Mitochondrial DNA sequence data support the allocation of Greek mainland chameleons to *Chamaeleo africanus*

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European chameleon populations have generally been referred to the species *Chamaeleo chamaeleon* (see e.g. Klaver, 1981; Blasco Ruiz, 1997), including those from the Greek islands Chios, Samos and Crete and the Greek mainland (Schreiber, 1912; Werner, 1938; Wettstein, 1953; Klaver, 1981; Böhme, 1989). Recently, however, Böhme et al. (1998) reported that a chameleon population from Gialova near Pylos in the south-western Peloponnesos should be morphologically assigned to the African species *Chamaeleo africanus*. The aim of the present study was to test this classification using molecular characters.

Tissue samples (hindleg muscle, either fresh or preserved in 98% ethanol) were available from the following species (one specimen, respectively): *Chamaeleo africanus*, Gialova near Pylos, Messenia, Greece, ZFMK 65651; *C. africanus*, Chad, ZFMK 65568; *C. chamaeleon*, captive bred-specimen (breeding stock originating from southern Portugal), ZFMK 68644; *C. gracilis*, Togo, from the pet trade, ZFMK 68645; *C. dilepis*, Grootfontein, Namibia (voucher not available); *C. quadricornis*, Manengouba Mountains, Cameroon, ZFMK 66735; *Rhampholeon brevicaudatus*, Uluguru mountains, Tanzania, ZFMK 68487;

Table 1. Pairwise substitution rates between taxa studied based on sequence differentiation of a 492 bp fragment of the mitochondrial 16S rRNA gene (gaps treated as fifth base). Below diagonal: absolute number of substitutions; above diagonal: relative substitution rates.

Species	1	2	3	4	5	6	7	8	9
1 *Brookesia* cf. *brygooi*	–	0.159	0.147	0.153	0.169	0.131	0.145	0.157	0.175
2 *B. peyrierasi*	79	–	0.181	0.181	0.197	0.167	0.173	0.173	0.193
3 *Chamaeleo africanus* (Greece)	73	90	–	0.012	0.056	0.070	0.109	0.107	0.119
4 *C. africanus* (Chad)	76	90	6	–	0.058	0.070	0.113	0.115	0.125
5 *C. chamaeleon*	84	98	28	29	–	0.093	0.127	0.137	0.131
6 *C. gracilis*	65	83	35	35	46	–	0.119	0.115	0.131
7 *C. dilepis*	72	86	54	56	63	59	–	0.103	0.115
8 *C. quadricornis*	78	86	53	57	68	57	51	–	0.123
9 *Rhampholeon brevicaudatus*	87	96	59	62	65	65	57	61	–

Brookesia cf. *brygooi*, Kirindy near Morondava, Madagascar, ZFMK 66707; *Brookesia peyrierasi*, Nosy Mangabe, Madagascar, ZFMK 66670.

DNA was extracted using QuiAmp tissue extraction kits (Quiagen). We used the primers 16SA (light chain; 5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16SB (heavy chain; 5′-CCG GTC TGA ACT CAG ATC ACG T-3′) of Kocher et al. (1989) to amplify a section of about 560 base pairs (bp) of the mitochondrial 16S gene. PCR cycling procedure was as follows. Initial denaturation step: 90 s at 94°C; 33 cycles: denaturation 45 s at 94°C, primer annealing for 45 S at 55°C, extension for 90 s at 72°C. PCR products were purified using Quiaquick purification kits (Quiagen). We sequenced a single-stranded 497 bp fragment homologous to the bp positions 4008-4611 of the *Xenopus laevis* mitochondrial genome (Roe et al., 1985), using an automatic sequencer (ABI 377). Sequences are available from GenBank (accession numbers AF121953-AF121961).

Sequences were aligned using the computer program SEQUENCE NAVIGATOR (Applied Biosystems). Alignments were subsequently adjusted manually. We omitted a short section (5 bp) from the original data set (bp 233-237 of the aligned sequences including gaps, corresponding to bp 229-233 of the unaligned *C. chamaeleon* sequence, not counting gaps) which was too variable to be reliably aligned. Sequences were analysed using PAUP 3.1.1. (Swofford, 1993). We calculated a maximum parsimony (MP) tree with gaps treated as a fifth character. Two thousand bootstrap replicates (Felsenstein, 1985) were run following Hedges (1992); only bootstrap supports of 70% and higher were considered as reliable, as such values were found to indicate 95% probability of correct topology by Hillis and Bull (1993). Two dwarf chameleon species of the genus *Brookesia* were used as outgroups.

In the resulting MP tree (fig. 1), the Greek and subsaharian specimens of *C. africanus* cluster together. *Chamaeleo chamaeleon* is the sister group of *C. africanus*, and *C. gracilis* is the sister group of the clade consisting of *C. africanus* and *C. chamaeleon*. These aspects of the tree topology are supported by high bootstrap values (> 80%). *Chamaeleo dilepis*, *C. quadricornis* and *Rhampholeon brevicaudatus* form a second branch of the tree. This

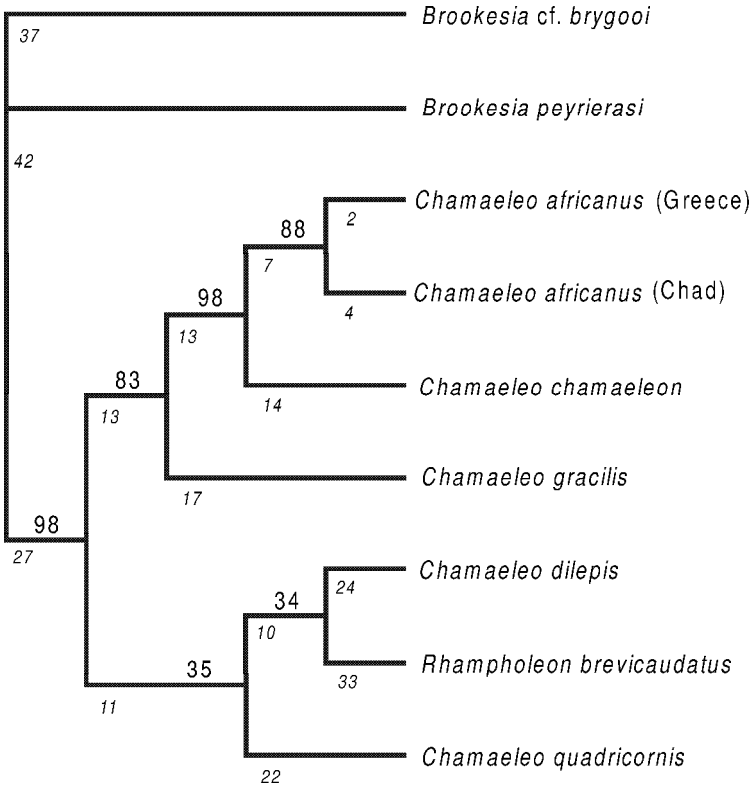

Figure 1. Phylogenetic tree of the taxa studied, produced by the maximum parsimony method using PAUP (gaps treated as fifth character state) and based on a 492 bp fragment of the mitochondrial 16S rRNA gene. Numbers are bootstrap values in percent (2000 replicates) and number of apomorphic changes (below each branch, in italics).

clade, however, is supported only by low bootstrap values (<35%). The ingroup clade containing all *Chamaeleo* species and the single *Rhampholeon* species was supported by a high bootstrap value of 98%, confirming that the two genera *Brookesia* and *Rhampholeon*, both containing terrestrial dwarf chameleons, are no monophyletic unit (Klaver and Böhme, 1997; see also Hofman et al., 1981). Sequence differentiation between the Greek and subsaharian *C. africanus* specimens was 1.2% (6 base pair differences with gaps taken into account), vs. 5.6-5.8% (28-29 base pair differences) between either *C. africanus* specimens and *C. chamaeleon* (table 1).

Considering the haplotypes of the studied chameleon specimens, the Greek specimen and the subsaharian *C. africanus* formed a distinct clade. This confirms that the Peloponnese chameleon belong to the species *Chamaeleo africanus*. To clarify the origin of the Greek chameleon population which, according to Böhme et al. (1998), may have been introduced in early historical times, a study including samples from a broader geographic range is necessary. The Peleponnesos specimens seem to differ from the subsa-

harian *C. africanus* by a markedly larger body size and a different colour pattern (Böhme et al., 1998). The observed sequence differences of the Greek specimen to the subsaharian *C. africanus* may indicate differentiation at the subspecific level. This is a further argument for the active protection of the Greek population (see Böhme et al., 1998; Bonetti, 1998). However, further studies involving the Nile Delta populations which appear to be morphologically similar to the Greek specimens (Böhme et al., 1998), and including other methodological approaches (e.g. allozyme electrophoresis), are necessary for taxonomic conclusions.

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References

Blasco Ruiz, M. (1997): *Chamaeleo chamaeleon* (Linnaeus, 1758). In: Atlas of Amphibians and Reptiles in Europe, p. 158-159. Gasc, J.P., Cabela, A., Crnobrnja-Isailovic, J., Dolmen, D., Grossenbacher, K., Haffner, P., Lescure, J., Martens, H., Martínez-Rica, J.P., Maurin, H., Oliveira, M.E., Sofianidou, T.S., Veith, M., Zuiderwijk, A., Eds, Paris, Societas Europaea Herpetologica and Muséum National d'Histoire Naturelle (IEGB/SPN).

Böhme, W. (1989): Neuer Nachweis von *Chamaeleo chamaeleon* (Linneaeus, 1758) vom Peloponnes, Griechenland. Herpetofauna **11**: 32-34.

Böhme, W., Bonetti, A., Chiras, G. (1998): The chameleons of the Greek mainland: taxonomic allocation and conservation needs of a second European species (Squamata: Sauria: Chamaeleonidae). Herpetozoa **11**: 87-91.

Bonetti, A. (1998): New life from Roman relics. BBC Wildl. **16** (7): 10-16.

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

Hedges, S.B. (1992): The number of replications needed for accurate estimation of the bootstrap P-value in phylogenetic studies. Mol. Biol. Evol. **9**: 366-369.

Hofman, A., Maxson, L.R., Arntzen, J.W. (1991): Biochemical evidence pertaining to the taxonomic relationships within the family Chamaeleonidae. Amphibia-Reptilia **12**: 245-265.

Hillis, D.M., Bull, J.J. (1993): An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. **42**: 182-192.

Klaver, C.J.J. (1981): Chamaeleonidae. *Chamaeleo chamaeleon* (Linnaeus, 1758) — Gemeines oder gewöhnliches Chamäleon. In: Handbuch der Reptilien und Amphibien Europas. Band 1, Echsen (Sauria) I, p. 217-238. Böhme, W., Ed., Wiesbaden, Akad. Verlagsgesellschaft.

Klaver, C.J.J., Böhme, W. (1997): Chamaeleonidae. Das Tierreich, Berlin, New York (de Gruyter) **112**: 1-85.

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

Roe, B.A., Din-Pow, M., Wilson, R.K., Wong, J.F. (1985): The complete nucleotide sequence of the *Xenopus leavis* mitochondrial genome. J. Biol. Chem. **260**: 9759-9774.

Schreiber, E. (1912): Herpetologia Europaea. 2nd ed. Jena, Fischer.

Swofford, D.L. (1993): PAUP: Phylogenetic Analysis Using Parsimony, version 3.1. Formerly distributed by Illinois Natural History Survey, Champaign, Illinois.

Werner, F. (1938): Die Amphibien und Reptilien Griechenlands. Stuttgart, Schweizerbarth.

Wettstein, O. (1953): Herpetologia Aegaea. Sitzungsber. österreich. Akad. Wiss., Wien, mathemath.-naturwiss. Klasse (1) **162**: 651-833.

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