

Comparative phylogeography and patterns of deep genetic differentiation of two gecko species, *Paroedura gracilis* and *Phelsuma guttata*, across north-eastern Madagascar

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Abstract. *Phelsuma guttata* and *Paroedura gracilis* are two species of geckos endemic to lowland and mid-elevation rainforests of the northern half of Madagascar's rainforest band. To test for concordant phylogeographic patterns in the two species and to assess intraspecific phylogeny and genetic diversity, we sequenced DNA from two mitochondrial genes (16S and ND4) and three nuclear genes (CMOS, PDC, RAG1). The mtDNA trees of both species suggest a strong phylogeographic split between phylogroups from the North East and Northern Central East biogeographic regions of Madagascar. However, this pattern is not mirrored in the nuclear gene dataset, where common alleles can be found across the two biogeographic regions. Private alleles characterize some of the northernmost and southernmost populations, especially the Montagne d'Ambre population of *P. gracilis*. In this species the two main mitochondrial clades agree with two distinct dorsal colour pattern phenotypes – striped versus cross-banded – separated at the junction of North East and Northern Central East. As no obvious geographic barrier to gene flow is apparent, the observed concordant phylogeographic pattern indicates that the border between these two regions might represent an area of secondary contact after divergence in refugia. Both nuclear and mitochondrial DNA reveal a higher genetic divergence among lineages of *P. gracilis* as compared to *Ph. guttata*, calling for a more detailed taxonomic assessment of the former species.

Key words. Squamata, Gekkonidae, comparative phylogeography, genetic diversity, Madagascar.

Introduction

Madagascar is known for high levels of lineage diversification in the various taxa composing its highly endemic and species-rich biota. In numerous studies, Malagasy amphibians and reptiles have served as focal groups to test hypotheses of species diversification and regional endemism, and therefore, the island might harbour one of the best studied and scientifically most accessible herpetofaunas in the tropics (BROWN et al. 2016). Diversification mechanisms proposed for the Madagascar system, among many others, include rivers as barriers (summarized in VENCES et al. 2009); mountain massifs (RAXWORTHY & NUSSBAUM 1995) or low-elevation watersheds (WILMÉ et al. 2006) functioning as refugia during dry periods; or the northern part of Madagascar acting as a species pump due to increased diversification in this geographically and bio-climatically complex part of the island (e.g. RATSOAVINA et al. 2011). A recent study has pointed towards the role of a complex mixture of all these factors leading to the currently observed high levels of species diversity on Madagascar (BROWN et al. 2014), yet the special role and high local endemism in northern Madagascar is a common theme across numerous groups of organisms (BROWN et al. 2016).

Small non-flying vertebrates, such as lizards, are often comparatively poor dispersers and therefore, landscape features and associated geological and climatic conditions can have substantial influence on their phylogeography (CAMARGO et al. 2010), which can lead to congruent phylogeographic patterns across species (AVISE 2000). In this study, we test the above hypothesis utilizing two focal species from the family Gekkonidae, the Graceful Madagascar Ground Gecko, *Paroedura gracilis* (BOULENGER, 1896) and the Speckled Day Gecko, *Phelsuma guttata* (KAU-

manically complex part of the island (e.g. RATSOAVINA et al. 2011). A recent study has pointed towards the role of a complex mixture of all these factors leading to the currently observed high levels of species diversity on Madagascar (BROWN et al. 2014), yet the special role and high local endemism in northern Madagascar is a common theme across numerous groups of organisms (BROWN et al. 2016).

DERN, 1922). These two lizard species are distributed in approximately similar ranges in the evergreen rainforests of the lowlands and mid-elevations of eastern and northern Madagascar.

Phelsuma guttata is a medium sized (total length up to 131 mm) diurnal gecko with a wide geographical distribution across the North East and Northern Central East geographical regions of Madagascar (as originally defined by BOUMANS et al. 2007; see also GLAW & VENCES 2007, BROWN et al. 2016), between Daraina in the north (RAKOTONDRAVONY 2006) and Ampasimanolotra (formerly known as Brickaville) in the south, although its southern distribution limit remains uncertain (GEHRING et al. 2010). It is mainly found in, or at the border of, undisturbed primary rainforests and littoral forests (GLAW & VENCES 2007, SCHÖNECKER 2008, HALLMANN et al. 2008), but has also been recorded from areas with degraded secondary vegetation (GEHRING et al. 2010) where it is commonly found on *Ravenala* traveler's palms and *Pandanus* screw pines (GLAW & VENCES 2007).

Paroedura gracilis is a medium sized (total length up to 120 mm) nocturnal gecko, mostly observed in primary low- and mid-altitude rainforests. It hunts at ground level and in the shrub layer up to a height of 100 cm. The species' distribution area, as that of *Ph. guttata*, encompasses the Northern Central East and North East regions, but also extends into the North region where it occurs in Montagne d'Ambre National Park, and reaches southwards to Anosibe An'Ala. Within its distribution range, the species is known to exhibit differences in colour pattern, but geographic differences and polymorphism within populations have not been adequately analysed yet.

Northern Madagascar, i.e., the area roughly delimited by a diagonal spanning from 15.5°S on the east coast to ca. 15.0°S on the west coast (BROWN et al. 2016), is known to harbour a high degree of endemism across different taxonomic ranks. This includes endemic intraspecific phylogroups, species, and supra-specific clades, most probably influenced by the area's high topographic and bioclimatic heterogeneity at small spatial scales (RAXWORTHY & NUSSBAUM 1995, WOLLENBERG et al. 2008, VENCES et al. 2009, BROWN et al. 2016). The limits of northern Madagascar, following this definition, correspond roughly to the borders between the Northern Central East and the North East on the one hand, and between the North-West and Sambirano regions on the other (BROWN et al. 2016). The diverse topology of the area includes several mountain massifs characterised by large expanses of rainforest such as Montagne d'Ambre, Tsaratanana or Marojeje, with intervening lowlands and drier limestone massifs such as Ankarana or Montagne des Français. Recent studies on different gecko genera such as *Uroplatus* (GEHRING et al. 2018), *Ebenavia* (HAWLITSCHKE et al. 2018) and *Phelsuma* (GEHRING et al. 2013) have documented profound phylogeographic splits corresponding to the limits between the Northern Central East and North East regions. As both of our focal species are found across these zones, we hypothesize that they also could concordantly exhibit a similar deep phylogeographic split. Therefore, in the present study, we aim to (i) compare

genetic diversity and phylogeographic patterns of these two largely co-distributed geckos, and in addition, (ii) document colour pattern variation across their ranges.

Materials and methods

Tissue samples were mainly collected by P.-S. GEHRING, F. M. RATSOAVINA, E. RAJERARISON, and F. RANDRIANASOLO in April and May 2009 and 2010 along an approximately 1,000 km north–south transect along Madagascar's east coast. Additional tissue samples were obtained during fieldwork in Madagascar conducted between 2000–2018 by M. VENCES, F. GLAW, M. D. SCHERZ and D. R. VIEITES. FGMV, FGZC, and ZCMV refer to F. GLAW and M. VENCES field numbers, PSG refers to field numbers of P.-S. GEHRING, MSZC and MSTIS refers to field numbers of M. D. SCHERZ, DRV to field numbers of D. R. VIEITES.

Species identification in the field was mainly based on diagnostic colouration patterns following GLAW & VENCES (2007) and HALLMANN et al. (2008). Tissue samples (tail clipping) were taken from geckos and preserved in 95–99% ethanol. Nearly all animals were immediately released after sampling. Some selected voucher specimens were euthanized by lidocaine overdose, fixed in 95% ethanol and preserved in 70% ethanol. Vouchers are held at the University of Antananarivo, Zoologie et Biodiversité Animale, Madagascar (UADBA-FGMV, UADBA) and the Zoologische Staatssammlung München, Germany (ZSM). Collecting localities were selected with the aim of covering the whole distribution area of *P. gracilis* and *Ph. guttata*. Altogether we sampled a total of 85 individuals from ca. 35 localities. Localities were geo-referenced with GPS receivers (see Supplementary Table S1 for precise coordinates).

DNA sequences were obtained for fragments of the following five genes: (i) the mitochondrial 16S rRNA gene (16S), (ii) the mitochondrial NADH Dehydrogenase Subunit 4 (ND4) gene, (iii) the nuclear protein coding gene for Oocyte Maturation Factor Mos (CMOS), a quite slowly-evolving conserved marker that can easily be amplified and sequenced across species and was included for additional information, (iv) the nuclear protein coding gene Phosducin (PDC), and (v) the gene encoding the Recombination Activation Protein 1 (RAG1), a single copy nuclear marker widely used in resolving relationships among vertebrate species (CHIARI et al. 2009), but also diagnostic between closely related gecko species (e.g., BAUER et al. 2011, GEHRING et al. 2018).

Total genomic DNA was extracted from the tissue samples using proteinase K digestion (10 mg/ml concentration) followed by a standard salt-extraction protocol (BRUFORD et al. 1998). Markers were amplified using polymerase chain reaction (PCR). For samples of *Ph. guttata*, we amplified the two mitochondrial markers 16S and ND4 and one nuclear marker, PDC. For samples of *P. gracilis*, we additionally amplified RAG1 and CMOS. All the primers used for the amplification of these markers are tabulated in Table 1.

Table 1. Information on the DNA markers amplified, primers used for their amplification, and source of the primers.

Marker	Primer name	Primer sequence	Reference
16S	16SAL	CGCCTGTTTATCAAAAACAT	PALUMBI et al. (1991)
	16SBH	CCGGTCTGAACTCAGATCACG	
ND4	ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	ARÉVALO et al. (1994)
	Leu	CATTACTTTTACTTTGGATTTGCACCA	
CMOS	C08	GCTTGGTGTTC AATAGACTGG	HAN et al. (2004)
	C09	TTTGGGAGCATCCAAAGTCTC	
PDC	PHOF 1	CCATCCAACATCTCAGCATGATGAA	BAUER et al. (2007)
	PHOR 1	CCCTCAGAATGATATTTGTCCTCA	
RAG1	Urop-Rag1-F1	GAAAACCTGGAGCGGTATGA	BAUER et al. (2011)
	Urop-Rag1-R1	GCAACTCTGCAAAACGTTGA	

All obtained PCR products were purified by Exonuclease I and Shrimp Alkaline Phosphatase digestion and sequenced on a 3130xl Genetic Analyser (Applied Biosystems) using Big Dye v3.1 cycle sequencing chemistry. Chromatograms were quality checked by eye, trimmed for low-quality stretches, heterozygote positions identified, and errors corrected using Codon Code Aligner (v5.1.5, Codon Code Corporation). Newly determined sequences have been deposited in GenBank (accession numbers in Supplementary Table S1).

We aligned sequences using the Muscle algorithm (EDGAR 2004) with default parameters set in MEGA 6.0 (TAMURA et al. 2013). All pair-wise sequence divergence values presented in the manuscript are uncorrected p-distances generated in MEGA 6.0 (Supplementary Table S2).

The final alignments of *Ph. guttata* were 527 bp (16S), 411 bp (ND4) and 338 bp (PDC) long, and those of *P. gracilis* were 616 bp (16S), 848 bp (ND4), 275 bp (PDC), 421 bp (RAG1) and 425 bp (CMOS) long.

To reconstruct mitochondrial phylogenetic relationships, we concatenated the two mitochondrial markers (16S and ND4). We then implemented a non-partitioned scheme to avoid over-parametrization in MrBayes 3.2 (HUELSENBECK & RONQUIST 2001, RONQUIST & HUELSENBECK 2003) using a Jukes-Cantor model of nucleotide substitution. We ran 50 million Markov Chain Monte Carlo (MCMC) generations in four chains each, and sampled trees every 200 generations, with a burn-in of 25%. Priors were set to variable rates of evolution and state frequencies remained unlinked. We visualized the log-likelihood values across time plot in Tracer version 1.6 (RAMBAUT et al. 2013) to verify convergence of parameters.

To visualize the geographical differentiation in nuclear genes separately from that of the mitochondrial genes, we constructed haplotype networks from individual nuclear gene datasets. We used the PHASE algorithm in DnaSP 10.01 (LIBRADO & ROZAS 2009) to separate nuclear sequences into alleles (haplotypes). This software was also used to calculate the number of haplotypes/alleles, number of variable sites in the alignment, nucleotide diversity (π), the average number of nucleotide differences (k) and

haplotype diversity (H_d) for all nuclear markers (Supplementary Text S3). From the nuclear alleles, we generated a Roehl Data Format (RDF) file in DnaSP and calculated Median Joining networks (BANDELT et al. 1999) in the software NETWORK 5.0 (fluxus-engineering.com). Loops in one of the networks (PDC of *P. gracilis*) were resolved by preferring connections to more central alleles and common alleles (POSADA & CRANDALL 2001). Networks were colour-coded geographically using Corel Draw Student X7 (Corel Corporation).

We gathered coloured photographs of live individuals (from our own field work) of the two species across their range and chose one representative photo of each species per site for graphical representation. Overall, we examined photographs of 28 individuals (Supplementary Table S4).

Sampled localities were plotted on a map using ArcGIS 10.13 (ESRI 2011) and colour-coded with identical location codes in the mitochondrial phylogenies and nuclear gene allele networks. The Madagascar outline shape file was obtained from www.maplibrary.org. Biogeographical regions within Madagascar were named following BOUMANS et al. (2007).

Results

The mitochondrial phylogeny of *Ph. guttata* revealed two major clades, one containing all individuals from the North East and the other containing all individuals sampled in the Northern Central East of Madagascar (Fig. 1). Uncorrected p-distances between these two clades ranged between 3.7–6.5% in 16S and 10.2–14% in ND4. Within each of these clades, values ranged between 0.0–6.0% in 16S and 0.0–11.9% in ND4. For the nuclear gene PDC, six alleles were identified in *Ph. guttata*. PDC alleles H1 and H3 were shared between localities belonging to the main mitochondrial clades; however, the five southernmost populations (Akanin'ny nofy, Mahaso, Sahafina, Tampolo and Vohibola) did not share haplotypes with any of the sites further north (Fig. 1).

The mitochondrial phylogeny of *P. gracilis* also revealed two major clades, one containing all individuals sampled from the Northern Central East and the other containing samples from the North East and the North (Fig. 2). The latter further split into a North and a North East clade. The Northern Central East clade differed from the North East and North clade by 6.3–11.2% in 16S and 11–14.9% in ND4; the North East and North subclades differed by 6.4–9.6% in 16S and 9.4–13.5% in ND4 (Supplementary Table S2). In the three nuclear genes studied for this species (CMOS, PDC and RAG1), widespread allele sharing occurred between populations of the two main mitochondrial clades (Fig. 2). A certain trend was observed for specimens of the southernmost populations to have unique alleles not shared with other populations (in PDC and RAG1; Fig. 2). Strikingly, the northernmost population (Montagne d'Ambre) had unique alleles in all three nuclear markers and did not show any allele sharing with any other location (Fig. 2). The gene PDC was more divergent among the individuals of *P. gracilis* as compared to *Ph. guttata*, with a nucleotide diversity of 0.82 and 14 recognized haplotypes (Fig. 2, Supplementary Text S3).

Based on examination of all photos available to us, individuals in all populations of *Ph. guttata* have a green or greenish-grey ground colouration dorsally, with a highly variable number of smaller and bigger red to light brown spots, and without obvious differences between sites belonging to genetic clades (Fig. 3). Some individuals show a blue hue in the neck (see Fig. 3E) and a dark red band extends from the nostrils through the eye until the ear opening in the neck in all the populations. The flanks and the tail show an irregular dark mottling. Juveniles and sub-adult individuals show a more distinct and contrasting pattern (see Fig. 3H).

In individuals of *P. gracilis*, the dorsal body colouration of all populations is light brown to greyish-yellow, also covering the upper half of the flanks laterally. Individuals photographed at night have a much lighter ground colour and therefore show a more contrasting pattern than those photographed during the day (Fig. 4). The lower half of the flanks and the ventral parts are whitish to dark-grey. A clear difference is observed between populations of the two main mitochondrial clades: populations of the first (northern) main clade have a dorsal pattern dominated by

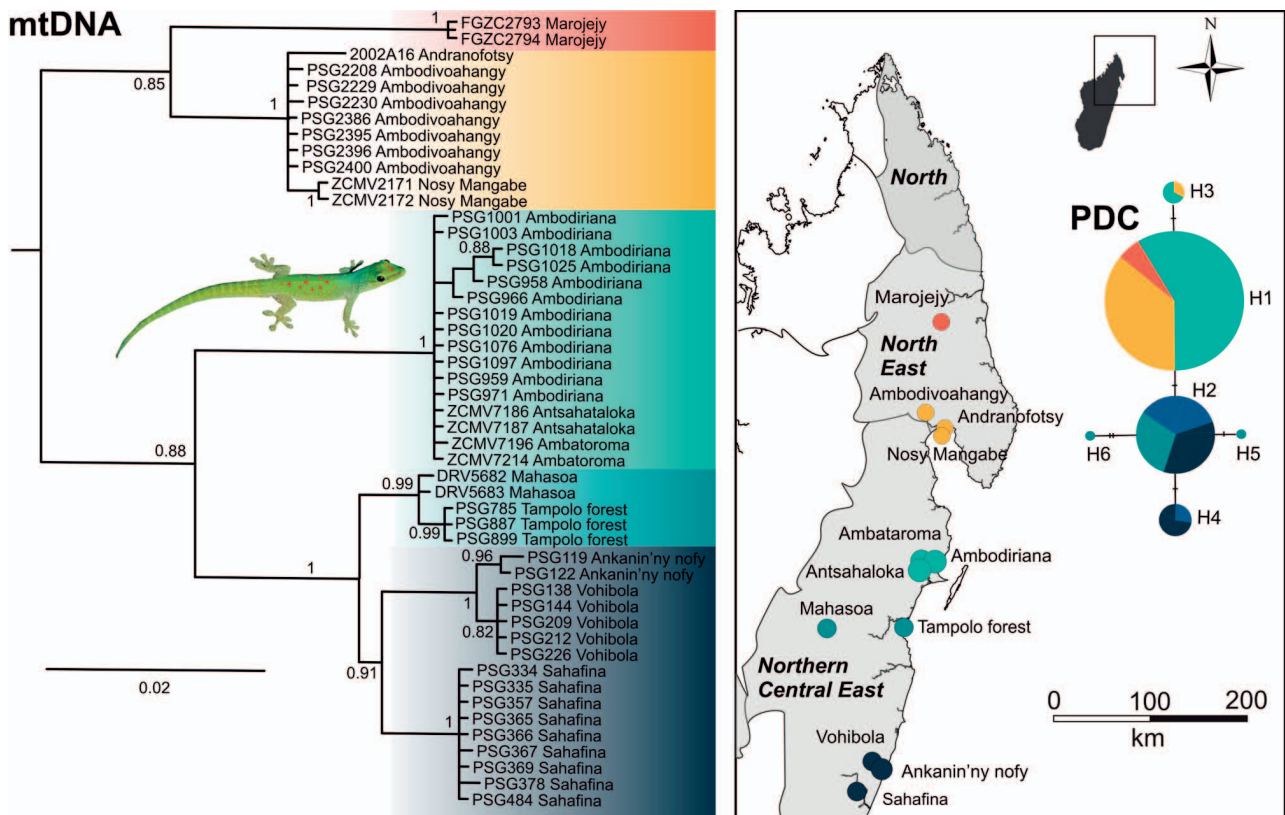


Figure 1. Phylogeographic variation of *Phelsuma guttata*. Left: Bayesian phylogeny from concatenated mitochondrial markers (16S and ND4). Values at nodes are Bayesian Posterior Probabilities (only shown if > 0.8). The tree was rooted with *Ph. madagascariensis* (not shown to allow for better graphical representation). Right: Map of Madagascar with coloured points indicating sampling points and allele (haplotype) network of the nuclear gene PDC; the size of the circles is associated with the frequency of the alleles (after phasing; smallest circle for allele H6 corresponds to N=1, largest circle for allele H1 corresponds to N=46). Cross bars between haplotypes represent one mutation each and colours in the network correspond to those in the map and mtDNA tree.

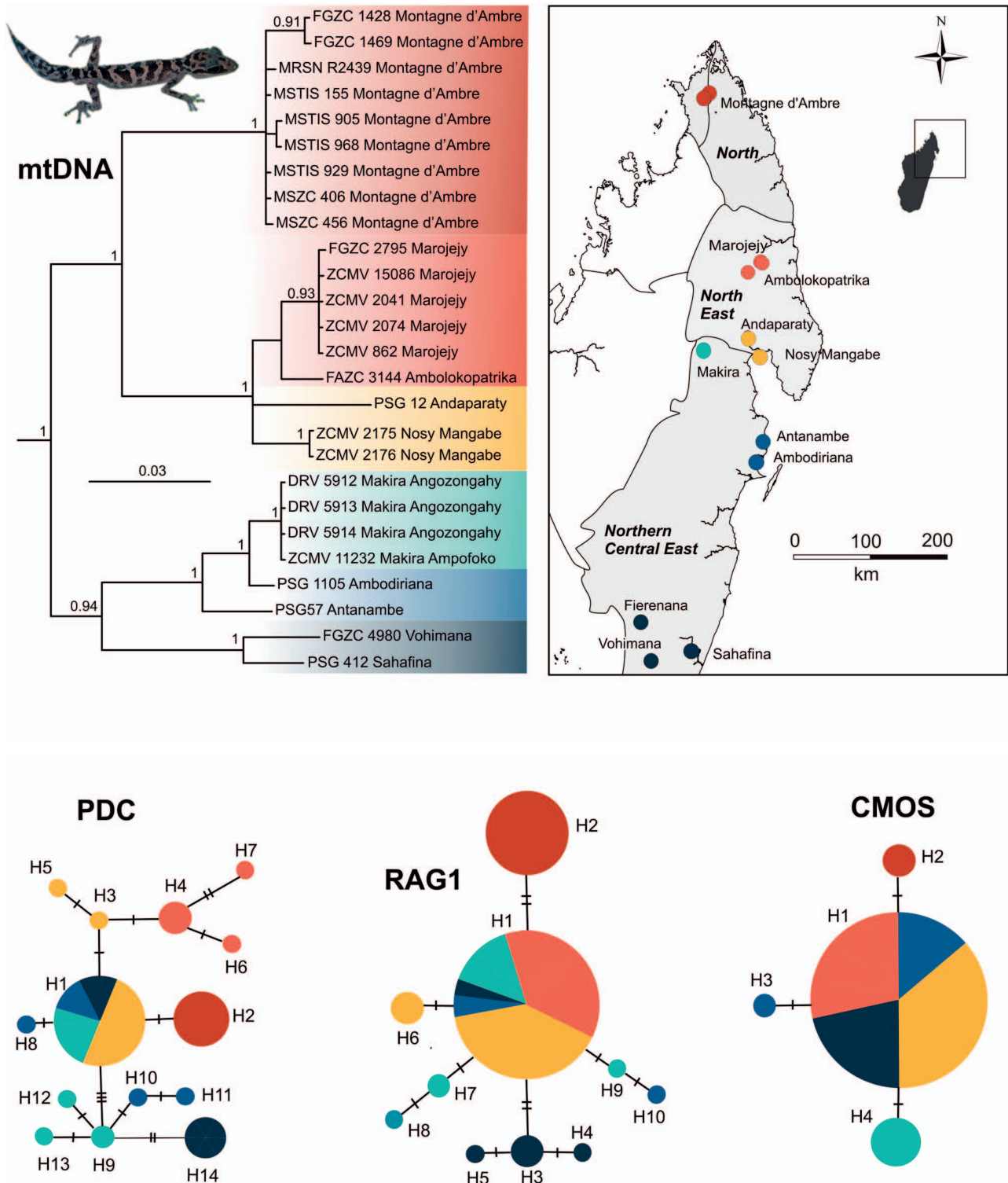


Figure 2. Phylogeographic variation of *Paroedura gracilis*. Upper left: Bayesian phylogeny from concatenated mitochondrial markers (16S and ND4). Values at nodes are Bayesian Posterior Probabilities (only shown if > 0.9). The tree was hierarchically rooted with *P. bastardi*, *P. masobe*, and *P. oviceps* (not shown to allow for better graphical representation). Right: Map of Madagascar with coloured points indicating sampling points (including Fierenana, a site sampled by APREA et al. 2013). Below: Allele (haplotype) networks of the nuclear genes PDC, RAG1 and CMOS; the size of the circles is associated with the frequency of the alleles (after phasing; smallest circles correspond to N=1, largest circle for allele H1 in CMOS corresponds to N=28). Cross bars between haplotypes represent one mutation each and colours in the networks correspond to those in the map and mtDNA tree.

crossbands whereas in the second (southern) clade all individuals are characterized by longitudinal stripes (Fig. 4). In the cross-banded phenotype dark horizontal crossbands extend from the neck until the base of the tail and can be continuous or ruptured by the ground colouration, and in the North East populations (e.g. Marojejy, Masoala, Nosy Mangabe) can be dissolved into a rather irregular pattern of separated dark spots (Figs 4 C, E). The striped pheno-

type observed in populations of the second main mitochondrial clade, occurring south of Antongil Bay, shows dorsally a distinct pattern of four roughly parallel longitudinal/vertical dark lines. The two outer longitudinal lines start from the eye socket and run along the upper flanks until the tail base where they merge. The inner lines start at the neck and run dorsally until the tail base. These longitudinal lines can be continuous or ruptured (see Figs 4 G, I).

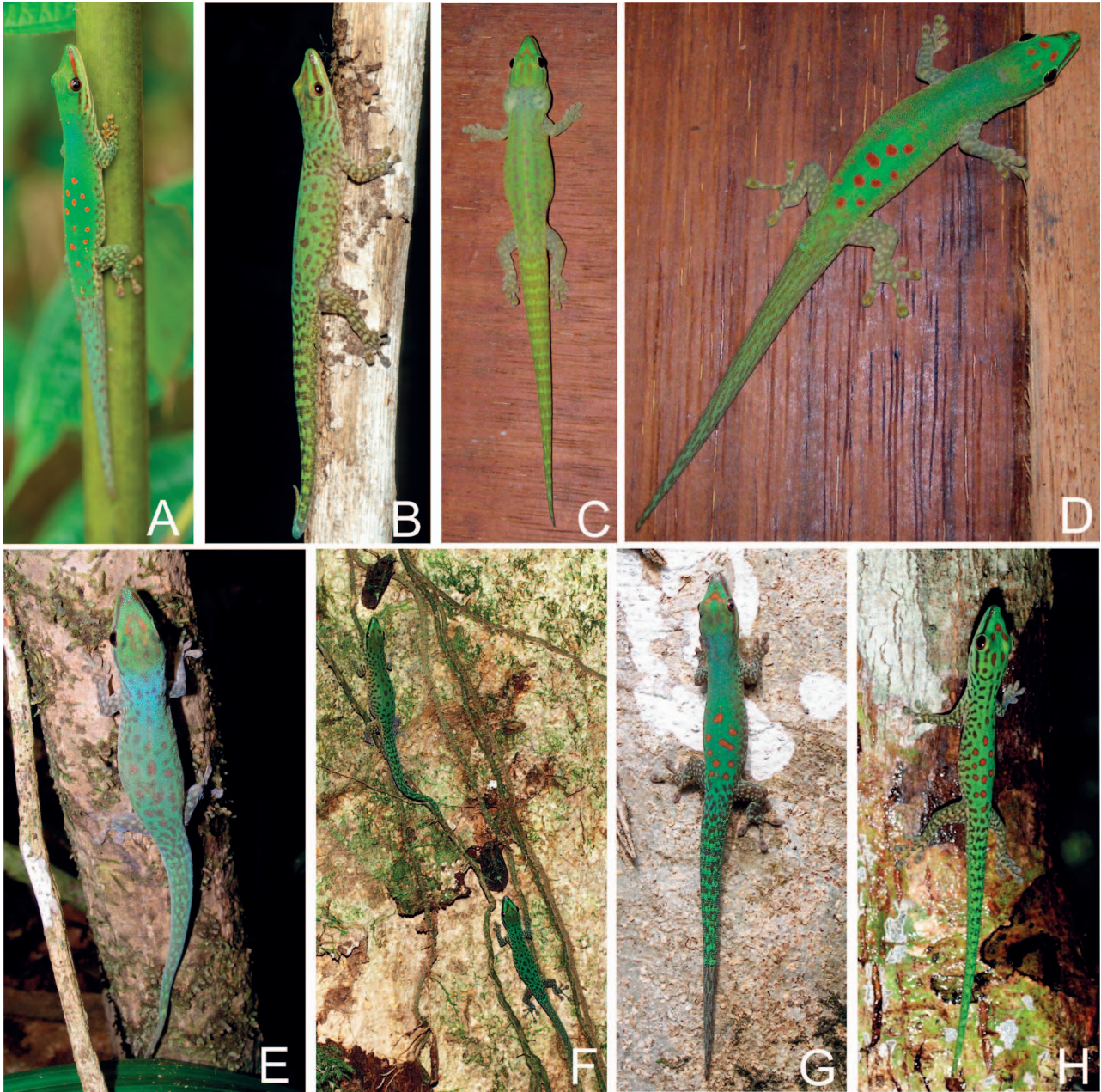


Figure 3. Colour variation in *Phelsuma guttata*; representative individuals from various populations, arranged from north to south: A) Marojejy, B) Marojejy, C) Nosy Mangabe (female), D) Nosy Mangabe (male), E) Masoala, F) Ambodiriana, G) Mananara-Nord, H) Vohibola (juvenile). All photographs by the authors.

Discussion

The two focal gecko species in this study, *Ph. guttata* and *P. gracilis* share a concordant phylogeographic pattern with a phylogeographic split at the juncture of Northern Central East and North East biogeographic regions. Their mitochondrial phylogenies also concordantly revealed deep phylogeographic structures, with strongly divergent subclades coinciding with geographically separate populations. Even localities geographically close to each other, and grouped in the same main subclade, often show a slight divergence in mitochondrial genes, which agrees with previous findings of distinct phylogeographical differentiation in numerous widespread species of Malagasy reptiles (e.g., BOUMANS et al. 2007, RATSOAVINA et al. 2010, 2011, FLORIO et al. 2012, 2016, GEHRING et al. 2012, 2013, 2018, HAWLITSCHKE et al. 2018, RUANE et al. 2018). Further, this study has identified mitochondrial subclades concord-

antly characterized by private nuclear alleles, particularly, the *P. gracilis* population from Montagne d'Ambre. This pattern agrees with some other geckos (e.g., *Uroplatus giganteus*: GEHRING et al. 2018) but not with others (e.g., the *Phelsuma lineata* complex: GEHRING et al. 2013). This detection of a diversifying lineage on the Montagne d'Ambre strengthens previous findings of presence of several micro-endemic lineages of amphibians and reptiles on this isolated northern massif (RATSOAVINA et al. 2011, RAKOTOARISON et al. 2017).

Our results corroborate the findings of GEHRING et al. (2012, 2018) and HAWLITSCHKE et al. (2018) showing a strong split at the junction of North East and Northern Central East regions roughly in the area of the Antongil Bay. However, the precise location of this phylogeographic break appears to be somewhat different among species. In the two geckos studied herein, the localities at the Antongil Bay (e.g., Ambodivoahangy, Andaparaty, Andrano-

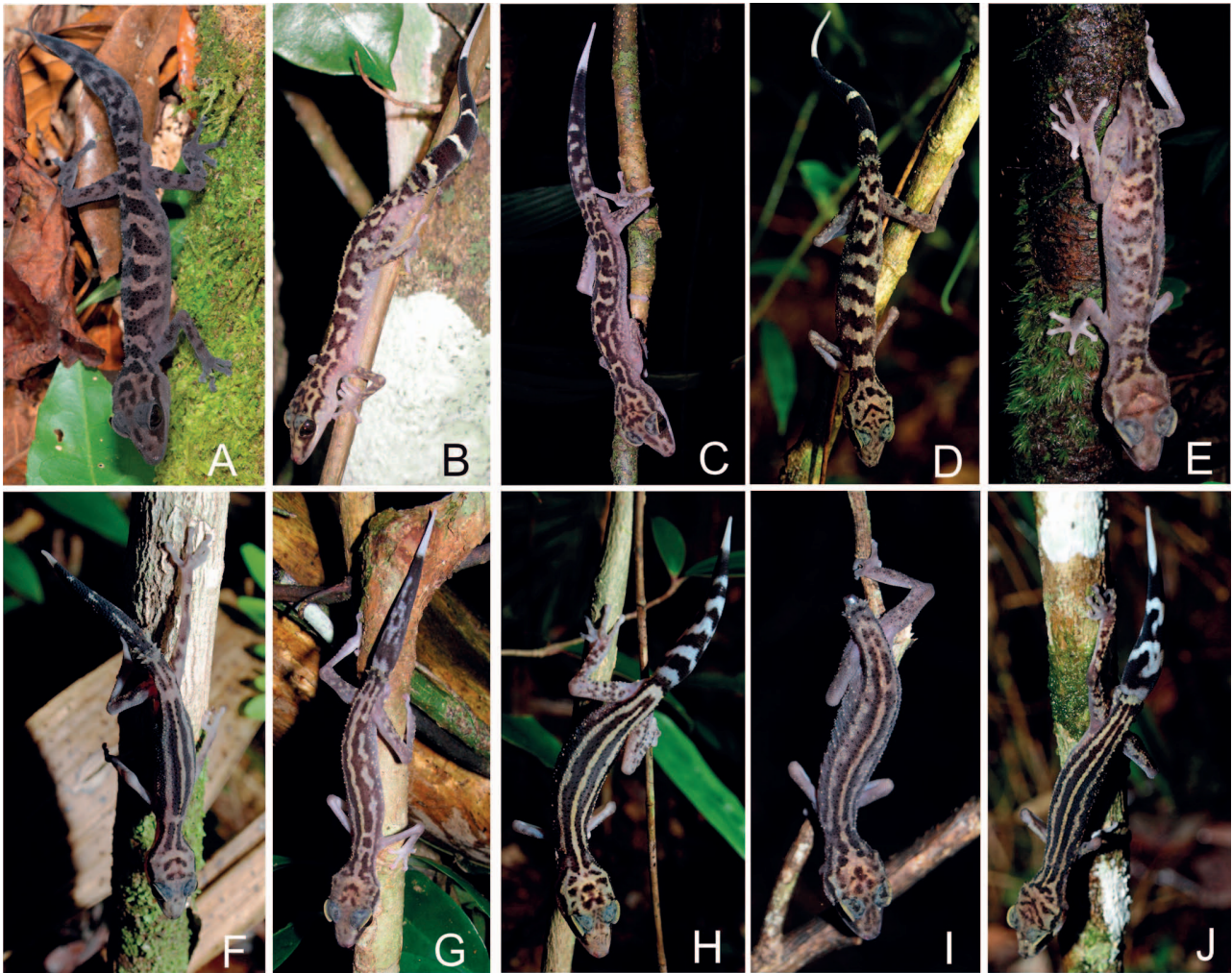


Figure 4. Colour variation in *Paroedura gracilis*; representative individuals from various populations, arranged from north to south: A) Montagne d'Ambre, B) Marojejy, C) Masoala, D) Ambodivoahangy, E) Nosy Mangabe, F) Ambodiriana, G) Mananara-Nord, H) Sahafina, I) Vohimana, J) Anosibe An'Ala. All photographs by the authors.

fotsy, Nosy Mangabe) are characterized by mitochondrial haplotypes of the northern subclade. Whereas, in the geckos of the *Uroplatus fimbriatus/giganteus* complex, this area harbours haplotypes of the southern subclade (i.e., of the species *U. fimbriatus*). A similar split can be recognized in the Bamboo Leaf-tailed Gecko (*Uroplatus lineatus*), with a distinct genetic clade north and south of the Antongil Bay (RATSOAVINA et al. 2013). *Phelsuma lineata*, a day gecko, does not occur in these lowland sites, but in the adjacent mid-elevations, populations with mitochondrial haplotypes of the northern subspecies '*punctulata*' extend even further southwards than Antongil Bay (GEHRING et al. 2013). KRÜGER (1996) and BERGHOF & HOESCH (2008) identified a contact zone between the day geckos *Phelsuma madagascariensis* and *Ph. grandis* along the river Laloana on the Masoala Peninsula, just a few kilometres north of Antongil Bay. In *Ebenavia*, the divergence zone is apparently north of Antongil Bay, with *E. boettgeri* occurring up to this area, and *E. safari* occurring from Marojejy northwest to Montagne d'Ambre (HAWLITSCHKE et al. 2018). Aside from geckos, other reptiles also exhibit a similar phylogeographic pattern. In the short-nosed chameleons of the *Calumma nasutum* complex (GEHRING et al. 2012), different populations from the lowland areas of north eastern Madagascar are consolidated within clade G (as defined by GEHRING et al. 2012). This cluster shows a profound split roughly in the area of the Antongil Bay (Ambodivoahangy and Marojejy populations vs. Ambodiriana, Tampolo and Makira populations; PRÖTZEL et al. submitted). In the lowland chameleon *Furcifer pardalis*, GRBIC et al. (2015) found relatively strong genetic differentiation between the populations from the North East and the Northern Central East, but with a boundary further north than the other examples we have given, around Bemanevika in the Sava region.

For lowland species, such as the two species studied herein and the *Uroplatus fimbriatus/giganteus* complex, no obvious geographical barriers exist between the North East and Northern Central East regions. The phylogeographic break observed at the border between these regions is therefore unlikely to reflect a primary divergence caused by ceasing gene flow in this area. It seems more plausible in such lowland species to hypothesize a past range contraction to smaller refugia, e.g. in drier periods (WILMÉ et al. 2006), genetic divergence among the now separated populations, and secondary contact upon subsequent range expansion. A comparative approach of statistical phylogeography, based on more extensive sampling of individuals and markers, would be needed to submit this hypothesis to a thorough test.

When comparing levels of genetic differentiation and genetic diversity, we observed that *P. gracilis* exhibits somewhat higher levels of genetic diversity in terms of pairwise uncorrected p-distances between the same locations as opposed to *Ph. guttata*; e.g., the 16S p-distance between Ambodiriana and Sahafina is 2.7% in *Ph. guttata* vs. 4.4% in *P. gracilis* (Supplementary Table S2). We also found higher genetic diversity in the gene PDC in *P. gracilis* as compared to *Ph. guttata*, with 14 recognized haplo-

types vs. six. Interestingly, the mitochondrial phylogeographic pattern in *P. gracilis* agrees with a clear phenotypic shift in dorsal banding patterns i.e. horizontal cross-bands vs. vertical/longitudinal stripes at the junction of North East and Northern Central East (see Fig. 4, A to E vs. F to J). This observation is based entirely on specimens photographed during own field work but agrees with other specimens we have seen in collections or photographed in the literature or web resources. Along with the deep genetic divergence, this might indicate taxonomic distinctness between the two main mitochondrial subclades. Interestingly the pattern of mitochondrial divergence is not paralleled by nuclear genes, with extensive nuclear allele sharing among populations of the two main mitochondrial clades, and with private alleles characterizing only the population of Montagne d'Ambre. APREA et al. (2013) have shown differences in the diploid chromosome numbers among *P. gracilis* populations, especially Montagne d'Ambre, Fierenana and Ambolokopatrika. This study also hypothesized that *P. gracilis* is an assemblage of several closely related species, and the data presented here appear to be congruent with this hypothesis. Despite these results, we refrain from translating this overall pattern into a novel species-level classification which in our opinion requires additional data to be convincing; *P. gracilis* as currently understood might represent a complex of two or several species. Deciding among these alternative hypotheses requires morphological studies and/or application of more fine-scale genetic methods to characterize nuclear gene flow at contact zones. It will also require clarification of the assignment of the name, as *P. gracilis* was described with the general type locality 'Madagascar' (BOULENGER 1896). On the other hand, for *Ph. guttata* we feel that the present classification as a single species, with various deep mitochondrial lineages, best reflects the pattern observed.

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Supplementary material

Supplementary Table S1. Sample voucher numbers with location (GPS coordinates) and respective GenBank accession numbers.

Supplementary Table S2. Pairwise genetic uncorrected p-distances between all individuals of both the species generated from mitochondrial markers 16S and ND4.

Supplementary Text S3. Additional information on alleles (haplotypes) identified from nuclear genes of *Phelsuma guttata* and *Paroedura gracilis*.

Supplementary Table S4. Numbers of photographed individuals of *Paroedura gracilis* and *Phelsuma guttata* per site, examined for analysis of colour and pattern variation.