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# Mitochondrial DNA sequence data suggests two independent colonizations of the Comoros archipelago by Chameleons of the genus *Furcifer*

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ABSTRACT. We used ND4 mtDNA sequences (815bp) to examine the relationships between *Furcifer* chameleons (Chamaeleonidae ; Reptilia) from the Comoro Islands. High genetic divergence between *F. cephalolepis* from Grand Comoro and *F. polleni* from Mayotte is hardly compatible with the hypothesis of them being sister-taxa given the young geological age of both islands. Thus, each island was independently colonized, presumably from Madagascar. Genetic diversity within both islands is similar, despite their very different geological ages. The degree of divergence found within a recent island like Grand Comoro may indicate that the molecular clock calibration typically applied to reptiles is not appropriate for this species.

KEY WORDS : Comoros, *Furcifer polleni*, *Furcifer cephalolepis*, Chamaeleonidae, colonization, ND4, molecular clock.

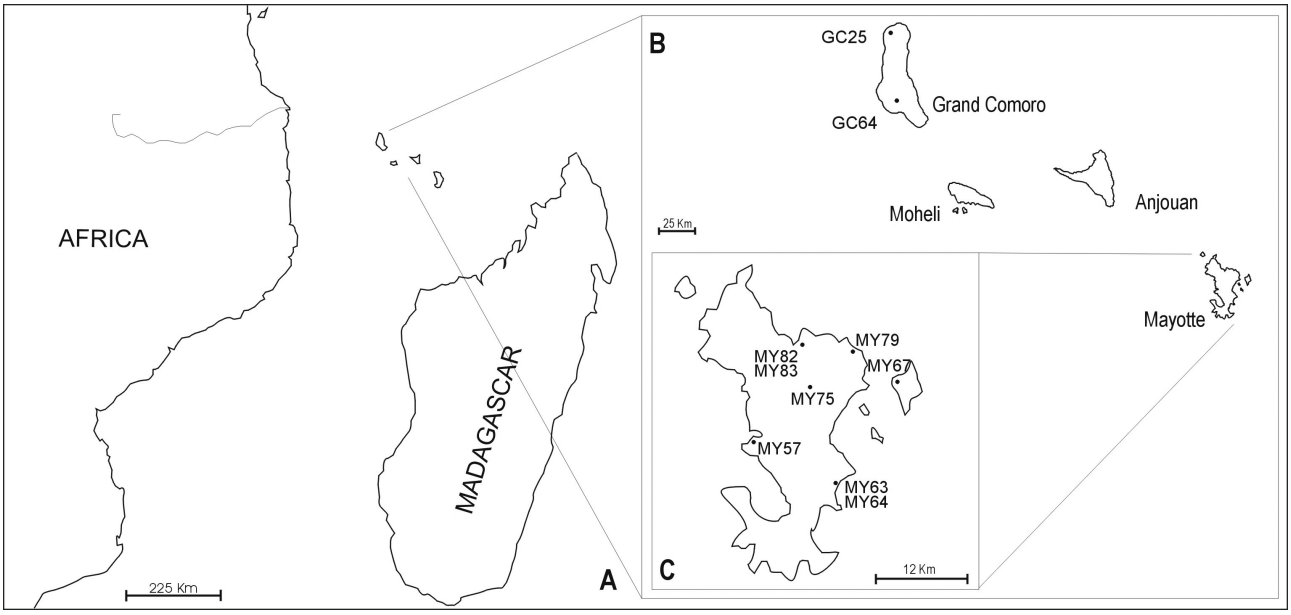


Fig. 1. – Maps showing : A) the position of Comoros archipelago relative to African mainland and Madagascar, B) the Comoros archipelago and the sampling sites in Grand Comoro and C) the sampling localities in Mayotte. Codes are given in Table 1.

## INTRODUCTION

The four major islands of the Comoros archipelago lie about 200 Km west of the northern tip of Madagascar, at the entrance of the Mozambique Channel (Fig. 1). After the separation of Madagascar from Mozambique, this volcanic chain of islands was formed, during the Miocene to the Late Pleistocene, and has been colonized by the flora and fauna of both Africa and Madagascar, which had already differentiated. The youngest of the Comoros is Grand Comoro (0.5 My), dominated by the volcano Karthala, which is still active, giving this island a uniform topography. Mayotte is the oldest, with 10-15 My, and harbours several volcanoes, being the result of the union of previously independent massifs. These dates correspond to the estimated age of the volcanic origin of the islands (MONTAGGIONI & NOUGIER, 1981 ; NOUGIER et al., 1986). The age of the oldest exposed lavas is considerably

more recent : 0.13±0.02 My for Grand Comoro and 7.7±1 for Mayotte. These islands never had contact with other landmasses and are separated from each other and from Africa by sea depths of more than 3600 m (EMERICK & DUNCAN, 1982; NOUGIER et al., 1986).

The genus *Furcifer* (Chamaeleonidae : Reptilia) is represented in these islands only by two endemic species; *Furcifer cephalolepis* Günther, 1880, in Grand Comoro and *Furcifer polleni* Peters, 1874, in Mayotte. The extant 14 species of this genus all inhabit Madagascar, with one, *F. pardalis* Cuvier, 1829, also present in Mauritius and Reunion Islands, probably representing another natural oceanic dispersal (RAXWORTHY et al., 2002).

In a previous study involving many Chamaeleonidae species, RAXWORTHY et al. (2002) found support for a Madagascan origin for chameleons with multiple "out-of-Madagascar" dispersal events, one of them being the colonization of the Comoros archipelago by *Furcifer* species. Based only on morphological data, these authors placed *F. cephalolepis* and *F. polleni* as sister-taxa and related to the *F. oustaleti* and *F. lateralis* groups from Madagascar. However, attempts to interpret a morphological phylogenetic tree in terms of colonization sequence are compromised by ecogenetic adaptation to current selective pressures influencing the tree (THORPE et al., 1994). This could be the case here, as they are placed together by two non-unique synapomorphies : both Comoros chameleons have reduced body size and lost lung diverticula, probably as function of body size. Dwarfism in island species is a fairly common evolutionary response, and is presumably adaptive, thus such adaptations may well have evolved in parallel. Therefore, to further assess the position of these species within the *Furcifer* clade on the basis of DNA sequence data, we obtained partial sequences of the ND4 gene, from the same region as RAXWORTHY et al. (2002). We also used ND4 sequences to assess intraspecific diversity within *F. cephalolepis* and *F. polleni.* Being a fast evolving gene, this is an adequate marker to use in recent evolutionary events. Considering the ages of the islands, and assuming just one colonization event, we expected minimal diversity within Grand Comoro, and also between *F. cephalolepis* and its presumed sister-taxa, *F. polleni*. Concerning Mayotte, its age and, in particular, its conglomerate nature, might have led to high genetic diversity within this island, as seen in reptiles from Tenerife, in the Canary Islands (THORPE et al., 1994, 1996; BROWN & PESTANO, 1998).

TABLE 1

Sample code, locality and accession numbers of *Furcifer* specimens used in this study. All other samples were from RAXWORTHY et al. (2002).

| Species | Locality | Code | Acession number |
|---|---|---|---|
| *Furcifer cephalolepis* | Foret de la Guille, Grand Comoro | GC 25 | DQ086038 |
| *Furcifer cephalolepis* | Belvedere, Grand Comoro | GC 64 | DQ086039 |
| *Furcifer polleni* | Sada road, Mayotte | MY 57 | DQ086040 |
| *Furcifer polleni* | Bandrélé | MY 63 | DQ086041 |
| *Furcifer polleni* | Bandrélé | MY 64 | DQ086042 |
| *Furcifer polleni* | Airport, Dzaouzi islet, Mayotte | MY 67 | DQ086043 |
| *Furcifer polleni* | Vahibé | MY 75 | DQ086044 |
| *Furcifer polleni* | Mahicavo | MY 79 | DQ086045 |
| *Furcifer polleni* | Longoni | MY 82 | DQ086046 |
| *Furcifer polleni* | Longoni | MY 83 | DQ086047 |

## MATERIALS AND METHODS

Tail tips from eight *F. polleni* and two *F. cephalolepis* were collected in Mayotte and Grand Comoro (geographic locations of the specimens are given in Table 1 and Fig. 1) and genomic DNA was extracted following standard high-salt protocols. A fragment including the terminal portion of the ND4 gene and the tRNA's for Serine, Histamine and Leucine was amplified by PCR using the primers published by ARÉVALO et al. (1994) and sequences from both strands were obtained on an automated sequencer (ABI 310). Alignment was performed using Clustal W 1.6 (THOMPSON et al., 1994; default parameters) and adjusted manually in BioEdit (HALL, 1999). Sequences from other *Furcifer* species from Madagascar and Reunion Island previously published by RAXWORTHY et al. (2002) were also included. *Chamaeleo jacksoni* and *Calumma cucullata* were used as outgroups. Ambiguous alignment regions (12 bp of the tRNA's) were excluded from all analyses. To select the model of nucleotide substitution that better fits our data set, the hierarchical likelihood-ratio test was carried out using Modeltest 3.06 (POSADA & CRANDALL, 1998). Sequences were then imported into PAUP*4.0b10 (SWOFFORD, 2003) and the chosen model used to perform Maximum Likelihood (ML) analysis with random sequence addition (10 replicate heuristic search). Maximum Parsimony (MP) analysis was also carried out with random sequence addition (100 replicate heuristic searches) and support for nodes was estimated through the bootstrap technique (FELSENSTEIN, 1985) with 1000 replicates. Bayesian analysis was implemented using MrBayes v.3.0 (HUELSENBECK & RONQUIST, 2001) with parameters estimated as part of the analysis and four incrementally heated Markov chains with the default heating values. All analysis started with randomly generated trees and ran for $10^6$ generations, saving one tree in each 10 generations. The log-likelihood values of the sample points were plotted against the generation time and all the trees prior to reaching stationarity were discarded, ensuring that burn-in samples were not retained. Combining the remaining trees, a 50% majority rule consensus tree was generated. The frequency of any particular clade of the consensus tree represents the posterior probability of that clade (HUELSENBECK & RONQUIST, 2001). Two independent replicates were conducted and inspected for consistency to check for local optima (HUELSENBECK & BOLLBACK, 2001). To assess variation within *Furcifer polleni* (from Mayotte), these sequences

(total length of 807 bp) were joined into a median network (BANDELT et al., 2000).

## RESULTS

Ten sequences were obtained and 22 sequences, representing 14 taxa, were included in the analyses, for an aligned length of 815 bp. The most appropriate model of evolution for this dataset was the GTR, with an estimate of invariable sites (0.4116) and a discrete approximation of the gamma distribution (1.1168). ML, MP and Bayesian analyses gave congruent estimates of relationships, with ML and Bayesian trees having identical topologies, and the MP tree having one difference in topology relative to these (Fig. 2). Concerning the intraspecific diversity, the two individuals from *F. cephalolepis* had distinct haplotypes, presenting 0.87% divergence (seven differences in 807 bp). Within *F. polleni*, five distinct haplotypes were found in a total of 8 individuals, with a maximum divergence of 0.74% (six differences in 807 bp) and without any clear geographic structure (Fig. 3).

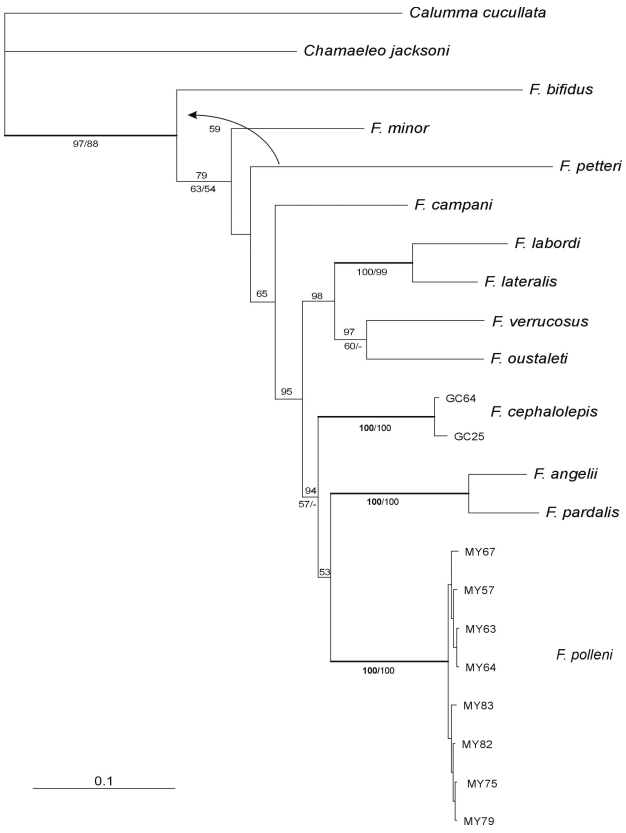


Fig. 2. – Tree derived from the Bayesian analysis of the 815 bp of the ND4 gene. Posterior probabilities are given above the branches with the bold branches having the value of 100%. Below the branches, bootstrap values for ML and MP are indicated (ML/MP). For both analyses, only bootstrap values above 50% are represented. The arrow indicates a variation in the position of one branch in the MP analysis and the respective bootstrap value is indicated below.

## DISCUSSION

Both *F. cephalolepis* and *F. polleni* represent distinct and very well supported branches. Their relative position and long branch lengths show that they are probably not sister-taxa, as previously suggested. Indeed, independently of the method used in the analyses, *F. polleni* always appeared as sister-taxa of the *F. angeli* and *F. pardalis* group from Madagascar, with *F. cephalolepis* splitting first from their common ancestor. This, points to independent colonization of both Comoro Islands. While we cannot exclude alternative hypotheses, like the existence of a very divergent unsampled lineage in Mayotte that could be the "sister-group" of *F. cephalolepis* from Grand Comoro, these are less likely due to our geographically widespread sampling in Mayotte. Clearly, further sampling is needed, especially from the extant *Furcifer* species from Madagascar (eight species from this genus are not included in this analysis), to clarify the relationships between them, and to better understand the process of colonization of the Comoros Islands.

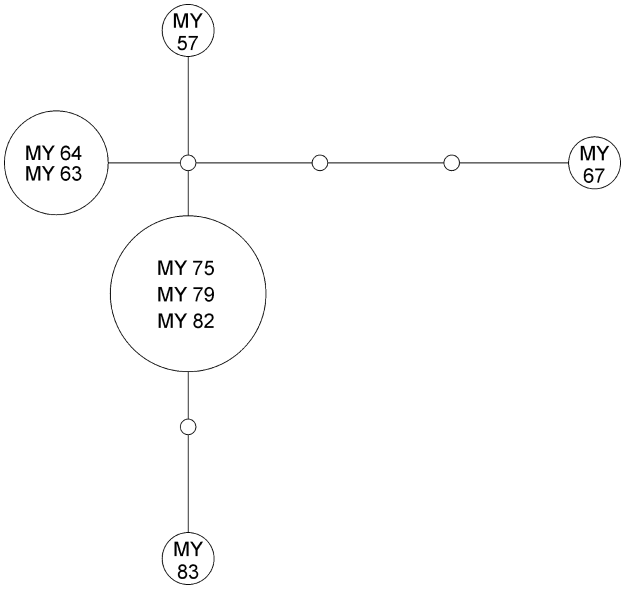


Fig. 3. – Network showing relationships between *Furcifer polleni* haplotypes found in Mayotte. Diameter of circles is proportional to the frequency of each haplotype and empty circles represent missing haplotypes.

The genetic divergence found inside *Furcifer cephalolepis* was surprisingly high, considering the age of the island. The two haplotypes from Grand Comoro have 0.87% genetic divergence (uncorrected p-distance), which, according to the vertebrate ectotherm mtDNA divergence rate – often used for this gene – of 0.4-0.6% per lineage per million year (RAND, 1994; CACCONE et al., 1999), would correspond to 0.73 to 1.1 million years divergence.

Even using a broader interval of sequence variation : 0.25-0.7% per lineage per million year (AVISE et al., 1992; CACCONE et al., 1997) we obtain estimates of divergence between 0.63 and 1.76 My, always higher than the oldest estimates for the age of the island – 0.5 My. So, the ND4 gene in *Furcifer* seems to be evolving faster that the rates generally used (at least at a value of 0.88% per lineage per million years if we use 0.5 My – age of Grand Comoro – as a calibration point. Moreover, these are minimum estimates of the divergence; based only in two individuals, as with more individuals even more divergent haplotypes could be found.

One possible explanation is that ND4 gene in *Furcifer* is evolving faster than predicted by these molecular clocks. However an alternative explanation for this result is that divergence within *F. cephalolepis* predates the colonization of Grand Comoro and this colonization was made by individuals including already differentiated mtDNA lineages.

Islands with known geological ages are often thought to be ideal for calibrating molecular clocks (CARRANZA et al., 2000) and the common procedure is that when sister-taxa are found on neighbouring islands to assume that the age of the younger island represents an approximate estimate for the maximum age of the split between the "offspring" population on the younger island and the "parental" population on the older island. However our results suggest that "universal" clocks are extremely inaccurate. Furthermore, precise phylogenies are needed – if divergence values between *F. polleni* and *F. cephalolepis* (assuming incorrectly they were sister taxa) were compared to the age of Grand Comoro we would obtain an erroneous estimated rate of evolution of at least 11% per lineage per million year. This type of calculation, focusing on the observed divergence between islands and using the age of the youngest one as a calibration point, is still commonly used (e.g. BROWN & PESTANO, 1998; WARREN et al., 2003). Our results highlight the importance of also assessing within-island diversity when estimating divergence rates.

In conclusion, our results suggest that the Comoros were independently colonized twice by *Furcifer* from Madagascar. They also suggest that this region of the ND4 gene and associated tRNA's may be evolving faster than that predicted by ectothermal vertebrate molecular clocks, which has implications for the estimated times of colonization of other island groups by chameleons. This further highlights the inaccuracies of generalized applications of molecular clocks, even when calibrated using known geological values such as the age of islands. The fast rate of evolution of this region of mtDNA makes it highly suitable for phylogeographic studies.

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