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The phylogenetic position of *Lygodactylus angularis* and the utility of using the 16S rDNA gene for delimiting species in *Lygodactylus* (Squamata, Gekkonidae)

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Abstract. The African genus *Lygodactylus* Gray, is composed of roughly 60 species of diurnal geckos that inhabit tropical and temperate Africa, Madagascar, and South America. In this study, we assessed the phylogenetic position of *L. angularis*, for which molecular data were so far lacking, by means of sequence analysis of the mitochondrial 16S rDNA gene. We also compared intraspecific *vs.* interspecific genetic divergences using an extended data set (34 species, 153 sequences), to determine whether a fragment of this gene can be useful for species identification and to reveal the possible existence of new cryptic species in the genus. The analysis placed *L. angularis* in a monophyletic group together with members of "*fischeri*" and "*picturatus*" groups. Nevertheless, the independence of the "*angularis*" lineage is supported by the high genetic divergence. Comparison of intraspecific vs. interspecific genetic distances highlights that, assuming an equal molecular rate of evolution among the studied species for the used gene, the threshold value useful for recognising a candidate new species can be tentatively placed at 7%. We identified four species that showed an intraspecific divergence higher than, or close to, the 7% threshold: *L. capensis* (8.7%), *L. gutturalis* (9.3%), *L. madagascariensis* (6.5%) and *L. picturatus* (8.1%). Moreover, two species, *L. mombasicus* and *L. verticillatus,* are paraphyletic in terms of gene genealogy. Thus, the study shows that a short fragment of the 16S rDNA gene can be an informative tool for species-level taxonomy in the genus *Lygodactylus.*

Keyword. African reptiles, biodiversity, DNA barcoding, genetic divergence, sister species, taxonomy.

INTRODUCTION

The genus *Lygodactylus* Gray, 1864 encompasses about 60 species of diurnal geckos that inhabit tropical and temperate Africa, Madagascar, and South America (Spawls et al.,

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2002). *Lygodactylus* has traditionally been divided into different "species groups" based on phenotypic characteristics (Loveridge, 1947; Pasteur, 1965; Jacobsen, 1992; Röll, 2000, 2004; Puente et al., 2009).

The phylogenetic relationships among species from Madagascar have been assessed by Puente et al. (2005) using a mitochondrial gene. Recently, Röll et al. (2010) provided a robust phylogeny of 28 species by using both mitochondrial and nuclear genes. This phylogeny mainly confirmed the monophyly of many of the species groups, previously identified on the base of morphology, as well as provided evidence for at least two additional lineages. Moreover, this study identified some divergent taxa which possibly belonged to additional undescribed species. In fact, the taxonomy of *Lygodactylus* at a species level is still uncertain, with some species known only from the type locality. Furthermore, the earlier identification of many species was based on scale features only and, consequently, due to their small sizes, the identification during field work is extremely difficult (Spawls et al., 2002).

A recent approach to studying species limits involves the use of a single short fragment of DNA that can help in the identification of an organism by its assignment to a previously described species (DNA barcoding). In the same way, it is possible to predict and describe new taxa using DNA (DNA taxonomy) (Blaxter, 2004). Moreover, this approach allows inclusion of additional information in a taxonomy based on morphological characters only (Padial et al., 2010). New candidate species can be identified on the basis of 1) elevated intraspecific genetic distances, similar to the ones found among different species and 2) the presence of paraphyletic species that suggests a need of taxonomic revision. Yet, this approach has some limitation, as in case of incomplete gene sorting, hybridization, different rates of molecular evolution at the same marker in a group of species or accidental amplification of nuclear mitochondrial pseudogenes (Frézal and Leblois, 2008).

Different genes are currently used as DNA barcodes in animals (e.g., cytochrome oxidase subunit I – COI, 16S rDNA – 16S, cytochrome b - cytb). Evidence suggests that no "universal" gene exists. To be useful for delimiting species, the gene must have one principal characteristic that is to exhibit limited overlap between intraspecific and interspecific divergence. For example, an accurate comparison of performances of COI and 16S in amphibians showed that 16S is well suited to distinguish between intraspecific and interspecific divergence, whereas, in birds, the best results were obtained using COI (Vences et al., 2005; Aliabadian et al., 2009).

In this study, we first analyse the phylogenetic position of *L. angularis*, a member of the "*angularis*" species group together with *L. grzimeki* Bannikov, using a mitochondrial molecular marker. At present, there are no other molecular data on the members of this species group neither to confirm that they both belong to a separate lineage, nor to place them in a phylogenetic context. Besides, *Lygodactylus* seems to be an appropriate genus for testing the utility of a single DNA fragment as a DNA barcode, because of the problems in identification and unstable taxonomy. A recent paper by Chiari et al. (2009), concerning species from Madagascar, highlighted that two of the eight analysed species (*L. madagascariensis* and *L. tolampyae*) showed a very high intraspecific genetic divergence using a fragment of the 16S rDNA. Consequently, in this paper, we extend the record with a dataset formed by a higher number of species in order to determine whether the same genetic marker can be useful for species identification. Finally, we reveal the possible presence of new cryptic species in *Lygodactylus* by providing evidence of genetic divergence and species paraphyly.

MATERIALS AND METHODS

We utilised almost all the available 16S rDNA sequences from *Lygodactylus* downloaded from GenBank (updated on February 2011; 34 species, 153 sequences; Table 1). Sequences from specimens with uncertain taxonomical attribution were excluded. Sequences from additional speci-

Table 1. Species, number of localities and number of individuals used for the comparative analysis done on the 16S rRNA gene fragment. *Lygodactylus* sp. 1 and sp.2 are undescribed species (Röll et al. 2010). Data from Chiari et al. (2009), Röll et al. (2010), Puente et al. (2005) and Rocha et al. (2009) and present data.

Species	Number of Localities	Number of specimens
L. angularis Günther (1893)	1	1
L. arnoulti Pasteur (1964)	1	5
L. bivittis (Peters, 1883)	1	4
L. blancae Pasteur (1995)	1	2
L. bradfieldi Hewitt (1932)	2	3
L. capensis (Smith, 1849)	6	9
L. chobiensis Fitzsimons 1932	1	2
L. conraui Tornier (1902)	1	2
L. gravis Pasteur (1964)	1	2
L. grotei Sternfeld (1911)	1	2
L. guibei Pasteur (1964)	2	3
L. gutturalis Bocage (1873)	2	5
L. keniensis Parker (1936)	1	3
L. kimhowelli Pasteur (1995)	1	4
L. laterimaculatus Pasteur 1964	1	3
L. lawrencei Hewitt (1926)	3	3
L. madagascariensis (Boettger, 1881)	3	6
L. miops Günther (1891)	1	4
L. mirabilis Pasteur (1962)	1	31
L. mombasicus Loveridge (1935)	2	4
L. montanus Pasteur (1964)	1	2
L. pauliani Pasteur and Blanc (1991)	1	1
L. picturatus (Peters, 1868)	2	4
L. pictus (Peters, 1883)	3	12
L. rarus Pasteur and Blanc (1973)	1	2
L. stevensoni Hewitt (1926)	1	2
L. thomensis (Peters, 1880)	1	1
L. tolampyae (Grandidier, 1872)	3	5
L. tuberosus Mertens (1965	1	11
L. verticillatus Mocquard (1895)	2	5
L. williamsi Loveridge (1952)	1	2
Lygodactylus sp. 1	1	4
Lygodactylus sp. 2	1	1

mens belonging to four species were added to the dataset (*L. mombasicus*, Nairobi, Kenya, 01°16'S - 36°49'E; *L. picturatus*, Morogoro, Tanzania, 06°49'S - 37°40'E ; *L. capensis*, Mutanda, Zambia, 12°22'S - 26°16'E; *L. angularis*, Mbeya, Tanzania, 12°33'S - 25°41'E; accession numbers HQ872459-63). The new 16S rRNA gene sequences were obtained from tissues fixed in ethanol 80%. DNA was extracted using the QIAmp tissue extraction kit (Qiagen). The primers 16SA-L (light chain; 59-CGC CTG TTT ATC AAA AAC AT-39) and 16SB-H (heavy chain; 59-CCG GTC TGA ACT CAG ATC ACG T-39) were used to amplify a section of the mitochondrial 16S ribosomal RNA gene (Palumbi et al. 1991).

The PCR cycling procedure was performed as follows: 34 cycles of denaturation for 90 sec at 95 °C, primer annealing for 60 sec at 50 °C, and extension for 90 sec at 72 °C. All sequences were aligned with Muscle, using default settings, and then adjusted manually. Sites including gaps and hypervariable regions, identified by visual inspection of the alignment, were removed. The final alignment was 432bp long. A preliminary NJ tree was built with this dataset, using Kimura 2-parameter distances (K2P) and 10000 bootstrap replicates generated by MEGA 4 (Kumar et al., 2008). We calculated the intraspecific distance for each species and for each species with 2 or more populations separately. Average K2P distances were computed based on pairwise comparisons of all sequences for each of these species. A correlation was made between number of localities and average intraspecific genetic distance. Information about the number of populations were obtained from the published articles. For interspecific distances, we calculated mean pairwise K2P distance among all pairs of species and, separately, between pairs of sister species. Sister species were identified according to our phylogenetic tree (see below) and by referring to the tree proposed by Röll et al. (2010). To study the phylogenetic position of *L. angularis,* the dataset was pruned to reduce the computation time of analyses, and only two or three sequences were retained for each species (80 sequences, 432bp, from 35 species). When possible, we kept sequences from different populations for each species. Phylogenetic relationships were assessed by Bayesian inference (BI), unweighted maximum parsimony (MP) and maximum likelihood (ML). We used the same outgroups used by Röll et al. (2010), i.e., *Phelsuma standingi* and *Rhoptropella ocellata*, since they are the closest relatives of *Lygodactylus* (Austin et al., 2004).

The appropriate model of substitution was chosen using the Model Test 3.7 program (Posada and Crandall, 1998). Models of evolution, which provide the best approximation to the data, were chosen for subsequent analysis according to the Akaike information criterion (AIC). The chosen model was the General Time Reversible (GTR) model with rate variation among sites (+G), a proportion of invariable sites I=0.2038 and a gamma distribution shape parameter of 0.3846. The models and parameters were used for ML trees in phyML (Guindon and Gascuel, 2003).

Maximum parsimony trees were obtained with PAUP 4.0b10 (Swofford, 2000) using a heuristic search and tree –bisection– reconnection and random addition of sequences. The robustness of the nodes was assessed using the bootstrap with 1000 replicates for MP and 500 replicates for ML.

For the BI we constructed the phylogeny using the software MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001) using the same model as in the ML analysis. Two independent Markov chain Monte Carlo analyses were run. We used 1 million generations, four chains and a burn-in of 10% of the generated tree.

Based on our phylogenetic results, the paraphyly of *L. mombasicus* and *L. verticillatus* was further investigated using a reduced dataset comprising sequences of species from the same species group. For *L. mombasicus* the dataset was composed of twenty-two sequences (482bp) belonging to seven species. For *L. verticillatus* the dataset was composed of twenty sequences (482bp) belonging to seven species. In this procedure, longer alignments could be used, since the internal hypervariable region is less extended when close species are compared, which offers greater power for phylogenetic resolution. Phylogenetic relationships were then assessed by BI, MP and ML.

RESULTS

Phylogenetic position of L. angularis. The phylogenetic analysis of the pruned dataset (432 bp, 80 sequences from 35 species) does not generally conflict with a previous phylogeny obtained by multigene analysis (Röll et al., 2010) and exhibits deep, weakly supported branches connecting well-supported species groups. The only species group that is not supported by present analysis is the "*pictus-mirabilis" group*, which is split into three lineages (not shown).

The analysis places *L. angularis* in a moderately supported clade, which includes species of the "*picturatus*" and "*fischeri"* groups. (Fig. 1A). Within this clade, the species of the "*fischeri"* group and of the "*picturatus*" group are well supported. However, basal relationships within this clade are not resolved.

Instances of paraphyletic species. Our phylogenetic analysis identified two putative instances of paraphyletic species (*L. mombasicus* and *L. verticillatus*). The paraphyly was further analysed with a dataset including species related to either of them. For the "*picturatus*" group, the dataset retrieved a topology (Fig. 1A) similar to the one obtained by Röll et al. (2010) that also included nuclear data. However, haplotype LYG1 from *L. mombasicus* has a basal position with respect to a cluster formed by the other sequences belonging to *L. mombasicus* and *L. kimhowelli*. This topology is well supported by all the methods used (Fig. 1A).

For *L. verticillatus*, the paraphyly is straightforward, since two distinct clusters within *L. verticillatus* have been found (min-max, 8.8-9.1% sequence divergence). One of the two clusters has high sequence similarity with the specimens identified as *L. heterurus* (2.3% sequence divergence) (Fig. 1B).

Intraspecific vs. interspecific genetic distance. Figure 2A shows the distribution of interspecific and intraspecific average pairwise genetic divergence. The two distributions overlap. The amount of overlap is between 4.9% and 9%. For intraspecific divergence, the range is from 0 to 9% (mean 1.8%; s.e. 2.6; N = 30), and four out of 30 values lie in the overlapping interval. Interspecific distances range from 4.9 to 37% (mean 24.2%; s.e. 6.0%), and eight out of 595 values lie in the overlapping interval. For interspecific pairwise divergence, we eliminated two pairs of close species that showed paraphyly (see above). The intraspecific genetic distance is strongly correlated with the number of localities ($R = 0.78$; $P<<0.0001$). Accordingly, the level of overlap between intra- and interspecific divergences can be inflated by the fact that we underestimated the intraspecific divergence: in many species, only one or a few populations were available for the analysis.

For this reason we compared (Fig. 2B) the distribution of intraspecific genetic distances for species including more than two populations with the distribution of genetic divergences between pairs of sister species. We used pairs of sister species in order to capture the divergence of recently emerged species. The intraspecific distances for species with more than 2 populations range between 0.6% to 9% (mean 4.4%, s. d. 2.8, N=11). The distribution of genetic divergences between pairs of sister species ranges between 4.9 and 15.7% (mean 10.5%, s.e. 4.0% N = 8). The two distributions overlap between 5% and 9%. The species with high average intraspecific genetic divergences are *L. capensis* (8.7%), *L. gutturalis* (9.3%), *L. madagascariensis* (6.5%), *L. tolampyae* (5.3%) and *L. picturatus* (8.1%). The sister species with the lowest intraspecific divergence are *L. arnoulti* and *L. pauliani* (4.9%).

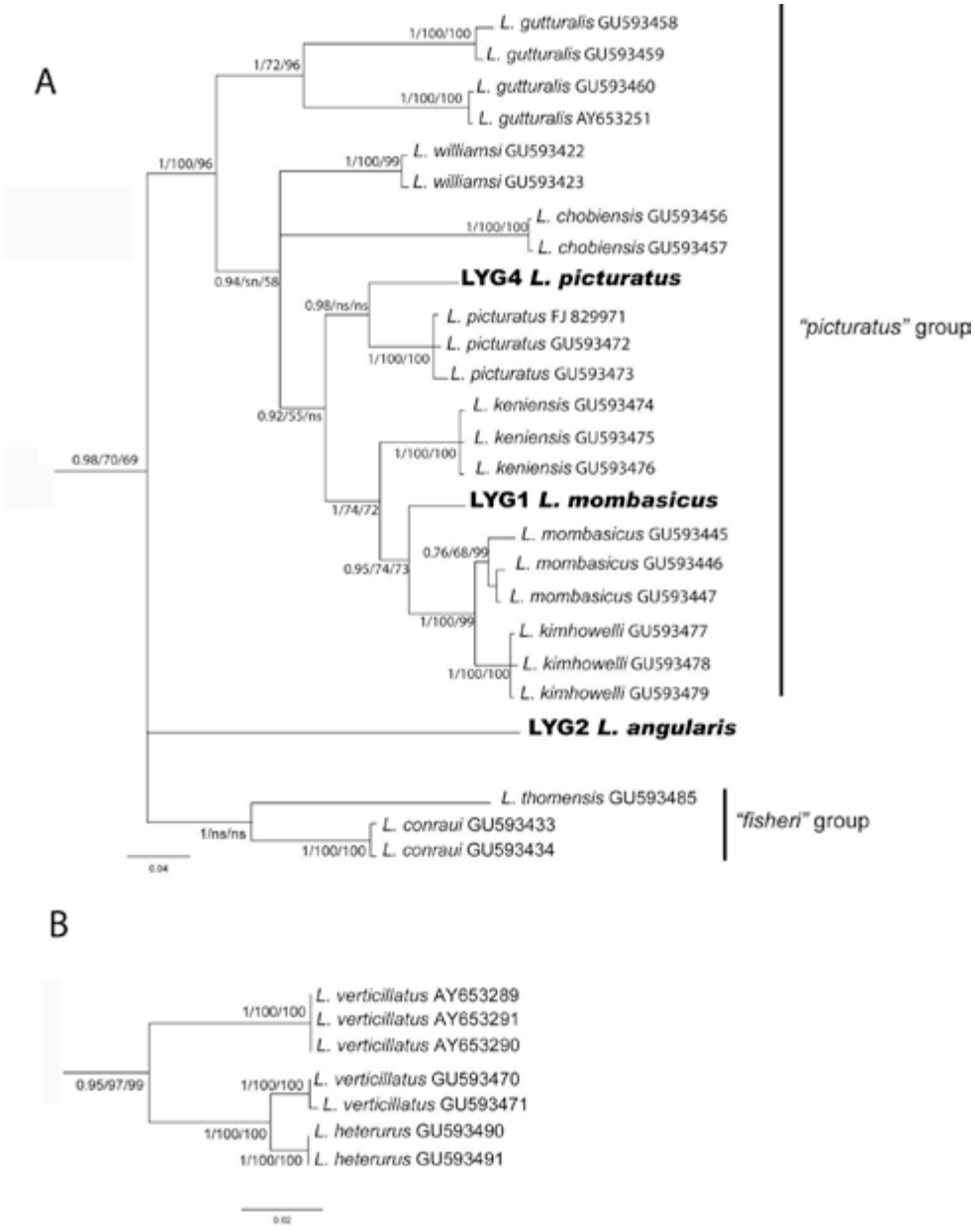

Fig. 1. Bayesian trees of A) the clade including the "*picturatus*" group, the "*fischeri*" group and *L. angularis* and B) the haplotypes belonging to *L. verticillatus* and *L. heterurus*. The haplotypes analysed are in bold. Numbers at the node correspond to the Bayesian posterior probabilities, the percentage of 1000 bootstrap replicates for maximum parsimony and over 500 replicates for Maximum Likelihood respectively.

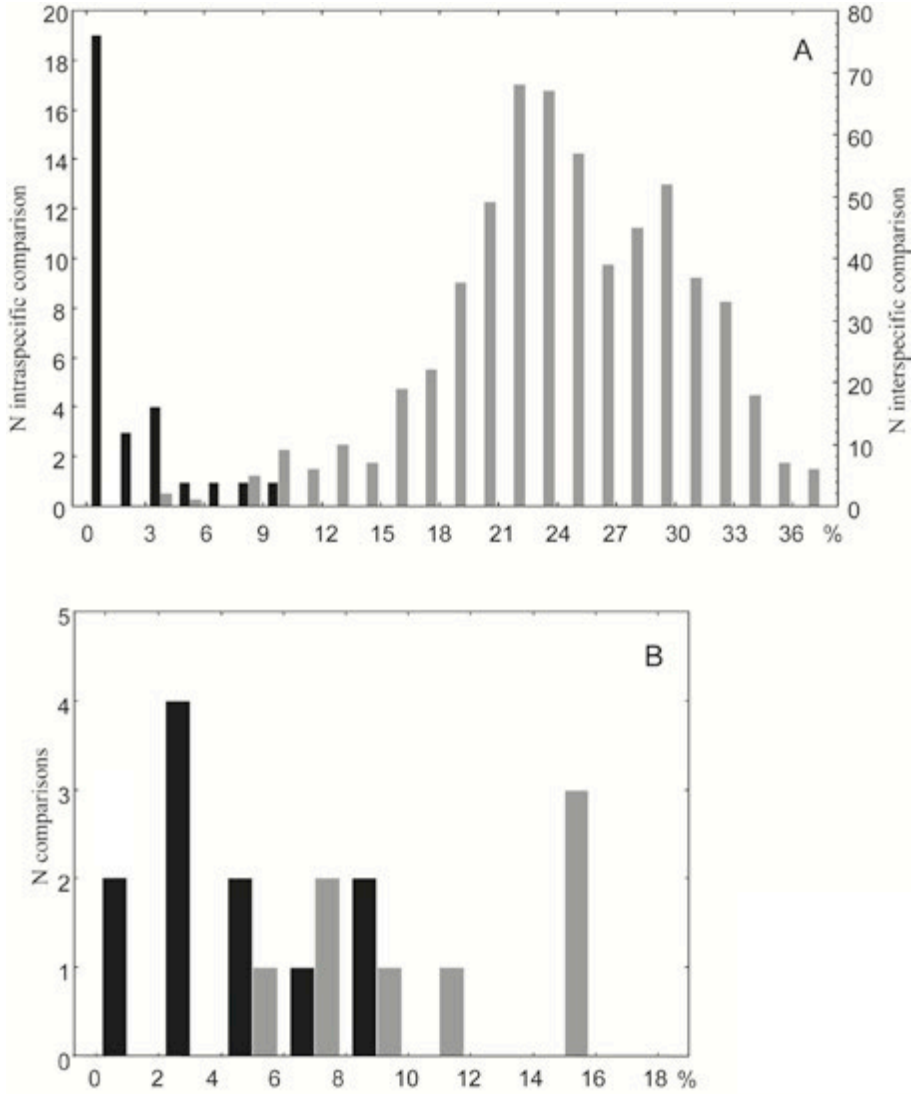

Fig. 2. Pairwise K2P pairwise distances in the 16S gene in *Lygodactylus.* A) Black bars are comparisons among conspecific sequences (left axis); grey bars represent comparisons among different species (right axis). In (B), black bars refer to comparisons of conspecific specimens for species with two or more populations. Gray bars refer to pairs of sister species only.

DISCUSSION

Phylogenetic position of L. angularis. Even if the basal nodes of the tree are not supported, the phylogenetic position of *L. angularis* is well-supported in our tree, which was built with a single mtDNA gene. This species resulted in a monophyletic group togeth-

er with members of "*fischeri*" and "*picturatus*" groups. The clustering of the members of these two species groups was previously supported by Roll et al. (2010). All these species share some scale characters, such as an undivided mental scale in most species. This character is also present in *L. angularis* (Loveridge, 1947), in accordance with the presently determined phylogenetic position of this species. The "*fischeri*" group contains West African species, and the "*picturatus*" group consists of mainly East African species. *L. angularis* shares a similar geographic distribution in eastern Africa with the member of the "*picturatus*" group. Moreover, *L. angularis* is also large-sized, as the species of the "*picturatus*" group, whereas geckos of the "*fischeri*" group are small and slender. Another character that may be in common between members of the "*picturatus*" group and *L. angularis,* is the presence of sexual dichromatism with coloured males. In fact, *L. angularis* males have rose pink ventral surface, while females are entirely lemon yellow. Nevertheless, the independence of the "*angularis*" lineage from the species of "*picturatus*" and "*fischeri*" groups is supported by the high genetic divergence (14-20%).

Inter- and intraspecific genetic divergence. The distribution of intraspecific and interspecific genetic divergence shows an overlap between 5% and 9%. The distribution of the interspecific divergence represents probably a good approximation of the real differences between species. However, we found that intraspecific divergence depends strongly on the number of localities sampled. For this reason, we suggest that the intraspecific diversity might be underestimated.

Despite this limitation, the threshold values useful for recognising a "good" species, according to our data, can be tentatively placed at 7% (K2P distance). In fact, only a couple of species shows an interspecific genetic divergence below this value (*L. arnoulti* and *L. pauliani*, 4.9% genetic divergence). Owing to scarcity of data on intraspecific variation, we cannot say whether the use of a lower threshold (5%) would produce an excessive number of false positives. Additional intraspecific data are needed to resolve this problem and ultimately to support a threshold shift from 7% to 5%. We identified four species that showed an intraspecific divergence higher than, or close to, the 7% threshold: *L. capensis* (8.7%), *L. gutturalis* (9.3%), *L. madagascariensis* (6.5%) and *L. picturatus* (8.1%). Present data confirm and extend previous results obtained with the same mtDNA marker on eight species of *Lygodactylus,* among which *L. madagascariensis* and *L. tolampyae* showed high intraspecific divergence (4.3% and 9.1%) (Chiari et al., 2009). Comparing the present and the previously published data, one can see that the differences in values of genetic divergences are most likely due to the different number of individuals (for in *L. tolampyae*) and the different length of the sequence used. In fact, the 16S rDNA gene has conserved regions, but also hypervariable ones. Consequently, the length of the fragment used for analysis affects easily the calculation of divergence. For a barcoding approach, obviously, the inter- and intraspecific divergence should be only compared by means of the same dataset.

The species with high intraspecific divergences, *L. gutturalis* and *L. capensis,* are widely distributed. The divergent sequences belong to distant populations (Guinea Bissau and Uganda for *L. gutturalis*; Zambia, Namibia, and South Africa for *L. capensis*). Additional nuclear markers and morphological characteristics should be used to infer the taxonomic status in these cases. The same concern arises for *L. picturatus*, a species in south-eastern Kenya and eastern Tanzania. The specimen of from central Tanzania studied here shows a

high divergence and results basal to the other sequences from the populations of the Kenyan and Tanzanian coasts. Current information on *L. madagascariensis*, distributed in the northwestern part of Madagascar, is too scant to allow any tentative discussion of species limits.

Two instances of paraphyletic species. We identified two paraphyletic species in terms of gene genealogy, namely *L. mombasicus* and *L. verticillatus*. In fact, the haplotype of *L. mombasicus* from Kenya, here analysed, is basal to other sequences from *L. mombasicus* and *L. kimhowelli*. According to Röll et al. (2010), *L. kimhowelli* and *L. mombasicus* are very similar morphologically. Their colour patterns differ, but they share all scale characters and the pattern of conspicuous black markings on head and neck (Röll, 2003). Genetic divergence between haplotypes of *L. mombasicus* and *L. kimhowelli* is low (2.6-3.1%), whereas the haplotype from Kenya diverges by 6% with respect to both of them. This value is only slightly lower than the threshold values identified above. It is evident that an accurate morphological and molecular study of specimens belonging to *L. mombasicus* and *L. kimhowelli* is needed before any conclusions regarding species status can be drawn.

Another species found to be paraphyletic is *L. verticillatus*. Röll et al. (2010) concluded that *L. verticillatus* and *L. heterurus*, small geckos that are very similar in morphology, are also quite similar genetically. For this reason, the authors proposed conspecificity of the two taxa. Our analysis includes two additional sequences (Puente et al., 2005) and reveals a more complex pattern. In fact, these sequences are the sister group of the other sequences belonging to *L. verticillatus* and *L. heterurus*, with a very high genetic divergence of 9%. This value is higher than the 7% threshold proposed above and suggests that *L. verticillatus* and *L. heterurus* are two different sister species.

CONCLUSION

This study shows that a short fragment of the 16S rDNA gene may be an informative tool for species-level taxonomy in the genus *Lygodactylus*. However, other genes should be tested, since no comparative results with other molecular markers are reported in this paper. Future studies should be planned to obtain a more accurate picture of intraspecific divergence for the studied gene, as well as for other molecular markers, and to include samples from underrepresented African regions. Finally, additional nuclear markers and other kind of data (for example, behavioural) should be analysed in those instance, where high intraspecific divergences were confirmed.

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