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

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**A B S T R A C T**

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.

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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 heterozygosity 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 heterozygosity 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
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 
that 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 varia-
tion can then be evaluated and the relative importance of the mag-
nitude 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 assess-
ment 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 coun-
terparts (Eldridge et al., 1999; Frankham, 1997, 1998; Hinten et al., 
2003). However, few studies have systematically attempted to dis-
entangle the effects of island area and age on levels of genetic var-
iation and almost all of these used allozyme markers (Capula, 1996; 
Capula and Cecarelli, 2003; Gorman et al., 1975; Soulé and Yang, 1997).

Island lizards have proved to be ideal organisms for studies of 
biogeographical and colonization history (Capula, 1996; Capula 
and Cecarelli, 2003; Pinho et al., 2008; Poulakakis et al., 
2005a,b), invasive species biology (Kolbe et al., 2004) and ecologi-
cal speciation (Butler et al., 2007; Losos et al., 2006; Ogden and 
Thorpe, 2002). Island lizard populations also make excellent mod-
els for studying the effects of fragmentation history on genetic var-
iation. For example, in a study on the effects of island history on 
patterns of allozyme variation in Lacertid lizards inhabiting off-
shore islands in the Adriatic, Gorman et al. (1975) argued that 
directional selection was responsible for the decline in genetic vari-
ation 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 km²). Similarly, in their study on the channel 
island lizard Uta stansburiana, Soulé and Yang (1973) hypothesized 
that low allozyme heterozygosity 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 po-
tential non-neutrality, making it difficult to determine whether ob-
served patterns of diversity are due to the effects of selection or 
drift, or a combination of both (Hinten et al., 2003). Another poten-
tial drawback of such markers is that unlike nuclear microsatel-
llites, allozymes show very low levels of polymorphism and hence 
do not possess the resolution needed to compare island populat-
ions with subtle differences in fragmentation history. 

The islands of the Aegean Sea in the north-eastern Mediterra-
nean basin represent an ideal system for exploring the effects of 
historical fragmentation on genetic variation in island lizards. Ae-
gean 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 se-
quence 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 frag-
mamentation 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 Ae-
gean 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 pre-
clude 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 dis-
tances 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 mag-
nitude and duration of the bottleneck resulting from island forma-
 tion 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 his-
tory on genetic variability in an island lizard and tease apart the ef-
fects 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 pat-
terns 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 popul-
ations, as predicted in a study system where drift should vastly out-
weigh 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 popula-
tion-specific measures of genetic differentiation. If island area and 
age are reliable predictors of population size and time since isola-
ton, then smaller, older islands will be more susceptible to drift 
and thus exhibit a greater level of genetic differentiation. Con-
versely, 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 (Kiklad-
hes) island group situated in the central Aegean Sea, Greece 
(Fig. 1). These islands span a wide range of sizes and periods of iso-
lation while still sharing a common geologic history and similar 
ecological and environmental conditions. All study islands are frag-
ments 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 frag-
mented 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 Consopo-
liatis, 2003).

Islands sampled in the present study fell into one of two very 
different size range categories: small islands of <1.64 km² and lar-
ger islands of 8.83–448 km². To calculate island age and hence pro-
vide 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 resolu-
tion information on the maximum depth of the underwater saddle 
connecting each island to the next largest land mass obtained from
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, Fl (Fira), GL (Glaronissi), IR (Irakleia), KE (Keros), KO (Koufonissi), KP (Kopria), LO (Louboundiaris), 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.

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 Peloponese 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
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’: GCTGTTAAGAAGAGCCTGTT; R-5’: CTTGCAATTGATATTGGTCC). 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 Mg2+, 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 with 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 (Huelsbeck 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 Akaiake 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 neighboring-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 samp 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 > 75% and posterior probabilities were > 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-
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
Mitochondrial cytochrome b gene sequence data were obtained from small 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 (*θ*) 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 Iatrkleia 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 (0.02220/0.8190 = 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*
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 Peloponnesian mainland, as well as the recently described *P. cretensis* populations from Crete, and *P. levendisi* populations from the small island of Poros (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/Eubois gulf (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 (79.85%) approached its maximum and supported the following island clusters: (1) Naxos, Makronissi and Ovriokastro (2) Andreas, Antikeros, Daskalio, Keros, Koufonissi, Louboudiaris and Megali Plaka (3) Agriolou, Glaronissi, Irakleia, Megalos Ambelas (4) Amorgos (5) Kopenia. 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 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 L1319 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, 2 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 L1319 and Pb10, L1319 and T434, Pb10 and T434, Pb10 and Pod8, and between T434 and Pod8. On the island of Nea Kameni, linkage disequilibrium was detected between L1319 and T434 and between Pb10 and Pod8. Single instances of linkage disequilibrium were also detected on the islands of Agriolou (L1319 and Pod8), Glaronissi (L1319 and T434), Koufonissi (L1319 and T434), Makronissi (L1319 and Pb10) and Ovriokastro (L1319 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 (Catta neo, 1984) may be the only surviving relic 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, Schinoussa and the small island of Antikythera, a discernible shift was detected in several small islands (Andreas, Agirlou, Daskalio, Galaronissi, Kornia, 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 heterozygosity \( (F_{1,12} = 8.69, p = 0.0122) \), 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 \( (F_{1,12} = 6.09, p = 0.0296; \text{Fig. 4b}) \), 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 heterozygosity \( (F_{1,12} = 1.01, p = 0.3357; \text{Fig. 4d}) \). 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 \((>0.85)\) for a model consisting of only area and the regression constant was consistently observed whereas substantially lower support \((<0.13)\) 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
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 Consopoliatis, 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 mutnt. 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 Hatchinson 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 landbridge 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 Fofopoulos 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).

Nucellar richness was a much more sensitive indicator of bottleneck history than expected heterozygosity 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, 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 Protocycladic 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 histocompatibility complex (Aguliar et al., 2004; Seddon and Baverstock, 1999; Hinten et al., 2003; Miller and Lambert, 2004).
this way, the potential fitness consequences of historical fragment-
tation 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.

References

Bromadi, K., Martin, O., Simon, J.C., Estoup, A., 2009. Development and cross-


Frankham, R., 1997. Do island populations have less genetic variation than mainland populations? Heredity 78, 311–327.

Boudjemadi, K., Martin, O., Simon, J.C., Estoup, A., 1999. Development and cross-


