Molecular Ecology (1998) **7**, 1407–1411

SHORT COMMUNICATION

Conservation genetics of insular *Podarcis* lizards using partial cytochrome *b* sequences

A. M. CASTILLA,*† V. FERNÁNDEZ-PEDROSA,‡ T. BACKELJAU,§ A. GONZÁLEZ‡ A. LATORRE,‡ and A. MOYA‡

**Department of Biology, University of Antwerp (UIA), B-2610 Wilrijk, Belgium, †Instituto Mediterráneo de Estudios Avanzados (C.S.I.C.), Km 7.5, Ctra. Valldemossa, E-07071 Palma de Mallorca, Spain, ‡Department of genetics. University of Valencia. Doctor Moliner 50. 46100-Burjasot, Valencia, Spain, §Royal Belgian Institute of Natural Sciences, Malacology Section, Vautierstraat 29, B-1000 Brussels, Belgium*

Abstract

Sequence data derived from a 306 bp fragment of mitochondrial cytochrome *b* and molecular variance estimates were used to investigate the genetic population structure of the endangered and endemic lizard *Podarcis atrata* of the Columbretes archipelago (Mediterranean, Spain). Our results show a very high and significant among-population genetic differentiation. F_{ST} values and phylogenetic analyses confirm the evolutionary distinctiveness of *P. atrata* populations, suggesting that the populations of these islands deserve special protection measures. The populations of the two islands Columbrete Grande and Mancolibre are less differentiated than those of Foradada and Lobo, and seem to have retained mainland haplotypes. This situation needs further attention as the origin of the mainland haplotypes is still unclear. If they are a result of recent introductions from mainland specimens, then they may represent a threat to the endemic lizards of the Columbretes islands.

Keywords: conservation genetics, cytochrome *b*, Mediterranean islands, *Podarcis hispanica atrata,* population genetic structure

Received 21 January 1998; revision received 23 March 1998; accepted 23 March 1998

Introduction

Podarcis atrata (Castilla *et al*. 1998) is an endangered and endemic species of the Columbretes islands (Castilla & Bauwens 1991). This Mediterranean archipelago is situated 51 km east of the Spanish mainland (39°54' N, 0°41' E) from which it is separated by a channel 90–100 m deep. The archipelago comprises four small islets (0.5–12 ha) (Fig. 1), whose ages (K-Ar dating) vary from 1 Myr (Columbrete Grande, Mancolibre) to 8–10 Myr (A. Aparicio, R. García, F. Bellido 1991, personal communication). In order to develop an appropriate conservation strategy for *P. atrata*, we analysed the population genetic structuring of these lizards as a first step in identifying evolutionarily significant units (ESU) as defined by Moritz (1994).

Correspondence: A. M. Castilla. E-mail: castilla@uia.ac.be

Materials and methods

Sampling

We examined five lizard populations: one pooled *Podarcis hispanica* collected along the coast of the Spanish mainland ($n = 8$) and four *P. atrata* populations from the Columbretes islands ($n = 20$). A tail tip of each specimen was clipped off and transferred into 100% ethanol. All animals were released again at their collecting site, except for three mainland specimens (Valencia) which were previously collected and preserved in 70% ethanol.

DNA isolation and amplification

Tail tips were kept in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 48 h, and subsequently homogenized in 196 μL of TE, 2 μL of proteinase K (20 mg/mL) and

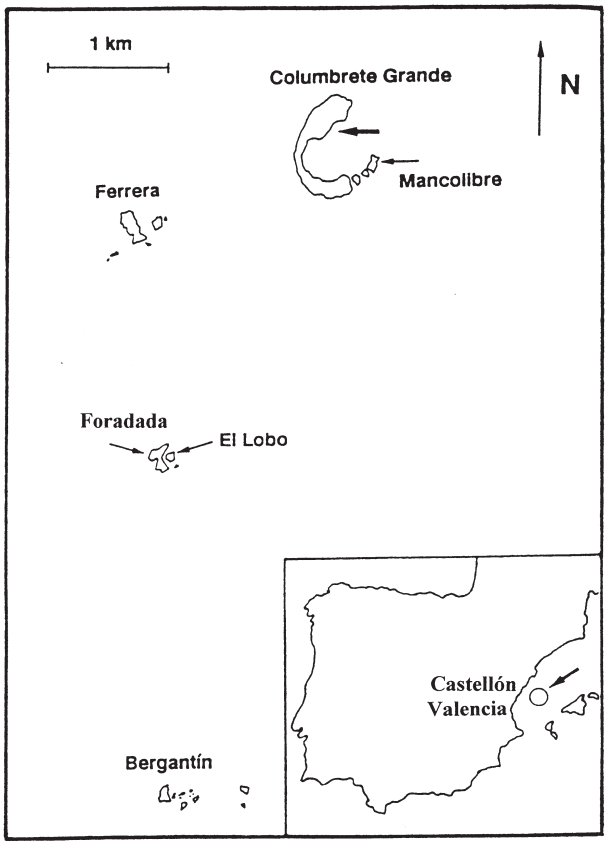

Fig. 1 World distribution of *Podarcis atrata*. Arrows indicate the islands where lizards are present. Figure modified from Castilla & Bauwens (1991).

2 μL of Triton X-100. Homogenates were incubated at 37 °C for 4.5 h and centrifuged for 10 min at 5000 **g**. Total DNA was extracted by chloroform/phenol, precipitated by ethanol, vacuum-dried and resuspended in 20 μL of TE. A 306 bp fragment of the mitochondrial cytochrome *b* gene was amplified using the universal primers L14841' and H15149' (Kocher *et al*. 1989). PCR reaction mixtures contained 3 μL of DNA, 10 μL of each dNTP, 100 nM primer and 2 units of *Taq* polymerase in a final volume of 100 μL. Reaction mixtures were subjected to the following PCR cycles protocol: 1 × (95 °C: 3–5 min), 40 × (95 °C: 30 s; 55 °C: 30 s; 72 °C: 60 s), 1 × (72 °C: 5–7 min). PCR products were precipitated with 5 M NaCL, 4 M $AcNH_4$ and 5 vols of 100% ethanol, and directly sequenced with an automated ABI sequencer using the Taq DyeDeoxyTM Terminator Cycle Sequencing kit (Perkin-Elmer). Sequences were submitted to the EBI/GenBank database (Accession nos AJ001773, AJ224406–9, AJ009994–6, AJ004904–11, AJ004978– AJ004983, AJ004986–87, AJ004989–AJ004992). CLUSTAL W version 1.5 was used for sequence alignment.

Sequence analysis

Nucleotide diversities ($Pi \pm SD$) were calculated using the Jukes–Cantor (JC) correction. Genetic differentiation between populations was assessed via F_{ST} values, and their significance tested by 10 000 permutations. Gene flow estimates (Nm) were derived from the pairwise F_{ST} values according to $F_{ST} = 1/(2Nm + 1)$ for haploid data. The partition of genetic variation among and within populations was assessed by an analysis of molecular variance (AMOVA) whose significance was tested with 10 000 permutations (Excoffier *et al*. 1992). Calculations were performed with the programs DNASP version 2.51 and ARLEQUIN version 1.0.

Phylogenetic relationships between haplotypes were inferred via neighbour-joining (NJ) of JC distances, maximum parsimony (MP) and maximum likelihood (ML) implemented by the programs MEGA version 1.02, RNA and PUZZLE version 2.5.1 All positions were equally weighted. Confidence estimates were obtained via bootstrapping or its equivalent with quartet puzzling (ML). This latter applied the Tamura–Nei (1993) model with transition/transversion (ts/tv) parameters estimated from the data.

Results and Discussion

Sequence alignment was straightforward and no indels had to be postulated. There were 95 polymorphic sites, 85 of which were parsimony informative. Pairwise sequence divergence varied from 0.3% to 20%, and the overall ts/tv ratio was 1.57.

The NJ and ML trees (Fig. 2A) showed significant bootstrap support (i.e. > 70%) for the monophyly of the clusters FO and LO, as well as for a group in which LO was joined with a nonsupported cluster of MA and CG. The four island populations formed a well-supported monophyletic group with PI as sister taxon. However, the Columbretes sequences CG3 and MA2 were significantly included in the mainland clade.

The MP analysis yielded 52 MP trees of length 217. The bootstrapped MP tree showed essentially the same groups as the NJ and ML trees, except for the clade uniting LO with CG + MA which in the MP tree was no longer significantly supported (Fig. 2B).

Because of the divergent placement of CG3 and MA2, all subsequent analyses were performed in parallel by including and excluding these two sequences. Given that there is little doubt that CG3 and MA2 were correctly identified and labelled during processing, the presence of the mainland haplotypes in the archipelago may be suggestive of: (i) ancestral polymorphisms; (ii) a recent introduction caused by the intensive human traffic between the mainland and CG-MA; or (iii) a historical colonization facilitated by eustatic sea-level changes (e.g. during the Würm

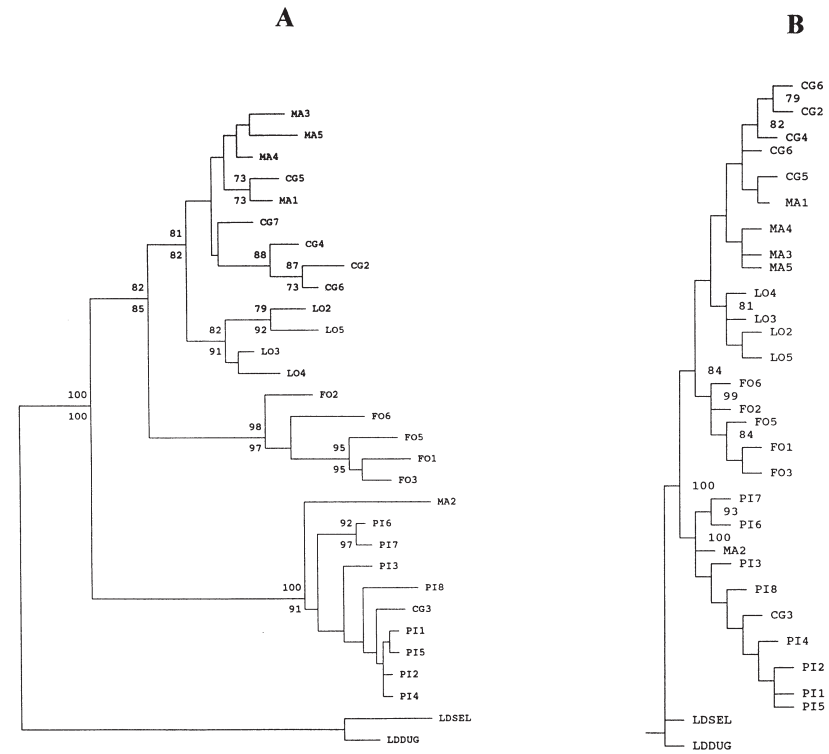

Fig. 2 Phylogenetic trees depicting relationships among insular and mainland *Podarcis* lizards. Collection sites for *P. hispanica* (PI) (Valencia: El Saler ($n = 2$), Burjasot ($n = 1$); Castellón: El Grao ($n = 5$)) and for *P. atrata* (Columbrete Grande (CG; $n = 6$), Mancolibre (MA; $n = 5$), Foradada (FO; $n = 5$) and El Lobo (LO; $n = 4$)). Bootstrap values are shown for neighbour-joining (tree A: above branches, 10 000 replicates) and maximum parsimony (tree B: 10 000 replicates), and quartet puzzling steps (1000) for the maximum likelihood tree (A, below branches). *Lacerta dugesii dugesii* and *L. d. selvagensis* (González *et al*. 1996) were used as outgroups.

glaciation when the sea level dropped 120 m). Our data are insufficient to distinguish among these alternatives.

Although the inclusion or exclusion of CG3 and MA2 produced different numerical values in the analyses (Tables 1 and 2), the overall conclusions were not affected. However, excluding CG3 and MA2 yielded more consistent and homogeneous population genetic parameters when CG-MA was compared with FO-LO.

The AMOVA showed that with CG3 and MA2, 58% ($P < 0.001$) of the variation occurred between populations.

Table 1 **A.** Nucleotide diversity ($Pi \pm$ SD based on the JC distances). Sample size is indicated (n). **B.** Pairwise F_{ST} values and their significance (in parentheses) between five Podarcis populations (abbreviations as in Fig. 2). Data involving CG and MA are presented for both the complete samples (all, above the diagonal) and for samples without CG3 and MA2 (out, below the diagonal)

	PI	CG	MA	FO	LO
A.					
all	0.02176 ± 0.004 ($n = 8$)	0.07059 ± 0.025 ($n = 6$)	0.07068 ± 0.029 ($n = 5$)	0.04071 ± 0.006 ($n = 5$)	0.03063 ± 0.00649 ($n = 4$)
out	– –	0.03285 ± 0.006 ($n = 5$)	0.02268 ± 0.004 ($n = 4$)	– –	– –
B.					
PI	–	0.6335 (0.0006)	0.6305 (0.0004)	0.7915 (0.0012)	0.8184 (0.0017)
CG	0.8033 (0.0002)	– –	0.0227 (0.4338)	0.5061 (0.0025)	0.2673 (0.0044)
MA	0.8183 (0.0015)	0.2849 (0.0243)	– –	0.4908 (0.0086)	0.2862 (0.0069)
FO	0.7915) (0.0005)	0.6522 (0.0077)	0.6568 (0.0082)	– –	0.6199 (0.0095)
LO	0.8184 (0.0027)	0.4354 (0.0084)	0.4900 (0.0293)	0.6199 (0.0070)	– –

Table 2 Estimates of effective numbers of migrants (*Nm*) per generation between Podarcis populations (abbreviations as in Fig. 2). Data involving CG and MA are presented for both the complete samples (above the diagonal) and without CG3 and MA2 (below the diagonal)

	PI	CG	MA	FO	LO
PI	–	0.2892	0.2930	0.1317	0.1109
CG	0.1224	–	21.5252	0.4879	1.3703
MA	0.1110	1.2547	–	0.5188	1.2471
FO	0.1317	0.2667	0.2613	–	0.3066
LO	0.1109	0.6483	0.5203	0.3066	–

Without CG3 and MA2 this figure was 73% ($P < 0.001$). This degree of among-island genetic differentiation in the Columbretes archipelago is very high in comparison to other lizard populations (e.g. 46% for *Podarcis tiliguerta*) (Capula 1996 and references therein). Although Capula used allozyme electrophoresis, these figures may provide crude provisional comparative data.

Except for CG3 and MA2, our analyses suggests that *P. atrata* from Columbretes is a monophyletic taxon consisting of at least two well-defined clades (FO and LO) that may be classified as evolutionary significant units (ESUs, Moritz 1994), and a paraphyletic grouping of CG-MA sequences. This latter observation is in line with the younger age of CG-MA, therefore lineage sorting may not be completed yet. The fact that CG-MA had the shortest divergence time, highest *Nm* estimates and smallest F_{ST} values supports this contention. The phylogenetic tree also suggests that FO is the basal branch in *P. atrata*. This is consistent with FO-LO being the oldest islands in the archipelago, thus implying an earlier colonization for FO-LO.

Although *Nm* values between islands were relatively low (< 1 in most cases), gene flow was higher between CG and MA (140 m apart) than between FO and LO (20 m apart). This unexpected observation may be due to the more accessible coasts of CG and MA (more gently sloping rocks with vegetation and inhabited by lizards) vs. the vertical cliffs without vegetation in FO and LO. Moreover, population densities in CG are high (>800 individuals/ha; Castilla & Bauwens 1991), so that dispersion to MA may be facilitated. In contrast, the FO and LO populations each comprises < 100 individuals (Castilla & Bauwens 1991) and these animals inhabit the inner parts of the islands, but not the coastal areas.

As a result of the higher gene flow found between CG and MA, genetic divergence between these populations is the lowest in the archipelago. This is also consistent with the recent geological time of separation of CG and MA (80 000 years ago, Hernández-Pacheco & Asensio 1966). However, FO and LO appear more differentiated despite the fact that these islands separated at the same time as CG and MA (A. Aparicio, personal communication). It is possible that the smaller populations of LO and FO have been more strongly influenced by genetic drift.

F_{ST} values and phylogenetic analyses confirm the evolutionary distinctiveness of *P. atrata* populations, and suggest the possibility that at least the populations of two islands (FO and LO) are separate ESUs which deserve special protection measures. However, further sampling of nuclear genes and analysis is needed to confirm this. The design of an integrated conservation program for this lizard should take into account the genetic isolation of these populations when considering *in situ* management decisions, such as re-introductions or translocations. Reinforcement is often cited as an answer to bring populations up to minimum viable size or to enhance local genetic variability. Therefore, careful planning of captive breeding programs should ensure that only *P. atrata* of the appropriate population is re-introduced onto each island, when required.

The populations of the two other islands (Columbrete Grande and Mancolibre) are less differentiated and seem to have retained mainland haplotypes. This situation needs further attention as the origin of the mainland haplotypes is still unclear. If they are due to recent introductions from mainland specimens, then they may represent a threat (e.g. hybridization, competition) to the endemic lizards of the Columbretes islands.

Our results emphasize the importance of preserving the genetic diversity of *P. atrata* and the habitat of the Columbretes islands. We highlight the need to continue with studies in the archipelago by increasing sample size and by using different genes of the mitochondrial and nuclear genome. Given the noninvasive nature of these techniques, such studies will allow a better understanding of the genetic structuring of the populations and will provide adequate knowledge to develop an appropriate conservation strategy for *P. atrata.*

Acknowledgements

We thank the director of the Natural Reserve, J. Jiménez, for permission, and Dirk, Valentín, Tony, Santi, and all guards in the Park for collecting lizards. We thank A. Aparicio, R. García (Depto. Vulcanología, Museo de Ciencias Naturales de Madrid, Spain) and F. Bellido (ITGE) for providing unpublished geological data, and to Pepelu and F. Palacios for logistic support. This work was supported by a Project of the Generalitat Valenciana, funds from the Exmo. Ayuntamiento de Castellón, by a contract of the Spanish National Science Foundation (C.S.I.C.) (to A.M.C.), and by Project DGICYT PB93–0697 (to A.M.).

References

Aparicio A, Mitjavila JM, Araña V, Villa IM (1991) La edad del volcanismo de las islas Columbrete Grande y Alborán (Mediterráneo occidental). *Boletín Geologico y Minero*, **102**, 562–570.

Capula M (1996) Evolutionary genetics of the insular lacertid lizard *Podarcis tiliguerta*: genetic structure and population heterogeneity in a geographically fragmented species. *Heredity*, **77**, 518–529.

Castilla AM, Bauwens D (1991) Observations on the natural history, present status, and conservation of the insular lizard *Podarcis hispanica atrata* on the Columbretes archipelago, Spain. *Biological Conservation*, **58**, 69–84.

Castilla AM, Fernández-Pedrosa V, Harris JD, González A, Latorre A, Moya A (1998) Mitochordrial DNA divergence suggests that *Podarcis hispanica atrata* (Squamata: Lacertidae) from the Columbretes islands merits specific distinction. *Copeia*, **1998**(4), 1037–1040.

Excoffier L, Smouse PE, Quattro JM (1992) Analyses of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

González P, Pinto F, Nogales M, Jiménez Asensio J, Hernández M, Cabrera VM (1996) Phylogenetic relationships of the Canary islands endemic lizard genus *Gallotia* (*Sauria*: *Lacertidae*), inferred from mitochondrial DNA sequences. *Molecular Phylogeny and Evolution*, **6**, 63–71.

Hernández-Pacheco F, Asensio IA (1966) Datos fisiográfico-sedimentológicos de la Columbrete Grande. *Boletín Real Sociedad Española Historia Natural (Geología)*, **64**, 179–198.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA*, **86**, 6196–6200.

Moritz C (1994) Defining 'evolutionarily significant units' for conservation. *Trends in Ecology and Evolution*, **9**, 373–375.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512–526.

This work is the result of a collaboration between three teams coordinated by A.M.C., who is a lizard evolutionary ecologist at the Spanish National Science Foundation (IMEDEA, CSIC). T.B. from the Royal Belgian Institute of Natural Sciences (Belgium) and Moya's group from the University of Valencia (Spain) are involved in studies of genetic differentiation in populations of vertebrate and invertebrate species.
