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First karyological analysis of the endemic Malagasy phantom gecko *Matoatoa brevipes* (Squamata: Gekkonidae)

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Abstract. The genus *Matoatoa* includes two Malagasy endemic species, *M. brevipes* and *M. spannringi*. Due to their cryptic behaviour, the two species are known only from a handful of specimens and have been included in few molecular studies. Here we carried out a molecular barcoding analysis using a fragment of the mitochondrial NADH dehydrogenase subunit 2 (ND2) and the first chromosomal analysis of *M. brevipes*. The molecular analysis confirmed the identity of the studied samples as *M. brevipes.* However, the level of genetic divergence (4% uncorrected *p*-distance) between our samples and other sequences of *M. brevipes*, suggests previously unrecognised diversity within the species. The karyotype of *M. brevipes* is composed of 2n = 34 chromosomes: the first pair is metacentric, while all the other pairs are telocentric and gradually decreasing in length (Arm Number, AN = 36). C-banding revealed little evidence of centromeric heterochromatin, while NOR-associated heterochromatin was found on the telomeres of a medium sized telocentric pair. No heteromorphic chromosome pairs were found in the karyotype of the species, suggesting that putative sex chromosomes are at an early stage of differentiation. Karyological comparisons with closely related species were performed with *Christinus marmoratus*, and representatives of the genera *Phelsuma*, *Ebenavia*, *Paroedura* and *Uroplatus*. Comparisons across genera suggest that chromosome diversification in this group of geckos probably occurred by means of chromosome fusions and inversions, leading to a reduction of the chromosome number and the formation of biarmed elements in different species.

Keywords. Chromosomes, leaf-toed geckos, *Matoatoa*, Madagascar, evolution, reptiles.

Madagascar is one of the hottest reptilian biodiversity hotspots, including more than 430 reptile species (Glaw and Vences, 2006; Uetz et al., 2019). The mostly endemic reptile fauna of Madagascar is still relatively poorly understood, despite significant progress in the last few decades (Glaw and Vences, 2006), with several new species described every year (Uetz et al., 2019). A significant number of these new reptile species are known only from a few specimens, and despite researchers applying molecular barcoding techniques (Nagy et al., 2012) with large numbers of comparative molecular sequences, the reptile diversity remains significantly underestimated.

Compared to other groups, only a small fraction of reptile species have been studied with cytogenetic methods, despite increasing evidence that species-level diversity is reflected at the karyotype level (e.g. Mezzasalma et al., 2016, 2018; Rovatsos et al., 2017). Malagasy vertebrates display dynamic patterns of chromosome evolu-

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tion including augmentation and reduction of the chromosome number and the independent diversification of sex chromosome systems (e.g. Mezzasalma et al., 2017a; Rovatsos et al., 2017), making the Malagasy reptile fauna an exciting study system for evolutionary cytogenetics.

The genus *Matoatoa* includes two endemic Malagasy gecko species, *M. brevipes* (Mocquard, 1900) and *M. spannringi* Nussbaum, Raxworthy and Pronk 1998. Species of *Matoatoa* are part of a clade including the southern African genera *Afrogecko*, *Cryptactites*, *Kolekanos* and *Ramigekko*, the Australian genus *Christinus* and the more distantly related genera *Afroedura*, *Goggia*, *Phelsuma*, *Paroedura*, *Ebenavia* and *Uroplatus* (Heinicke et al., 2014). Karyological data are currently available for *Christinus marmoratus* and different species of *Phelsuma*, *Paroedura*, *Ebenavia* and *Uroplatus* (King and Rofe, 1976; King and King, 1977; Aprea et al., 1996, 2013). However, there are no available chromosome data from the genus *Matoatoa*. Here we present the results of the first karyological study on the endemic Malagasy phantom gecko *M. brevipes* and a preliminary mitochondrial analysis to provide a taxonomic identification of the sample studied. Finally, we compared our newly generated karyotype data to those from closely related gecko genera and hypothesise how karyotype evolution occurred in the group.

We used a sample from a female specimen of *M. brevipes* collected 20 km south of Tulear (the cell suspension and tissue sample are deposited in the Natural History Museum, London, UK, and in the Department of Biology, University of Naples Federico II, with voucher GA436). The sample was preliminary treated with colchicine (1mg/ml; 0.1 ml/10 gr body weight) and, after two hours, tissue samples with high mitotic indices (intestine and gonads) were placed for 30 min in hypotonic solution (sodium citrate 0.50% + KCl 0.56%) and then transferred in Carnoy's fixative (Methanol + glacial acetic acid, 3:1).

We performed a phylogenetic analysis using a fragment of 561 bp of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2). Genomic DNA was extracted using the standard phenol-chloroform method (Sambrook et al., 1989). For the ND2 analysis we used the primers and PCR parameters reported in Heinicke et al. (2014). After sequencing, the obtained chromatogram (accession number MT579309) was manually corrected and aligned with homologous ND2 segments of *M. brevipes* deposited in GenBank using Chromas Lite 2.2.1 and BioEdit 7.2.6.1 (Hall, 1999). Phylogenetic analysis was performed with Bayesian inference (BI) using MrBayes 3.2.5 (Ronquist et al., 2012), and Maximum likelihood (ML) using MEGA 10.1.7 (Tamura et al., 2013), with 2,000,000 generations and 1000 bootstrap replicates, respectively. In both analyses we included homologous ND2 sequences of representatives of phylogenetically closely related gecko genera: *Christinus alexanderi* (KF666813), *Christinus guentheri* (KF666801), *Cryptactites peryngueyi* (KF666814), *Ramigekko swartgergensis* (KF666793) and *Afrogecko porphyreus* (KF666772). We used as outgroup two homologous sequences of the phylogenetically closely related *Kolekanos plumicaudus* (KF666791, JX041304).

In the cytogenetic analysis, chromosomes were prepared by the scraping + air drying method (see Mezzasalma et al., 2016), and a combination of traditional staining (Giemsa staining at pH7) and banding techniques (sequential C-banding + Giemsa + CMA_3 + DAPI; see Mezzasalma et al., 2013, 2015, 2017b).

In our phylogenetic analysis, the studied female (GA436) is the sister taxon of the other three available ND2 sequences of *M. brevipes* (KF666815, KF666816, and EF490777) (Fig. 1). The uncorrected *p*-distance of ~4%, between our newly generated ND2 sequence and those available from GenBank, highlights the occurrence of intraspecific diversity. In turn, the other three available ND2 sequences of the species do not show any appreciable nucleotide variability (Fig. 1). Potential explanations for the intraspecific divergence in *M. brevipes* may be related to geographical patterns of genetic diversity, however, all three ND2 sequences of the species available from GenBank (KF666815, KF666816, and EF490777) are not associated with specific sampling localities (see Nussbaum et al., 1998; Heinike et al., 2014) and more

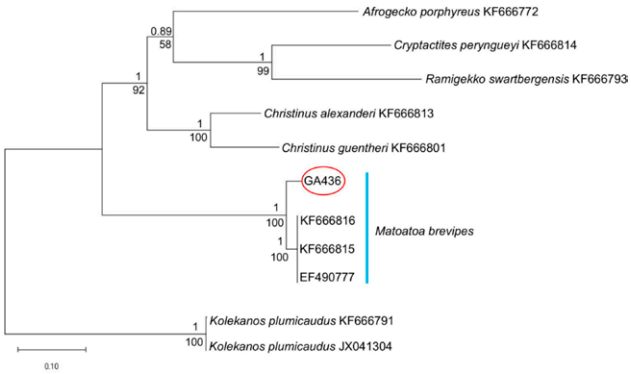

Fig. 1. Phylogenetic tree with ML (1000 bootstrap replicates) and BI (2,000,000 generations), with our newly generated ND2 sequence (GA436) and available homologous sequences of *Matoatoa brevipes* (KF666815, KF666816, EF490777). We included homologous sequences of *Christinus alexanderi* (KF666813), *Christinus guentheri* (KF666801), *Cryptactites peryngueyi* (KF666814), *Ramigekko swartgergensis* (KF666793), *Afrogecko porphyreus* (KF666772) and *Kolekanos plumicaudus* (KF666791, JX041304). Bootstrap and Bayesian posterior values are reported below and above tree branches, respectively.

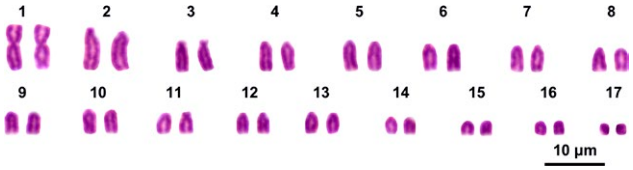

Fig. 2. Giemsa stained karyotype of *Matoatoa brevipes* with 2n = 34 chromosomes.

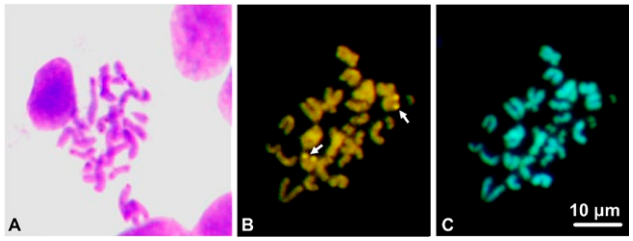

Fig. 3. C-banding + Giemsa (A), CMA_3 (B) and DAPI (C) on metaphase plates of the female specimen of *M. brevipes* GA436, showing low heterochromatin content and absence of completely or largely heterochromatic chromosomes. White arrows in (B) point at NOR-associated heterochromatic blocks. Scale bar = 10 µm.

data are needed to advance hypotheses on the intraspecific molecular diversity of *M. brevipes*.

We found that *M. brevipes* has a karyotype composed of 2n = 34 chromosomes, with one metacentric pair (1st) and 16 telocentric pairs, gradually decreasing in length and without any clear distinction between macro- and micro-chromosomes (Fig. 2). Sequential C-banding revealed a scarce heterochromatin content in the genome of *M. brevipes*, without any completely or largely heterochromatic chromosome (Fig. 3). Centromeric and telomeric heterochromatin was more easily viewed with C-banding + CMA_3. Furthermore, the telomeric heterochromatin of two medium sized telocentric chromosomes was relatively brighter with CMA_3, suggesting the co-localization of the NOR associated heterochromatin (Fig. 3). In fact, C-banding + CMA3 can be a useful technique to localize NORs. Unambiguous co-localization of CMA_3 signals after C-banding and Ag-NOR staining and NOR-FISH signals have been consistently documented in different papers and taxa (see e.g. Pardo et al., 2001; Suman and Kaur, 2013). This is especially true for karyotypes showing low heterochromatin content like squamates and lacking other paired CMA_3+ heterochromatic blocks. Karyological comparisons with closely related genera were possible with *Christinus marmoratus* and different species of *Phelsuma*, *Paroedura*, *Ebenavia* and *Uroplatus*. *Christinus marmoratus* has accentuated chromosome polymorphism, with different populations possessing different chromosome number (2n = 32, 34 and 36) and mor-

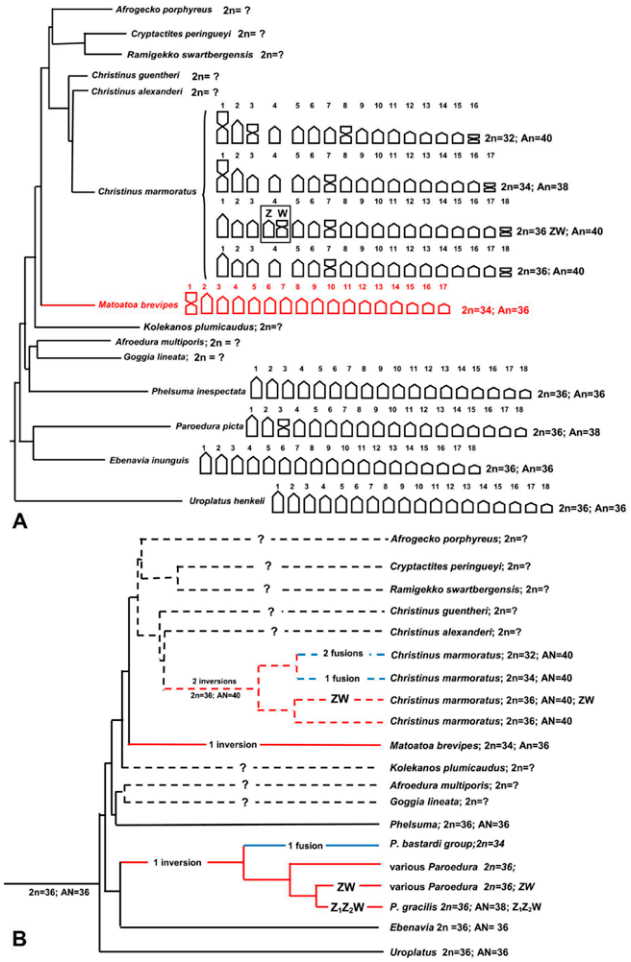

Fig. 4. (A) Phylogenetic relationships of circum-Indian Ocean leaf toed geckos (redrawn from Heinicke et al., 2014) superimposed with available haploid karyograms (King and Rofe, 1976; Aprea et al., 1996, 2013; this study). **(B)** Karyotype evolutionary hypothesis and relative chromosome rearrangements.

phology, with or without differentiated ZW sex chromosomes, and an invariable Arm Number (AN) of 40 (King and Rofe, 1976; King and King, 1977). *Christinus marmoratus* probably includes cryptic species (Kay, 2008), and the variable karyotypes found in different clades are similar to those found in other geckos of the same group in chromosome number (2n = 32-36) and the prevalence of telocentric elements, with one (as in *M. brevipes* and *P. picta*) or more bi-armed chromosomes occurring on different chromosome pairs.

We superimposed the updated chromosomal data on the phylogeny of Heinicke et al. (2014) to explore possible scenarios of chromosome diversification in the group (Fig. 4). Based on the distribution of karyotypes, we hypothesise a putative ancestral karyotype with 2n =

36 and all telocentric elements (AN = 36). In fact, higher numbers of chromosomes and telocentric elements are considered primitive features of lizard karyotypes (see Deakin and Ezaz, 2019). Furthermore, the hypothesised ancestral karyotype of 2n = 36 is the most common karyotype in the focal gecko species and is conserved in all *Uroplatus*, *Ebenavia*, and *Phelsuma* species studied so far (Aprea et al., 1996, 2013 and our unpublished data). Starting from an ancestral 2n = 36 karyotype, multiple fusion and inversion events likely led to the formation of biarmed elements and the reduction of the chromosome number in different clades (Fig. 4). In particular, one fusion between two medium sized telocentric pairs probably originated the karyotype of *M. brevipes* (with 2n = 34 and one metacentric pair). One inversion involving two primitive telocentric pairs may explain the karyotypic variation across species of *Paroedura* (Aprea et al., 2013; Koubová et al., 2014) and a subsequent centric fusion probably shaped the karyotype of *P. bastardi*, reducing the chromosome number to 2n = 34 (see also Aprea et al., 2013; Koubová et al., 2014). In *Christinus marmoratus*, two inversions involving pairs 7 and 18, likely formed the karyotype of 2n = 36 populations, and one and two centric fusions of telocentric elements formed the karyotype of 2n = 34 and 2n = 32 populations, respectively. Similar chromosome rearrangements have been hypothesized in different phylogenetic lineages within the family Gekkonidae, with distinct karyotype formulas progressively diverging from the hypothesized ancestral condition by means of independent chromosome fusions, fissions and inversions (see also Trifonov et al., 2011; Srikulnath et al., 2014).

Concerning sex determination systems, differentiated ZW chromosomes are common in squamates, and have been observed in various *Paroedura* species (Koubová et al., 2014), including a complex $Z_1Z_1Z_2Z_2/Z_1Z_2W$ sex chromosome system in *P. gracilis* (Aprea et al., 2013). However, the female specimen of *M. brevipes* studied here did not possess heteromorphic or largely heterochromatic chromosomes. This suggests that the ZW sex chromosomes are at an early differentiation stage, as has been observed in a 2n = 36 population of *C. marmoratus* (King and King, 1977). Variability in sex chromosome morphology is not unexpected as squamate reptiles can have either XY or ZW sex chromosomes and these conditions can evolve and differentiate rapidly, sometimes occurring within the same taxon (e.g. Gamble, 2010; Koubová et al., 2014; Mezzasalma et al., 2019).

Our results indicate that the NOR loci of *M. brevipes* are probably located on the telomeres of a medium sized telocentric chromosome pair. This contrasts with NOR location in related taxa indicating a remarkable variability in their chromosomal localization in geckos. For example, NORs are on the first chromosome pair in *E. inunguis*, on the last pair in *Paroedura* and on different chromosome pairs in *Phelsuma* and *Uroplatus* (Aprea et al., 1996, 2013 and our unpublished data), highlighting a high genomic mobility of these elements.

In conclusion, the karyotype of *M. brevipes* is composed of 2n = 34 chromosomes with one metacentric and 16 telocentric pairs. Comparison with the available karyotypes of closely related gecko genera allowed us to hypothesise an evolutionary scenario where a karyotype of 2n = 36 with all telocentric chromosomes was the ancestral state of the group. This ancestral state would necessitate chromosome diversification occurring by means of fusions and inversions, leading to a reduction of the chromosome number and the formation of biarmed elements.

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