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The double origin of Iberian peninsular chameleons

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There is considerable controversy concerning the origin of Iberian populations of the Mediterranean chameleon, *Chamaeleo chamaeleon*. Current opinion dictates that Spanish populations result from introductions during the 18th and 19th centuries, with subsequent translocations from the original populations to other parts of Spain. The Portugese population in the Algarve is believed to have been introduced from Africa or Spain during the 1920s. However, Holocene remains of chameleons suggest that the Malaga population at least could have a much older origin. Analysis of sequences from the mitochondrial 16S ribosomal RNA gene of samples from the Iberian Peninsula and North Africa revealed a double origin for the Iberian population. The Mediterranean Iberian (Malaga) population is closely related to Mediterranean North African populations, with Atlantic Iberian populations more closely related to populations of the Atlantic coast of North Africa. The overall genetic differentiation and diversity observed was very low, preventing precise dating of the colonization events. However this low level of differentiation is not consistent with Plio-Pleistocene colonization, the assumed timing for a natural colonization event and suggests that chameleons were probably introduced twice by man in the recent past. *Biological Journal of the Linnean Society*, 2002, **75**, 1–7.

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INTRODUCTION

Chameleons in the Iberian Peninsula are distributed across six distinct provinces: five in Spain (Almeria, Granada, Malaga, Cadiz and Huelva) and one in Portugal (Algarve). The origin of the species in Iberia, and the origin of each population within the peninsula, is one of the most intriguing questions regarding European chameleons. These populations form the Northern tip of a wide circum-Mediterranean distribution, from North Africa to Turkey and the Peloponnese. The species also occurs on the Mediterranean islands of Cyprus, Crete, Samos, Chios, Malta and Sicily. Iberian chameleons belong to the subspecies *Chamaeleo chamaeleon chamaeleon* (Linnaeus), one of a number of subspecies that collectively have a distribution stretching from Europe and North Africa to the Middle East (Hillenius, 1978; Klaver, 1981; Klaver & Böhme, 1986).

Linnaeus was the first author to mention the existence of chameleons in the Iberian Peninsula in his *System Naturae* of 1766; 'Habitat in Africae, Asiae, Hispaniae australis arboribus'. Later, other authors reported the species in southern Spain (Martínez y Montes, 1852), Cadiz (Machado, 1859), and Malaga (Bosca, 1877). Gadow (1901) considered that the species had been introduced into the Malaga region, and since then it has been thought that all Iberian populations were introduced relatively recently by

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man. Blasco (1997a, 1997b) suggested a chronology for the introduction process. According to this, the Cadiz population was a 19th century introduction and the Malaga population was founded much earlier, probably some centuries before. Later, the species was introduced from either Africa or Spain into the Algarve in Portugal between 1920 and 1931 or 1920–50 and into the Huelva region between 1940 and 1950, probably from a Cadiz population (Blasco *et al*., 1985). Some other populations in the Cadiz region result from introductions from 1960 to 1980 (Blasco *et al*., 1985). For the Algarve it was Themido (1945), and not Oliveira (1931) as usually cited (Blasco, 1997a, 1997b), who first mentioned the presence of chameleons brought by workers that went to work in Moroccan and Spanish factories in the 1920s. The Granada population was explained by an expansion of the Malaga population, while that of Almeria was thought to originate from a contemporary introduction from other Spanish population. However, the discovery of Holocene remains that may be *Chamaeleo chamaeleon* (Talavera & Sanchíz, 1983) challenged this established scenario of relatively recent introductions (Bons, 1973; Busack, 1977; Blasco, *et al.* 1979). It has been suggested that the Malaga population at least could have a natural origin (Talavera & Sanchíz, 1983; Crespo & Oliveira, 1989; Blasco, 1997a, 1997b).

Previous comparative studies on the morphology and karyology of populations in North Africa and Southern Europe showed no statistically significant difference (Blasco *et al*., 1985). Moreover, biochemical evidence, based on electrophoretic comparison of 21 loci, showed that the Spanish and Portuguese specimens are genetically similar to the Moroccan population (Hofman *et al.,* 1991). Both sets of results suggest a recent colonization process.

However, because of the long history of geological and cultural links between the Iberian Peninsula and North Africa (the putative origin of the Iberian populations), it is possible that chameleons were found on the Iberian Peninsula before recent times. The current populations could then either be the descendants of a natural ancestral colonization or alternatively, if climatic oscillations led to an historical extinction, be the product of more recent, human-mediated colonizations.

Three different aspects of the question of origin of Iberian chameleons can be addressed: (1) the timing of colonizations, (2) their synchrony, and (3) the source population(s) for each Iberian colony.

Considering first the timing of the colonizations, four different hypotheses can be postulated: (1) a natural, ancestral colonization (between 2 Mya and 200 000 BP), (2) a natural, but more recent colonization (between 200 000 BP and 5000 BP), (3) a prehistorical or historical colonization (5000 BP–200 years ago), or (4) recent colonizations (<200 years ago).

With regard to synchrony, one of a number of combinations might be true. A scenario in which the Malaga population was established by a natural or prehistoric event and the other populations resulted from more recent colonizations is perhaps the most commonly accepted. Morocco seems to be the logical source population for both natural and human-driven colonization. However, the location of the source population is the subject of debate. The aim of this paper is to address these three aspects of the origin of the Iberian chameleons. In order to achieve this, sequences of a fragment of the mitochondrial 16S rRNA gene, from a selection of chameleon populations, were examined.

MATERIALS AND METHODS

Population samples

Individuals were sampled under licence, and were free-living animals directly collected for this study, or samples were collected as road kills. Muscle tissue was sampled from dead animals and blood was taken from live animals. Live animals were immediately released in the wild after being sampled, and none were deliberately or accidentally sacrificed during the course of this work. Thirty-seven individuals from eight populations were used in the study. The locations of the eight populations are shown in Figure 1. One population is from Portugal (Algarve), three are from Spain (Huelva, Cadiz and Malaga), and four are from Morocco (Al Hoceima, Essaouira, El Jadida, and Erfoud). Previously published sequences of 16S rRNA from two closely related species *Chamaeleo africanus*, *Chamaeleo dilepis* and from the Algarve population of *Chamaeleo chamaeleon* (Kosuch *et al.,* 1999) were taken from GenBank (accession numbers AF121960, AF121957 and AF121956, respectively) and used as outgroups and an additional ingroup.

DNA extraction, amplification and sequencing

Total DNA extraction was carried out using standard protocols (Sambrook *et al.,* 1989). Polymerase chain reaction (PCR) amplifications of a fragment of the mitochondrial DNA (mtDNA) 16S ribosomal RNA gene was carried out using published primers: L02510 5′-CGCCTGTTTATCAAAAACAT-3′(Palumbi, 1996) and H03063 5′-CTCCGGTTTGAACTCA GATC-3′ (Rassmann, 1997). These primers are named according to their occurrence on the heavy (H) or light (L) strand and the position of their 3′ base in the human mitochondrial DNA sequence (Anderson *et al*., 1981). Detailed PCR procedures and conditions were used as described previously (Paulo, 2001). PCR products were then cleaned and concentrated with

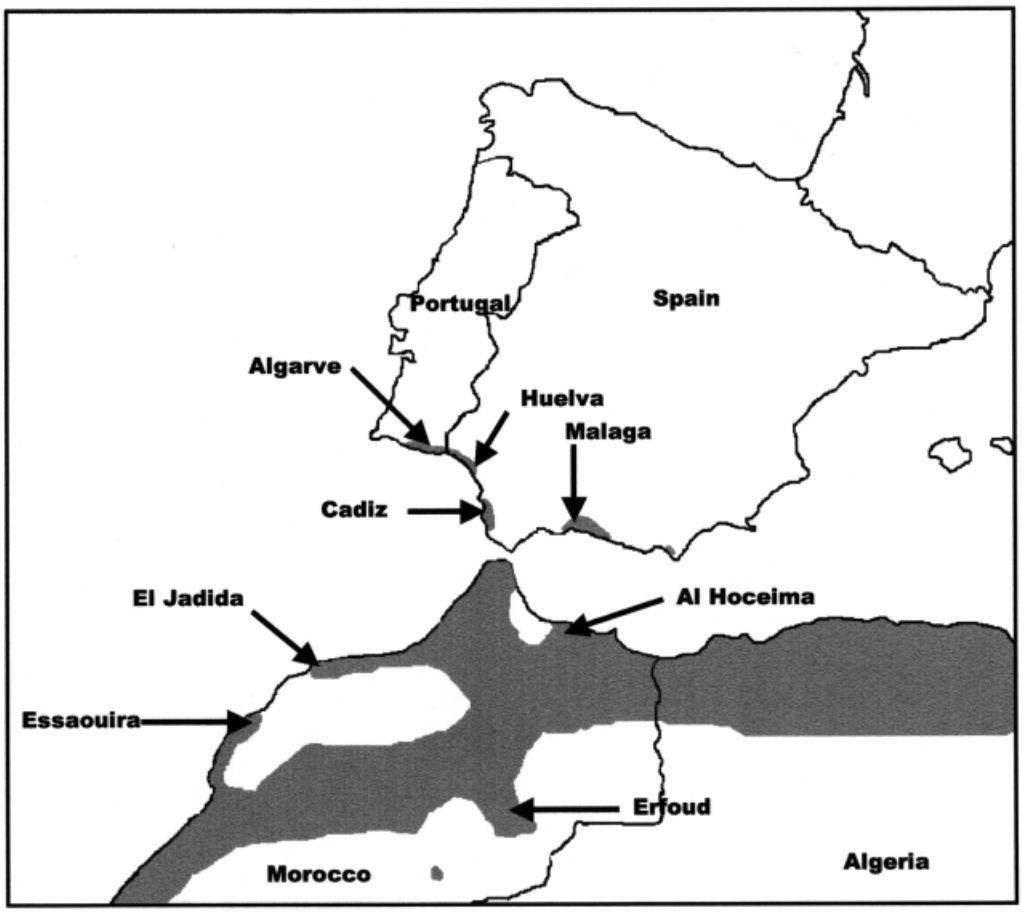

Figure 1. Map of the Iberian Peninsula and part of North Africa, with the approximate distribution of chameleons shown in grey. The positions of sampled chameleon populations are indicated.

Geneclean (Bio 101) and 25–50 pg of purified DNA template was used for dRhodamine terminator cycle sequencing (ABI Prism) following the manufacturer's instructions. Sequencing products were resolved on a semiautomatic genetic analyser (ABI Prism model 377). Both strands were sequenced for all samples.

Sequences were aligned first using SEQUENCHER software and then checked by eye. They are deposited in GenBank under the accession numbers AF372127–AF372133.

Analytical Methods

Phylogenetic analysis using maximum parsimony, maximum likelihood and distance-based criteria was performed using PAUP*4.0.B4A (Swofford, 2000). Gaps were treated as a fifth character state for the parsimony analysis or as missing data for other analyses.

For unweighted maximum parsimony, the optimal tree was found by a heuristic search with tree-bisection-reconnection as the branch-swapping algorithm. Initial trees were obtained via stepwise addition with 100 replicates of random addition sequence. The g1-statistic was calculated from the frequency distribution of lengths of a thousand random trees and the tree length of the optimal tree compared with this distribution (Hillis & Huelsenbeck, 1992). Ensemble indices, consistency index (Kluge & Farris, 1969), retention index (Farris, 1969) and homoplasy index (Archie, 1989), were calculated to describe the amount of homoplasy of the tree.

For maximum likelihood and distance-based phylogenetic analysis, MODELTEST 3.0 software (Posada & Crandall, 1998) associated with PAUP* was used to select an appropriate model of sequence evolution. The selected model was then used to calculate the maximum likelihood phylogenetic tree and a neighbour-joining tree (Saitou & Nei, 1987). The likelihood calculation had parameters describing the variation in substitution rate among sites (the shape parameter of a gamma distribution split into four categories), the

proportion of invariant sites, and the relative rate of the two types of transition and transversion.

In all forms of analyses, bootstrapping with 1000 pseudo-replicates was performed to evaluate the robustness of the nodes of obtained phylogenetic trees.

RESULTS

Of the 478 base pairs analysed, 70 were variable but only nine were parsimony informative. The differences between haplotypes were generally small. The maximum difference between any two haplotypes was seven substitutions, when each gap was considered as a character (Table 1). Thirty-seven samples were analysed, and seven different haplotypes were detected.

The maximum parsimony tree is presented in Figure 2. As the phylogenetic trees produced by maximum likelihood and neighbour-joining had the same topology, only the bootstrap values for those analyses are shown. The tree length of the maximum parsimony tree was 84, exceeding the minimum (for the observed number of substitutions) of 80 because of homoplasies (consistency index = 0.952, retention index = 0.733, and homoplasy index = 0.048). The random generation of trees produced a highly skewed distribution suggesting a phylogenetically informative data set ($g1 = -1.052$; $P < 0.01$) (Hillis & Huelsenbeck, 1992).

The most appropriate evolutionary model calculated through MODELTEST and PAUP software was the Tamura-Nei model (Tamura & Nei, 1993) with equal rates of variation for all the sites, and a specific substitution rate for G→A transition of 1.28 and for C→T transition of 3.73.

TREE TOPOLOGY AND CLADE DIFFERENTIATION

Two distinct clades were formed by the seven ingroup haplotypes. These clades were separated by only four substitutions. Clade A (haplotypes H1, H2 and HGB1) had higher bootstrap support than clade B (haplotypes H3, H4, H5, H6, H7) in all forms of analyses. The average pairwise genetic distance between the two clades, calculated with the Tamura-Nei correction, was 0.8% and the maximum distance between any two *C. chamaeleon* haplotypes was 1.2%. These distances were lower than the 3% difference that was found between *C. africanus* and *C. chamaeleon* sequences.

Out of a total of 24 individuals from the populations of Algarve, Huelva, Cadiz, El Jadida and Essaouira, only two haplotypes were found (H1 and H2), both haplotypes clustering in clade A (Fig. 2, Table 2). The three populations in Iberia and two in Africa shared the most common haplotype (H2). The other haplotype in this group (H1), was detected in five out of six individuals from the El Jadida population, with the sixth individual having the shared haplotype H2 (Table 2). The GenBank sequence of *C. chamaeleon* from the Algarve (HGB1) included as an ingroup sample in this analysis clustered with the other Algarve haplotype (H1), and differed from each other by two base pairs. The remaining populations (Malaga, Al Hoceima and Erfoud) contained the remaining five haplotypes that were clustered in clade B (Fig. 2, Table 2).

Haplotypes H3, H4, and H5 were private to the Erfoud population while haplotype H6 was shared between the North African population of Al Hoceima and the Iberian population of Malaga. The Al Hoceima population also had a private haplotype, H7 (Table 2).

Table 1. Informative sites of the haplotypes detected, with the sequence of each specific haplotype, H1 to H7 and HBG1. The informative sites correspond to the positions: 2734, 2735, 2757, 2758, 2837, 2840, 2926, 2987 and 3004, according to their occurrence in the light strand in the human mitochondrial DNA. The dash represents gaps in the sequences and the period represents the same base as in the reference sequence

Haplotype	Bases										
H1	T	T	A	A	T	G	C	G	A	C	G
HGB1	-	-	.	.	.	.	.	.	G	.	A
H2	-	-	.	.	.	.	.	.	A	.	G
H3	-	-	-	.	-	.	G	A	A	T	G
H4	-	-	-	.	.	A	G	A	A	T	G
H5	-	-	-	.	.	G	G	A	A	T	G
H6	-	-	.	.	.	G	G	A	A	T	G
H7	-	-	.	T	.	G	G	A	A	T	G

Table 2. Haplotype distribution by population. Each column represents one particular haplotype from H1 to H7, the main area of the table indicates the numbers of individuals at each locality with a specific haplotype. Column totals show the overall occurrence of each haplotype and the row totals show the number of samples analysed per population

Population	H1	H2	H3	H4	H5	H6	H7	*N*
Algarve		5						5
Huelva		4						4
Cadiz		4						4
El Jadida	5	1						6
Essaouira		5						5
Erfoud			4	2	1			7
Al Hoceima						1	1	2
Malaga						4		4
Total	5	19	4	2	1	5	1	37

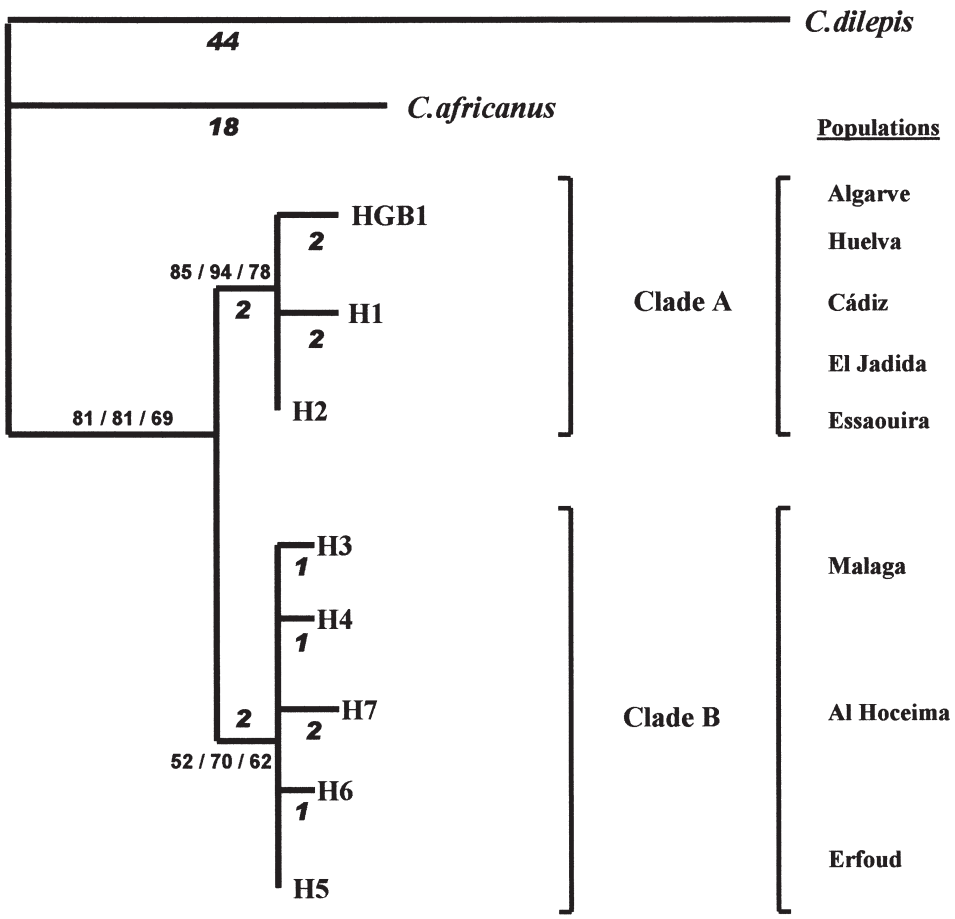

Figure 2. Unweighted maximum parsimony tree showing relationships between the seven haplotypes of detected in the chameleon mtDNA 16S ribosomal RNA gene (H1 to H7). Haplotype HGB1 is the GenBank sequence of a chameleon sample from the Algarve population. The two outgroups are also GenBank sequences. Numbers in italics under branches indicate the number of substitutions along that branch and numbers above branches are the bootstrap support values of maximum parsimony, neighbour-joining (with distances corrected by the Tamura-Nei model) and maximum likelihood trees, respectively. The populations in which haplotypes from the two clades were found are also indicated.

DISCUSSION

Origin of Iberian populations

In our sample of 17 individuals of Mediterranean chameleons from the Iberian Peninsula, only two mtDNA haplotypes were found, while seven haplotypes were detected in 20 individuals from North African populations. Such a pattern of relative diversity strongly suggests that the Iberian populations were founded by individuals from North African populations. Furthermore, the presence of two distinct clades, with Iberian and North African individuals represented in each, supports a model of dual colonization of Iberia from North Africa. The Malaga population of chameleons (on the Mediterranean side of the Iberian peninsula) appears to originate from the Mediterranean populations of North Africa as haplotypes found in Malaga are also found in the Al Hoceima population and these cluster strongly with haplotypes from the desert population of Erfoud, south of the Atlas Mountains (Figs 1 and 2).

In contrast, the populations of chameleons near the Atlantic coast of Iberia (i.e. Cadiz, Huelva and Algarve) were probably founded by animals that came from the Atlantic coast of Morocco. Moreover, it is probable that these Iberian populations originated from the south-western part of Morocco. All five individuals sampled from the Essaouira population possessed a single haplotype (H2), which is identical to the haplotype fixed in the Atlantic Iberian populations. The H2 haplotype is also found in the north-west Moroccan El Jadida population, but at a much lower frequency. Unfortunately, we were not able to obtain samples from the intermediate populations of

the Rabat-Tanger coast, so we cannot exclude this region as a potential source for the Atlantic Iberian populations. If an Essaouiran origin for the Atlantic Iberian populations of chameleon is confirmed, a human-driven colonization model is strongly favoured, as this region is not geographically close to Iberia. However, Essaouira was a historically important trading port, as far back as the 7th century BC when the Phoenicians discovered the area, followed by latter-day colonization and trade by the Romans and Portuguese.

Divergence times

The level of genetic differentiation detected between clade A and B was much lower than the levels detected among species of chameleons (Kosuch *et al*., 1999). Moreover, these levels are much reduced compared to the levels detected in a group of ocellated lizard species (*Lacerta lepida*/*Lacerta tangitana*) from both sides of the Strait of Gibraltar, where comparable genetic data are available (Paulo, 2001).

From our ocellated lizard data (*L. lepida*/*L. tangitana*) it was possible to derive calibration rates of sequence evolution in mtDNA 16S rRNA gene. These data suggested that the substitution rate in the 16S rRNA gene is ~0.42% sequence divergence per million years. The average difference between chameleon clades is 0.8%. Assuming a similar rate for the provisional molecular clock between the ocellated lizard and chameleons, the divergence between the two clades corresponds to the Plio-Pleistocene transition. However as proportionately larger errors are associated with smaller divergences, the uncertainty associated with this estimate is considerable.

Within clades, the sharing of haplotypes between North African and Iberian populations suggests relatively recent divergence, and the rejection of the hypothesis of natural ancestral colonization close or after the Plio-Pleistocene transitions. However, the three alternative hypotheses previously described (natural more recent, prehistorical/historical or recent event) cannot be distinguished, but the two human-mediated hypotheses seem to be more probable than the second hypothesis. Similarly, the relative timing of colonization or introduction of the various Iberian populations is yet to be resolved, and it remains possible that the events occurred close to each other as previously suggested (Blasco, 1997a, 1997b).

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