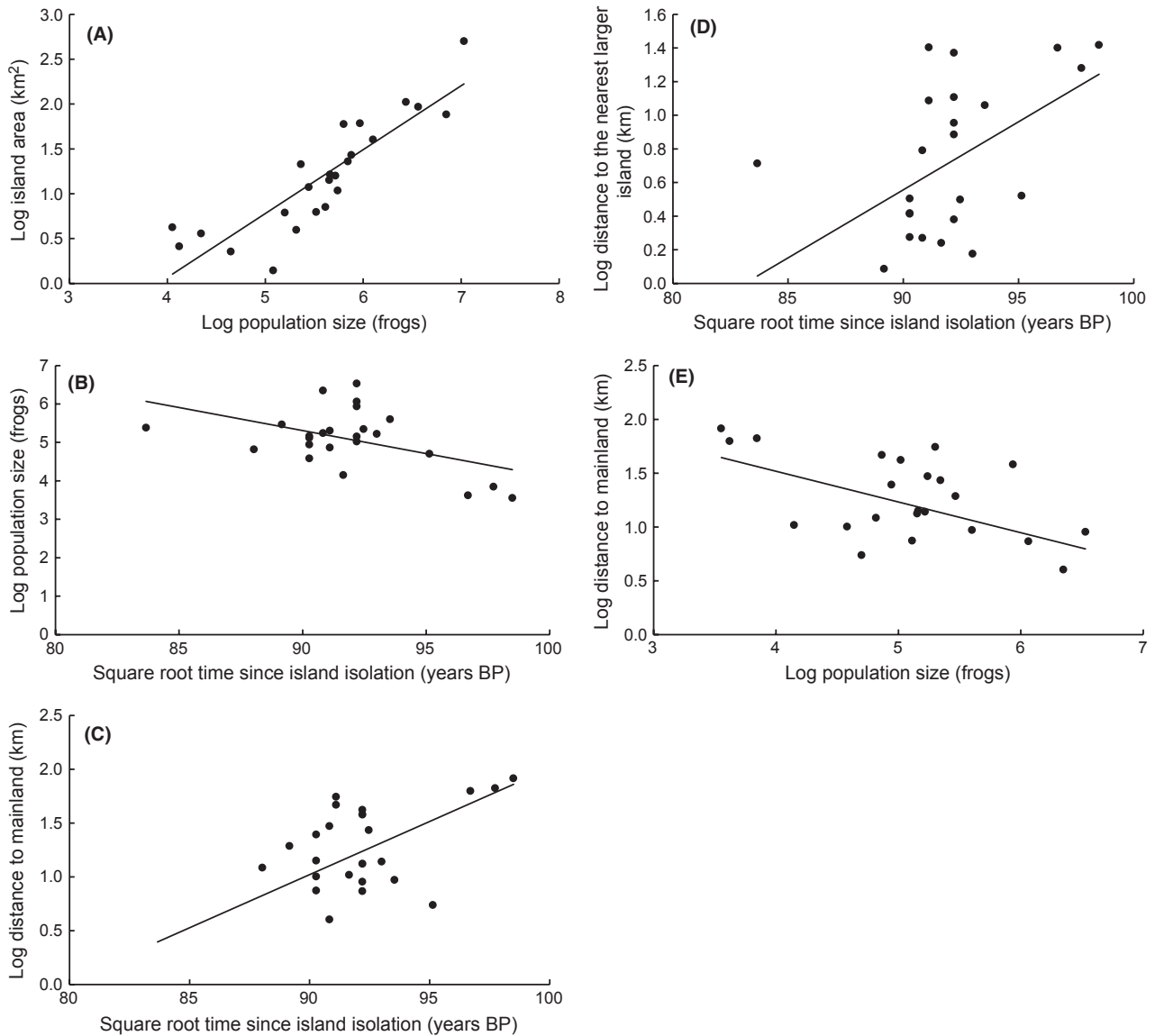


Fig. 2 The relationship between island features on 24 islands of the Zhoushan Archipelago, China. (A) Population size and island area. (B) Time since island isolation and population size. (C) Time since island isolation and distance to mainland. (D) Time since island isolation and distance to the nearest larger island. (E) Distance to mainland and population size.

populations on the islands (Table 2). All of the remaining variables (i.e. island area, distance to the mainland and distance to the nearest larger island) only explain 27% of the variations in genetic diversity, and these relationships were not significant. The results were still similar (Table S4, Supporting Information) when two furthest islands were excluded from our data set.

Discussion

We found reduced genetic diversity in the island populations, compared with the mainland populations. This loss in genetic variation was related to island population

size and island isolation time. We detected no gene flow between the mainland and the islands or among the islands. Furthermore, genetic variation within the island populations was not correlated with the distance to the mainland or the distance to the nearest larger island. These results suggest the absence of gene flow between the mainland and the Zhoushan islands, as well as among the islands, after the islands were separated from the mainland and from each other by the rise in the sea level. Reduced genetic variation and significant population differentiations on the islands were most likely due to random genetic drift of the finite insular populations. As a result, genetic diversity

Table 2 The unique contribution of island features to expected heterozygosity (H_e) of pond frog populations on the Zhoushan Archipelago, China, by hierarchical partitioning analysis

Variables	Independent deviance explained (%)
Time since island isolation (years BP, square-root-transformed)	43.93*
Population size (frogs, log-transformed)	29.07*
Island area (km ² , log-transformed)	13.84
Distance to mainland (km, log-transformed)	9.41
Distance to large island (km, log-transformed)	3.75

*Significant at 0.05.

reduced with increasing island isolation time and reduced population size. The neutral theory for finite populations predicts that retained genetic diversity in a population due to random genetic drift increases with effective population size and reduces with the number of generations (Frankham 1996, 2012). The island isolation time is a measure of the number of generations that have occurred after the isolation of the island populations. Because the density of insular animal populations increases with reduced island area (MacArthur & Wilson 1967; Adler & Levins 1994; Rodda & Dean-Bradley 2002; Wang *et al.* 2009; Li *et al.* 2011), population size may better represent the effective population size (N_e) than island area itself. Therefore, our study provides strong evidence for relationships between retained genetic diversity and population size and island isolation time for pond frogs on the islands, consistent with the prediction of the neutral theory for finite populations (Kimura 1983).

Several studies have documented that agricultural development such as crop fields can destroy the habitats of animals (Joly *et al.* 2001; Benton *et al.* 2003; Biedrzycka & Konopinski 2008), resulting in population bottlenecks that may cause genetic diversity loss. For example, Biedrzycka and Konopinski (2008) found that populations of spotted suslik (*Spermophilus suslicus*) in the western region of Ukraine and eastern Poland exhibited genetic bottlenecks due to habitat destruction by agricultural development, while those in the eastern region did not exhibit population bottlenecks because their habitats were not destroyed (Biedrzycka & Konopinski 2008). Although agriculture on the Zhoushan islands has developed extensively in the last two centuries (Committee 1992), these agricultural developments are unlikely to have had negative effects on the genetic variation in insular populations for pond frogs. Both irrigated fields (containing many ditches) and rice fields

are suitable habitats for the frogs and provide high-quality breeding habitats for frog reproduction (Knutson *et al.* 2004; Wang *et al.* 2009). Furthermore, high densities of pond frogs have often been observed in arable land (Pope *et al.* 2000; Johansson *et al.* 2005; Wang 2006), compared with other habitats, such as grassland and woods. These findings indicate that agriculture might not result in genetic bottlenecks of pond frog populations on the Zhoushan Archipelago.

Reduced genetic diversity has been widely reported in insular populations (Frankham 1997; White & Searle 2007; Furlan *et al.* 2012). Positive relationships have been detected between island size (or habitat island size) and genetic diversity for various taxa, such as mammals, birds, reptiles and insects (Frankham 1996; Cheylan & Granjon 1998; Hanfling & Brandl 1998; Hanfling *et al.* 2002; Knaepkens *et al.* 2004). Other factors, such as population size (White & Searle 2007), island age and distance to large island (Bottin *et al.* 2005; Hurston *et al.* 2009), were also found to affect insular genetic diversity. However, some studies did not detect a correlation between genetic diversity and distance from the mainland or the time since isolation (Hinten *et al.* 2003). To the best of our knowledge, our study is the first to identify the effects of both population size and time since island isolation on retained genetic diversity of insular populations. Our results clearly show that genetic drift has contributed to the genetic diversity pattern of pond frog populations in the Zhoushan Archipelago.

Seawater may be a significant barrier to the dispersal of pond frogs due to the species' intolerance of seawater, and thus, gene flow might have been limited among the islands of the Zhoushan Archipelago since the rise of sea levels during the Holocene (Guo 1979). However, seawater is not always a strict limit to the dispersal of some amphibians (Vences *et al.* 2003, 2004; Measey *et al.* 2007). Some species, such as *Rana cancrivora*, *Bufo viridis* and *Xenopus laevis*, have some degree of salt tolerance (Balinsky 1981). Amphibians may also disperse from the mainland to islands through brackish water present seasonally or throughout the year (Seppa & Laurila 1999). Additionally, movements among islands via rafting have been suggested as a possible mechanism of long-distance overwater dispersal (Hedges *et al.* 1992; Kaiser *et al.* 1994). For example, there is evidence to suggest that marine environments can, at least occasionally or periodically, provide freshwater paths for amphibians from the mainland of Africa to drift on floating rafts to remote oceanic islands and colonize them (Measey *et al.* 2007). The seawater concentration in the East China Sea is normal (3%) and varies little seasonally (Committee 1992). Adult frogs of pond frogs in such seawater would die in 60 min (Yang 2003).

Furthermore, there is no evidence of colonization of amphibian species by rafting or introductions by people from the mainland to the islands of the Zhoushan Archipelago (Jiang *et al.* 1991; Li *et al.* 1998; Yang 2003; Committee 2007). Therefore, the insular populations of pond frogs seem not to have undergone subsequent migration following isolation, even over the short overseas distance (600 m) between Dapengshan Island and Jintang Island (Table 1).

Amphibian populations on land-bridge islands are hypothesized to mainly come from two sources: population isolation before the formation of the islands and overseas colonization or introductions by human from adjacent mainland populations (Velo-Anton *et al.* 2012). Our result favours the hypothesis that the Zhoushan Archipelago populations of pond frogs originated in populations that lived in the coastal highlands before they became isolated from the mainland due to sea level rise, *c.* 7000–10 000 years ago (Guo 1979). Similar cases were recently reported for populations of slender salamanders (*Batrachoseps attenuatus*) on the islands of the San Francisco Bay (Martinez-Solano & Lawson 2009) and fire salamanders (*Salamandra salamandra*) on the islands of the Iberian Peninsula (Velo-Anton *et al.* 2012). Some studies have detected genetic structure in herpetological populations on large islands (Johansson *et al.* 2008; Moore *et al.* 2008). Our study includes some large islands, such as Zhoushan (502 km²) and Daishan (105 km²) (Committee 1992). Whether there is the genetic structure of pond frog populations on such islands remains to be explored. In the mainland, we observed population differentiations between Yuanhua, Xiepu and Guoju (Table S1, Supporting Information), and no gene flow was detected among all three sites (Table S2, Supporting Information). This arose possibly because amphibians have relatively poor dispersal ability (Olalla-Tarraga *et al.* 2011), which can result in highly structured populations with restricted gene flow (Shaffer *et al.* 2000).

Loss of genetic variation in small populations that have been isolated for prolonged periods of time may compromise fitness and evolutionary potential and also make such populations more susceptible to extinction. These possibilities have been the focus of intense investigation in conservation biology (Frankham 1997, 2012; Alsos *et al.* 2009; Dixo *et al.* 2009; Hurston *et al.* 2009; Bruggeman *et al.* 2010; Landguth *et al.* 2010). Understanding the factors responsible for loss of genetic variation is crucial for managing fragmented populations in the wild. Our study highlights the importance of the size and isolation time of populations in understanding the mechanisms of genetic diversity loss and differentiation in insular or fragmented wild populations. Because predictors of genetic diversity are often correlated to some extent, the identification of isolation time

as a significant factor may be crucial to understanding the mechanisms of genetic diversity loss. Island area or the area that a fragmented population occupies may be a poorer surrogate of its effective population size than population size because both population density and the proportion of suitable habitat area may vary among islands or fragmented habitats. However, population size estimation would require intensive field work and in many cases cannot be achieved due to financial limitations or other reasons. Consequently, island size may still be a valuable measurement to use in the absence of population size data (Frankham 1996; Cheylan & Granjon 1998; Hanfling & Brandl 1998; Hanfling *et al.* 2002; Knaepkens *et al.* 2004). Alternatively, suitable habitat area has been used in calculating population size (White & Searle 2007) and is more closely related to population size than island area. Our study showed that suitable habitat area (log-transformed) explained more variation in population size for pond frogs (Pearson's correlation, $r = 0.965$, $P < 0.001$) than island area (log-transformed) ($r = 0.872$, $P < 0.001$). Compared with population size, suitable habitat area can be more easily estimated by GIS (geographical information system) technology (White & Searle 2007), or land cover map (like this study). It should be a better surrogate for population size than for island area if data on suitable habitat area are available. In short, our study of the pond frog populations in the Zhoushan Archipelago increases our understanding of the relationship between the loss of genetic diversity, population size and isolation time in a land-bridge archipelago.

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Y.L. designed the study; S.W., W.Z., X.L., X.G., Y.L., Z.G. and X.L. collected samples; S.W. and S.Y. performed experiments; S.W., Y.L. and J.Y. analysed data; S.W. and Y.L. wrote the manuscript.

Data accessibility

Sample locations and multilocus microsatellite genotypes are available at DRYAD entry doi:10.5061/dryad.dq4 g5.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Pairwise F_{ST} for all studied populations.

Table S2 Asymmetric migration rates between pond frog populations.

Table S3 Coefficients and probabilities (in parentheses) of the Pearson's correlation between expected heterozygosity (H_e) and island characteristics for pond frogs on 22 islands [excluding two furthest islands (Huaniao and Shengshan) from the mainland] of the Zhoushan Archipelago, China.

Table S4 The unique contribution of island features to expected heterozygosity (H_e) of pond frog populations on 22 islands (excluding two furthest islands (Huaniao and Shengshan) from the mainland) of the Zhoushan Archipelago, China, by hierarchical partitioning analysis.