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# Effects of fragmentation on genetic diversity in island populations of the Aegean wall lizard *Podarcis erhardii* (Lacertidae, Reptilia)

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ABSTRACT

Landbridge islands offer unique opportunities for understanding the effects of fragmentation history on genetic variation in island taxa. The formation of islands by rising sea levels can be likened to a population bottleneck whose magnitude and duration is determined by island area and time since isolation, respectively. The Holocene landbridge islands of the Aegean Sea (Greece) were formed since the last glacial maximum and constitute an ideal system for disentangling the effects of island area, age and geographic isolation on genetic variability. Of the many reptile species inhabiting this island system, the Aegean wall lizard *Podarcis erhardii* is an excellent indicator of fragmentation history due to its widespread distribution and poor over-water dispersal abilities. In this study, we utilize a detailed record of Holocene fragmentation to investigate the effects of island history on wall lizard mitochondrial and nuclear microsatellite diversity. Findings show that the spatial distribution of mitochondrial haplotypes reflects historical patterns of fragmentation rather than geographic proximity *per se*. In keeping with neutral bottleneck theory, larger and younger islands retain more nuclear genetic variation than smaller, older islands. Conversely, there is no evidence of an effect of isolation by distance or effect of distance to the nearest larger landmass on genetic variability, indicating little gene flow between islands. Lastly, population-specific measures of genetic differentiation are inversely correlated with island area, suggesting that smaller islands exhibit greater divergence due to their greater susceptibility to drift. Taken together, these results suggest that both island area and time since isolation are important predictors of genetic variation and that these patterns likely arose through the progressive fragmentation of ancestral diversity and the ensuing cumulative effects of drift.



## 1. Introduction

Understanding how fragmentation and associated demographic bottleneck events affect genetic diversity and extinction probabilities in wild populations has been a major focus in conservation biology (e.g. Gibbs, 2001; Hanski, 1998). Small, isolated populations subjected to sustained demographic bottlenecks will rapidly lose genetic variation through drift (Frankham et al., 2002; Wright, 1931). This loss in variation may compromise fitness and evolutionary potential (Eldridge et al., 1999; England et al., 2003), making populations more susceptible to extinction (Frankham et al., 2002; Westemeier et al., 1998). Population genetic theory predicts that demographic bottlenecks will reduce both heterozygozity and allelic variation (Nei et al., 1975). Of these two measures, allelic richness appears to be the more sensitive indicator of bottleneck history (Leberg, 1992; Nei et al., 1975; Spencer et al., 2000). However, levels of genetic variability can also recover due to rapid post-bottleneck population growth (Nei et al., 1975) or even small amounts of immigration (Keller et al., 2001).

Whereas loss in heterozygozity in finite populations is relatively easy to predict (Frankham et al., 2002; Hedrick, 2000; Wright, 1931), forecasting the loss in allelic variation over more than a single generation bottleneck is much more difficult (England and Osler, 2001; Watterson, 1984). Furthermore, despite numerous studies on genetic consequences of bottleneck events in natural populations (e.g. Bouzat et al., 1998; Glenn et al., 1999; Kaeuffer et al., 2007; Keller et al., 2001; Saccheri et al., 1998), few have systematically tested predictions of neutral bottleneck theory in a series of naturally replicated island populations of known history (Hinten et al., 2003). These predictions are well established (Wright, 1931; Nei et al., 1975; Chakraborty and Nei, 1977; Leberg, 1992; reviewed in Frankham et al., 2002) and can be summarized as follows: (1) genetic diversity will be progressively lost through time due to the cumulative effects of drift in finite populations (2) the greater the magnitude of the population bottleneck the

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greater the magnitude of loss in genetic variation (3) the longer the duration of the bottleneck the greater the amount of diversity lost (4) allelic variation will be a better indicator of bottleneck effects than heterozygosity.

Continental landbridge islands make ideal candidates for the study of the genetic consequences of population bottlenecks. The fragmentation of populations formerly inhabiting contiguous land masses by rising sea levels during the Holocene can be likened to a sustained population bottleneck whose magnitude is a function of area and whose duration is a function of island age. Using this information, the effects of demographic history on genetic variation can then be evaluated and the relative importance of the magnitude of the bottleneck (island area), its duration (island age) and susceptibility to migration (island isolation) can be systematically assessed. Moreover, this study system also lends itself to an assessment of island biogeography theory (MacArthur and Wilson, 1967; Shafer, 1990) at the population genetic level. Previous studies have demonstrated that island populations have lower levels of genetic variation and higher levels of inbreeding than their mainland counterparts (Eldridge et al., 1999; Frankham, 1997, 1998; Hinten et al., 2003). However, few studies have systematically attempted to disentangle the effects of island area and age on levels of genetic variation and almost all of these used allozyme markers (Capula, 1996; Capula and Ceccarelli, 2003; Gorman et al., 1975; Soulé and Yang, 1973).

Island lizards have proved to be ideal organisms for studies of biogeographical and colonization history (Capula, 1996; Capula and Ceccarelli, 2003; Pinho et al., 2008; Poulakakis et al., 2005a,b), invasive species biology (Kolbe et al., 2004) and ecological speciation (Butler et al., 2007; Losos et al., 2006; Ogden and Thorpe, 2002). Island lizard populations also make excellent models for studying the effects of fragmentation history on genetic variation. For example, in a study on the effects of island history on patterns of allozyme variation in Lacertid lizards inhabiting offshore islands in the Adriatic, Gorman et al. (1975) argued that directional selection was responsible for the decline in genetic variation in smaller and older islands. In contrast, it was postulated that drift only reduced levels of genetic variation in the smallest of islands (<0.01 $km^2$). Similarly, in their study on the channel island lizard *Uta stansburiana*, Soulé and Yang (1973) hypothesized that low allozyme heterozygozity observed on smaller islands was due to the cumulative effects of directional selection over time. As these studies however demonstrate, one of the difficulties of using allozyme markers to assess population history is their potential non-neutrality, making it difficult to determine whether observed patterns of diversity are due to the effects of selection or drift, or a combination of both (Hinten et al., 2003). Another potential drawback of such markers is that unlike nuclear microsatellites, allozymes show very low levels of polymorphism and hence do not possess the resolution needed to compare island populations with subtle differences in fragmentation history.

The islands of the Aegean Sea in the north-eastern Mediterranean basin represent an ideal system for exploring the effects of historical fragmentation on genetic variation in island lizards. Aegean archipelagos are comprised of more than 3,000 land bridge islands that became isolated by rising sea levels since the last glacial maximum ~20,000 years ago (Fairbanks, 1989; Van Andel and Shackleton, 1982). Detailed bathymetric data for the Aegean Sea are available and have been used to determine the specific sequence and the exact duration of island isolation (Foufopoulos and Ives, 1999).

Of the many reptile species inhabiting this island system, the Aegean wall lizard *Podarcis erhardii* is an excellent indicator of fragmentation history due to its widespread distribution and poor over-water dispersal abilities. A number of factors suggest that there has been no substantial over-water dispersal between island populations of this species during the Holocene. Firstly, most Aegean wall lizard populations are morphologically distinct and a large number of subspecies (>25) have been described (Gruber, 1986). Such a diversity of distinct island forms therefore suggests that gene flow is not an important factor in this system. Secondly, several aspects of the life history of the Aegean wall lizard also preclude extensive over-water dispersal in this species, namely its poor floating abilities and aversion to laying eggs in vegetation. Lastly, in contrast to other island lizard studies (see Carlsbeek and Smith, 2003), the cold waters, relatively large inter-island distances and absence of substantial vegetation cover available for rafting in the Mediterranean make over-water dispersal unlikely. Therefore, for such poor dispersing species, the impact of the magnitude and duration of the bottleneck resulting from island formation can be inferred. Assuming island area is a reliable indicator of reptile population size (King, 1987), this system therefore provides a unique opportunity to directly test the effects of bottleneck history on genetic variability in an island lizard and tease apart the effects of bottleneck magnitude (area), duration (age) and migration (geographic isolation) on different measures of genetic variation.

The present study first sets out to test whether the distribution of mitochondrial cytochrome *b* variation among islands reflects patterns of historical fragmentation. Microsatellite data are then used to test the prediction that genetic variation is positively correlated with island area and negatively correlated with island age. These data are also used to test the hypothesis that distance to the nearest largest landmass has no effect on genetic diversity and that there is no significant isolation-by-distance effect between island populations, as predicted in a study system where drift should vastly outweigh the effects of gene flow (Hutchison and Templeton, 1999). Lastly, a novel Bayesian method (Foll and Gaggiotti, 2006) was used to examine the effects of island area, age and isolation on population-specific measures of genetic differentiation. If island area and age are reliable predictors of population size and time since isolation, then smaller, older islands will be more susceptible to drift and thus exhibit a greater level of genetic differentiation. Conversely, if gene flow is an important component of the present study system, then population genetic differentiation between islands will be positively correlated with geographic distance.

## 2. Material and methods

### 2.1. Study sites

The islands selected for study are part of the Cyclades (Kikladhes) island group situated in the central Aegean Sea, Greece (Fig. 1). These islands span a wide range of sizes and periods of isolation while still sharing a common geologic history and similar ecological and environmental conditions. All study islands are fragments separated over the course of the Holocene from a large ancestral landmass (the so called 'Protocycladic block'). Over the span of the last 15,000 years, rising sea levels progressively fragmented the original ancestral population of lizards living on this landmass into smaller populations, each of which was subject to the equivalent of a sustained bottleneck dating from their time of separation (Foufopoulos and Ives, 1999; Perissoratis and Conispoliatis, 2003).

Islands sampled in the present study fell into one of two very different size range categories: small islands of <1.64 $km^2$ and larger islands of 8.83–448 $km^2$. To calculate island age and hence provide a proxy for bottleneck duration, we estimated the time since separation from the next largest land mass using published global and local Holocene eustatic sea level change curves (Erol, 1981; Fairbanks, 1989). These records were combined with high resolution information on the maximum depth of the underwater saddle connecting each island to the next largest land mass obtained from

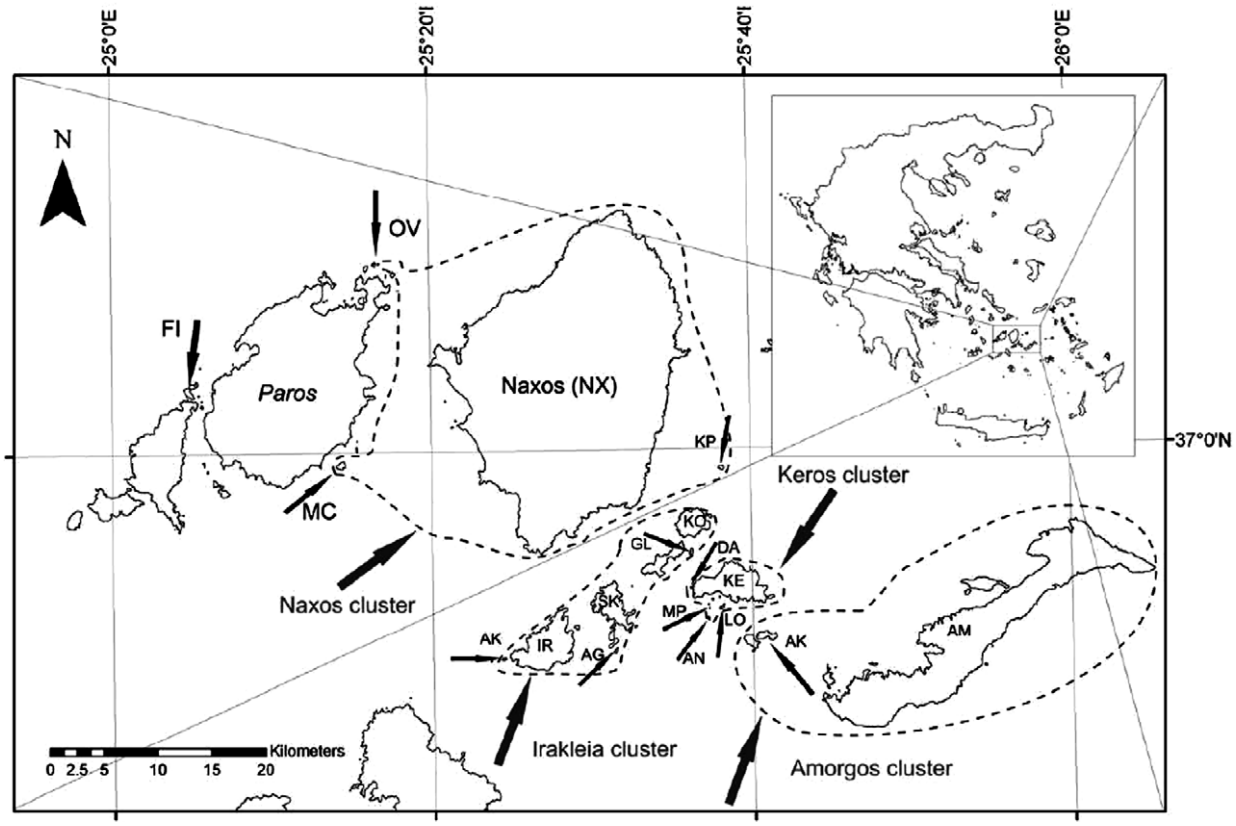


**Fig. 1.** Map of central Cyclades in the Aegean Sea. Islands sampled are labeled with the two letter code given in Supplementary Table 1. Two letter codes for 18 of the 20 study islands in this study are as follows: AG (Agrilou), AK (Antikeros), AM (Amorgos), AN (Andreas), DA (Daskalio), FI (Fira), GL (Glaronissi), IR (Irakleia), KE (Keros), KO (Koufonissi), KP (Kopria), LO (Loumboudiaris), MA (Megalos Ambelas), MC (Makronissi), MP (Megali Plaka), NX (Naxos), (OV) Ovriokastro, SK (Schoinoussa). Note that the small island of Nea Kameni (NK) and the larger volcanic island of Santorini (SA) are to the south of the islands depicted in this map.

navigation charts (USDMA, 1991) and from field sonar soundings conducted by one of the authors (J. Foufopoulos, unpublished data). Using these data, island ages and hence estimates of bottleneck duration, were obtained for each of the island populations. One exception is the very large island of Naxos which is the largest remnant of the Protocycladic block. As this island never experienced a population bottleneck of the magnitude experienced in other islands in this study, it was given an age of 0.

Both island area and distance to the nearest largest land mass were compiled from the published literature and from local maps (USDMA, 1991; Foufopoulos and Ives, 1999). As Naxos is the largest island that historically formed the core of the Protocycladic block and there are no larger nearby landmasses to act as colonist sources, it was also given a geographic distance of 0. Pair-wise geographic distances between study islands were also calculated using a web-based great distance calculator (www.gb3pi.org.uk/great.html) in order to test for a significant association between inter-island genetic and geographic distances (Mantel, 1967).

Islands were grouped into four clusters based on their common fragmentation history (Foufopoulos and Ives, 1999). The first cluster consists of the main island of Naxos, and three neighboring islets (Ovriokastro, Makronissi, and Kopria). The second cluster is centered on Keros, to the Southeast of Naxos and includes four adjacent islands (Andreas, Daskalio, Loumboudiaris and Megali Plaka). The third cluster encompasses the moderately sized islands of Irakleia, Schoinoussa and Koufonissi and surrounding satellite islets (Agrilou, Glaronissi, Megalos Ambelas). The last cluster consisted of the larger islands of Amorgos and Antikeros to the Southeast of Keros. Sequence data from populations inhabiting the peripheral islands of Fira (west of Paros) and the volcanic islands of Santorini and Nea Kameni were included in the phylogenetic study. However, these islands were excluded from analyses of fragmentation effects owing to either their independent volcanic history (Santorini and Nea Kameni) or distant isolation relative to other islands under study (Fira). Table 1 provides information on island area, inferred age, geographic distance to the nearest larger island and sample sizes used for cytochrome *b* sequencing and microsatellite genotyping.

### 2.2. Sampling

The Aegean wall lizard is a small, heliothermic, generalist lacertid that is widespread across the mainland and the islands of the Western Aegean sea region. The populations from the Peloponnese were early on described as a separate species, *P. peloponnesiacus*, making *P. erhardii* a paraphyletic taxon (Fig. 2) (see discussion in Poulakakis et al., 2003). However the recent elevation (Lymberakis et al., 2008) of the populations from Crete and the ancient islet of Pori (Southwest Aegean Sea) into distinct taxa *P. cretensis* and *P. levendisi* removed this incongruence, reserving the name *P. erhardii* for the mainland and Central Aegean island populations (shaded in dark gray box in Fig. 2). Lizards were captured from islands using hand-held silk nooses, sticky traps or mealworm baits and were held in cloth bags before body measurements were recorded later in the day. Small tissue samples were obtained either through tail or toe clips or from autotomized tails and stored in screw-top vials containing 95% ethanol.

### 2.3. DNA extraction

DNA was extracted from tissue samples using a high salt, phenol–chloroform procedure outlined by Sambrook et al. (1989). A

**Table 1**
Physical and geographic characteristics and corresponding sample sizes of the islands examined in this study.

| Island | Area ($km^2$) | Inferred age (years) | Distance (km) | *N* (cyt *b*) | *N* (msat) | Code |
|---|---|---|---|---|---|---|
| Agrilou | 0.090 | 9650 | 0.400 | 12 | 25 | AG |
| Antikeros | 1.640 | 13,500 | 6.880 | 13 | 19 | AK |
| Amorgos | 123.000 | 13,800 | 25.12 | 5 | NA | AM |
| Andreas | 0.050 | 8550 | 0.050 | 10 | 17 | AN |
| Daskalio | 0.015 | 1500 | 0.100 | 12 | 30 | DA |
| Fira | 0.900 | 1500 | 0.200 | 5 | 19 | FI |
| Glaronissi | 0.188 | 5650 | 0.550 | 10 | 26 | GL |
| Irakleia | 18.078 | 9800 | 5.355 | 9 | 17 | IR |
| Keros | 15.050 | 9150 | 8.925 | 9 | 13 | KE |
| Koufonissi | 13.000 | 8350 | 4.46 | 10 | 16 | KO |
| Kopria | 0.138 | 11,700 | 4.200 | 11 | 27 | KP |
| Loumboudiaris | 0.123 | 6450 | 0.652 | 3 | NA | LO |
| Megalos Ambelas | 0.070 | 7850 | 0.350 | 14 | 24 | MA |
| Makronissi | 0.042 | 5850 | 1.050 | 9 | 24 | MC |
| Megali Plaka | 0.030 | 6450 | 0.400 | 15 | 25 | MP |
| Naxos | 448.000 | 0 | 0 | 16 | 30 | NX |
| Nea Kameni | 3.400 | 57[a] | 73.658 | 10 | 30 | NK |
| Ovriokastro | 0.220 | 5750 | 0.630 | 7 | 25 | OV |
| Santorini | 71.200 | >200,000[b] | 18.100 | 1 | NA | SA |
| Schoinoussa | 8.830 | 6250 | 5.350 | 9 | 21 | SK |

NA, microsatellite data is not available from these islands because of small sample size.
[a] Portions of Nea Kameni are older but we assume here that the most recent volcanic eruption of 1950 eradicated any pre-existing resident lizard populations.
[b] Santorini is separated by waters >150 m deep from the other islands and was never part of the Protocycladic block.

447 bp fragment of the mitochondrial cytochrome *b* locus was amplified by polymerase chain reaction (PCR) using primers (Palumbi, 1996) modified to better match the lacertid cytochrome *b* data: (F-5′GGCCTGAAAAACCACCGTTG; R-5′: CCCTCAGAATGATATTTGTCC). These primers amplify the 5′ end of the cytochrome *b* gene and amplify the same region used in numerous previous studies of *Podarcis* phylogeography (Poulakakis et al., 2003; 2005a,b). The forward primer is located at positions 14113–14132 within the tRNA-Glu of the *P. muralis* whole mitochondrial genome (NC_011607) whereas the reverse primer is located at the corresponding positions 14564–14584 of the *P. muralis* cytochrome *b* gene.

PCR amplification was carried out using the following conditions: an initial denaturation step for 3 min at 94 °C followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 47 °C and extension for 30 s at 72 °C, followed by a final extension step for 10 min at 72 °C. PCR reactions were carried out in a 50 μl reaction volume containing 1× enzyme buffer (200 mM Tris, pH 8.4, 500 mM KCl), 1.5 mM $Mg^{2+}$, 0.2 μM of each primer, 0.2 mM dNTPs, 0.5U of *Taq* (Invitrogen) and 15–30 ng DNA. Sequencing reactions were carried out using the BigDye v1.1 kit (ABI) and run on an ABI 3100 automated sequencer. As nuclear translocations of mitochondrial DNA (numts) have been previously diagnosed in *Podarcis* spp. (Pinho et al., 2006; Podnar et al., 2007), sequences were inspected for hallmarks commonly associated with nuclear contamination (Bensasson et al., 2001; Triant and DeWoody, 2007). The cytochrome *b* gene coding region (positions 24–448) of the mitochondrial DNA alignment included in the present study was translated and checked for frameshifts and indels using the program MEGA v3.1 (Kumar et al., 2004). Differences in transition–transversion rates and synonymous to non-synonymous substitution rates between *P. erhardii* mitochondrial sequences were compared to those of a mitochondrial (DQ001019) and numt (DQ001021) sequence pair from *P. sicula* using the program DnaSP (Rozas et al., 2003).

### 2.4. Phylogenetic reconstruction

Phylogenetic analysis of unique mitochondrial cytochrome *b* haplotypes was carried out using maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods. *P. erhardii* sequences obtained from this and previous studies (Poulakakis et al., 2003; 2005a,b) are listed in Supplementary Table 1. MP and ML analyses were carried out using PAUP 4.0b10 (Swofford, 2000) whereas Bayesian analyses were carried out using the Monte Carlo Markov Chain (MCMC) method implemented in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). For parsimony analyses, a starting tree was obtained using the stepwise addition option and heuristic searches were conducted using the tree-bisection-reconnection (TBR) heuristic algorithm. All character changes were considered unordered and unweighted. The strength of support for individual nodes was assessed by 300 bootstrap replicates of the data.

For ML analysis, model parameters were initially estimated from the best-fitting model of substitution identified by MODELTEST v3.6 (Posada and Crandall, 1998) using the Akaike Information Criterion. MODELTEST identified the transversion model (Zharkikh, 1994) as the model that best explained the data, with an alpha shape parameter value for the gamma distribution of 1.4228 and proportion of invariant sites equal to 0.5598. Subsequent searches were performed using an iterative procedure similar to that of Sullivan et al. (2005): (1) An initial starting tree using these initial MODELTEST parameters was built using the neighbor-joining method; (2) model parameters were then estimated and fixed to perform searches using the TBR heuristic search algorithm; (3) the resulting tree was then used as a starting tree and searches were performed using the nearest neighbor interchange branch swapping algorithm, estimating all parameters simultaneously. Steps 2 and 3 were then repeated until there was no change in tree topology and ML values. Support for individual branches in the ML tree was assessed with 300 bootstrap replicates using the re-estimated parameter values and the starting tree resulting from the above iterative procedure.

For Bayesian analyses, a general time-reversible model (Tavaré, 1986) was adopted that allowed for among-site rate variation and a proportion of sites to be invariant. Prior probabilities for model parameters were not defined *a priori* and were left at their default values. In order to ensure that the MCMC had not been trapped in local optima (Leaché and Reeder, 2002), output from each of two separate analyses, consisting of three heated chains and a cold chain, was compared to each other using the program TRACER (Rambaut and Drummond, 2007). The proportion of samples to be discarded as "burn in" was assessed by looking at the output from the *sump* command in MrBayes and by examining the MCMC trace files. In each case, runs were only accepted if the effective sample size (ESS) was greater than 500 for all model parameters. Convergence among runs and across analyses was assessed by verifying whether different runs attained the same stationary distribution and average log likelihood values. Chains were run for 20,000,000 iterations and were sampled every 10,000th generation. Support for a specific node was accepted if the relevant bootstrap value was $\geqslant$75% and posterior probabilities were $\geqslant$0.95.

### 2.5. Microsatellite isolation and amplification

Twenty-seven microsatellite primer pairs previously isolated from other lacertid lizards were tested on *P. erhardii* (Boudjemadi et al., 1999; Nembrini and Oppliger, 2003; Pinho et al., 2004). Of these, only 13 primer pairs amplified fragments of the expected size: Lv319 and Lv2145 (Boudjemadi et al., 1999); A7, B7, C8, D1 and C24 (Nembrini and Oppliger, 2003); Pb10, Pb20, Pb50 and Pb66 (Pinho et al., 2004); Pod1a and Pod8 (Poulakakis et al., 2005c). These candidate loci were screened for allele length poly-

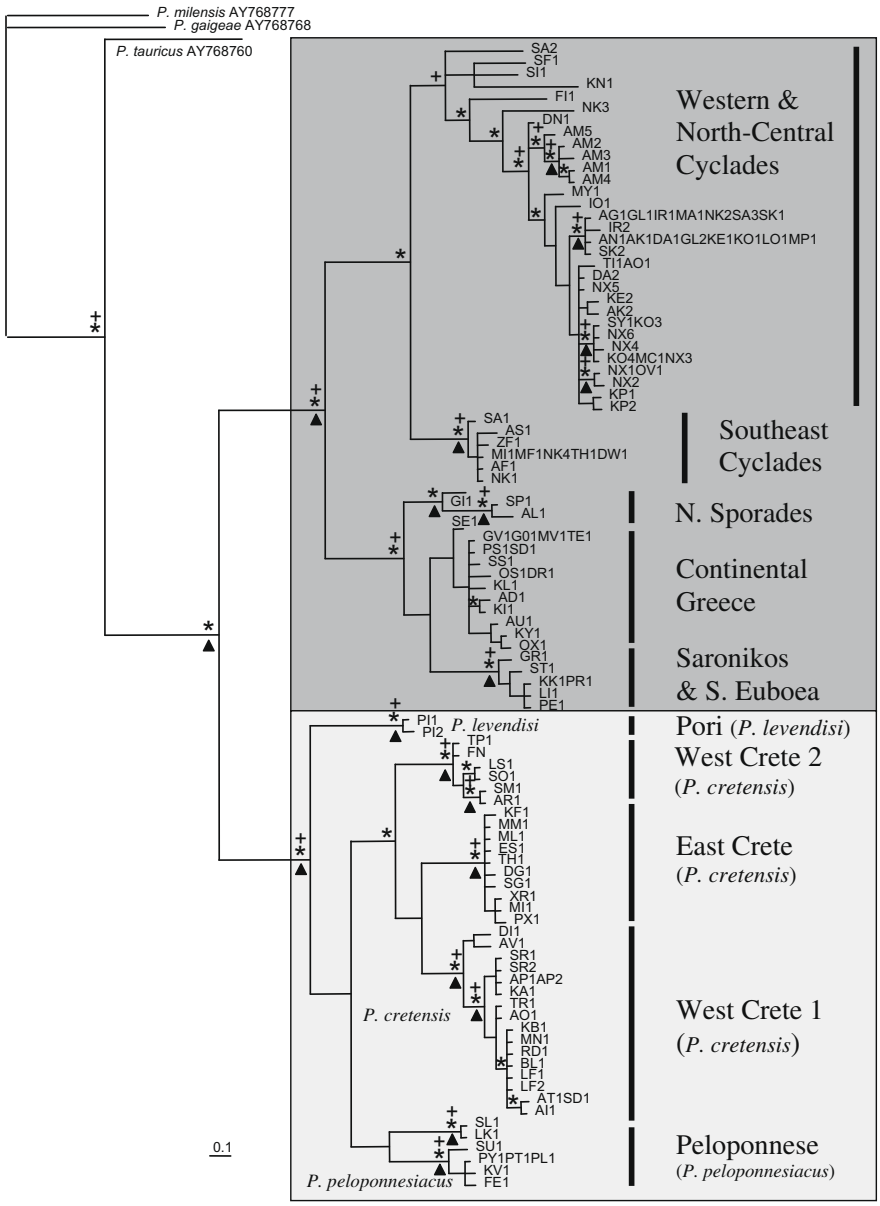


**Fig. 2.** Bayesian analysis of *Podarcis* mtDNA cytochrome *b* sequences. Posterior probability values ⩾0.95 are indicated (*) adjacent to the node of interest. Nodes supported by either maximum parsimony (+) or maximum likelihood (▲) are also indicated above and below the relevant branch. Haplotype identification codes correspond to the island codes given in Supplementary Table 1. Note that branch labels are identical to those in Poulakakis et al. (2005a). The two major geographic lineages are indicated by the two shaded boxes.

morphism(s) on a 5% polyacrylamide gel using the Protean IIxi Cell electrophoresis system (BIORAD). The six loci that were found to be variable were sequenced to verify the presence of a microsatellite repeat. Of these six loci, only five contained microsatellite repeats. All five loci were subsequently co-amplified using the multiplex kit (Qiagen) and analyzed on an automated sequencer using the Genemapper v.4.0 software (ABI).

### 2.6. Statistical analyses

The program ARLEQUIN V3.11 (Excoffier et al., 2005) was used to estimate cytochrome *b* haplotype and nucleotide diversity. The program TCS version 1.21 (Clement et al., 2000) was used to construct a network between all mitochondrial haplotypes sampled from islands in the present study within the North-central and Southeastern Cyclades based on a 95% maximum parsimony criterion.

A Spatial Analysis of Molecular Variance or SAMOVA (Dupanloup et al., 2002) was also carried out on the mitochondrial dataset in order to independently assess whether maximally differentiated island clusters reflected the known fragmentation history of the islands selected for this study. The islands of Fira, Nea Kameni and Santorini were excluded from this analysis because of their independent history relative to the other islands in the dataset.

Microsatellite alleles were classified into bins using the program FLEXIBIN (Amos et al., 2007). Exact tests for deviations from Hardy–Weinberg equilibrium were carried out using the program ARLEQUIN V3.11. A Holm–Bonferroni correction was employed to determine the appropriate critical value for rejection of the null hypothesis. The possibility of Null alleles and other genotyping errors (short allele dominance and misclassification of alleles due to stutter) was assessed using the program MICRO-CHECKER (Van Oosterhout et al., 2004). Lastly, linkage

disequilibrium between loci was assessed using a likelihood ratio test implemented in ARLEQUIN v3.11 (Excoffier and Slatkin, 1998). The null hypothesis of linkage equilibrium was rejected at a critical value consistent with the relevant Holm–Bonferroni correction.

ARLEQUIN V3.11 was also used to estimate expected microsatellite heterozygozity ($H_E$), average number of alleles ($A$), the bottleneck statistic ($M$) (Garza and Williamson, 2001) and construct a pair-wise microsatellite $F_{ST}$ distance matrix between islands. A Mantel test was carried out in order to assess whether there was a significant association between genetic and geographic distances between islands using the program IBDWS (Jensen et al., 2005). Bottleneck detection was carried out using both the $M$ statistic which varies from 0 to 1.0 (Garza and Williamson, 2001) and observed shifts in allele frequency distribution relative to a large, outbred population (Luikart et al., 1998). The $M$ statistic is obtained by dividing the number of alleles by their total size range. In large populations, allele frequency distributions are expected to have a high proportion of rare alleles. However, following a bottleneck event many rare alleles are lost and the distribution of alleles is often shifted towards higher frequency classes. It then follows that a large, stable population with many alleles is expected to have an $M$ value close to 1.0 whereas populations subject to a bottleneck event will be closer to 0. Simulations have shown that this reduction in $M$ is proportional to the severity and duration of the bottleneck (Garza and Williamson, 2001).

Multiple linear regression analysis was used to determine what proportion of the variation in each of the three genetic variables ($A$, $H_E$ and $M$) was explained by island age, area and distance to the nearest larger land mass. Island area was log-transformed and island age was square-root transformed to normalize the distribution of values (Vittinghoff et al., 2005). To test for the effects of immediate ancestry on levels of genetic variation, an additional variable coding for whether an island directly descended from another island (0) or from the original Protocycladic landmass (1) was included in a subset of multiple regression analyses. Seven islands (Andreas, Daskalio, Glaronissi, Koufonissi, Megali Plaka, Megalos Ambelas and Schoinoussa) separated from other islands while the rest separated directly from the Protocycladic block. The effect of human activity on genetic variation was also assessed by including a variable coding for the absence (0) or presence (1) of a permanent human population on an island. All multiple regression analyses were conducted in SAS v9.1 (SAS Institute, Cary NC) or SPSS 14.0 (SPSS Inc., Chicago, IL).

A generalized linear model approach based on a hierarchical Bayesian method implemented in the program GESTE (Foll and Gaggiotti, 2006) was carried out to examine the effects of age, area and isolation on population-specific estimates of $F_{ST}$. Posterior probabilities for all nine possible models (based on two environmental variables and their interaction term) were compared in order to identify the best combination of environmental factors that explains the distribution of island $F_{ST}$ values. Due to limitations of the program, only two factors could be considered at once so models were tested with the following combination of environmental variables and their associated interaction term: area and age; area and distance; age and distance. Using MCMC methods, 10 pilot runs of 200 iterations in length were used as burn-in prior to drawing samples from a chain of 100,000 in length, separated by a thinning interval of 10. The length of this chain was extended 10-fold and ran a further three times. In order to check for convergence, traces for each parameter were compared within individual runs to verify that the samples had been drawn from the stationary phase of the posterior distribution.

## 3. Results

### 3.1. Mitochondrial data

Mitochondrial cytochrome *b* gene sequence data were obtained from 190 samples of *P. erhardii* collected from 20 islands distributed throughout the Cyclades (Table 1). All sequences were deposited in GenBank and are accessible as FJ895617-FJ895806. When data were combined with sequences from previous work (Poulakakis et al., 2003; 2005a,b), a total of 285 sequences were obtained, of which a total of 99 unique haplotypes were identified. The following sequences were used as outgroups in all phylogenetic analyses: *P. milensis* AY768777, *P. gaigeae* AY768768 and *P. tauricus* AY768760. Within the haplotype network for the Cyclade island populations sampled in the present study, 22 unique haplotypes were found. Many island lizard populations were fixed for a single mitochondrial haplotype (Table 2). The low overall nucleotide diversity ($\pi$) within islands with two or more haplotypes reflects the shallow divergence among sequences. The greatest amount of haplotype diversity was found in the relatively large islands of Naxos, Nea Kameni, Koufonissi and Irakleia whereas almost all small island populations, excluding the young island of Daskalio and the old island of Kopria, were fixed for a single haplotype.

Translation of mitochondrial sequences including those published in earlier work (Poulakakis et al., 2003; 2005a,b) did not reveal any frameshifts or indels, as might be expected to be found in nuclear pseudogenes that are no longer functional. Similarly, there was no evidence for apparent double banding patterns in sequence chromatograms indicating that only a single sequence type was amplified in each PCR reaction. The ratio of non-synonymous (dN) to synonymous (dS) substitutions was higher in the *P. sicula* numt-mitochondrial comparison (0.0220/0.8190 = 0.026862) than for all *P. erhardii* mitochondrial sequence comparisons (0.00904/0.52507 = 0.017213), reflecting the greater functional constraints likely placed on coding (mitochondrial) sequences. Lastly, transition:transversion ratios were higher in mitochondrial–mitochondrial comparisons (30/7 = 4.2) compared to the *P. sicula*

**Table 2**
Mitochondrial and microsatellite diversity of the populations included in this study.

| | $H_d$ | $\pi$ | $A$ | $H_E$ | $M$ |
|---|---|---|---|---|---|
| Agrilou | 0.00000 | 0.00 | 4.2 | 0.5450 | 0.3202 |
| **Amorgos** | **0.90000** | **0.00564** | ~ | ~ | ~ |
| Andreas | 0.00000 | 0.00 | 2.8 | 0.3990 | 0.2903 |
| Antikeros | 0.15384 | 0.00036 | 6.2 | 0.5836 | 0.3650 |
| Daskalio | 0.16667 | 0.00078 | 6.0 | 0.6119 | 0.3966 |
| **Fira** | **0.00000** | **0.00** | **1.4** | **0.1542** | **0.7905** |
| Glaronissi | 0.00000 | 0.00 | 5.0 | 0.6402 | 0.3280 |
| Irakleia | 0.38889 | 0.00182 | 9.4 | 0.7676 | 0.3631 |
| Keros | 0.22222 | 0.00057 | 6.4 | 0.6074 | 0.4574 |
| Kopria | 0.18182 | 0.00042 | 4.2 | 0.5096 | 0.3703 |
| Koufonissi | 0.43636 | 0.00336 | 4.4 | 0.5835 | 0.2572 |
| **Loumboudiaris*** | ~ | ~ | ~ | ~ | ~ |
| Makronissi | 0.00000 | 0.00 | 5.0 | 0.6047 | 0.5081 |
| Megali Plaka | 0.00000 | 0.00 | 2.4 | 0.3917 | 0.4264 |
| Megalos Ambelas | 0.00000 | 0.00 | 4.0 | 0.5835 | 0.3836 |
| Naxos | 0.69853 | 0.00679 | 12.6 | 0.7691 | 0.4899 |
| **Nea Kameni** | **0.56364** | **0.03240** | **8.6** | **0.7144** | **0.4058** |
| Ovriokastro | 0.00000 | 0.00 | 6.2 | 0.5518 | 0.4595 |
| **Santorini*** | ~ | ~ | ~ | ~ | ~ |
| Schoinoussa | 0.00000 | 0.00 | 8.0 | 0.7543 | 0.4248 |

*Note:* Islands in **bold** were not included in multiple regression and isolation by distance analyses.
$H_d$, mitochondrial haplotype diversity; $\pi$, nucleotide diversity; $H_E$, mean expected heterozygozity; $A$, mean allelic richness averaged over all microsatellite loci; $M$, bottleneck statistic of Garza and Williamson (2001).
* Haplotype diversity estimates were omitted for sample sizes <5.

mitochondrial–nuclear comparison (43/14 = 3.1), consistent with the transitional bias expected in mitochondrial sequences.

All three phylogenetic methods provided strong support for two major geographically distinct lineages: (1) a group containing *P. peloponnesiacus* from the Peleponnesian mainland, as well as the recently described *P. cretensis* populations from Crete, and *P. levendisi* populations from the small island of Pori (see Lymberakis et al., 2008), and (2) a group containing *P. erhardii* populations from the Greek mainland, as well as the Cyclades islands, N. Sporades, Euboea and the Holocene landbridge islets of the Saronikos/Euboikos gulfs (Fig. 2). Within the *P. erhardii* lineage, there is a Northwest–Southeast split in the distribution of haplotypes with samples from continental Greece, Euboea, and nearshore landbridge islets, as well as the N. Sporades group constituting one sub-group and sequences from the Cyclades archipelago constituting another. Within the Cyclades, there are two discernable groups consisting of sequences within the Western/North-Central Cyclades and the ancient deepwater islands of the Southeastern Cyclades. Whereas sequences from the Western Cyclades group (Serifos, Sifnos, Kythnos) in Poulakakis et al. (2005a) are basal to the rest of the Cyclades, these sequences group with the North-Central Cyclades cluster in the present study. In both studies, however, there is no strong support for the Western Cyclades as a diagnosable cluster which coincides with the fact that these islands are separated by relatively deep waters. Many major branches within the tree were consistent across all methods and the overall topologies are very similar, with the exception of the position of the Southeastern Cyclades haplogroup. In Bayesian analyses, this haplogroup is sister to the rest of the Cyclades whereas in both MP and ML analyses this group is nested within the main North-central Cycladic haplogroup. The difference in the tree topologies obtained using these methods may be due to the short time window in which separation of the Western and South eastern island groups occurred. Differences among haplotypes within the Southeastern group are relatively shallow although individuals sampled from Santorini (SA) and Nea Kameni (NK) islands share a common haplotype with lizards from both South eastern and West/North-Central Cycladic haplogroups, consistent with the possibility of rare, anthropogenic long-distance dispersal events.

Close examination of the network of haplotypes from the Cyclades (Fig. 3) illustrates weak regional structuring consistent with the fragmentation history shared by the four island clusters associated with (1) Naxos, (2) Keros, (3) Amorgos and Antikeros, and (4) Irakleia, Schoinoussa and Koufonissi. Haplotypes from Santorini and Nea Kameni are found in disparate locations across both the Central and Southern Cyclades. One class of haplotypes from these two islands was so divergent that they could not be connected to the network because the number of mutational steps was greater than the maximum number allowed for reconstructions with 95% confidence. Similarly, haplotypes from Fira could not be joined to other haplotypes sampled in the Cycladic network.

SAMOVA identified clusters of islands that for the most part reflect the known fragmentation history of the region. With the number of clusters set to five, the among-group component of the total variance (79.89%) approached its maximum and supported the following island clusters: (1) Naxos, Makronissi and Ovriokastro (2) Andreas, Antikeros, Daskalio, Keros, Koufonissi, Loumboudiaris and Megali Plaka (3) Agrilou, Glaronissi, Irakleia, Megalos Ambelas (4) Amorgos (5) Kopria. Increasing the cluster number to six further increased the among-group variance component to its apparent maximum. Although these findings largely reflect the hypothesized historical fragmentation history of the islands under study, there are a few cases where the bathymetric history of an island differs from the observed pattern of genetic clustering: (1) The clustering of Antikeros with its sister island Keros and other islands

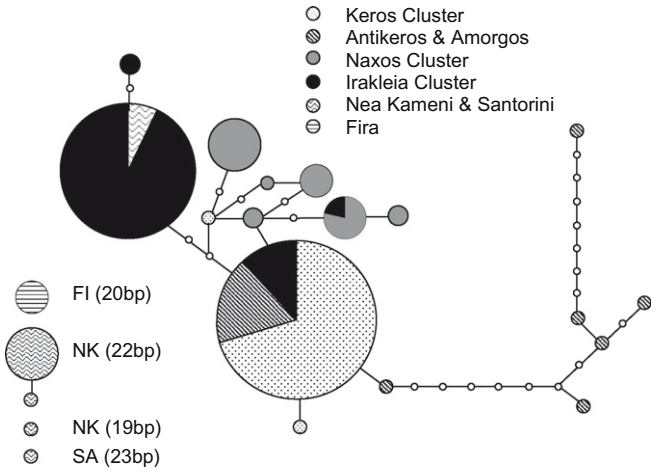


**Fig. 3.** Minimum spanning network of the 22 unique mitochondrial haplotypes of *P. erhardii* found within the Cyclades islands in the Aegean Sea. The size of the circle is proportional to the number of individuals possessing a given haplotype. Islands were placed into clusters based upon their inferred shared history and are as follows: Keros Cluster (AN, DA, KE, LO, MP), Amorgos Cluster (AK, AM), Naxos Cluster (NX, MC, OV, KP), Irakleia Cluster (AG, GL, IR, MA, KO, SK) and Santorini (SA) and its associated islet Nea Kameni (NK).

in this group instead of the larger, more ancient island of Amorgos (2) the clustering of Koufonissi with islands in the neighboring Keros cluster rather than with the moderately sized islands of Irakleia and Schoinoussa to the south (3) the failure of Kopria to cluster with the Naxos group.

### 3.2. Microsatellite data

There were 15 instances of significant deviations from HWE, all of which were due to heterozygote deficiency. Of these, 12 cases were observed for the locus T434, two cases for Lv319 and one case for locus Pb10. MICRO-CHECKER also found overwhelming evidence for the presence of null alleles (Pemberton et al., 1995) at locus T434 in 12 out of 17 populations. Owing to problems associated with this locus, initial statistical analyses were conducted with and without this locus. Results from multiple regression and isolation by distance analyses however did not differ so results for these analyses and those of GESTE are reported for the complete dataset. Linkage disequilibrium (LD) or non-random association of alleles can arise as a consequence of mutation, random genetic drift, selection and population admixture (Hartl and Clark, 2007). Only two islands (Andreas and Nea Kameni) exhibited significant LD at more than one pair of loci after Holm–Bonferroni correction. On the island of Andreas, linkage disequilibrium was detected between Lv319 and Pb10, Lv319 and T434, Pb10 and T434, Pb10 and Pod8, and between T434 and Pod8. On the island of Nea Kameni, linkage disequilibrium was detected between Lv319 and T434 and between Pb10 and Pod8. Single instances of linkage disequilibrium were also detected on the islands of Agrilou (Lv319 and Pod 8), Glaronissi (Lv319 and T434), Koufonissi (Lv319 and T434), Makronissi (Lv319 and Pb10) and Ovriokastro (Lv319 and T434). As the likelihood ratio test assumes Hardy–Weinberg genotype proportions, it is likely that many of these observed deviations from linkage equilibrium are due to heterozygote deficiencies at one or more loci in these populations.

Excluding Fira, the average number of alleles (*A*) varied from 2.4 to 12.6 with the large island of Naxos containing the greatest number of alleles (Table 2). The least number of alleles was observed in Fira, which together with the small Antiparos population (Cattaneo, 1984) may be the only surviving relict of a population that previously inhabited the island of Paros. In general, there appeared

to be no relationship between island history and the bottleneck statistic *M*.

Shifts in allele frequency distributions appeared to reflect the known bottleneck history of the islands under study. Whereas there was no apparent shift in allele frequency distribution in the larger islands of Naxos, Schoinoussa and the small island of Antikeros, a discernible shift was detected in several small islands (Andreas, Agrilou, Daskalio, Glaronissi, Kopria, and Megalos Ambelas). In these cases, the increase in the number of high frequency alleles and the reduction in the number of rare alleles, suggests that these island populations may have experienced a particularly severe historical bottleneck. There was no apparent significant isolation-by-distance effect, despite the fact that $F_{ST}$ values were generally high (Supplementary Table 2).

### 3.3. Multiple regression analyses

In multiple linear regression analyses, neither distance to nearest landmass nor any of the possible interaction terms between the three explanatory variables in the model were significant ($p > 0.10$) and they were subsequently removed from the model. There was a significant positive relationship observed between log (area) and both allele number ($F_{1,12} = 25.60$, $p = 0.0003$) and heterozygozity ($F_{1,12} = 8.69$, $p = 0.0122$), suggesting that populations inhabiting bigger islands are larger in size and thus retain more genetic variation (Fig. 4a and c). A significant negative relationship was also observed between the square root transformation of island age and allele number ($F_{1,12} = 6.09$, $p = 0.0296$; Fig. 4b), indicating that populations that are isolated for longer periods of time lose more variation due to the cumulative effects of drift. However, this relationship was not significant for heterozygozity ($F_{1,12} = 1.01$, $p = 0.3357$; Fig. 4d). The *M* statistic was not significantly related to island age, area or distance ($p > 0.05$). However, when one problematic locus with a limited allele range was removed (Pod1a), a negative correlation was observed between island age and *M* ($F_{1,13} = 4.99$, $p = 0.0437$). There was also no significant effect ($p > 0.05$) of either immediate island ancestry or human presence on any of the genetic response variables and these dummy variables were subsequently discarded from the regression model.

Results from replicate runs of the generalized linear model implemented in the program GESTE gave consistent findings. Moreover, lengthening the chain did not result in any substantial changes to the results. Strong posterior support ($\geqslant 0.85$) for a model consisting of only area and the regression constant was consistently observed whereas substantially lower support ($\leqslant 0.13$) was observed for models that included area with either of the other two variables (age, distance). Similarly, posterior support was moderately weak (<0.55) for a model containing both age and distance and negligible for either of these factors alone. The relationship between population-specific $F_{ST}$ and area was always negative, suggesting that smaller islands are more highly differentiated than larger islands in the study system. Although support for an age or distance effect was weak, $F_{ST}$ values were always negatively correlated with age and positively correlated with distance, suggesting that older and/or more distant islands are more highly differentiated.

## 4. Discussion

This study examines the effect of island fragmentation history on genetic variation in a widespread species of Aegean lizard. Overall, mitochondrial data shows greater sub-structuring than previous studies have shown (Poulakakis et al., 2003; 2005a,b) due to more intensive sampling efforts within islands and the addition of previously un-sampled islands. Although mitochondrial genetic variation within islands was low, patterns of variation between

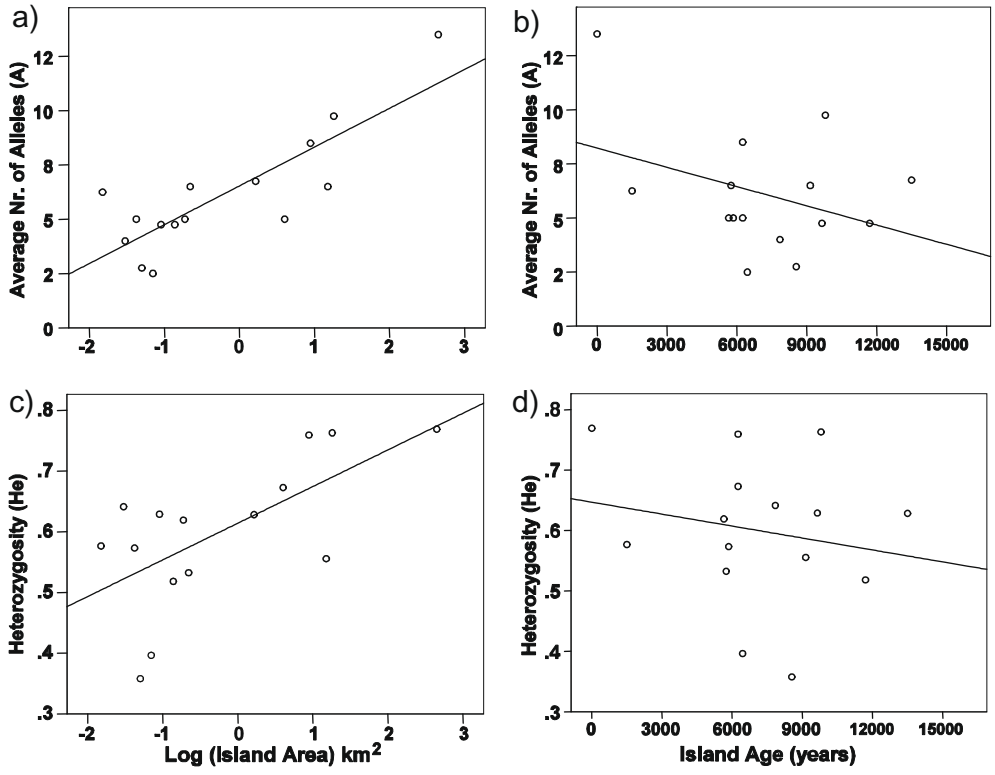


**Fig. 4.** The effect of island area and age on two measures of microsatellite diversity. The top row depicts the relationships between the average number of alleles and either (a) log-transformed island area ($km^2$) or (b) inferred island age (years). The bottom row similarly illustrates the relationship between heterozygosity and (c) log-transformed island area or (d) inferred island age.

islands suggest that the distribution of haplotype diversity has been shaped by their fragmentation history. Our results indicate that while high haplotype diversity appears to have persisted on the large island of Naxos, other smaller islands have been drifting towards fixation since isolation. In general, islands that share a recent common history (i.e. were recently connected to one another) possess closely related haplotypes.

Of the islands examined in the present study, the islands of Fira, Santorini and Nea Kameni have experienced a very different history from the remainder of the islands under study. The isolated Fira population possesses a haplotype that is highly divergent from others within the region, despite its shared history with the Protocycladic block (Van Andel and Shackleton, 1982; Perissoratis and Conispoliatis, 2003). This genetically distinct population may have survived as a relict of the ancestral population present at one time but now extinct on the island of Paros. Alternatively, the Fira haplotype might be another product of a long-distance dispersal event from another, as yet un-sampled, island in the region or is a numt. Further sampling in the region should help validate or refute this hypothesis.

Nea Kameni is volcanic in origin and is located inside the submerged Santorini caldera. Neither Santorini nor Nea Kameni share a common fragmentation history with any of the other islands included in this study and have been isolated for more than 200,000 years from the Protocycladic block (Beerli et al., 1996). However, both Santorini and Nea Kameni share a common haplotype with lizards within the Irakleia island cluster more than 50 km to the north. This disjunct haplotype distribution is likely due to one or more long range dispersal events between these regions. Nea Kameni and Santorini are unusual in that both constitute major tourist destinations so that the dense boat traffic between Santorini and other Cycladic islands may have also facilitated long-distance dispersal.

With the exception of this apparent long-distance colonization event, there is little indication of gene flow between islands. Support for this observation is provided by the apparent historical partitioning of mitochondrial haplotypes, absence of any effect of distance on genetic variability and lack of any significant isolation-by-distance relationship. The pattern observed in the present study fits well with the Case III model described by Hutchison and Templeton (1999) and indicates that drift is much more influential than gene flow in shaping patterns of observed genetic variation between neighboring islands. Interestingly, a previous study of lizard distributions on land bridge islands found distance to mainland to be the weakest explanatory variable of island species composition (Case, 1975), thus also suggesting that in a system with very little gene flow, inter-island distance poorly predicts species dispersal. A more recent study by Foufopoulos and Ives (1999) also found that present reptile distributions on Aegean islands reflect differences in traits associated with survival in small areas of habitat, and that extinction rather than colonization is the main process structuring the distribution of species in the archipelago.

Many of the small island populations in the present study showed distortions in their allele frequency distributions that are consistent with genetic signatures expected under a population bottleneck (Luikart et al., 1998; Spencer et al., 2000). However, the ratio of allele number to range in allele size ($M$) did not appear to be a reliable indicator of bottleneck history except when the locus Pod1a was removed. The exclusion of Pod1a indicated an inverse relationship between island age and $M$. This conforms to earlier theoretical work that predicts that the $M$ statistic should decrease with increasing bottleneck duration (Garza and Williamson, 2001). Another previous study also found that the $M$ statistic failed to detect a known population bottleneck (Whitehouse and Harley, 2001). Two potential explanations have been offered for these results: (1) that the failure to detect a bottleneck signature could be due to a non-stepwise mutation (mutation events favoring the increase or decrease of allele length by more than one repeat unit instead of only a single step), or (2) constraints on the range in allele size limit the utility of this statistic, as was observed with the locus Pod1a. The $M$ ratio is also very sensitive to the mutation rate and may be affected by changes in the mutational steps used to construct the expected distribution of this statistic (Williamson-Nateson, 2005).

Findings from this study also show that microsatellite variation is positively correlated with area and negatively with age. However, allelic richness ($A$) was the only measure that exhibited a significant association with both of these island characteristics. Moreover, results from the program GESTE also indicate that area is a key determinant of genetic differentiation, consistent with the theoretical expectation that among population differentiation will increase as the effects of drift accumulate (Hartl and Clark, 2007).

Allelic richness was a much more sensitive indicator of bottleneck history than expected heterozygozity as both theoretical (Nei et al., 1975; Spencer et al., 2000) and experimental (Leberg, 1992) studies have suggested. Specifically, bottleneck theory predicts that in the absence of immigration and mutation, rare alleles will be lost much more rapidly than heterozygosity following a population bottleneck event (Frankham et al., 2002). Although the high rates of mutation in microsatellite loci (Whittaker et al., 2003) may have increased the likelihood of new alleles arising in larger and older island populations, many of the islands in this study are very small in size and therefore less likely to have gained alleles through mutation since their isolation. In the present study, only four islands contained private alleles: Irakleia, Makronissi, Naxos and Ovriokastro. Although Irakleia and Naxos are large compared to the other islands in the study, Makronissi and Ovriokastro are small in size and of moderate age, making it difficult to speculate as to whether the private alleles on these islands are due to accumulation of *de novo* mutations or simply due to the fact that we failed to sample these alleles in other islands.

Results from the multiple regression models also suggest that levels of population genetic variation are not affected by immediate ancestry, i.e. whether a given island broke off from the main Protocoycladic block or from another smaller island. Our findings also indicate that there was no effect of human presence on lizard genetic diversity, suggesting that human activities have not promoted over-water transport of lizards. This may in part be due to the lack of asexual reproduction in *P. erhardii* and the fact that the species lays its eggs only in deep soil crevices rather than transportable vegetation matter (Gruber, 1986).

In conclusion, both island age and area appear to exert an important influence on the retention of population genetic diversity whereas the impact of gene flow appears negligible. To the best of our knowledge, the drift-related effects of age on genetic variation have not yet been convincingly documented in naturally replicated island populations. In contrast to observations by Gorman et al. (1975), findings here demonstrate convincingly the importance of drift in our system. These results also demonstrate the utility of landbridge islands as model systems for exploring the effects of habitat fragmentation (Shafer, 1990) and interactions between demographic history and genetic variation (Lande, 1988). Loss of genetic variation in small populations that have been isolated for protracted periods of time may potentially compromise future evolutionary potential. Our data also suggest that reptile populations isolated on small habitat fragments due to anthropogenic activities will likely lose genetic variation over the long term. Future work should contrast findings based on neutral markers with loci potentially under selection such as loci of the major histo-compatibility complex (Aguilar et al., 2004; Seddon and Baverstock, 1999; Hinten et al., 2003; Miller and Lambert, 2004). In

this way, the potential fitness consequences of historical fragmentation can be more comprehensively assessed.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.03.028.

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