Genetic variation within endemic *Podarcis* lizards from the Balearic Islands inferred from partial Cytochrome *b* sequences

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Abstract. Sequences of the cytochrome *b* gene were analysed for 47 samples of two species of *Podarcis* from the Balearic Islands (*P. pityusensis* from Pityuses and *P. lilfordi*, from Gymnesies archipelago). The average uncorrected distance between the two species studied was 9.7%. The sampled individuals from each species form reciprocally monophyletic units. Assuming an overall rate of change for cytochrome *b* of 2% per million years the nucleotide divergence of 9.7 ± 1.9% between *P. lilfordi* and *P. pityusensis*, could correspond to a divergence time of 4.95 ± 0.95 million years. The separation time between Gymnesies and Pityuses archipelagos according to geological hypotheses is around 5 million years, thus our results suggest the ancestors of the two present forms became isolated during this event.

The most parsimonious networks suggest that currently accepted subspecies do not form monophyletic groups, and so should be reassessed. Despite our limited sampling the level of variability is much higher in *P. lilfordi* than *P. pityusensis*. Since they are sister taxa the relative age of each species is equal, therefore the differences might be due to historical population structure differences. However it seems that the forms on the islands are not genetically distinct units, implying that morphological differences are recent adaptations to their environments.

Introduction

Wall lizards, *Podarcis* (Reptilia; Lacertidae), are the predominant reptile fauna across much of central and southern Europe. *Podarcis* has been the subject of extensive ecological and behavioural studies, and there is an acknowledged need to put these into an historical context through the estimation of a phylogeny (Bauwens et al., 1995). Recent data from mitochondrial DNA partial gene sequences has supported the monophyly of the genus and provided support for some relationships between species (Harris and Arnold, 1999; Oliverio et al., 2000). However more extensive sampling within Iberian *Podarcis* has

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revealed a complex of cryptic species (Harris and Sá-Sousa, 2002; Harris et al., 2002), and the same may also be true of *Podarcis sicula* in Italy (Oliverio et al., 1998, 2000, 2001). There is therefore an additional need for sampling within species to determine if this is a widespread phenomenon in the genus.

Two endemic species of *Podarcis* inhabit the Balearic archipelago, *Podarcis lilfordi* in Gymnesies islands (Mallorca, Menorca, Cabrera and associated islets) and *Podarcis pityusensis* in Pityuses islands (Ibiza, Formentera and surrounding islets). Predation by introduced weasels, hedgehogs, and feral cats probably eliminated these species from the main islands of Menorca and Mallorca. Only a recent introduced population (Murada) of *P. pityusensis* has been described in Mallorca island. A high number of subspecies have been described, many restricted to extremely small islets, although most are not accepted by all taxonomists (Muller, 1927, 1928; Eisentraut, 1928, 1950; Salvador, 1984; Pérez-Mellado, 1997, 1998; Cirer, 1987). Harris and Arnold (1999) and Oliverio et al. (2000) suggest these species are sister taxa, but both were based on analysis of a single individual of each species. Other studies have questioned whether they deserve species rank at all (Capula, 1997).

The purpose of this work was to determine sequence variability in a region of the cytochrome *b* gene in *Podarcis* from populations in the Balearic islands, and to compare this with subspecific taxonomy and against levels of variation within other *Podarcis* species.

Materials and methods

Specimens of *Podarcis lilfordi* and *P. pityusensis* were captured from islands and islets of the Balearic Archipelago, including individuals from five subspecies of *P. pityusensis* and six of *P. lilfordi* (table 1 and fig. 1). Two to five individuals were analyzed for each locality. The tail tip of each individual was clipped off and stored at −20°C. Live animals were released again in the same capture place. Total genomic DNA was extracted following standard protocols (González et al., 1996). A 306 bp fragment of the mitochondrial cytochrome *b* gene was amplified using the primers L14841 and H15149 (Kocher et al., 1989). Thermocycling consisted of 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. Both strands of the amplified DNA were sequenced on an automated ABI 310 sequencer using a Taq DyeDeoxy™ Terminator Cycle sequencing kit (Applied Biosystems). Sequences were submitted to Genbank (accession numbers AY046281 to AY046312).

Cytochrome *b* sequences were aligned against the single published sequences from each of these species *P. lilfordi* (AF052639) and *P. pityusensis* (AF052640) (Castilla et al., 1998a). Since relationships within *Podarcis* have not been well supported in previous analyses of the genus (Harris and Arnold, 1999; Oliverio et al., 2001) we included several outgroup taxa: *Podarcis muralis* (Harris et al., 1998; AF080278), *P. hispanica* (AF052634 and Castilla et al., 1998a), *P. tiliguerta* (AF133457; Harris and Arnold, 1999), *P. filfolensis* (AF1333443; Harris and Arnold, 1999) and the more distantly related *Lacerta agilis* (AF080299; Harris et al., 1998). The data was then imported into PAUP*4.0b10 (Swofford, 2002) for phylogenetic analysis. We used the approach outlined by Huelsenbeck and Crandall (1997) to test 56 alternative models of evolution, employing PAUP*4.0b5 and Modeltest (Posada and Crandall, 1998). Once a model of evolution was chosen, it was used to estimate a tree using neighbor joining (NJ). Support for nodes was estimated using the bootstrap (Felsenstein, 1985) technique, with 1000 replicates. A maximum parsimony (MP) analysis was also carried out (100 replicate heuristic search) and support for nodes estimated by bootstrapping with 1000 replicates. We also estimated relationships using bayesian analysis as implemented in MrBayes (Huelsenbeck and Ronquist, 2001) which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) sampling approach.

Table 1. The *P. pityusensis* and *P. lilfordi* populations sampled.

Species	Subspecies	Locality[a]	Samples[b]
Podarcis pityusensis			
	P. p. pityusensis (Boscá, 1883)	1	PpE (4)
	P. p. formenterae (Eisentraut, 1928)	2	PpFx (5)
	P. p. grueni (Müller, 1928)	3	PpFt (5)
	P. p. formenterae (Eisentraut, 1928)		
	P. p. espalmadoris (Müller, 1928)	4	PpEr (3)
	P. p. formenterae (Eisentraut, 1928)		
	P. p. espardalensis (Müller, 1928)	5	PpEl (3)
	P. p. formenterae (Eisentraut, 1928)		
	P. p. pityusensis (Boscà, 1882)	6	PpMu (2)
Podarcis lilfordi			
	P. l. kuligae (Müller, 1927)	7	PlCp (4)
	P. l. kuligae (Müller, 1927)	8	PlCf (4)
	P. l. muelleri (Eisentraut, 1928)		
	P. l. giglioli (Bedriaga, 1879)	9	PlD (4)
	P. l. pobrae (Salvador 1980)	10	PlPo (4)
	P. l. fahrae (Müller, 1927)	11	PlFo (4)
	P. l. conejerae (Müller, 1927)	12	PlCo (3)

[a]The numbers correspond with those of fig. 1; [b]number of individuals in brackets.

Bayesian analyses were conducted with random starting trees, run 1×10^6 generations, and sampled every 100 generations using a general-time-reversible model of evolution. In both searches stationarity of the Markov Chain was determined as the point when sampled log likelihood values plotted against generation time reached a stable mean equilibrium value; "burn-in" data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a summary phylogeny. Two independent replicates were conducted and inspected for consistency to check for local optima (Huelsenbeck and Bollback, 2001).

Within species, bifurcating trees are often not the most appropriate way to represent relationships between haplotypes (reviewed in Posada and Crandall, 2001). Therefore we also used the statistical parsimony algorithm (Templeton et al., 1992) performed in TCS (Clement et al., 2000) to estimate the maximum number of differences among haplotypes as a result of a single substitution with a 95% confidence level.

Results and discussion

Sequences of the cytochrome *b* fragment were analysed for 52 samples of *Podarcis* and one *Lacerta*. No indels were observed. Within the ingroup there were 50 variable positions, 47 of which were parsimony informative; 34 were variable within *P. lilfordi*, but only 7 sites within *P. pityusensis*. In most cases the substitutions occurred in the third codon position. Only six substitutions gave aminoacid replacements, five of which were found in *P. lilfordi* and one in *P. pityusensis*. The average uncorrected distance between the two species studied was 0.097. This is within the range typically observed between reptile sister taxa (Harris, 2002).

All phylogenetic analyses gave trees with a similar topology. Using Modeltest (Posada and Crandall, 1998) we concluded that the HKY model (base frequencies A 0.27,

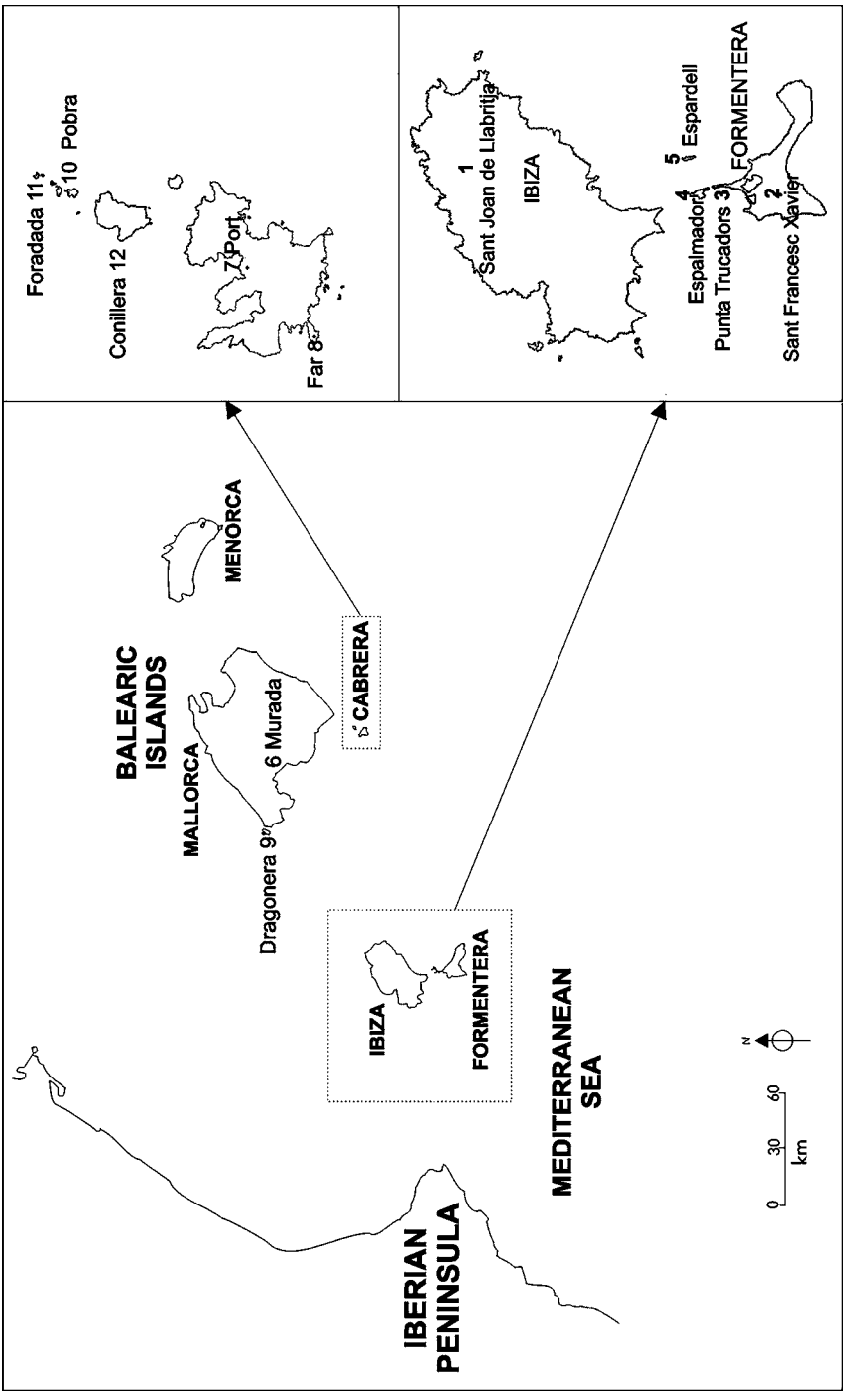

Figure 1. Map showing the Balearic Islands, and the localities sampled in this study.

C 0.32, G 0.10, T 0.30, 4.52 transition/transversion ratio), with a gamma distributed rate heterogeneity model (4 rate categories, $\alpha = 0.901$) and an estimated proportion of invariable sites (0.51) was the most appropriate model of evolution for these data. Maximum parsimony analysis found 620 trees of 256 steps that were similar to the NJ trees (fig. 2). Eighty-three characters were parsimony informative. Bayesian analyses similarly supported the same groups found by NJ and MP (fig. 2).

The sampled individuals from each species form reciprocally monophyletic units. Assuming an overall rate of change for cytochrome *b* of 2% per million years (Carranza et al., 2000), the nucleotide divergence of 9.7±1.9% between *P. lilfordi* and *P. pityusensis*, could correspond to a divergence time of 4.95±0.95 million years. The separation time between Gymnesies and Pityuses archipelagos according to geological hypotheses is around 5 million years, thus our results suggest the ancestors of the two present forms became isolated during this event.

Using TCS several statistically separate groups were produced (fig. 2). One comprises of all *P. pityusensis* haplotypes. Within *P. lilfordi*, the haplotypes formed three different networks. The networks suggest that currently accepted subspecies do not form monophyletic groups, and so should be reassessed. The level of variability is much higher in *P. lilfordi* than *P. pityusensis*. Since they are sister taxa, the age of each species is equal. Therefore, assuming the same mutation rate, the differences might be due to historical population structure differences or an effect of not having sampled across all the existent genetic variability of *P. pityusensis*. The fact that *P. lilfordi* was only recently made extinct on the main island of Mallorca may not have had an effect on the population substructuring on the small islands. It is likely that *P. lilfordi* had a high degree of population substructure on Mallorca, as this island is ecologically heterogeneous. Thus the small islands would probably have been colonized by various main island populations multiple times. These islands therefore would probably have included individuals from multiple genetically distinct (and now extinct) mainland forms. Variation within *P. lilfordi* of up to 4.8% demonstrates that small insular populations of *Podarcis* lizard can contain high genetic diversity, as reported in *P. atrata* (Castilla et al., 1998b). In some cases (i.e. samples of Cabrera island), the intralocality diversity was very high, near to the maximum detected for this species. For a more accurate explanation to this fact and to be able to evaluate of it could be due to introductions, more individuals are necessary to be sampled. Within *P. pityusensis* variation is low implying less population substructure, which is not surprising as Ibiza is smaller and more ecologically uniform than Mallorca. The fact that in this network there are no missing internal nodes could indicate no evidence of a recent bottleneck; if that was the case some rarer alleles would go extinct leading to gaps between present haplotypes, an equivalent example to the mean ratio of the number of alleles to the range in allele size, M, calculated for microsatellites to detect populations size reductions (Garza and Williamson, 2001). The differences between the two species could be explained in this way, but more variable markers such as microsatellites, would be needed to confirm this. However it seems

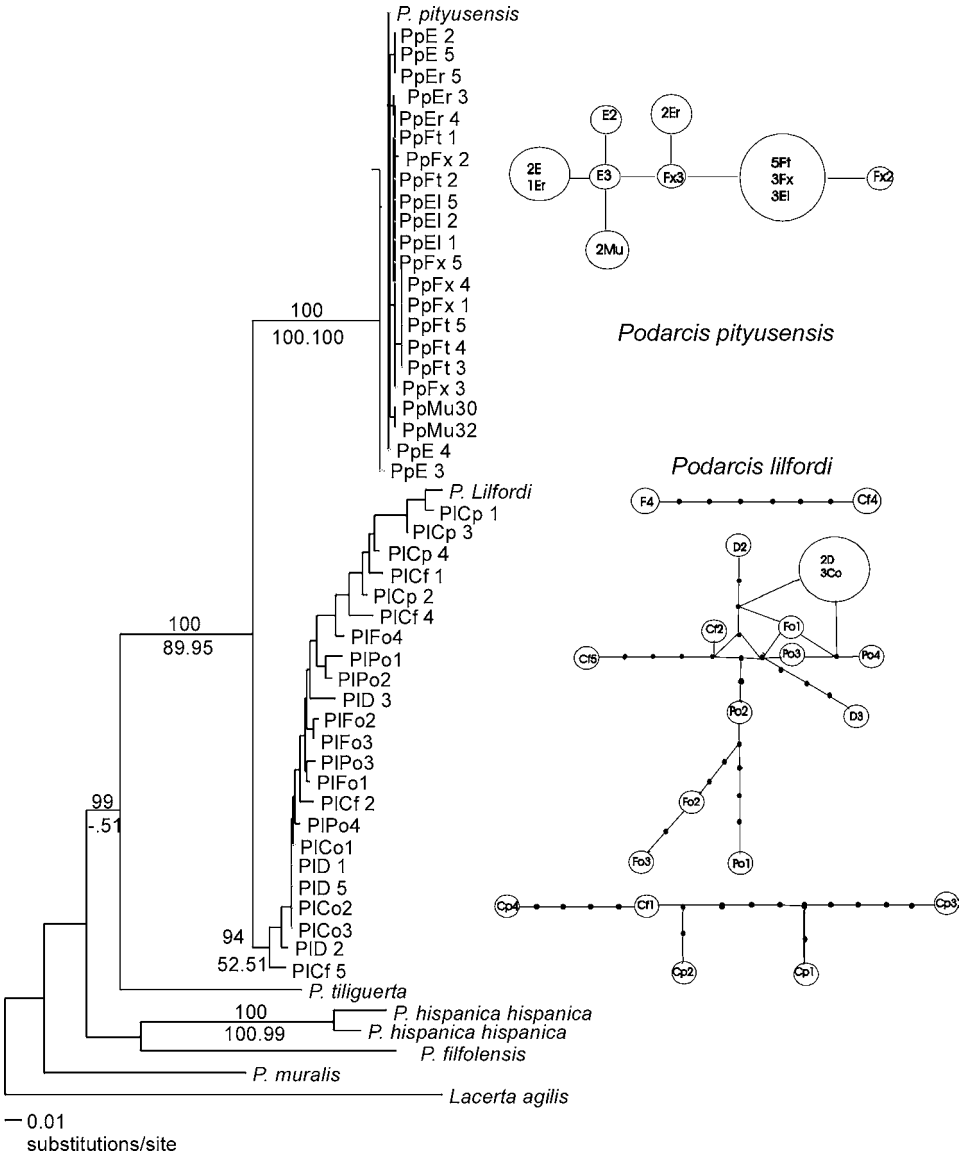

Figure 2. Tree derived from a NJ analysis using the model described in the text. Bayesian analyses produced identical estimates of between-species relationships as the NJ analysis, and average posterior probabilities are shown above nodes. MP also produced the same estimate of between species relationships. Bootstrap support (>50%) from the NJ and MP (1000 pseudoreplicates) analyses, respectively are shown below nodes separated by a dot. Where a method did not have >50% support, a dash is given. Sample code names refer to table 1. Where no code name is given the sequences were already published elsewhere. Networks produced by TCS are shown next to the respective parts of the tree. Size of circles is related to the number of individuals with a given haplotype, and dots indicate predicted missing haplotypes.

that islands subspecies are not genetically distinct units, implying that their morphological adaptations are very recent.

We point out the need to continue with the studies in the archipelago by increasing the number of samples (other islands and/or islets) and by using different genes of the mitochondrial and nuclear genome to better understand the genetic structure of the populations. Moreover, this knowledge will be very important to develop an appropriate conservation program for these endemic *Podarcis* lizards.

Acknowledgements. We thank the directors and guards of the National Park of Cabrera and the National Reservation of Ses Salinas (Ibiza) for their logistics support. Also we thank the responsibles of the Natural Park of Dragonera and the *Conselleria de Mediambient (Govern de les Illes Balears)* for their permissions for the captures.

References

Bauwens, D., Garland, T., Castilla, A.M., Van Damme, R. (1995): Evolution of sprint speed in Lacertid lizards: morphological, physiological, and behavioural covariation. Evolution **49**: 848-863.

Bedriaga, J.V. (1879): Herpetologische Studien. Archiv für Naturgeschichte **45**: 243-339.

Boscà, E. (1882): Exploración herpetological de las islas de Ibiza. *Bol. Soc. Española* Hist. Nat. **12**: 246-255.

Capula, M. (1997): Genetic divergence and phylogenetic inferences in the genus *Podarcis*. In: Abstracts of the Third Herpetological Congress of Herpetology, Rocek, Z., Hart, S., Eds, Prague, Third World Congress of Herpetology.

Carranza, S., Arnold, E.N., Mateo, J.A., López-Jurado, L.F. (2000): Long-distance colonization and radiation in gekkonid lizards, *Tarentola* (Reptilia: Gekkonidae), revealed by mitochondrial DNA sequences. Proc. Roy. Soc. Lond. B **267**: 637-649.

Castilla, A.M., Fernandez-Pedrosa, V., Harris, D.J., González, A., Latorre, A., Moya, A. (1998a): Mitochondrial DNA divergence suggest*s Podarcis hispanica atrat*a from the Columbretes Islands merits specific distinction. Copeia **1998**: 1037-1040.

Castilla, A.M., Fernández-Pedrosa, V., Backeljau, T., González, A., Latorre, A., Moya, A. (1998b): Mitochondrial DNA variability and conservation of an endangered lizard from the Columbretes islands. Mol. Ecol. **7**: 1047-1051.

Cirer, A.M. (1987): Revisión taxonómica de las subespecies del lacértido *Podarcis pityusensis* Boscà 1883. Ph.D. University of Barcelona, Barcelona.

Clement, M., Posada, D., Crandall, K.A. (2000): TCS: a computer program to estimate gene genealogies. Mol. Ecol. **9**: 1657-1660.

Eisentraut, M. (1928): Vörlaufige Diagnosen einiger neuen Rassen der Balearischen Inseleidechse *Lacerta lilfordi*. Das Aquarium **1928**: 121-124.

Eisentraut, M. (1950): Eidechsen der Spanischen Mittelmeerinseln ind ihre rassennaufspaltung im lichte der evolution. Mitteilungen aus dem Zoologischen Museum in Berlin **26**: 1-228.

Felsenstein, J. (1985): Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**: 783-791.

Garza, J.C., Williamson, E.G. (2001): Detection of reduction in population size using data from microsatellite loci. Mol. Ecol. **10**: 305-318.

González, P., Pinto, F., Nogales, M., Jiménez-Asensio, J., Hernández M., Cabrera, V.M. (1996): Phylogenetic relationships of the Canary islands endemic lizard genus *Gallotia* (Sauria: Lacertidae), inferred from mitochondrial dna sequences. Mol. Phylogenet. Evol. **6**: 63-71.

Harris, D.J. (2002): Reassessment of comparative genetic distance in reptiles from the mitochondrial cytochrome *b* gene. The Herpetological Journal **12**: 85-86.

Harris, D.J., Arnold, E.N., Thomas, R.H. (1998): Relationships of the lacertid lizards (Reptilia: Lacertidae) estimated from mitochondrial DNA sequences and morphology. Proc. R. Soc. Lond. B **265**: 1939-1948.

Harris, D.J., Arnold, E.N. (1999): Relationships and evolution of wall lizards, *Podarcis* (Reptilia, Lacertidae) based on partial mitochondrial DNA sequences. Copeia **1999**: 740-754.

Harris, D.J., Sá-Sousa, P. (2002): Molecular phylogenetics of Iberian Wall lizards (*Podarcis*), is *Podarcis hispanica* a species complex? Mol. Phyl. Evol. **23**: 75-81.

Harris, D.J., Carranza, S., Arnold, E.N., Pinho, C., Ferrand, N. (2002): Complex biogeographic distribution of genetic variation within *Podarcis* Wall lizards across the Strait of Gibraltar. J. Biogeog. **29**: 1257-1262.

Huelsenbeck, J.P., Crandall, K.A. (1997): Phylogeny estimation and hypothesis testing using maximum likelihood. Ann. Rev. Ecol. Syst. **28**: 437-466.

Huelsenbeck, J.P., Bollback, J.P. (2001): Empirical and hierarchical Bayesian estimation of ancestral states. Syst. Biol. **50**: 351-366.

Huelsenbeck, J.P., Ronquist, F. (2001): MR-BAYES: Bayesian inference of phylogeny. Bioinformatics **17**: 754-755.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., Wilson, A.C. (1989): Dynamics of mitochondrial evolution in animals: amplification and sequencing with conserved primers. Proc. Nat. Acad. Sci. USA **86**: 6196-6200.

Müller, L. (1927): Beitrag zur Kenntnis der rassen von *Lacerta lilfordi*. Zoologischer Anzeiger **73**: 257-269.

Müller, L. (1928): Die Inselrassen der Lacerta lilfordi (Gthr). Verbandlungen der deutschen Zoologischen Gesellschaft **1928**: 333-337.

Oliverio, M., Bologna, M.A., Monciotti, A., Annesi, F., Mariottini, P. (1998): Molecular phylogenetics of the Italian Podarcis lizards (Reptilia, Lacertidae). Ital. J. Zool. **65**: 315-324.

Oliverio, M., Bologna, M.A., Mariottini, P. (2000): Molecular biogeography of the Mediterranean lizards *Podarcis* Wagler, 1830 and *Teira* Gray, 1838 (Reptilia, Lacertidae). J. Biogeography **27**: 1403-1420.

Oliverio, M., Burke, R., Bologna, M.A., Wirz, A., Mariottini, P. (2001): Molecular characterisation of native (Italy) and introduced (USA) *Podarcis sicula* populations (Reptilia, Lacertidae). Ital. J. Zool. **68**: 121-124.

Pérez-Mellado, V. (1997): *Podarcis lilfordi* (Gunther, 1874). In: Atlas of Amphibians and Reptiles in Europe, pp. 280-281. Gasc, J.P., Ed., Paris, Societas Europaea Herpetologica, Muséum National d'Histoire Naturelle.

Pérez-Mellado, V. (1998): Género *Podarcis* Wagler, 1830. In: *Reptiles*. Salvador A. (Coordinador) Fauna Iberica, 10, pp. 242-243. Ramos et al., Eds, Madrid, Museo de Ciencias Naturales CSIC.

Posada, D., Crandall, K.A. (1998): Modeltest: testing the model of DNA substitution. Bioinformatics **14**: 817-818.

Posada, D., Crandall, K.A. (2001): Intraspecific gene genealogies: trees grafting into networks. Trends Ecol. Evol. **16**: 37-45.

Salvador, A. (1980): Materiales para una herpetofauna baleárica. 4. Las poblaciones de lagartija balear (*Lacerta lilfordi*) del archipiélago de Cabrera. I Reunión Iberoamericana de Zoología de Vertebrados. La Rábida: 401-454.

Salvador, A. (1984): A taxonomic study of the Eivissa wall lizard, *Podarcis pityusensis* Boscà (1883). In: Biogeography and Ecology of the Pityusic Islands, pp. 393-427. Kubier, H., Alcover, J.A., Guerau d'Arellano, C., Eds, The Hague, Dr. W. Junk Publ.

Swofford, D.L. (2002): PAUP*: Phylogenetic Analysis Using Parsimony 4.0. Sunderland, Massachusetts, USA, Sinauer Associates.

Templeton, A.R., Crandall, K.A., Sing, C.F. (1992): A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data III. Cladogram estimation. Genetics **132**: 619-633.

Received: November 1, 2003. Accepted: January 28, 2004.