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Eastern Mediterranean chameleons (*Chamaeleo chamaeleon*, *Ch. africanus*) are distinct

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Abstract. Based on mitochondrial 16S rRNA sequences, we suggest that the founder individuals of the introduced Greek population of *Chamaeleo africanus* originated in the Nile Delta region of Egypt. In *Ch. chamaeleon*, we discovered in the eastern Mediterranean new 16S rRNA haplotypes, being highly distinct from previously published western Mediterranean haplotypes. Eastern Mediterranean haplotypes were found in samples from northern Syria, Cyprus, Crete, Samos, Malta and Tunisia. The occurrence of an eastern Mediterranean haplotype in Tunisia and of distinct haplotypes in Morocco could argue for a phylogeographic break in northwestern Africa.

Keywords: 16S rRNA gene, *Chamaeleo africanus*, *Chamaeleo chamaeleon*, Eastern Mediterranean, phylogeography.

Introduction

In Europe occur two chameleon species, the Common Chameleon, *Chamaeleo chamaeleon* (Linnaeus, 1758), distributed in the southern Iberian Peninsula, Greece, Cyprus, Sicily and Malta (Klaver, 1981; Blasco, 1985), and the African Chameleon, *Ch. africanus* Laurenti, 1768, restricted to the vicinity of Pylos, Peloponnese, Greece (Böhme et al., 1998). While the Common Chameleon is widely distributed in the Mediterranean and the Middle East, *Ch. africanus* is a central African species that has reached Egypt via the Nile River (Anderson, 1898; Joger, 1981; Klaver, 1981; Böhme, 1985; Lutzmann, 2003).

In contrast to many other Mediterranean amphibians and reptiles, the phylogeography of chameleons is badly understood. Using the 16S rRNA gene, Kosuch et al. (1999) confirmed that the chameleons occurring near Pylos represent *Ch. africanus* and found that *Ch. africanus* from Chad are genetically clearly distinct from the Greek population. Paulo et al. (2002) analyzed Moroccan and Iberian *Ch. chamaeleon* populations using the same gene and discovered two haplotype clades, one occurring in Moroccan and Iberian sites along the Atlantic coast and another comprising sequences from Erfoud and Al Hoceima (Morocco) and Malaga (Spain). Paulo et al. (2002) concluded that chameleons are not native in the Iberian Peninsula and were probably introduced in the recent past from two distinct regions of Morocco. In the present paper we use the published known-locality sequences from Kosuch et al. (1999) and Paulo et al. (2002) for comparison with new sequences of *Ch. africanus* and *Ch. chamaeleon* from the eastern Mediterranean to gain a better insight in the phylogeographic structure of these endangered species.

Materials and methods

Sampling and laboratory techniques

Tissue and saliva samples were obtained from three *Ch. africanus* and 11 *Ch. chamaeleon* (table 1), including for the first time an Egyptian individual of *Ch. africanus* and Tunisian, Maltese and eastern Mediterranean specimens of *Ch. chamaeleon*. Tissues were collected from dead specimens found in the field (now deposited in the Goulandris Natural History Museum, Kifissia); three samples were provided from the Natural History Museum of Crete, Irakleio. For allowing a comparison with previously published data of Kosuch et al. (1999) and Paulo et al. (2002), we decided

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Table 1. Haplotype distribution of *Chamaeleo africanus* and *Ch. chamaeleon*, based on the results of Kosuch et al. (1999), Paulo et al. (2002) and from this study. For accession numbers of previously published haplotypes, see references. Accession numbers of haplotypes reported in this study are: FM162016-FM162019.

Species	Site	Ca1	Ca2	K1	P1	P2	P3	P4	P5	P6	P7	D1	D2	D3	D4	*n*	Reference
Ch. africanus	Pylos, Greece	3	–	–	–	–	–	–	–	–	–	–	–	–	–	3	Kosuch et al. (1999), this study
	Chad	–	1	–	–	–	–	–	–	–	–	–	–	–	–	1	Kosuch et al. (1999)
	Assuan, Egypt	1	–	–	–	–	–	–	–	–	–	–	–	–	–	1	This study
	Total	4	1	–	–	–	–	–	–	–	–	–	–	–	–	5	
Ch. chamaeleon	Algarve, Portugal	–	–	1	–	5	–	–	–	–	–	–	–	–	–	6	Kosuch et al. (1999), Paulo et al. (2002)
	Huelva, Spain	–	–	–	–	4	–	–	–	–	–	–	–	–	–	4	Paulo et al. (2002)
	Cadiz, Spain	–	–	–	–	4	–	–	–	–	–	–	–	–	–	4	Paulo et al. (2002)
	El Jadida, Morocco	–	–	–	5	1	–	–	–	–	–	–	–	–	–	6	Paulo et al. (2002)
	Essaouria, Morocco	–	–	–	–	5	–	–	–	–	–	–	–	–	–	5	Paulo et al. (2002)
	Marrakech, Morocco	–	–	–	–	1	–	–	–	–	–	–	–	–	–	1	This study
	Erfoud, Morocco	–	–	–	–	–	4	2	1	–	–	–	–	–	–	7	Paulo et al. (2002)
	Al Hoceima, Morocco	–	–	–	–	–	–	–	–	1	1	–	–	–	–	2	Paulo et al. (2002)
	Malaga, Spain	–	–	–	–	–	–	–	–	4	–	–	–	–	–	4	Paulo et al. (2002)
	near Tunis, Tunisia	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1	This study
	Malta	–	–	–	–	–	–	–	–	–	–	1	1	–	–	2	This study
	Crete, Greece	–	–	–	–	–	–	–	–	–	–	–	–	1	–	1	This study
	Samos, Greece	–	–	–	–	–	–	–	–	–	–	–	–	3	–	3	This study
	Cyprus	–	–	–	–	–	–	–	–	–	–	–	–	2	–	2	This study
	Samaan Mts, Syria	–	–	–	–	–	–	–	–	–	–	1	–	–	–	1	This study
	Total	–	–	1	5	20	4	2	1	5	1	2	1	6	1	49	

to sequence the mitochondrial 16S rRNA gene. Using approximately 1-3 mm^3 of tissue or oral swab material, total genomic DNA was extracted by overnight incubation at 55°C in lysis buffer (10 mM Tris, pH 7.5, 25 mM EDTA, 75 mM NaCl, 1% SDS) including 0.5 mg of proteinase K (Merck) and subsequent purification with a standard chloroform protocol. DNA was precipitated from the supernatant with 0.2 volumes of 4M LiCl and 0.8 volumes of isopropanol, centrifuged, washed, dried and resuspended in TE buffer. Two fragments of the 16S rRNA gene were amplified using the primers 16SA (Kocher et al., 1989) and H03063 (Rassmann, 1997). PCR was performed in a 50 μL volume (50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.5) containing 1 unit of *Taq* DNA polymerase (Bioron), 10 pmol dNTPs (Eppendorf or Fermentas), and 10 pmol of each primer. After initial denaturing for 90 s at 94°C, 35 cycles were performed with denaturing 45 s at 94°C, annealing 45 s at 48°C, and primer extension for 90 s at 72°C, followed by a final elongation of 5 min at 72°C. PCR products were purified by precipitation under the following conditions: 1 volume PCR product (30 μL), 1 volume 4 M NH_4Ac (30 μL) and 12 volumes EtOH (100%; 360 μL). DNA was pelleted by centrifugation and the pellet washed with 70% ethanol. The pellet was dissolved in 20 μL H_2O. PCR products were sequenced with the primer 16SA on an ABI 3130 sequencer (Applied Biosystems) following standard procedures. Sequences were individually checked and aligned using BioEdit 7.0.5.2 (Hall, 1999).

Phylogenetic and network analyses

As outgroups we downloaded from GenBank the same sequences as used in Kosuch et al. (1999), *Chamaeleo gracilis*, *Ch. dilepis*, *Ch. quadricornis*, *Brookesia* cf. *brygooi*, *B. peyrierasi* and *Rhampholeon brevicaudatus* (for accession numbers, see Kosuch et al., 1999).

Sequence data were analysed using the optimality criteria Maximum Parsimony (MP; equal weighting) and Maximum Likelihood (ML) as implemented in PAUP* 4.0b10 (Swofford, 2002; setting swap = TBR). The best evolutionary model for the data was selected by the Akaike information criterion (AIC) using Modeltest 3.06 (best-fit model: GTR + G; Posada and Crandall, 1998). Under ML, the starting tree was obtained by stepwise addition. Bootstrap support was calculated with PAUP* 4.0b10 for MP with both means of gap treatment calculated separately (gapmode = missing or gapmode = newstate; other settings: maxtre = 1000, def hs add = cl and nreps = 1000) and ML (maxtre = 10, nreps = 100).

As intraspecific gene genealogies are not necessarily well-represented by software enforcing dichotomous splits (Posada and Crandall, 2001), we calculated also parsimony networks using TCS 1.21 (Clement et al., 2000), for which gaps were coded as fifth character state.

Results

Our Egyptian and Greek samples of *Chamaeleo africanus* yielded the same haplotype as the

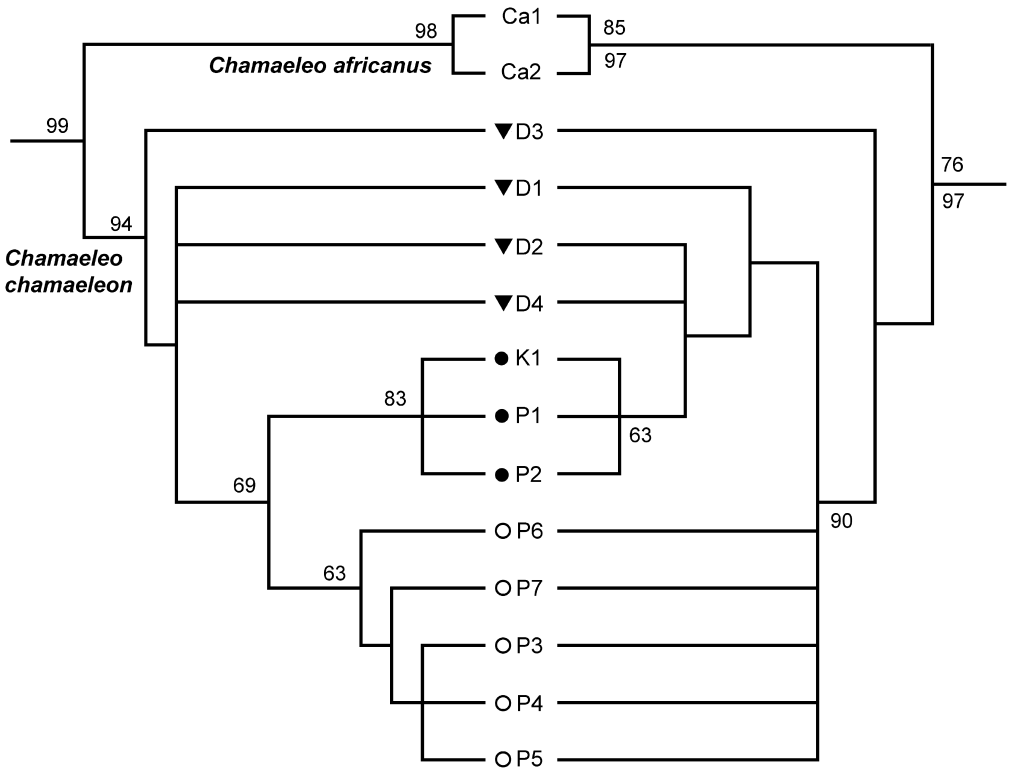

Figure 1. Phylogenetic hypotheses for the relationships of haplotypes of *Chamaeleo chamaeleon* and *Ch. africanus*. Left, 50% majority rule consensus of 27 equally parsimonious trees (CI = 0.7035, RI = 0.6655; 313 steps) with gaps coded as fifth character state (in the strict consensus, haplotypes P3-P7 of *Ch. chamaeleon* occur in a polytomy together with a clade comprising haplotypes K1, P1, P2). Right, topology as revealed by strict consensus trees under ML (24 trees) and MP (52 trees) when gaps are coded as missing (CI = 0.7088, RI = 0.5870; 256 steps). Numbers along nodes are bootstrap support values greater than 50; left, top: ML (maxtree = 10, 100 replicates), bottom: MP (maxtree = 1000, 1000 replicates). Outgroups removed for clarity.

individual from the vicinity of Pylos, Greece, studied Kosuch et al. (1999). This haplotype (Ca1) is highly distinct from another *Ch. africanus* haplotype identified by Kosuch et al. (1999) from Chad (Ca2; table 1). In parsimony network analysis, both *Ch. africanus* haplotypes differ by six mutational steps and are not connected with *Ch. chamaeleon* haplotypes when 90-95% probability thresholds are applied for the connection limit. Despite their distinctness, both haplotypes occur under all tree-building methods in a well-supported clade being the sister of *Ch. chamaeleon* (fig. 1).

As for *Ch. chamaeleon*, our sample from the vicinity of Marrakech, Morocco, contained a haplotype (P2) previously identified from Morocco and the Iberian Peninsula by Paulo et al. (2002). Our other samples represent four new haplotypes (D1-D4; table 1). All of these eastern Mediterranean haplotypes differ from Moroccan and Iberian haplotypes consistently in the occurrence of gaps at positions 231-236 and 280 of our alignment. Due to the occurrence of these gaps, the eastern Mediterranean haplotypes form a clearly distinct cluster in parsimony network analysis. Western Mediterranean haplotypes are placed in two clusters being separated by a minimum of three steps (fig. 2). These clusters correspond with the two clades of western Mediterranean haplotypes of Paulo et al. (2002).

In all of our phylogenetic analyses, the monophyly of *Ch. chamaeleon* haplotypes is well-supported; however, the interrelationships

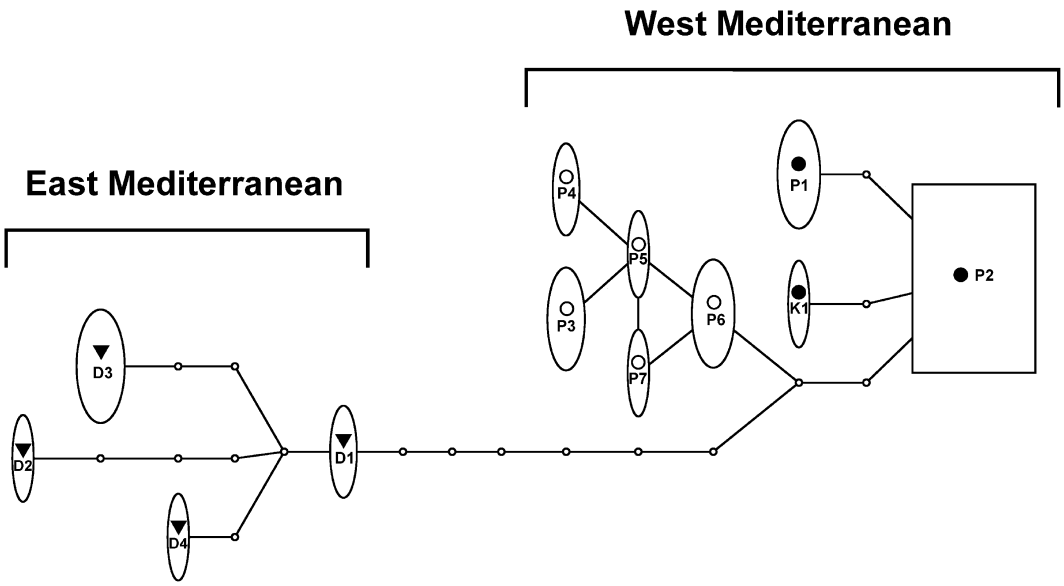

Figure 2. Parsimony network of 16S rRNA haplotypes of *Chamaeleo chamaeleon* (gaps coded as fifth character state). Symbol size corresponds with haplotype frequency; small circles indicate missing intermediate haplotypes. Each line between haplotypes and circles stands for one mutational step. The haplotype with the greatest outgroup probability (0.3390) is shown on the right as rectangle. Symbols correspond with fig. 1. For geographic distribution and frequency of individual haplotypes, see table 1 and fig. 3.

of individual haplotypes are weakly resolved. All methods confirm that one of the eastern Mediterranean tip-haplotypes (D3) is highly distinct, placing it as sister to a clade containing all other *Ch. chamaeleon* haplotypes. Moreover, the monophyly of the three haplotypes K1, P1 and P2 is weakly to well-supported, depending on the tree-building method. The monophyly of eastern Mediterranean haplotypes is not supported, and the second western Mediterranean clade (P3-P7) revealed by Paulo et al. (2002) is only recovered with weak support by MP when gaps are coded as fifth character state. Many branching patterns are contradictory and mostly weakly supported when the different methods are compared (fig. 1).

Discussion

For a long time, it was thought that all European chameleons represent the Common Chameleon, *Chamaeleo chamaeleon* (Klaver, 1981). However, Böhme et al. (1998) discovered that a second species, the African Chameleon (*Ch. africanus*), occurs near Pylos, Peloponnese. The European populations of both species are generally considered to be introduced by man (Böhme et al., 1998; Kosuch et al., 1999; Paulo et al., 2002). While northwestern Africa is the likely source for the Spanish and southern Portuguese populations of *Ch. chamaeleon* (Paulo et al., 2002), the geographic origin of the founder individuals of Greek *Ch. africanus* remained unclear. A sub-Saharan individual of *Ch. africanus* from Chad, studied by Kosuch et al. (1999), was clearly distinct from an African Chameleon from the vicinity of Pylos. Based on external morphological characters, Böhme et al. (1998) speculated that the Greek population could be derived from African Chameleons from the Nile Delta. This hypothesis is supported by our sequence data, providing evidence that the same haplotype occurs in the Egyptian Assuan region and in Greece. Based on an analysis of 16 morphological variables and 10 character ratios, Dimaki (2007) came to the same conclusion. She found that *Ch.*

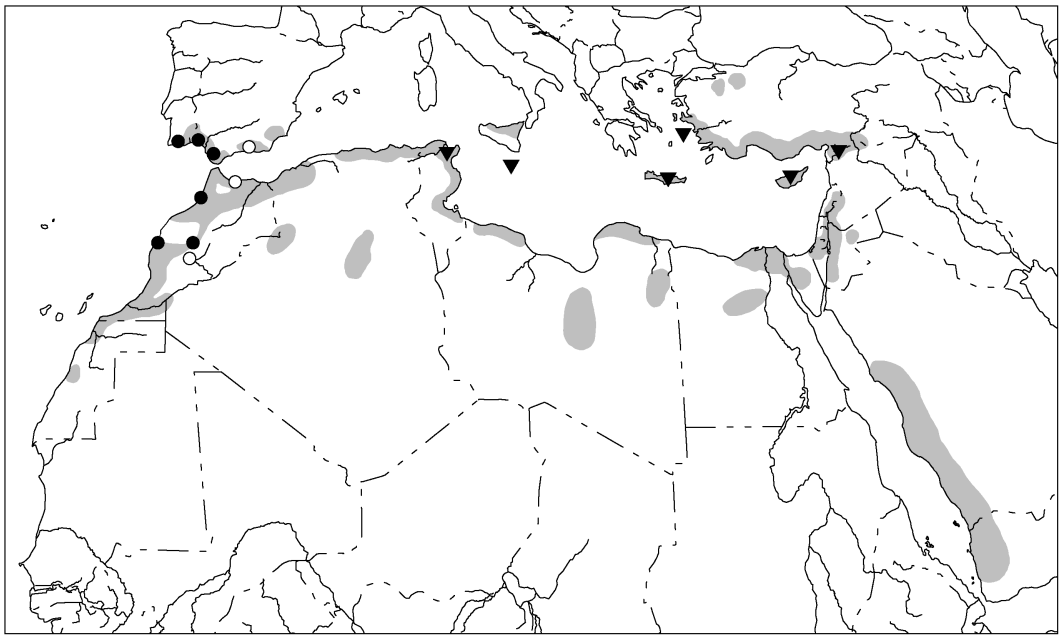

Figure 3. Geographic distribution of haplotype groups of *Chamaeleo chamaeleon* in the Mediterranean. Range shaded; solid and open circles: western Mediterranean haplotypes; triangles: eastern Mediterranean haplotypes. Symbols correspond with figs 1-2. For exact localities and individual haplotypes, see table 1.

africanus from Pylos and Egypt clustered together while central and east African specimens were clearly distinct.

All previously published genetic data of *Ch. chamaeleon* were based on western Mediterranean individuals. In eastern Mediterranean chameleons we discovered clearly distinct 16S rRNA haplotypes, suggestive of considerable phylogeographic variation and of subspecific variation (fig. 3). The discovery of a Tunisian haplotype (D4) resembling the eastern Mediterranean haplotypes (fig. 2) could indicate a phylogeographic break in northwestern Africa, like in several other amphibians and reptiles (e.g., newts: Carranza and Arnold, 2003; Carranza and Wade, 2004; Veith et al., 2004; tree frogs: Recuero et al., 2007; terrapins: Fritz et al., 2006; snakes: Guicking et al., 2006).

For a complete understanding of the phylogeography of the common chameleon, a range-wide sampling is needed. In the present study we have chosen the slowly evolving 16S rRNA gene as marker to enable a comparison with previously published sequence data. However, it is obvious from our phylogenetic trees that additional and more variable markers, like for instance the mitochondrial cytochrome *b* gene, are needed for obtaining a better resolved phylogeographic structure and for shedding new light on the badly understood subspecific differentiation of Common Chameleons. Already from our preliminary data it is obvious that the allocation of all European populations of *Ch. chamaeleon* to the same subspecies, as suggested by Klaver (1981) and other authors, is unlikely when the highly distinct haplotypes of Iberian, Maltese and Greek chameleons are considered. This point of view is also supported by the finding of Dimaki (2007) that the Common Chameleons on Samos are morphologically clearly distinct from chameleons from North Africa, the Iberian Peninsula, southern Arabia and the Middle East.

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