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High haplotype diversity in a microendemic Malagasy gecko species, *Lygodactylus mirabilis* (Pasteur, 1962)

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Abstract

Among Malagasy montane reptiles, the diurnal gecko *Lygodactylus mirabilis* has one of the most restricted distribution ranges, occurring only on the Tsiafajavona mountain on the Ankaratra massif. Here we report data on the current distribution of this species and its genetic diversity. Mitochondrial data based on samples collected in the only previously known distribution area (the Tsiafajavona peak) showed numerous haplotypes at low frequencies, suggesting a past population expansion and a relatively high within-species genetic diversity in an extremely small distribution area. Our field survey also revealed that the range of the species is larger than previously thought, but still is extremely small and restricted to the Ankaratra massif.

Key words: Madagascar, *Lygodactylus*, 16S rRNA, Cytochrome *b*, Mountain microendemism

Introduction

Madagascar has been proposed as a good study system for understanding the patterns and processes of species diversification (Vences *et al*. 2009). Recent publications (Vences *et al*. 2009; Vieites *et al*. 2009) have revealed an astonishingly high number of undescribed species on the island (see Vences *et al*. 2009 for a list of recent publications on cryptic Malagasy species). Previous studies have shown that tropical montane habitats are especially important to herpetofaunal diversity in Madagascar (e.g., Raxworthy & Nussbaum 1996), because they exhibit a large degree of local endemism, with some species occurring only at high elevation with extremely limited distribution areas (Raxworthy 2003).

Out of the about 90 currently described Malagasy gecko species, 22 belong to the genus *Lygodactylus* (Glaw & Vences 2007; Puente *et al*. 2009). This genus includes 60 recognized species mostly distributed in Africa and Madagascar, with a few species in South America (Bauer 2003). *Lygodactylus* are small, diurnal geckos, normally less than 40 mm in snout-vent length (Pasteur 1964). In Madagascar, six *Lygodactylus* species are known to live above 1500 m altitudes, on a mountain chain that spans the island longitudinally and is otherwise known as the high plateau. *Lygodactylus mirabilis* (Pasteur 1962), only known to live in the highest elevation area on Tsiafajavona mountain on the Ankaratra massif, seems to be the species with the most adapted morphology to its environment among the montane dwarf geckos of Madagascar, with strongly

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keeled scales, a reduced first finger, and polymorphic coloration. It occurs at high elevations above 2000 m and attains a maximum snout-vent length of 29 mm (Puente *et al.* 2009; Vences *et al.* 2002). The Ankaratra massif is characterized by remnants of primary rainforest between 1700-2000 m, while above 2000 m the forest is replaced by grasslands (Goodman *et al.* 1996; Raxworthy & Nussbaum 1996; Vences *et al.* 2002). Reproduction of *L. mirabilis* appears to occur throughout the year, including the warm-wet austral summer and the cold-dry austral winter (Vences *et al.* 2002). Besides this limited information, little is known about this microendemic species, including the amount of genetic diversity in its population. We here present the first data on the distribution and genetic diversity of this species.

Materials and methods

Sampling and distribution

During a three day survey carried out by seven people in February 2006 on the Ankaratra Massif in central Madagascar, geographic coordinates and altitude above sea level (asl) were recorded by GPS instruments for each individual of *L. mirabilis* encountered as well as for egg deposition sites of this species (non-glued small eggs found under stones, see Vences *et al.* 2002). Presence/absence along all the surveyed localities was recorded for this animal and its deposited eggs. The surveyed localities extend along the known distribution range of the species on the mountain-top of Tsiafajavona and the surrounding mountain peaks. Geographic coordinates were used to construct distribution maps with ArcView GIS (ver. 3.2.a, Esri® 1992-2000; ESRI, Redlands, CA). Moreover, 31 samples for DNA analysis were collected through the only currently known distribution area (Fig. 1B and C for range of the sampling). Tissue samples for DNA analysis were not collected for the other surveyed localities. Tissues of the collected samples have been deposited in the Peabody Museum at Yale University and in the collection of M. Vences in the Technical University of Braunschweig.

Molecular protocols

Genomic DNA was extracted from tailclip tissues fixed in ethanol 99% using the DNeasy Blood & Tissue Kit from Qiagen. Fragments of the mitochondrial genes cytochrome *b* and 16S rRNA were amplified via the polymerase chain reaction (PCR). A fragment of 761 base pairs (bp) of the cytochrome *b* gene was amplified using the primer combination CBL14753 and CB3H (Austin *et al.* 2004) or the newly designed primer Lygo_cytb (5' CAAATAGGAAGTATCATTCAG 3') in combination with CBL14753. A fragment of 490 bp of the 16S rRNA was also amplified using the universal primers 16SA-L and 16SB-H (Palumbi *et al.* 1991). PCRs and purification of PCR products followed standard protocols. Sequences (in both directions) were resolved on a 3730 DNA Analyzer (Applied Biosystems). Random individual samples were amplified and sequenced again to verify for possible PCR errors. Sequences were deposited in Genbank; accession numbers: GQ910795-GQ910852.

Molecular analyses

Sequences were checked and aligned with SEQUENCHER (GeneCodes Corporation, Ann Arbor, MI, USA) using the Clustal algorithm. Alignment was subsequently refined by eye.

The number of variable and parsimony-informative sites were calculated using MEGA v.4.0 (Tamura *et al.* 2007), while nucleotide diversity (π, Nei & Li 1979) and gene diversity (*h*, Nei & Tajima 1981) were obtained with ARLEQUIN v.3.1 (Excoffier *et al.* 2005). MEGA was used to calculate the sequence divergence (as uncorrected p-distances) and base frequencies for the complete dataset (both markers analysed together) and for each marker independently, with gaps or missing data deleted in pairwise comparison. R (The R Development Core Team 2008) was used to test for normality of the p-distances of each marker and to run an analysis of variance (ANOVA) F test on the regression between the two used markers.

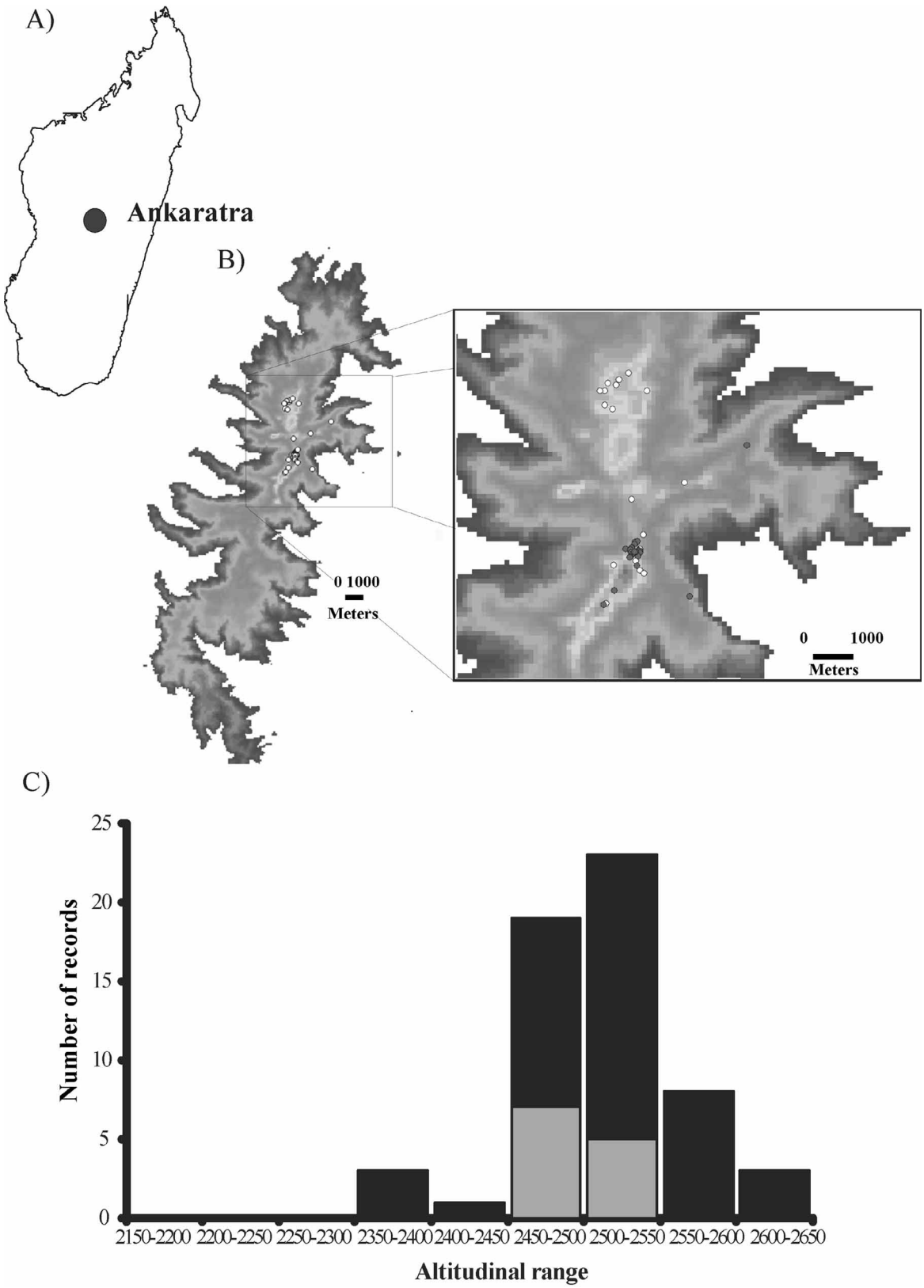

FIGURE 1. A) Map of Madagascar with indicated the location of the Ankaratra Massif. B) Distribution map of the surveyed area along the mountain peaks of the Ankaratra Massif in Madagascar (see Material and Methods for further explanations). White points indicate where *Lygodactylus mirabilis* specimens have been found; grey points indicate the locations of the individuals sampled and used for the genetic analysis. C) Altitudinal range of the recorded individuals of *L. mirabilis* found. Grey areas are proportional to the number of *L. mirabilis* eggs found at specific altitudes.

To compare genetic diversity (as uncorrected p-distances) of the 16S rRNA fragment of *L. mirabilis* for 409 homologous bp with other *Lygodactylus* species, 35 sequences belonging to seven species were downloaded from Genbank (for a list of the species and Genbank accession numbers see Table 1). Moreover, to compare the genetic diversity (as uncorrected p-distances) observed at the cytochrome *b* gene in *L. mirabilis* with the homologous sequences of other species of geckos, other Gekkonidae cytochrome *b* sequences were downloaded from Genbank (for a list of species and Genbank accession numbers see Table 2). Only species for which more than six individuals per species were available were used.

Sequences of the 16S rRNA and cytochrome *b* fragments were collapsed (each marker alone and concatenated) into haplotypes using the program DNAcollapser v.1.0, available online. Haplotype sequences of the concatenated dataset were used to construct a haplotype network using TCS (Clement *et al.* 2000), with the gaps coded as a fifth state.

We tested the neutrality of the mutations of the cytochrome *b* data comparing rates of non-synonymous and synonymous mutations using the online website datamonkey.org. We used two independent codon-biased maximum likelihood methods (FEL and IFEL) to detect codons under selection, using the HKY85 model as estimated to be the best fitting model to the data (best fitting model found using the same website). The IFEL method is an extension of the FEL method, allowing the estimation of the ratio between non-synonymous and synonymous substitution rates separately on internal and terminal branches of the tree built using the data (see Kosakovsky Pond *et al.* 2006 for additional information).

Furthermore, after confirming the lack of recombination using the above mentioned website (datamonkey.org), Fu's F*s* neutrality test (Fu 1997) was run in ARLEQUIN on the concatenated dataset of 27 sequences (for which both gene fragments were available) and on each single gene to detect population growth, using 1000 simulated samples to obtain the F*s* statistic. Episodes of population growth generally lead to negative values of F*s*. The same software was also used to perform a mismatch distribution analysis on the same dataset to characterize population expansion. A mismatch distribution corresponds to the distribution of the observed number of nucleotide differences between pairs of haplotypes. The distribution may have different shapes depending on the different scenarios (e.g., unimodal shape for a population gone through a recent demographic expansion, Slatkin & Hudson 1991). ARLEQUIN uses the non-linear least-square approach to find the values of the demographic parameters, by minimizing the sum of square deviation (SSD) between the observed and the expected distribution following a stepwise expansion model. The expansion model is rejected if 95% (α=0.1) or more of the simulated mismatch distribution have a lower SSD of the observed under the hypothesis that the estimated parameters are the true ones. The raggedness index (*r*, Harpending 1994), corresponding to the magnitude of change in the frequencies of the mismatch classes, is also used to understand if the population experienced an expansion. Larger values of *r* are characteristic of a stationary population with a multimodal distribution, while smaller values indicate unimodal distribution typical of an expanding population. For our mismatch distribution analysis, we used a bootstrap number of 100.

Results

Distribution

Fig. 1 shows the distribution and the altitudinal level of occurrence of *Lygodactylus mirabilis*. Despite the large searching area and effort, animals were found only on two mountain peaks (Fig. 1B) indicating an extremely restricted distribution area. However, the occurrence of the second population on a mountain neighboring the Tsiafajavona peak to the north is a new record (corresponding to the peak of 2607 m asl that bears the same name as the entire massif, Ankaratra). Altitudinal distribution range spans from 2350 to over 2600 m asl, with a higher concentration of individuals observed between 2450 and 2550 m asl (Fig. 1C), where we also found eggs. In the egg deposition sites we observed up to six eggs laid under the same stone (data not shown).

Genetic diversity

Sequences were obtained from a total of 31 individuals of *Lygodactylus mirabilis* for the 16S rRNA fragment and from a subset of 27 individuals for the cytochrome *b* fragment. For the 16S rRNA fragments we needed to add eight gaps to correctly align the sequences. Of the 761 bp of the cytochrome *b* fragment analysed, 41 sites were variable, 16 were parsimony-informative, and 19 haplotypes were recovered. Out of the 490 bp of the 16S fragments, 13 sites were variable and 11 parsimony-informative and 15 haplotypes were found. Fig. 2 shows the relationship existing between the pairwise p-distances of the cytochrome *b* and 16S markers for the 27 individuals for which both markers were available. Correlation between the two markers was not linear (R^2= 0.25), indicating that they do not evolve in a similar way; however, this could be due to the small sample size used. In fact, the results of the ANOVA indicate that the p-distances of the two markers are not statistically different (degree of freedom=1, F value= 118.67, p= 2.2^{e-16}). All sequences included in this study experienced typical characteristics of mitochondrial DNA. Base composition showed a deficiency of guanine, especially in the cytochrome *b* gene (values are approximated for excess; cytochrome *b*: T= 28%, A= 31%, C= 26%, G= 15%; 16S rRNA: T= 20%, A= 30%, C= 28%, G= 22%; concatenated dataset: T= 25%, A= 27%, C= 30%, G= 18%) as already reported for reptiles (Harris 2002), and an open reading frame was maintained for the cytochrome *b* marker. This, together with the lack of double peaks observed in the sequences suggests that we did not amplify nuclear pseudogenes (numts) instead of the mitochondrial markers.

Haplotype and nucleotide diversity values were h=0.912±0.032 and π=0.008±0.005 and h=0.966±0.021 and π=0.007±0.004 for the 16S and cytochrome *b* respectively and of h=0.986±0.014 and π=0.006±0.003 for the concatenated data set (we considered only the 27 individuals for which data from both markers were obtained). When compared with the other species of *Lygodactylus* available on Genbank for 409 homologous bp of the 16S rRNA gene, *L. tolampyae* shows the highest intra-specific p-distance value (Table 1). Table 2 shows the average p-distances for the same homologous fragment of cytochrome *b* in different species of geckos, indicating that the average p-distances found in *L. mirabilis* are lower than the ones observed in other species (but probably also sampled on larger geographic areas).

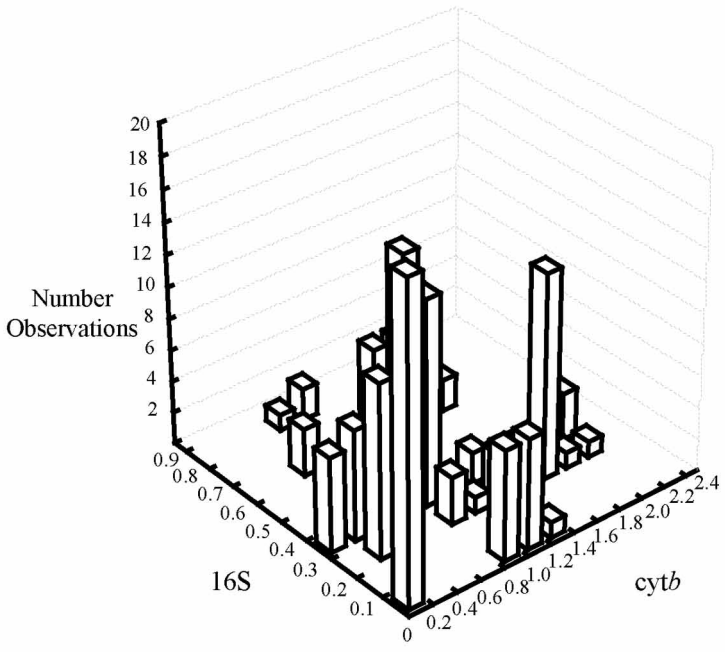

FIGURE 2: Three-dimensional plot of pairwise uncorrected p-distances for the cytochrome *b* (x-axis) and 16S rRNA (y-axis) markers. The number of observation (z-axis) indicates the number of times that a defined combination of cytochrome *b* – 16S distances has been observed.

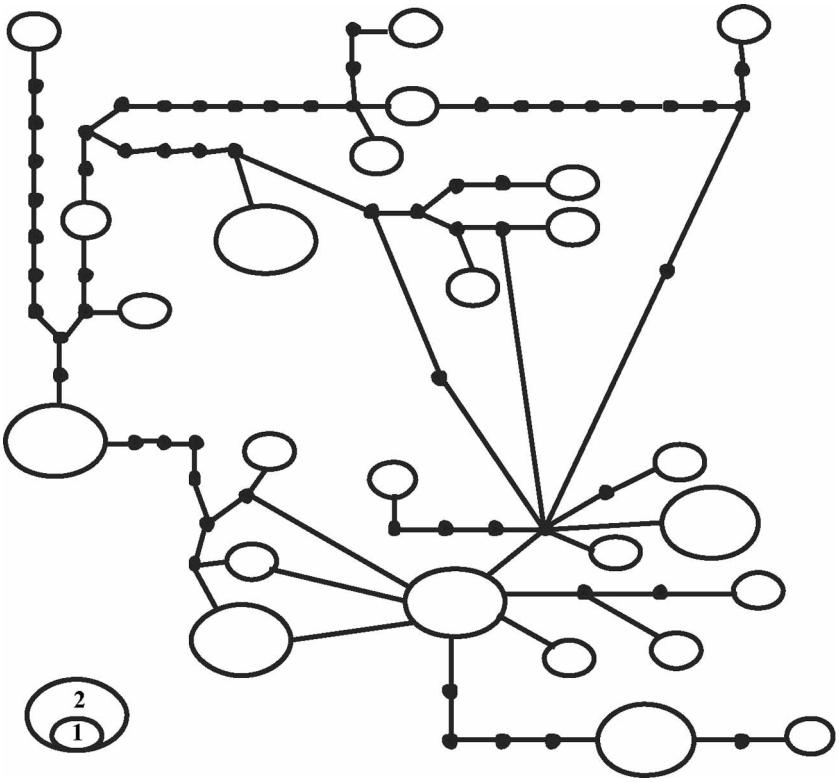

FIGURE 3: Haplotype network of *L. mirabilis* using all the 31 sampled individuals (1251 bp, cytochrome *b* and 16S rRNA genes). Circle size indicates the frequency of the haplotype, as indicated by the circles on the left side of the figure. Black dots indicate missing haplotypes. Straight lines between two haplotypes indicate that they differ by one mutation.

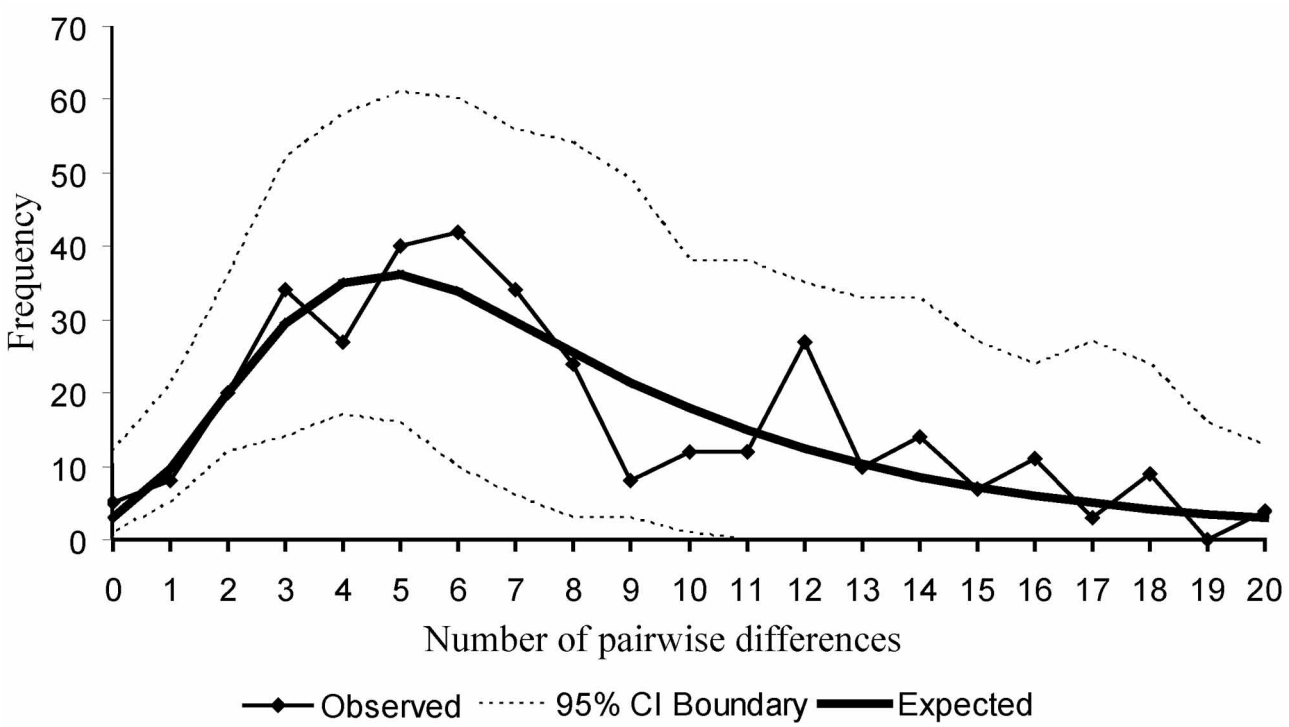

FIGURE 4: Mismatch distribution graph of the 27 mtDNA sequences (1251 bp, cytochrome *b* and 16S rRNA genes) in *L. mirabilis*. The solid line with diamonds represents the observed distribution curve and the diamonds are the relative frequencies of nucleotide differences between pair of individuals. The tick solid line represents the distribution expected from an expanding population. Dashed lines indicate the 95% confidence interval. Additional information can be found in the Materials and Methods.

The analysis of the 1251 bp of both gene fragments concatenated resulted in a total of 25 haplotypes out of 31 individuals. The haplotype network constructed from these 25 haplotypes shows the absence of any high frequency haplotype (Fig. 3). The absolute genetic distance between the different sequences goes from a minimum of 0 to a maximum of 18 substitutions for the concatenated data set of 27 individuals (average absolute distance: 7), between 0 and 16 for the cytochrome *b* (average absolute distance: 5), and from 0 to 9 for the 16S rRNA (average absolute distance: 2). Average p-distances are 0.7% for the complete dataset of 27 individuals (range 0.0-1.9%) and for cytochrome *b* (range 0.0-2.1%) and 0.5% for the 16S rRNA gene (range 0.0-0.8%). Numerous missing haplotypes were found.

We did not detect selection in our dataset. Only two amino acid sites of the cytochrome *b* gene (sites 119 and 145) resulted to be under negative selection (dN/dS<0) with significant p-value (0.04 and 0.01 for the sites 119 and 145, respectively). However, the same sites did not appear to be under selection according to the IFEL method, which is more suitable for the analysis of population data. Finally, the Fu's F*s* test indicated episodes of population growth (F*s*= -10.62216, p= 0.002 for the concatenated dataset; F*s*= -8.87486, p= 0.001 for the cytochrome *b* gene and F*s*= -4.64407, p= 0.03 for the 16S), further confirmed by the mismatch analysis showing a distribution that follows the one expected under an expansion model (Fig. 4). This result is confirmed by both SSD and raggedness index tests (SSD= 0.006 and p (SSD simulated > SSD observed)= 0.51; *r*=0.015 and p(*r* simulated> *r* observed)= 0.31).

TABLE 1. Species, Genbank accession numbers, number of individuals (N), and average p-distances (in %) used for the comparative analysis done on 409 bp of the 16S rRNA gene fragment.

Species	Accession number	N	p-distances
L. mirabilis	GQ910795-GQ910825	31	0.5
L. verticillatus	AY653289-91	3	0.0
L. tuberosus	AY653283-88	6	0.2
L. tolampyae	AY653278-80	3	9.1
L. pictus	AY653259 AY653265-72 AY653276	10	1.6
L. miops	AY653260-63	4	0.0
L. madagascariensis	AY653239 AY653254-58	6	4.3
L. bivittis	AY653242-44	3	0.0

Discussion

Boumans *et al.* (2007) and Münchenberg *et al.* (2008) found high levels of 16S rRNA variation in a number of widespread lizard species throughout Madagascar. This variation, however, mostly corresponded to differences between geographical clusters of populations whereas specimens from the same site normally shared the same haplotype. In contrast, in *L. mirabilis*, haplotypes are shared by a maximum of two individuals and only 38% of individuals had shared haplotypes (concatenated 16S rRNA and cytochrome *b*). The haplotype network presented in this study was built based on concatenated sequences of two genes, 16S and cytochrome *b*, which increases the chance of finding differences between haplotypes. But even if 16S is analyzed separately, 15 haplotypes are found in 31 individuals. Our molecular data therefore reveal a remarkable level of genetic diversity in *L. mirabilis* with numerous low frequency haplotypes and no main haplotype occurring in a larger number of specimens. The observed average genetic distance between individuals observed in *L. mirabilis* is not especially high when compared to the within species average genetic distances found in other gecko species (Table 2). However, our genetic results pertain to a species that

occurs in an extremely small range of only a few square km and the lack of population genetic studies on other gecko species with similarly restricted population distribution areas precludes any rigorous comparison of within-population genetic diversity.

TABLE 2. Genus, species, number of individuals (N), and average p-distances (in %) used for the comparative analysis done on a fragment of the cytochrome *b* gene. Bp indicates the number of homologous base pairs considered for each genus and *L. mirabilis*.

Genus	Species	Bp	N	Within species average p-distances
Lygodactylus	*L. mirabilis*	302	27	0.4
Hemidactylus	*H. frenatus*		10	8.8
	H. bouvieri		15	6.7
	H. turcicus		41	1.9
	H. angulatus		25	7.9
	H. mabouia		32	1.7
Lygodactylus	*L. mirabilis*	713	27	0.7
Phelsuma	*P. lineata*		9	9.0
	P. kochi		6	8.3
	P. abbotti		7	2.9
Lygodactylus	*L. mirabilis*	306	27	0.4
Coleodactylus	*C. septentrionalis*		33	10.3
	C. amazonicus		68	19.8
	C. brachystoma		7	18.2
	C. meridionalis		47	11.5
Lygodactylus	*L. mirabilis*	732	27	0.7
Gekko	*G. japonicus*		6	0.1
	G. wenxianensis		6	0.4
	G. gecko		10	5.2
Lygodactylus	*L. mirabilis*	356	27	0.5
Pachydactylus	*P. weberi*		8	2.2
	P. montanus		20	9.1
Lygodactylus	*L. mirabilis*	543	27	0.7
Tarentola	*T. boettgeri*		40	8.0
Lygodactylus	*L. mirabilis*	340	27	0.5
Phyllurus	*P. ossa*		32	3.1

One possible explanation for the syntopic occurrence of highly divergent mtDNA haplotypes observed in *L. mirabilis* could be the presence of cryptic species. However, we did not find two or more well-differentiated and genetically homogeneous clades in our dataset. Thus, not one but several cryptic species would need to be invoked to account for the variation observed. Furthermore, the maximum 16S rRNA and cytochrome *b* genetic divergence (9 as absolute distance and 0.8% for p-distances and 16 and 2.1% for the 16S rRNA and cytochrome *b* respectively; see results for average distances) is below the divergence between geographical variants of the same species in geckos (e.g., Boumans *et al*. 2007; Lamb & Bauer 2000) and lower than the genetic divergence observed among distinct species of geckos (e.g. Rocha *et al*. 2009 and references therein). Therefore, it would be more conservative and in line with current knowledge of gecko

phylogeography to view all individuals as belonging to *L. mirabilis*. Discussion should therefore turn to other factors that may have led to the high haplotype mtDNA diversity observed.

Our results support a scenario in which *L. mirabilis* experienced a population growth in which multiple haplotypes were generated with the following extinction of some of these haplotypes. Some authors previously proposed a high mitochondrial substitution rate in geckos (Jesus *et al.* 2002; Harris 2002; Blair *et al.* 2009) as estimated by very high divergences in mitochondrial genes along with low divergences in nuclear genes. The pattern found in *L. mirabilis* could be explained in terms of high mitochondrial substitution rate if this species recently expanded. However, the haplotype network and evidence for a past demographic expansion from Fu's Fs test suggests a more complex scenario for *L. mirabilis*, with multiple subgroups of haplotypes. This could be in agreement with a population history characterized by multiple periods of isolation during climate shifts, followed by population growth and consequent genetic diversity among these isolated populations (e.g., on different mountain tops) and secondarily contact with subsequent genetic admixture (in a similar way to what is described for ice age refugia and secondary contacts e.g., in Hewitt 1996). As our results are based on two maternally inherited markers, additional nuclear data and the inclusion of samples from the newly found population would help to better estimate the history of this species.

Whether geckos generally have a particularly high mitochondrial mutation rate, or whether our results can be explained by a species-specific pattern of population history and population structure, would have profound consequences for the usage of mitochondrial data in the discovery of species diversity in the Gekkonidae. In fact, divergence thresholds of mitochondrial genes are increasingly used to identify candidate species of organisms (e.g., Vieites *et al.* 2009) although due to multiple restrictions they need to be applied with caution and only to provide preliminary assessments, never as exclusive argument to actually describe new species (e.g., see Rubinoff *et al*. 2006 on the risks of using mitochondrial genes for species identification). This might even more be true in geckos if high mitochondrial divergences in these lizards may indeed turn out to reflect a mitochondrial substitution rate higher than in other squamates.

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