

Genetic divergence between and within two subspecies of *Laudakia stellio* on islands in the Greek Cyclades

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The study of genetic differentiation between allopatric island populations should ideally account for regional palaeogeography, as this can often help to explain current distribution patterns. Here we present a study of two subspecies of the lizard *Laudakia stellio* in the Greek Cyclades, an excellent model for studying vicariant speciation over a relatively short geological timescale: *L. s. stellio* on the islands of Mykonos and Delos; and *L. s. daani* on Paros and Naxos. Using AFLP techniques, we demonstrate a high degree of genetic differentiation both between and within the two subspecies, and relate this to the known palaeogeography of this region. Our results suggest that the genetic differences between the populations of the two subspecies can be explained by a more recent colonization of Paros and Naxos by *L. s. daani*, with *L. s. stellio* having been established in the Cyclades for a longer period of time.

Key words: amplified fragment length polymorphism (AFLP), allopatric speciation, population structure, lizard

INTRODUCTION

Traditionally, phenotypically distinct allopatric populations of a species have been labelled as subspecies (as opposed to being identified as new species), under the assumption that if they were to come into secondary contact they would be sufficiently genetically and behaviourally similar to interbreed and produce viable offspring (Boggs, 2001; Coyne & Orr, 2004; Mallet, 2006). However, this assumption does not seem particularly reliable. Tests of the ability to interbreed are rare (Ford, 1985; Gyllensten et al., 1985), and data are therefore probably too sparse to allow general rules to be drawn up. In this context, phenotypic divergence between previously allopatric subspecies that have come into secondary contact can be misleading, because phenotypic plasticity can either exaggerate or minimize the apparent divergence between allopatric populations. The question of secondary contact is also difficult to assess, because the timescales over which speciation is likely to occur are often long enough for phenomena such as global cooling and warming, as well as sea level fluctuations. Profound geological changes have the potential to alter the distribution of animals on a large scale, potentially splitting or reuniting populations (Brown et al., 2000; Macey et al., 2000; Mathee & Flemming, 2002; Oliverio et al., 2000).

Lizards of the genus *Laudakia* living on islands in the Mediterranean provide an excellent model with which to investigate how geological processes have impacted on genetic differentiation and speciation. *Laudakia stellio* (Linnaeus 1758) is up to about 300 mm long (including the tail) with a dorsoventrally flattened body, triangular head, well-defined neck and long legs and tail. The body is covered in small interlocking scales and there are rows of tubercles across the dorsal surface which, coupled with

the pointed scales on the limbs and tail whorls, act to give this species a somewhat spiky appearance (Arnold & Ovenden, 2002). Sharp claws allow for purchase on the rocks and tree trunks that are its natural environment. The species can change colour quite rapidly in response to temperature, becoming lighter when warm and darker when cold. *Laudakia stellio* is a sit-and-wait ambush predator, with a diet consisting largely of arthropods (predominantly hymenopterans), but vegetation, smaller lizards and even hatchling birds are also taken. The reproductive season is from March until early June. Females lay up to two clutches of between six and eight eggs in nests they dig in the ground, and the eggs hatch after about two months (Arnold & Ovenden, 2002).

Allopatric populations of two subspecies, *L. s. stellio* (Linnaeus 1758) and *L. s. daani* (Beutler & Frör 1980), occur on islands in the Greek Cyclades. They are distinguished by clear morphological, chromatic and behavioural differences. Thus, Almog et al. (2005) analysed 35 phenotypic variables from 118 preserved museum specimens of *L. s. stellio* and *L. s. daani* collected from sites in mainland Greece and Turkey, and on a number of Aegean islands. The aim of their study was to test the contested validity of the *L. s. daani* subspecies. As part of their study they performed a number of cluster analyses based on morphological characters in order to confirm the distribution of the two subspecies in this region. They found that the two subspecies are morphologically divergent, with males differing in more variables than females; that *L. s. stellio* is more colourful than *L. s. daani* and has an orange head; that in *L. s. stellio* sexual dimorphism was mainly chromatic as opposed to mensural in *L. s. daani*; and that *L. s. stellio* is restricted to the Mykonos archipelago (Delos, Mikro Rhematiaris, Mykonos, Rinia and Tinos), whereas all other populations of *L. stellio* in

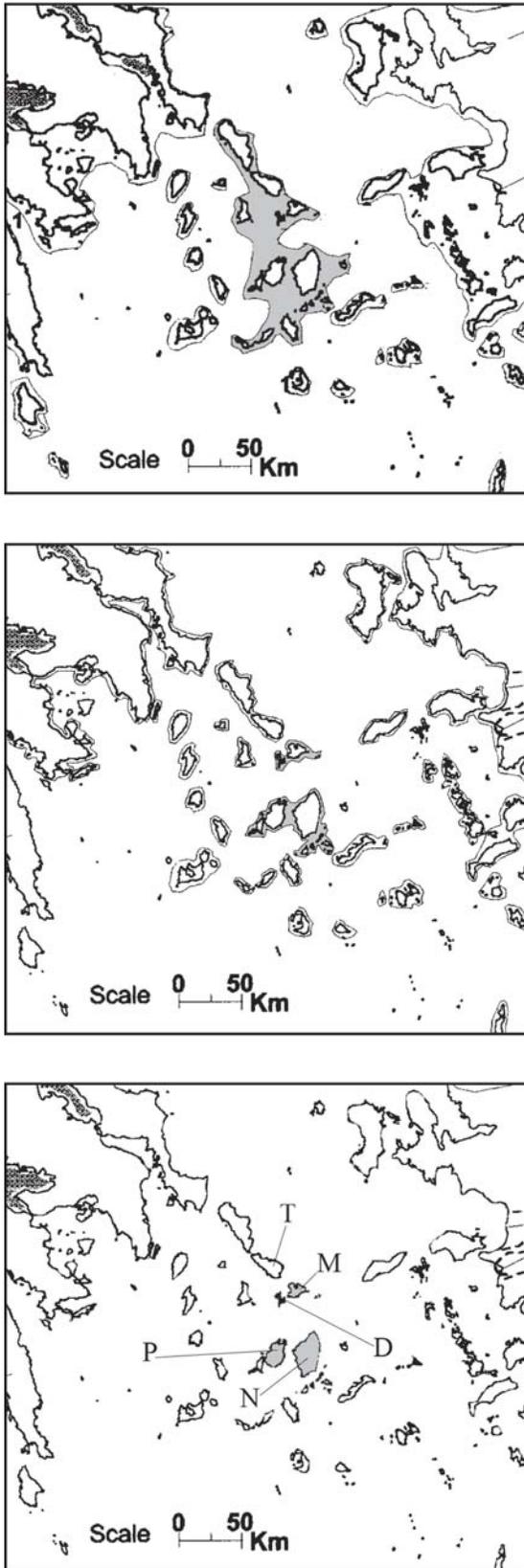


Fig. 1. Historical maps of the Cyclades (adapted from Perissoratis & Conispoliatis, 2003). From top to bottom the maps show coastal outlines for 21,500 years ago, 11,500 years ago and the present day. The landmasses containing the islands in this study have been highlighted in grey. The locations of Mykonos, Delos, Paros, Naxos and Tinos (M, D, P, N and T respectively) are shown in the bottom map for reference.

Greece and Turkey belong to the subspecies *L. s. daani* (Almog et al., 2005).

Brammah (2007) studied extant populations of lizards on four Greek islands, Mykonos, Delos, Paros and Naxos, two carrying *L. s. daani* and two with *L. s. stellio*. He found extensive morphological, chromatic and behavioural differences between these populations. Male *L. s. daani* are larger than male *L. s. stellio* with more callus scales and tail whorls, and longer hind toes. Female *L. s. daani* had deeper heads, more tail whorls and longer femurs than female *L. s. stellio*. On both Mykonos and Delos, *L. s. stellio* had orange heads, whereas on Paros and Naxos, *L. s. daani* had grey heads. In addition, differences in male display structure were also recorded. Males of both subspecies perform push-up displays with a series of large up-and-down movements (bobs), each of which could contain several small amplitude movements (peaks). Each male was capable of both a simple and a complex display, those of *L. s. daani* having significantly more peaks than those of *L. s. stellio*. In addition to these between-subspecies differences, smaller intra-subspecies differences were noted in morphology, head colour and display structure between islands (Brammah, 2007).

The level of phenotypic differentiation observed between lizards on different islands suggests that these populations are currently completely isolated, raising the question of how the islands became populated in the first place. One hypothesis predicting how the islands became populated considers historical changes in sea level: the Protocycladic Block, on which Mykonos, Delos, Paros and Naxos were located, has been isolated from the Greek mainland for over 200,000 years (Beerli et al., 1996). For more recent periods it has proved possible to reconstruct in detail how changes in sea level have impacted on the ways land areas have been linked over the last 21,500 years (Foufopoulos & Ives, 1999; Perissoratis & Conispoliatis, 2003). As a result of rising sea levels, this landmass has since fragmented to form the Cycladic archipelago (Fig. 1). Thus, Mykonos, Delos, Paros and Naxos were all part of the same landmass 21,500 years ago. Since then, the landmass that gave rise to Mykonos and Delos became isolated 12,100 years ago and the islands became isolated from one another 9,500 years ago. The isolation of Naxos occurred 7,450 years ago and that of Paros more recently, the island probably becoming isolated around 1,950 years ago.

To explore the relationship between phenotypic and actual genetic divergence, set in the context of the known geological history of the islands on which they live, we decided to conduct a genetic study based on amplified fragment length polymorphisms (AFLPs) (Mueller & Wolfenbarger, 1999). AFLPs allow the relatively rapid generation of large numbers of anonymous bands without the need for specific marker development, and have proved an effective tool for studying population differentiation in a wide range of species (Bensch et al., 2002; Giannasi et al., 2001; Ogden & Thorpe, 2002; Svensson et al., 2004). All of the data in this study were collected from extant *L. s. stellio* from Mykonos and Delos, and *L. s. daani* from Naxos and Paros.

Table 1. Primer combinations used for the AFLP selective amplification and numbers of AFLP polymorphisms generated.

<i>TaqI</i> primer (5'-3')	<i>EcoRI</i> primer (5'-3')	No. of polymorphic loci scored
GATGAGTCC TGA CCG A\CTG	GAC TGC GTA CCA ATT C\ATG	20
GATGAGTCC TGA CCG A\CGA	GAC TGC GTA CCA ATT C\ACA	11
GATGAGTCC TGA CCG A\CAG	GAC TGC GTA CCA ATT C\ACA	15
GATGAGTCC TGA CCG A\CCA	GAC TGC GTA CCA ATT C\ATG	12
GATGAGTCC TGA CCG A\CCA	GAC TGC GTA CCA ATT C\AGC	7
GATGAGTCC TGA CCG A\CCA	GAC TGC GTA CCA ATT C\AAC	10
GATGAGTCC TGA CCG A\CAC	GAC TGC GTA CCA ATT C\AGC	6
	Total	81

MATERIALS AND METHODS

Tissue sampling

DNA samples were obtained under licence from the Greek Ministry of Agriculture from males, females and unsexed juvenile lizards captured from 17 April to 9 June 2005 on the Greek Cycladic islands of Mykonos, Delos, Paros and Naxos. Samples took the form of a tail clipping approximately 0.5 cm in length, stored in 100% ethanol. Animals were sexed morphologically at sampling, males having a row of thickened callus scales down the centre of the belly and a patch of callus scales just anterior to the cloaca, while females lack these entirely (Almog et al., 2005). In total we collected 136 samples: 18 male, 11 female and four juvenile *L. s. stellio* from Mykonos; 14 male, 11 female and 10 juvenile *L. s. stellio* on Delos; 10 male, 14 female and nine juvenile *L. s. daani* on Paros; 13 male, 12 female and 10 juvenile *L. s. daani* on Naxos. Sampling sites were located so as to collect samples that were representative of each island population as a whole.

AFLP genotyping

AFLP methodology largely followed established methodologies (Vos et al., 1995) and is described in detail by Dasmahapatra et al. (2009). DNA was first extracted using a standard phenol-chloroform extraction protocol (Sambrook et al., 1989). DNA was then quantified on 0.7% agarose gels stained with ethidium bromide. Approximately 100–400 ng of DNA were digested with *TaqI* (5 units in a 10 µl volume, 2 h at 65 °C) followed by *EcoRI* (5 units in a 20 µl volume, 2 h at 37 °C). *EcoRI* and *TaqI* adapters were then ligated on to the digested DNA using T4 DNA ligase (1 unit in a 50 µl reaction volume, 3 h at 37 °C) and the mixture diluted 10-fold with TE. For the pre-amplification, 5 µl of the ligated DNA was added to a 50 µl PCR reaction mix containing Tris-HCl (10 mM, pH 8.3), MgCl₂ (1.5 mM), KCl (50 mM), dATP, dTTP, dCTP and dGTP (0.2 mM of each), *Taq* polymerase (1 unit) and *EcoRI* and *TaqI* primers carrying one selective nucleotide (50 ng of each). The *EcoRI* primer sequence was 5'-GACTGCGTACCAATTCA-3' and the *TaqI* primer sequence was 5'-GATGAGTCCTGACCGAC-3'. Reactions were covered with mineral oil, subjected to 30 cycles (30 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C) and subsequently diluted 10-fold with TE. For the selective amplification, 2.5 µl of the diluted pre-amplification prod-

uct was added to a 12.5 µl PCR reaction mix containing Tris-HCl (10 mM, pH 8.3), MgCl₂ (1.5 mM), KCl (50 mM), dATP, dTTP and dGTP (0.2 mM of each), dCTP (0.04 mM), *Taq* polymerase (1 unit), $\alpha^{32}\text{P}$ -dCTP (0.1 mCi), *EcoRI* selective primer (5 ng) and *TaqI* selective primer (30 ng). PCR products were resolved on 6% polyacrylamide gels and visualized by autoradiography. Samples that showed either unusually faint or absent bands at all loci or occasional spuriously strong bands were not scored for that primer combination. The resulting bands were scored by eye as presence or absence (Table 1). Any samples that failed at three or more of the seven primer combinations were excluded from the analysis.

Data analysis

AFLP-SURV (Vekemans et al., 2002) was used to calculate the proportion of polymorphic loci in each population, gene diversity, overall genetic differentiation among populations (F_{ST}) and pairwise F_{ST} between each of the populations. The program STRUCTURE 2.2.3 (Pritchard et al., 2000) was then used to infer the most likely number of groups present (K) within the AFLP dataset. This program essentially attempts to subdivide the dataset in a way such that any departures from Hardy–Weinberg equilibrium and linkage equilibrium are minimized within the resulting clusters (see Pritchard et al., 2000 for details). We ran five independent runs for $K=1-5$ setting the option RECESSIVEALLELES to 1 (Falush et al., 2003) and using 10^6 MCMC iterations after a burn-in of 10^5 to allow convergence of the MCMC chain prior to data collection. We also specified the default correlated allele frequencies model and assumed admixture. The replicate runs for each value of K were highly concordant for their output log likelihood values. The most likely number of groups was evaluated using both the maximal value of $\ln P(D)$, a model-choice criterion that estimates the posterior probability of the data and ΔK , a statistic based on the second order rate of change of the likelihood function with respect to K (Evanno et al., 2005).

RESULTS

We scored 136 individuals at 81 polymorphic AFLP loci. Populations sampled from Mykonos and Delos had a significantly higher proportion of polymorphic loci and gene diversity than those on Paros and Naxos (proportion of polymorphic loci $\chi^2_3 = 96.67$, $P < 0.001$; gene diversity,

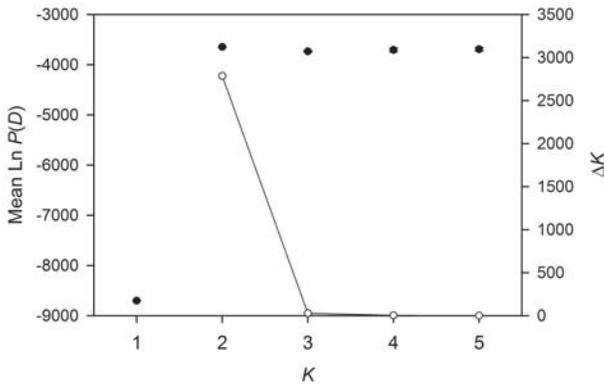


Fig. 2. Results of the STRUCTURE (Pritchard et al., 2000) analysis showing mean \pm SE $\ln P(D)$ and ΔK values (filled and open circles respectively) based on five replicates for each value of K .

ANOVA, $F_{(3,127)}=92.2$, $P<0.001$, Table 2). The four island populations were genetically highly distinct overall, with an overall F_{ST} of 0.62. The permutation test for genetic differentiation among populations based on 500 random permutations of individuals among populations also indicated a strong deviation from the null hypothesis of no difference among the islands ($P<0.0001$). Pairwise F_{ST} values between pairs of populations (Table 3) indicate a high degree of genetic differentiation between the two subspecies, with Paros and Naxos populations also showing marked differentiation.

Applying the program STRUCTURE (Pritchard et al., 2000) to ask whether the same populations could be identified without *a priori* knowledge of sample origin, both $\ln P(D)$ and ΔK peaked at $K=2$ (Fig. 2), providing strong support for the presence of two populations. The two subspecies are readily distinguished (Fig. 3; light grey = *L. s. stellio*, dark grey = *L. s. daani*), STRUCTURE indicating that *L. s. stellio* on Mykonos and Delos form a separate genetic cluster to the *L. s. daani* populations on Paros and Naxos.

Having identified these two major genetic clusters, the STRUCTURE analysis was repeated for each major cluster individually to determine whether or not sub-groups were present within the clusters. For the Mykonos and Delos populations, both $\ln P(D)$ and ΔK peaked at $K=1$, indicating that STRUCTURE was unable to find sub-groups within this cluster. For the Paros and Naxos

populations, both $\ln P(D)$ and ΔK peaked at $K=4$. The resulting plot of cluster membership coefficients reveals some admixture, but the Paros/Naxos split is readily apparent (Fig. 4).

Finally, we investigated the likelihood that *L. s. stellio* evolved from an ancestral population of *L. s. daani* that became isolated when the Mykonos archipelago became isolated from the Protocycladic Block 12,100 years ago. To do this we used the following equation:

$$F_{ST} = 1 - \left(1 - \left(\frac{1}{2Ne} \right) \right)^t$$

where Ne is the effective population size and t is time in generations since isolation (Hartl & Clark, 1997). We specified a generation time (i.e. the time from an individual's birth to the birth of its middle offspring) of three years and an F_{ST} of 0.70 (roughly equivalent to the genetic differentiation between the two subspecies) (Table 3). We then calculated (to the nearest 100 individuals) the population size below which an F_{ST} of 0.70 could be achieved within 12,100 years. This gave a population size of 1,700 individuals (Fig. 5).

For comparison, we estimated approximate current population sizes. This calculation is inevitably simplistic and ignores variation in habitat quality in both space and time. We include it not because we believe it is accurate but to give the reader an idea of the densities this species lives at and how these translate into ball-park estimates of population size given the land-areas of each island. Nine line transects were carried out on Mykonos, each 150 metres long and surveying approximately 1.5 km². Extrapolating from the maximum number of lizards seen on any given transect to the area of each island gives estimates of 4,242, 202, 7,878 and 17,331 animals for Mykonos, Delos, Paros and Naxos respectively. These are certainly only rough estimates, because lizard densities on Mykonos may not correspond to densities on the other three islands, because the transects represent mainly adult territorial males who display in open positions, and because we don't account for the effects of variable male reproductive success or the fact that population sizes are likely to have varied considerably over the last 12,100 years. Nonetheless they give an impression of relative and minimum population sizes. These estimates of current population size suggest that 12,100 years of isolation is insufficient for an F_{ST} of 0.70 to have arisen on any island pair not including Delos (Fig. 5).

Table 2. AFLP polymorphism characteristics for four lizard populations.

Population	No. of samples	No. of loci	No. of polymorphic loci	Proportion of polymorphic loci	Nei's gene diversity (SE)
Delos	34	81	59	72.80	0.229 (0.021)
Mykonos	30	81	55	67.90	0.264 (0.022)
Paros	33	81	17	21.00	0.103 (0.018)
Naxos	34	81	10	12.30	0.049 (0.011)

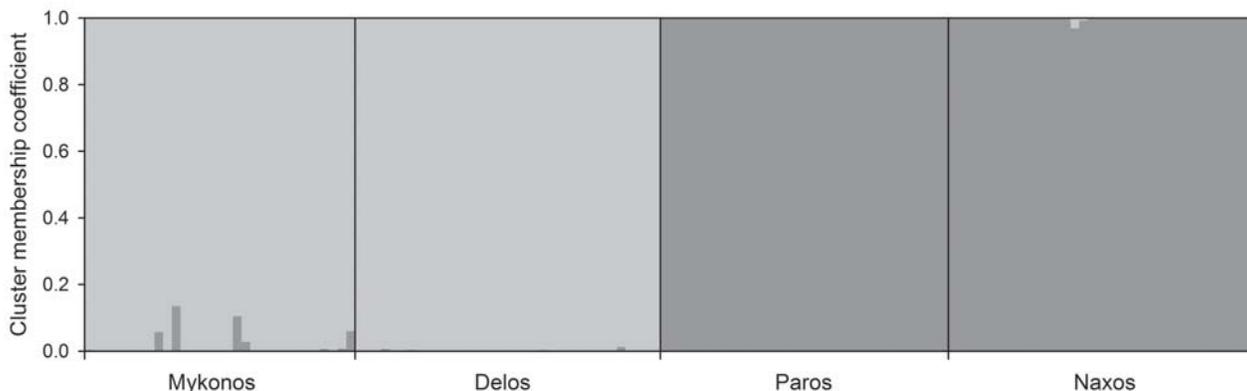


Fig. 3. Summary plot of four lizard populations, showing the estimated membership coefficients for each individual in each cluster. The vertical axis is the proportion of each individual's genome assigned to each cluster. Each individual is represented by a single vertical line and the proportion of light and dark grey represents the proportion of that individual's genome assigned to clusters 1 and 2 respectively.

DISCUSSION

We have analysed AFLP polymorphism data for lizards sampled from four Cycladian islands. We found evidence of clear differentiation between the two putative subspecies, originally named based on slight morphological differences, but also between two island populations carrying members of the same subspecies. Unexpectedly, the greatest differentiation within a subspecies occurred between the largest pair of populations, where neutral drift should act more slowly.

Knowledge of the likely historical changes in sea level in the Mediterranean allows a rather accurate reconstruction of how long different island populations of lizards have been isolated. Within this framework, it is possible to construct a range of competing hypotheses for how the current populations arose (see also, for example, Beerli et al., 1996). The first of these is that the

Protocycladic Block was initially inhabited by a single ancestral population that subsequently evolved into the two subspecies through allopatric speciation. However, this seems unlikely, given the levels of differentiation seen between the different islands. Naxos and Paros, the two largest islands with larger populations, show lower levels of diversity and much greater differentiation than exists between the two smaller islands, Mykonos and Delos. Under neutral drift, the converse is expected, with smaller populations having less diversity and diverging faster than equivalent larger ones (e.g. Coyne & Orr, 2004). Modern populations may not reflect historical events, and it is possible that Naxos and Paros both underwent an extreme bottleneck, reducing diversity and allowing faster differentiation.

The opposite hypothesis is that each of the populations represents a recent colonization from the mainland. As we were unable to sample mainland

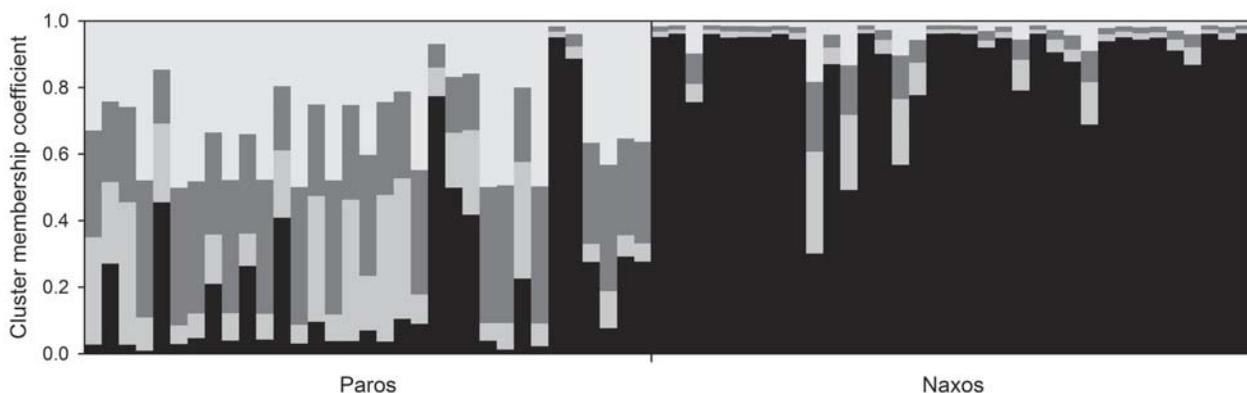


Fig. 4. Summary plot of lizard populations from Paros and Naxos, showing estimated membership coefficients for each individual in each cluster. The vertical axis is the proportion of each individual's genome assigned to each cluster. Each individual is represented by a single vertical line and the proportion of the four different shades of grey represents the proportion of that individual's genome assigned to the four clusters identified by STRUCTURE. The proportion of black in individuals from Naxos indicates genetic differentiation between lizards sampled from these two islands.

Table 3. Pairwise F_{ST} among lizard populations.

	Mykonos	Paros	Naxos
Delos	0.073	0.728	0.775
Mykonos	*	0.685	0.735
Paros	*	*	0.163

populations this hypothesis will remain difficult to disprove, though it again seems unlikely given the generally high genetic homogeneity within each island. This suggests a single colonization event, combined with the relatively small distances between islands, which would tend to promote colonization from the same source(s). If all islands were colonized separately, why not from the same source and, if from different sources, why is there no evidence of animals from two different sources on the same island? Based on the above, it seems more plausible that colonization events are rare, and that the two subspecies probably arose at or before sea level rises divided the Protocycladic Block into its various islands.

To infer more about the history of these lizards we need to consider the current levels of differentiation in more detail. Of the two subspecies, the animals on Paros and Naxos appear closest to what we might expect based on isolation followed by divergence due to drift. These populations also carry the relatively low levels of diversity expected of small populations that have remained isolated for almost 10,000 years. If we assume an effective population size of 10,000, the observed value of F_{ST} of 0.163 would on average be expected after about 3,500 generations, suggesting an entirely plausible generation length of three years. In contrast, the populations on Mykonos and Delos show roughly half the level of differentiation of Paros and Naxos ($F_{ST} = 0.073$), despite being much smaller in size. Using the same equation with an average effective population size of 1,000 suggests only 200 generations would be needed to achieve the observed F_{ST} . Coupled with their relatively high genetic diversity, these calculations suggest a much more recent colonization, taking place well after the two islands separated. Two possibilities exist: either a more ancient event brought lizards to both islands from the same source, or perhaps the close proximity of the islands has allowed for Delos to be repeatedly recolonized and/or augmented

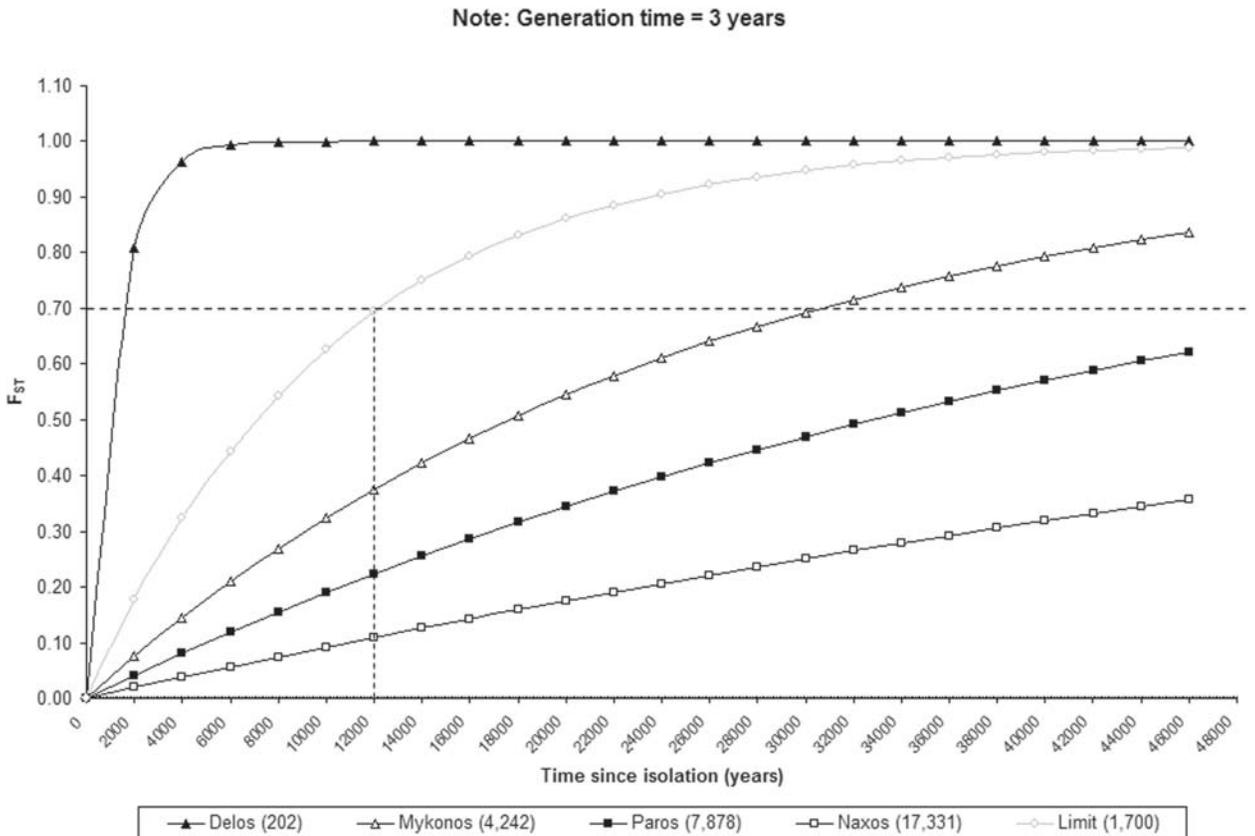


Fig. 5. Chart plotting the curve for the estimated population sizes of Delos, Mykonos, Paros and Naxos according to the equation $F_{ST} = 1 - (1 - (1/2Ne))^t$, where generation time = 3 years. The number of years required to achieve an F_{ST} of 0.70 decreases with declining effective population size (Ne). The curve in grey represents the population size below which an F_{ST} of 0.70 is achievable within 12,100 years.

from Mykonos, thereby reducing divergence. Either seems plausible, but the high levels of diversity relative to island size argue that these lizards arrived more rather than less recently.

Placing these results in the context set by previous morphological studies suggests the two subspecies pre-date the break-up of the Protocycladic Block (see also Almog et al., 2005). Of the two, *L. s. daani* is the most widespread, occurring on the mainland of Greece and Turkey as well as other islands. It is therefore plausible that *L. s. daani* existed on Naxos and Paros as they became separated, these populations subsequently diverging and losing diversity. In contrast, *L. s. stellio* is restricted to the Mykonos archipelago. Thus, while the lizards on Mykonos and Delos were probably of the *L. s. stellio* type as rising sea levels created these islands, the continuing high levels of genetic diversity and low differentiation suggest strongly that these populations have either gone extinct and been reseeded rather recently, or perhaps simply been augmented by immigrants from a larger island, presumably Tinos.

We have been unable to address two important questions: when the two subspecies arose and, more importantly, why their distributions do not overlap. The two “standard” explanations would be competitive exclusion and occupation of niches that differ in some as yet undetermined way. A further possibility is that they evolved their distinctness during the period when the Protocycladic Block was dividing into a northern and southern half, the northern population then diverging more from the ancestral morphology. The clear prediction is that both subspecies should be equally divergent from the mainland *L. s. daani*, something that could be tested should appropriate samples become available. That the time scales involved are sufficient to create the observed differences is suggested by similarity of F_{ST} values between the subspecies and between Naxos and Paros. Similarly, Brammah (2007) revealed significant inter-island differences within each subspecies in terms of display structure, morphology and coloration, suggesting that differences arise rapidly in allopatry.

In conclusion, our study of four populations of lizards on different Cycladic islands reveals a pattern of considerable morphological, behavioural and genetic divergence. The relative magnitudes of these suggest a pattern whereby a single species on the Protocycladic Block evolved into two subspecies as rising sea levels created a northern and southern island. Populations on Naxos and Paros then became permanently isolated and gradually diverged as they lost variability. In contrast, populations on Mykonos and Delos appear much more recent, showing less divergence and higher diversity, and hence probably arrived after these islands became separated from the larger land mass of Tinos.

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