Assessing the spatial patterns of genetic diversity of species of the genera *Calumma* and *Furcifer*

Internship Report 2022/2023

Bachelor's Degree in Biology from the University of Porto

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Acknowledgements

I sincerely thank CIBIO for providing me with a place where to work, for giving me the opportunity to learn more about new research areas such as bioinformatics and programming, and for improving my knowledge in various fields such as phylogeography, genetics and data analysis.

A special "thank you" to my supervisor Angelica Crottini for her guidance during this year, for everything she has taught me, making me a better professional and especially for the incredible opportunity to work together. A special "thank you" also to my co-supervisor Silvia Carvalho, for all her help with bioinformatics and data analysis, which I was a total stranger to. Finally, a big thank you to Maria João Paúl, who was always willing to help me and answer my questions, giving up her time to do so.

At last, thank you to my parents for giving me the support I so desperately needed during this difficult process.

Summary

One of the most important components of biodiversity is genetic diversity. Over the years and due to extreme anthropogenic pressure, genetic diversity has been decreasing rapidly. Madagascar hosts a great diversity of reptile species and high level of endemicity, but at the same time, there is still a large information gap on the spatial patterns of their genetic diversity to fill. The available data remain scarce and several of the metadata associated to the collection of samples is unprecise or incomplete. We revised the sampling information of all sequences of the mitochondrial ND2 gene that have been generated for a specific set of species of the genus *Calumma* (*C. boettgeri*, *C. fallax*, *C. gallus*, *C. guibei*, *C. nasutum* and *C. vohibola*) and of the genus *Furcifer* (*F. lateralis*, *F. major*, *F. oustaleti*, *F. verrucosus*, *F. viridis*) (sequences downloaded from GenBank). An ND2 alignment was made for each species, and we used this alignment and a semi-automated software to infer the spatial patterns of nucleotide diversity. This was done to understand the areas expected to contain the highest levels of genetic diversity for each species. Finally, we diagnosed the possible bias that may have occurred in our dataset using this method. We described the possible errors and solutions and showed the importance of storing metadata information.

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1. Introduction

1.1. Biodiversity and its Downfall

The term “Biodiversity” is one of the most important notions used in Biology. Its most accepted definition is the one that was proposed at the 1992 Convention on Biological Diversity, where it was stated that “Biological Diversity means the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems. “ (Gaston & Spicer, 2004). Biological diversity is not evenly distributed across the planet, however. It follows a specific spatial pattern, being higher as we approach the equator (Singh, 2002). The patterns of endemicity of species, together with patterns of high deforestation rates lead to the proposal of the establishment of several biodiversity “hotspots” (Myers et al., 2000). Featuring a high richness of unique and threatened species, these biodiversity hotspots are extremely important for a better understanding of how species diversity is distributed worldwide (Reid, 1998). Also, since the proposal of these areas as priority areas for conservation, there has been increased conservation efforts in these sites, (Reid, 1998).

Pressures on these sites have arisen due to increased human expansion and its impacts on habitats and ecosystems across the globe (Singh, 2002). Habitat fragmentation due to human occupation, the overexploitation of non-renewable resources, landscape changes, increased extinction rates and pollution are just a few examples in a panoply of problems (Singh, 2002).

1.2. Genetic Diversity

Genetic diversity is one of the many levels of biological diversity. Throughout this research, the definition of genetic diversity that was used was the one provided by Gaston & Spicer (2004). The authors affirm that genetic diversity comprises the

components of the genetic coding that structures organisms (nucleotides, genes, chromosomes) and variation in the genetic make-up between individuals within a population and between populations. Despite efforts to conserve biodiversity, it was only after many years that conservation of genetic diversity began to be considered part of the equation (Hoban et al., 2021). The lack of synthesis on the spatial pattern of genetic diversity can lead to a lack of understanding of how vital it is this type of diversity for the well-being of all species (Hoban et al., 2021).

Studying the effects of genetic diversity at the ecological level remains challenging, as there is an enormous number of related variables that can determine the extent of genetic diversity and its spatial patterns. The extent of genetic diversity has effects at the population level (e.g. determining increased productivity and behavioural changes); at the community level (e.g. determining its maintenance); and at the ecosystem level (e.g. affecting nutrient cycling and variations in decomposition) (Hughes et al., 2008).

The loss of genetic diversity worldwide has been a recurring problem (Amos & Harwood, 1998). This loss is usually the results of phenomena such as habitat fragmentation that provide the conditions for genetic drift, bottleneck effect or inbreeding effects to happen (Amos & Harwood, 1998).

1.3. Madagascar

Madagascar is one of the most emblematic hotspots in the planet, hosting high rates of endemic vascular plants (ca. 82%) and having lost more than the 90% of its native forest cover (Myers et al., 2000). In addition, there are a huge number of animal and plant species yet to be formally described, often outnumbering the number of species already described (Vences et al., 2009).

Deforestation for subsistence of local populations continues, leading to even more severe fragmentation of ecosystems, which are the main reasons for the degradation of Malagasy ecosystems (Ralimanana et al., 2022). This has led numerous endemic species to the brink of extinction, such as the flagship Malagasy giant jumping rat, *Hypogeomys*

antimena, which has seen a decline in the number of individuals in its populations and is considered an highly threatened species (Ganzhorn et al., 2001).

1.4. Reptile Biodiversity in Madagascar

Madagascar hosts high level of endemism, and reptile species are no exception. In a study carried out by (Antonelli et al., 2022), 427 species of reptiles were catalogued, of which 98% are endemic to the island. The highest concentration of reptile species is present in the eastern humid forest, but also can be found in the dry biome of the western coast and in the subarid biome in the southwest (Antonelli et al., 2022). When looking at regional endemism, reptiles show variable values between clades, but in most clades and subclades higher endemicity is found in northern Madagascar (Brown et al., 2016; Antonelli et al., 2022).

In this study, we focus on two genera of chameleons, the genus *Calumma* and the genus *Furcifer*.

The genus *Calumma* is composed of more than 40 species, and it is entirely endemic to the island of Madagascar (Glaw & Vences, 2007). It is thanks to an increased and recent taxonomic effort that several new species have been described (e.g. Prötzel et al., 2020).

The genus *Furcifer* is composed of 24 species which are endemic to the islands of Madagascar, the Comoros and Mayotte (Glaw et al., 2009). Higher species richness in the genus *Furcifer* is found in the dry and subarid biomes, with few species colonizing the rainforest, while higher endemism rates are found in the northwest and in the coastal areas in the southwest (Brown et al., 2016).

1.5. Network of Protected Areas of Madagascar

Madagascar's biodiversity has been declining (Ganzhorn et al., 2001). This has led to a growing effort in preserving the remaining areas covered in primary vegetation (Goodman et al., 2019), and there are currently 98 protected sites throughout

Madagascar. Efforts in protecting these sites aims not only to protect Madagascar's unique biodiversity, but also to provide ecological services to its population (Goodman et al., 2019).

Of all the species of the genera *Calumma* and *Furcifer*, only two - one species from each genus – currently have no part of their distribution included in any protected area (Jenkins et al., 2014; Antonelli et al., 2022). These are: *Calumma hafahafa,* which is currently classified as Critically Endangered following the classification of the IUCN Red Listing criteria (IUCN, 2011a), and *Furcifer tuzetae,* which is classified as Data Deficient (IUCN, 2011b).

1.6. Objectives

The aim of this work is to compile existing information on the distribution of each mitochondrial sequence generated for individuals of the genera *Calumma* and *Furcifer* and, with this information, infer the spatial distribution of the genetic diversity of each species. An additional objective was to optimize the methodology used to carry out these steps, as well as to check for errors in this process and to think about possible solutions. In this way, it would be possible to expand the use of this approach to the study of all reptiles of Madagascar. Capitalizing on the data generated by previous contributions, we aim to obtain an overview of the distribution of the genetic diversity of the species of these two genera.

2. Methods

2.1. Data Mining

Despite the chameleons in Madagascar having received recent taxonomic interest (e.g. Prötzel et al., 2020; Prötzel et al., 2015), research on their phylogeographic patterns falls short, limiting what is known both in term of their geographical distribution on this island and the extent of their genetic diversity. Once this information will be available for each species, it will be possible to better plan the conservational goals established for the ecosystems they occupy (Rosauer et al., 2016).

We retrieved publicly available sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) running a customized R script of the package 'rentrez' (Winter, 2017). Information was gathered for all species of the genera *Furcifer* and *Calumma* [*C. boettgeri*, *C. fallax*, *C. gallus*, *C. guibei*, *C. nasutum*, *C. vohibola, F. lateralis*, *F. major*, *F. oustaleti*, *F. verrucosus*, *F. viridis*]: their sequences were obtained in the form of FASTA files, while metadata associated to each sequence, such as species name, GenBank/Bold accession number, sequence length, gene, voucher, geographic coordinates, the country and geographic descriptors where the sample was collected, *etc.* were automatically compiled in an Excel file (see Table 1 for an example). In cases where the coordinates were not present (only the geographical descriptor and the country), we tried to automatically retrieve the coordinates with the help of two R packages, "ggmap" and "geonames".

After gaining all this data, we filtered it by selecting only species that had at least 10 sequences for at least one gene. This filtering resulted in a final database of 11 species (Table 2).

Table 1. A fragment of the Excel table automatically obtained using the customized R script of the package 'rentrez' used for retrieving metadata.

sp.name	title	gene	voucher	slen	country	country_c	lat	long	lat2	long2	locality
Calumma l	Endangere	ND2	FGMV 200	389	Madagasc	MG	NA	NA	-15.06541	47.687344	Manongar
Calumma l	Molecular	ND1	NA	1733	NA	NA	NA	NA	NA	NA	NA
Calumma l	A tarzan y	ND4	FGMV 200	537	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Large-scal	PRLR	NA	543	NA	NA	NA	NA	NA	NA	NA
Calumma l	Large-scal	RAG1	NA	1482	NA	NA	NA	NA	NA	NA	NA
Calumma l	Large-scal	cmos	NA	765	NA	NA	NA	NA	NA	NA	NA
Calumma l	Large-scal	ND2	NA	948	NA	NA	NA	NA	NA	NA	NA
Calumma l	Large-scal	16S rRNA	NA	469	NA	NA	NA	NA	NA	NA	NA
Calumma l	Hiding dee	ND2	ACZC 1369	280	Madagasc	MG	NA	NA	NA	NA	Nosy Be L
Calumma l	Hiding dee	ND2	ACZC 1370	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Hiding dee	ND2	ACZC 1372	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Hiding dee	ND2	ACZC 1544	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Hiding dee	ND2	ACZC 1558	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Hiding dee	ND2	ACZC 1578	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Hiding dee	ND2	FGMV 200	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Hiding dee	ND2	FGMV 200	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Hiding dee	ND2	FGMV 200	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be
Calumma l	Hiding dee	ND2	FGMV 200	280	Madagasc	MG	NA	NA	-13.90516	48.015113	Nosy Be

2.2. Relevant Genes

After selecting the 11 species of interest, we built a table summarizing the information on the number of sequences available for each molecular marker (Table 2). ND2 was the molecular marker chosen as it had the most complete dataset.

Table 2. List of species analyzed in this study, associated to the number of sequences available in public repositories for two genes.

genus	species	Candidate	spalias	ND2	CMOS
Calumma	*Calumma boettgeri*		calboe	51	36
Calumma	*Calumma fallax*		calfal	34	0
Calumma	*Calumma gallus*		calgal	30	4
Calumma	*Calumma guibei*		calgui	10	0
Calumma	*Calumma nasutum*		calnas	107	23
Calumma	*Calumma vohibola*		calvoh	11	0
Furcifer	*Furcifer lateralis*		furlat	34	1
Furcifer	*Furcifer major*		furmaj	41	2
Furcifer	*Furcifer oustaleti*		furous	90	92
Furcifer	*Furcifer verrucosus*		furver	45	44
Furcifer	*Furcifer viridis*		furvir	38	0

2.3. Analyzed Species

2.3.1. *Calumma*

***Calumma boettgeri* (Boulenger, 1888), Fig. 1:**

A species distributed in northern Madagascar, is classified as Least Concern, LC, following the IUCN Red Listing criteria (IUCN, 2015). They are known for being one of the smallest species of Chameleon having a total length of about 8 to 10 centimetres (Glaw & Vences, 2007).

***Calumma fallax* (Mocquard, 1900), Fig. 1:**

Individuals of the *Calumma fallax* species are known by having three bright blue dorsoventral stripes on the body that can be crossed by a broad white lateral stripe. In terms of distribution this species is present in eastern Madagascar from Andohahela in the south to Mandraka (Prötzel et al., 2020). It is classified as Data Deficient, DD, following the IUCN Red Listing criteria (IUCN, 2011c).

***Calumma gallus* (Günther, 1877), Fig. 1:**

This species is classified as Endangered, EN (IUCN, 2011d), following the IUCN Red Listing criteria. It is distributed in east and northeast Madagascar (IUCN, 2011d). The males are known for having a long, pointed and flexible nose appendage that has blue, violet and green spots (Glaw & Vences, 2007).

***Calumma guibei* (Hillenius, 1959), Fig. 1:**

Very little is known on this species. It has a very short rostral appendage and it is found at high altitudes at Mt. Tsaratanana (Prötzel et al., 2017). It is classified as Near Threatened, NT, following the IUCN Red Listing criteria (IUCN, 2011e).

***Calumma nasutum* (Duméril & Bibron, 1836), Fig. 1:**

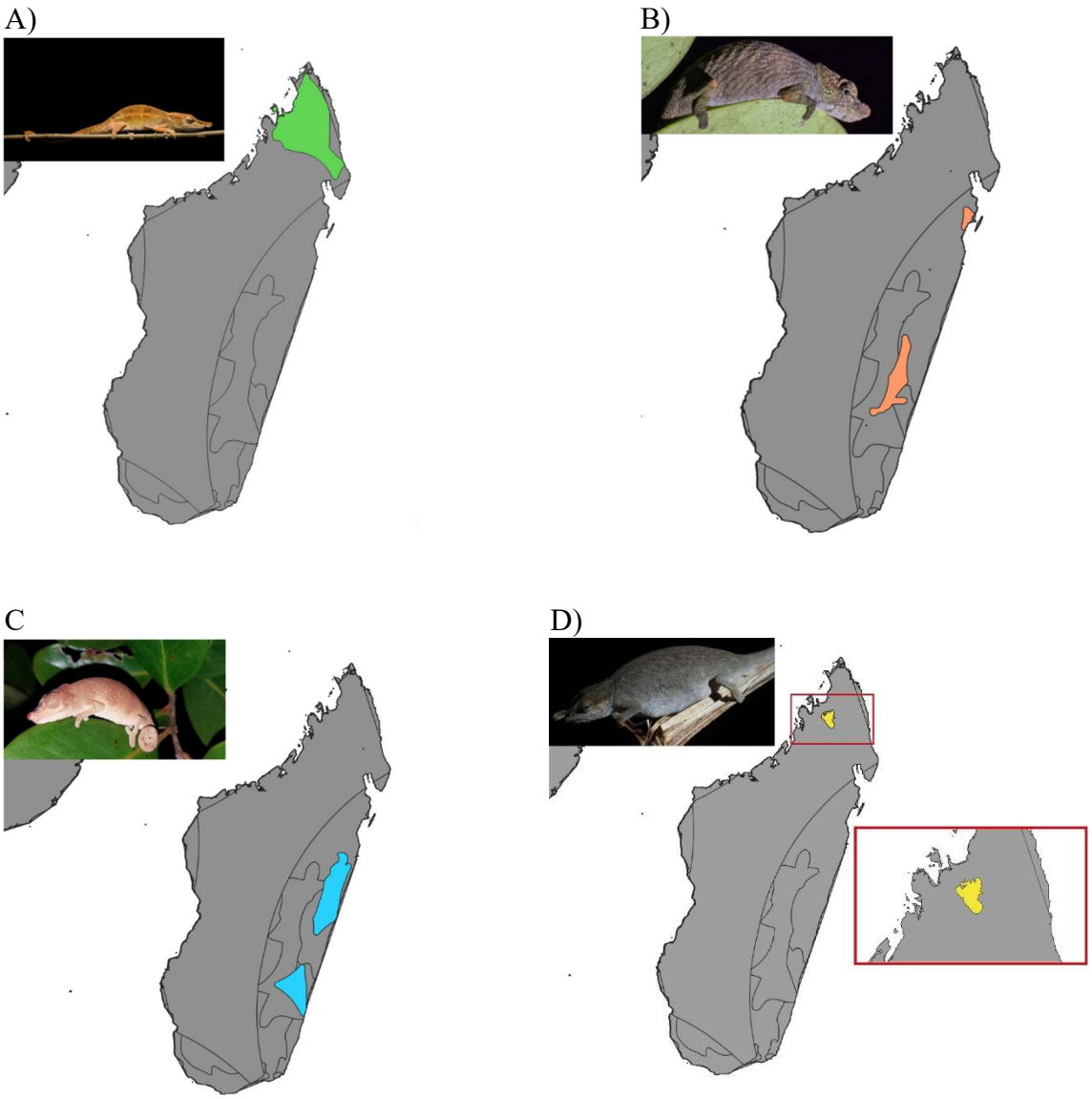
It has a remarkable rostral appendage, and it is distributed from eastern Madagascar between Anosibe An'ala and Andasibe in the central east to Sorata in the north of the

island (Prötzel et al., 2020). It's status is classified as LC following the IUCN Red Listing criteria (IUCN, 2011f).

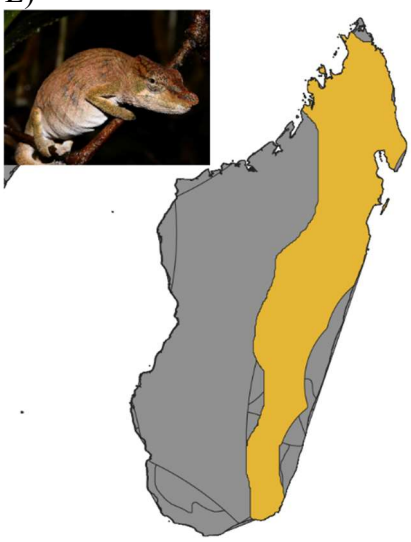
***Calumma vohibola* (Gehring, Ratsoavina, Vences & Glaw, 2011), Fig. 1:**

Calumma vohibola is distributed across a few localities within a 60 km-long stretch along the coastal area between Ivoloina and the island of Ille aux Prunes in the north and the Vohibola forest in the south (Gehring et al., 2011). It has a very dark reddish ground coloration with many irregular blue spots (Gehring et al., 2011). It is classified as EN following the IUCN Red Listing criteria (IUCN, 2014a).

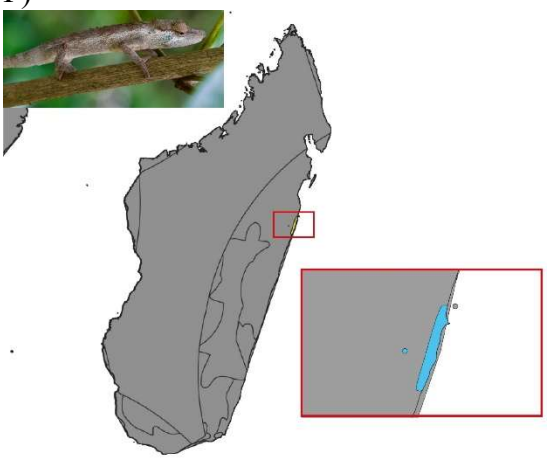
Figure 1. Maps showing the known range and a representative picture of: A) *Calumma boettgeri,* B) *Calumma fallax*, C) *Calumma gallus*, D) *Calumma guibei*, E) *Calumma nasutum*, F) *Calumma vohibola*. Information retrieved from (Roll et al., 2017)



E)



F)



2.3.2. *Furcifer*

***Furcifer lateralis* (Gray, 1831), Fig. 2:**

This species is characterized by having a white ventral line, a lively green colouration throughout the body and often three dark circles on the lateral body which are intercepted by a lateral white line. This species occurs in western and northern Madagascar (Florio et al., 2012). It is classified as LC following the IUCN Red Listing criteria (IUCN, 2014b).

***Furcifer major* (Brygoo, 1971), Fig. 2:**

Furcifer major is classified as LC following the IUCN Red Listing criteria (IUCN, 2014c). It is distributed along the south of Madagascar (IUCN, 2014c). This species has a pale green-coloured body, with white markings all over it (Glaw & Vences, 2007).

***Furcifer oustaleti* (Mocquard, 1894), Fig. 2:**

Furcifer oustaleti is distributed throughout all Madagascar. It is one of the largest species of chameleons worldwide, males can reach 70 centimetres long and females can go up

to 40 centimetres long (Florio & Raxworthy, 2016). It is classified as LC following the IUCN Red Listing criteria (IUCN, 2011g).

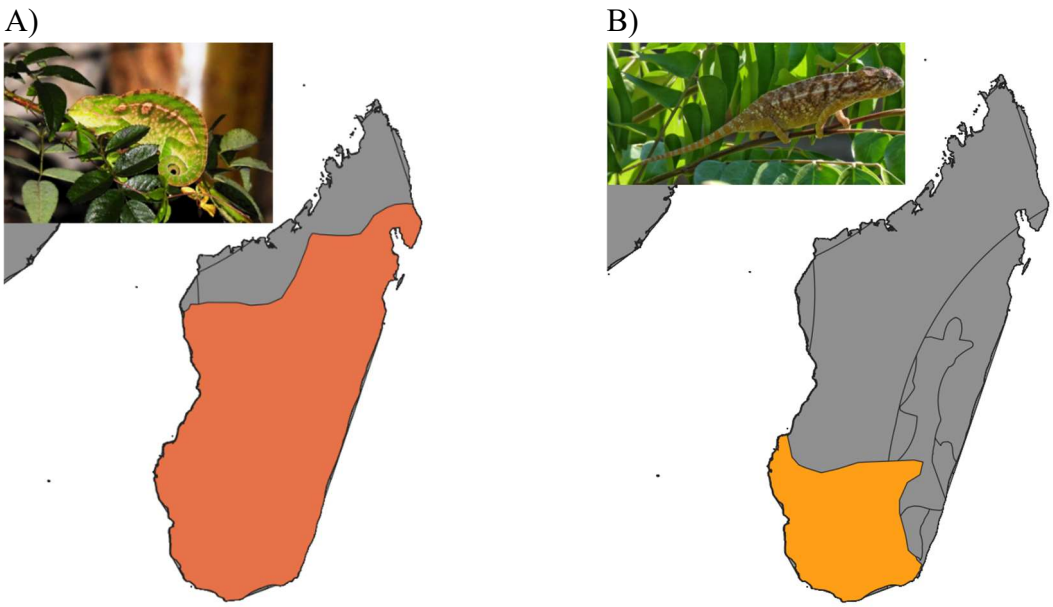
***Furcifer verrucosus* (Cuvier, 1829), Fig. 2:**

The range of *F. verrucosus* is confined to the extreme south and southwest of Madagascar. Alongside *F. oustaleti* it is one of the biggest species of chameleons in the world, with males reaching lengths of 57 centimetres and females 21 centimetres long (Florio & Raxworthy, 2016). It is classified as LC following the IUCN Red Listing criteria (IUCN, 2011h).

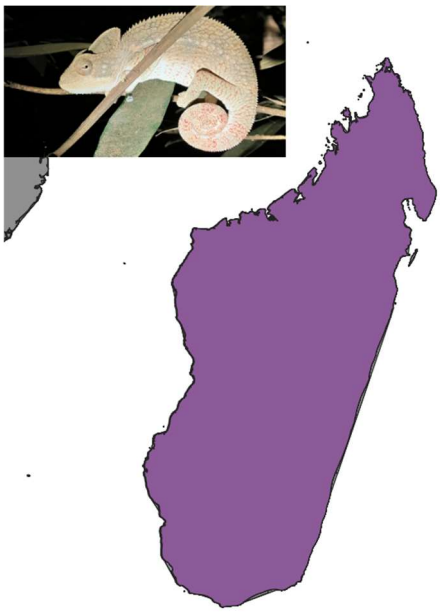
***Furcifer viridis* (Florio, Ingram, Rakotondravony, Louis Jr., Raxworthy, 2012 et al., 2012), Fig. 2:**

This species is recognizable by having a double row of scales along the dorsal body ridge, 14-18 tubercles on the parietal crest and a typical green colouration in adults (Florio et al., 2012). It is classified as LC following the IUCN Red Listing criteria (IUCN, 2014d) and it is distributed from north-west to south Madagascar (Florio et al., 2012).

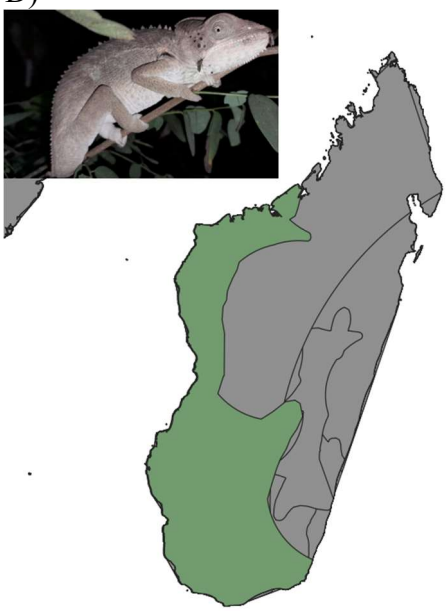
Figure 2. Maps showing the known range and a representative picture of: A) *Furcifer lateralis*, B) *Furcifer major*, C) *Furcifer oustaleti*, D) *Furcifer verrucosus*, E) *Furcifer viridis*. Information retrieved from (Roll et al., 2017)



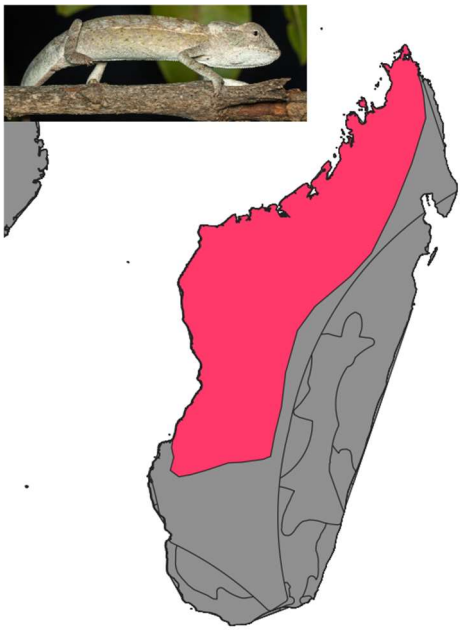
C)



D)



E)



2.4. Compilation of Additional Coordinates

Once the species of interest were selected, we confirmed that the ND2 sequences were lacking several sampling coordinates. We compiled this with an automated process

responsible for retrieving Digital Objects Identified (DOI's) numbers associated with the ND2 sequences. To retrieve these DOI's, the process would use an R Script that would refer to the packages 'bib2df', 'bibtex', 'easyPubMed', 'RefmanagerR' and 'Rscopus'; it would then compile the references and their bibliographic data on a BibTeX file.

By opening this file with a reference manager (e.g., 'ZOTERO'), it was possible to retrieve, in a PDF format, most of the papers that contained the referenced ND2 sequences. For the GenBank accession numbers for which with this step it was not possible to fill the coordinate information, we manually compiled them following this approach: the original paper referring to each sequence was found, analyzed and its coordinates collected (Table 3).

Table 3. Excel table which provides an example of the information which has been compiled manually.

sp.name	title	gene	voucher	slen	country	country_c	lat	long	lat2	long2	locality
Calumma l	Untangling	ND2	FGZC 5291	525	Madagasc	MG	NA	NA	-18,9133	47,9145	Mandraka
Calumma l	Untangling	ND2	FGZC 5238	488	Madagasc	MG	NA	NA	-18,9289	47,8936	Mandraka
Calumma l	Untangling	ND2	FGZC 5227	488	Madagasc	MG	NA	NA	-18,9289	47,8936	Mandraka
Calumma l	Untangling	ND2	FGZC 5226	488	Madagasc	MG	NA	NA	-18,9122	47,9144	Mandraka
Calumma l	Untangling	ND2	FGZC 4588	488	Madagasc	MG	NA	NA	-19,7103	47,8182	Tsinjoariv
Calumma l	Untangling	ND2	FGZC 4575	488	Madagasc	MG	NA	NA	-19,68	47,7706	Tsinjoariv
Calumma l	Untangling	ND2	FGZC 4352	488	Madagasc	MG	NA	NA	-18,4629	47,9381	Tsinjoariv
Calumma l	Untangling	ND2	FGMV 200	525	Madagasc	MG	NA	NA	-21,22	47,37	Ranomafa
Calumma l	Hiding dee	ND2	DRV 6284	506	Madagasc	MG	NA	NA	-14,3464	49,10278	Andrevore
Calumma l	Hiding dee	ND2	DRV 6286	506	Madagasc	MG	NA	NA	-14,3464	49,10278	Andrevore
Calumma l	Hiding dee	ND2	PSG 2604	506	Madagasc	MG	NA	NA	-19,68	47,77063	Tsinjoariv
Calumma l	Hiding dee	ND2	PSG 2610	506	Madagasc	MG	NA	NA	-18,5782	47,06014	Tsinjoariv
Calumma l	Hiding dee	ND2	PSG 2655	506	Madagasc	MG	NA	NA	-21,2439	47,42622	Ranomafa
Calumma l	Hiding dee	ND2	FGMV 200	506	Madagasc	MG	NA	NA	-21,2262	47,3696	between V
Calumma l	Hiding dee	ND2	FGMV 200	506	Madagasc	MG	NA	NA	-21,2262	47,3696	Ranomafa
Calumma l	Hiding dee	ND2	FGMV 200	506	Madagasc	MG	NA	NA	-21,2262	47,3696	between V
Calumma l	Hiding dee	ND2	FGMV 200	506	Madagasc	MG	NA	NA	-21,2262	47,3696	Ranomafa

In situations where only the toponym was available, a database of toponyms (Table 4) available in the research group was used to identify coordinates and add them to our database. All coordinates were then converted into decimal degrees.

Table 4. Table with toponyms and associated coordinates available in one of the original contributions analyzed (from Crottini et al., 2008).

Locality/population	Latitude (S)	Longitude (E)	INP grp.	Sample size		Haplotypes		Π	
				S. got.	*M. exp.*	*S. got.*	*M. exp.*	*S. got.*	*M. exp.*
Ambatovaky	23°24.18′	45°06.16′	Out	–	2	–	2 (E11, E12)	–	0.00332
Ambovo	22°30.48′	45°21.15′	In	2	5	1 (S1)	3 (E1–E3)	0	0.00166
Amparambatomavo	22°18.11′	45°21.36′	In	4	2	1 (S1)	2 (E2, E3)	0	0.00166
Ampasibe	23°02.35′	45°16.57′	Out	–	7	–	4 (E1, E8, E10, E15)	–	0.00221
Andohaosy	22°31.00′	45°20.00′	In	–	2	–	1 (E3)	–	0
Andohasahenina	22°50.00′	45°11.28′	Out	35	3	3 (S1, S2, S4)	3 (E1, E5, E7)	0.00133	0.00332
Andranombilahy	22°48.51′	45°14.16′	Out	1	–	1 (S2)	–	nc	–
Andranomena	22°44.41′	45°16.50′	Out	7	19	2 (S1, S2)	3 (E1, E5, E8)	0.00078	0.00091
Antambonoa	22°22.31′	45°17.46′	In	1	5	1 (S1)	2 (E2, E3)	nc	0.00100
Bemenara	22°48.07′	45°15.00′	Out	9	6	4 (S1–S3, S5)	5 (E1, E6, E7, E10, E13)	0.00183	0.00310
Bevato	22°30.36′	45°21.35′	In	2	–	1 (S1)	–	0	–
Grotte des Portugais	22°18.06′	45°18.37′	In	–	1	–	1 (E2)	–	nc
Iambahatsy	22°24.35′	45°16.13′	In	–	8	–	3 (E1–E3)	–	0.00160
Isalo Oasis	22°37.37′	45°21.12′	Out	–	4	–	2 (E1, E15)	–	0.00083
Lola	22°55.54′	45°19.48′	Out	4	10	3 (S1, S6, S7)	2 (E1, E5)	0.00165	0.00059
Malaso	22°35.31′	45°21.32′	Out	7	8	5 (S1, S8–S11)	3 (E1, E2, E15)	0.00235	0.00113
Morahariva	22°46.12′	45°18.42′	Out	–	10	–	3 (E1, E5, E9)	–	0.00126
Petit Nazareth	22°33.25′	45°21.23′	In	3	3	2 (S1, S11)	2 (E1, E3)	0.00110	0.00221
Reine de l'Isalo	22°37.41′	45°20.38′	Out	–	2	–	1 (E1)	–	0
Sahanafa*	22°18.35′	45°17.48′	In	–	3	–	2 (E2, E3)	–	0.00111
Sakamalio	22°26.09′	45°15.31′	In	–	5	–	3 (E2–E4)	–	0.00199
Sakavato*	23°29.01′	44°56.09′	Out	–	1	–	1 (E10)	–	nc
Tsianerena*	22°52.38′	45°18.26′	Out	–	6	–	1 (E1)	–	0
Tsimanolabero	22°34.59′	45°23.00′	Out	–	4	–	2 (E1, E16)	–	0.00083
Tsiombivositra	22°18.15′	45°21.50′	In	2	8	1 (S1)	2 (E2, E3)	0	0.00071
Vohitanana	22°38.12′	45°20.46′	Out	1	3	1 (S1)	1 (E1)	nc	0
Zahavola	22°37.38′	45°21.52′	Out	24	21	1 (S1)	4 (E1, E5, E14, E15)	0	0.00095
Total				102	148	11	16	0.00127	0.00202

2.5. Coordinate Mapping

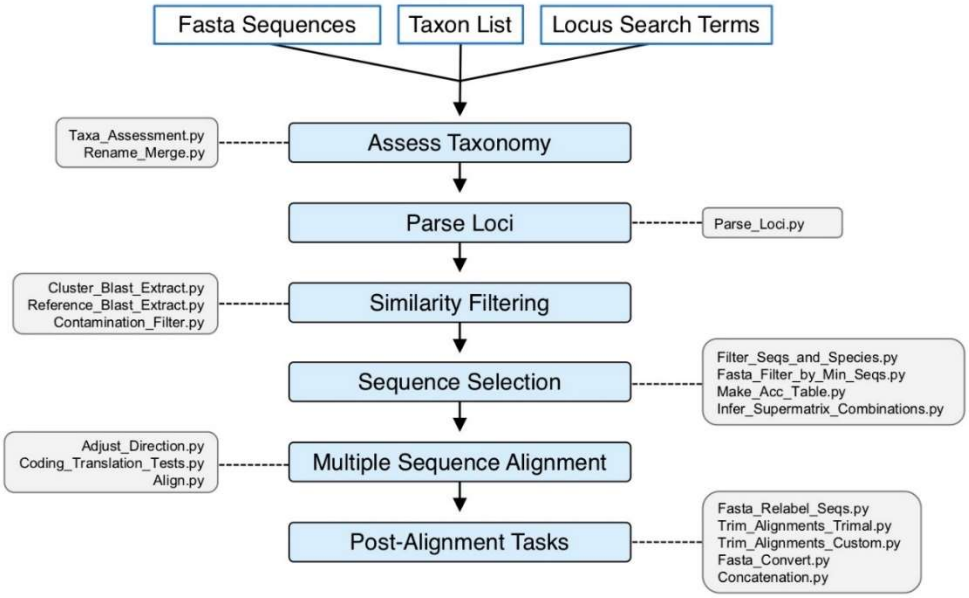
An R script using the "rgdal" and "raster" packages processed all the coordinates associated with each sequence for each species. This information was converted into a map to check the spatial distribution of each sampling locality for each sequence for each species. The distribution area of each species was obtained from the database of (Roll et al., 2017) (see also Figs. 1 and 2). We compared the distribution of the sampling localities with the sequences associated with the species' distribution areas and checked whether all the records were accurate (i.e., whether they fell within the species' predicted distribution area) or whether they came from localities confirmed by recent publications.

2.6. Sequence Alignment

SuperCRUNCH (Portik & Wiens, 2020), a Python toolkit, was used to perform species-specific ND2 alignments. This tool was used to assemble genetic datasets and can use

unpublished sequences, sequences retrieved automatically from GenBank, or a combination of the two, thus automating the selection, organization and alignment of genetic sequences (Figure 3). All the steps taken by this toolkit are shown in Figure 3. In the first step, "Assess taxonomy", the different species in the FASTA files are identified. In the second step, "Parse Loci", the software identifies the different gene fragments by species and divides them into separate FASTA files. This step took place even though we only had one gene, ND2. In the third step, "Similarity Filtering", similar sequences are combined, and homologous regions identified. After this, it removes non-homologous regions and non-target data sets. The next step is "Sequence Selection" where the toolkit automatically produces super matrices of different molecular markers for each individual. The dataset analyzed in this study was restricted to sequences of a homologous fragment of the ND2 mitochondrial gene, for comparison purposes. As such, this step was not used. The fifth step, called "Multiple Sequence Alignment", involves aligning the sequences and adjusting the direction of all the sequences in each locus-specific FASTA file. Finally, the "post-alignment tasks" phase was used to export the produced alignments. To ensure that there were no obvious errors, the ND2 alignments for each species were also manually checked.

Figure 3. Representation of the SuperCRUNCH analysis workflow used in this study.



2.7. Mapping Genetic Diversity

With the help of two R packages, "phylin" and "ape", species-specific nucleotide diversity was mapped using the aligned sequences and the associated information on the sampling localities. The measurement of the degree of genetic variability is calculated using nucleotide diversity, defined by the average nucleotide dissimilarity per site between two sequences (Nei & Li, 1979). This calculation was carried out using the "ape" package. Since nucleotide diversity is calculated on the basis of a set of sequences and is sensitive to the number of sequences in the used dataset, we determine a radius within which the samples associated to the sequences were collected. We defined the radius as the maximum distance between a location and its nearest neighbour, so there would be at least two samples in each set. To take into account the differences in sample sizes, we identified the haplotypes that occurred in each neighbourhood and iteratively resampled two of the complete set of haplotypes and calculated the nucleotide diversity (Nei & Li, 1979) of the two haplotypes.

The resampling was repeated 1000 times and the average was calculated. Using the "phylin" package, nucleotide diversity was interpolated for the entire range of species using inverse distance-weighted interpolation (Tarroso et al., 2015). Two types of maps were drawn up for each species: one map contained the projection of the inferred genetic diversity for each species in a square, encompassing all the sampling localities; the other map cut out the projection of the inferred genetic diversity for each species in the respective species distribution area. It should be noted that, in some cases, the databases used as references for this type of research are known to contain outdated versions of species distributions. Techniques like the latter, by having a dual approach, tend to minimize this risk.

3. Results

Three different maps were compiled for each species: 1) The map depicting all the sampling localities associated with each ND2 sequence that is available for each species over imposed to the species' range, as available on Roll et al. (2017). 2) The map containing the information depicted on the first map and the information on the inferred genetic variation throughout the respective distribution area. 3) The third map represents the inferred genetic diversity of each species in a rectangular area covering the species-specific distribution area and the sampling localities of the sequences assigned to that specific species.

As it can be seen in the maps obtained for the *Calumma boettgeri*, there is a decrease in genetic diversity from southwest to the north. All sampled localities used for this analysis are included in the range obtained (Fig. 4A).

As for *Calumma fallax* and *Calumma gallus*, it is possible to observe a mismatch between the localities sampled and for which we have ND2 sequences, and the ranges presented in the retrieved database (Fig. 4B, C). This is even more pronounced for *Calumma fallax*. For *Calumma fallax* we observe a higher inferred genetic diversity in the North (Fig. 4B), while for *Calumma gallus* the inferred genetic diversity is higher in the South (Fig. 4C).

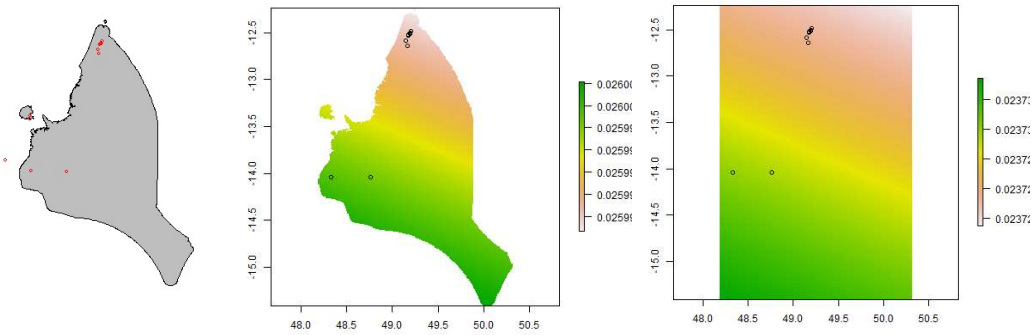
With *Calumma guibei* we observe an increased inferred genetic diversity from southwest to northeast (Fig. 4D). The localities from which there are ND2 sequences available correspond to a small fraction of the known species distribution.

In the case of *Calumma nasutum* the majority of the sample's localities fall inside the given range, and the genetic diversity is inferred to be the highest in the North (Fig. 4E).

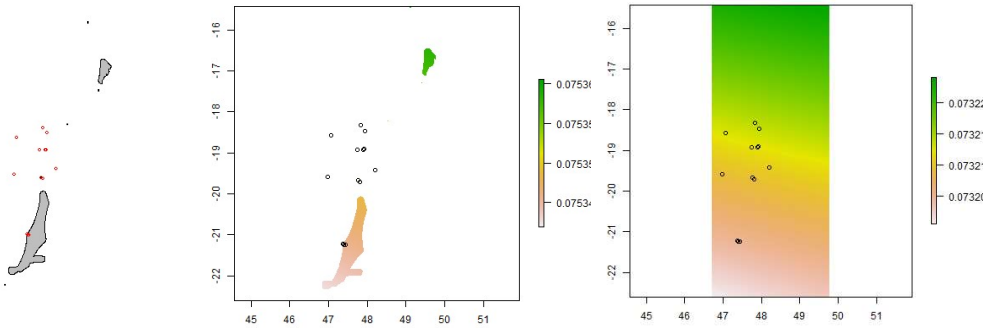
For *Calumma vohibola* we had a low number of sampling localities, and that correspond to a small fraction of the known species distribution. However, the inferred genetic diversity is highest in the East (Fig. 4F).

Figure 4. Maps showing the spatial distribution of the inferred nucleotide diversity for each species of the genus *Calumma* analyzed in this study: A) *Calumma boettgeri*, B) *Calumma fallax*, C) *Calumma gallus*, D) *Calumma guibei*, E) *Calumma nasutum*, F) *Calumma vohibola*.

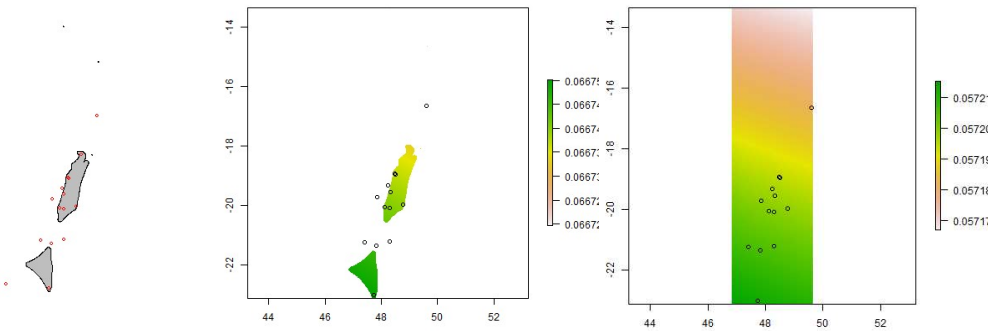
A)



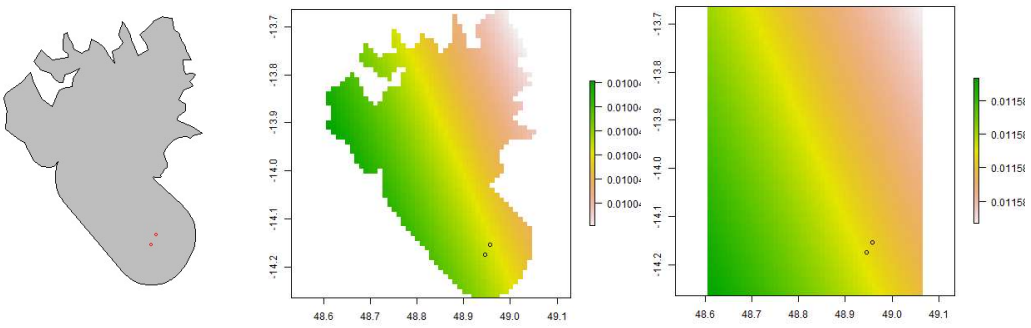
B)



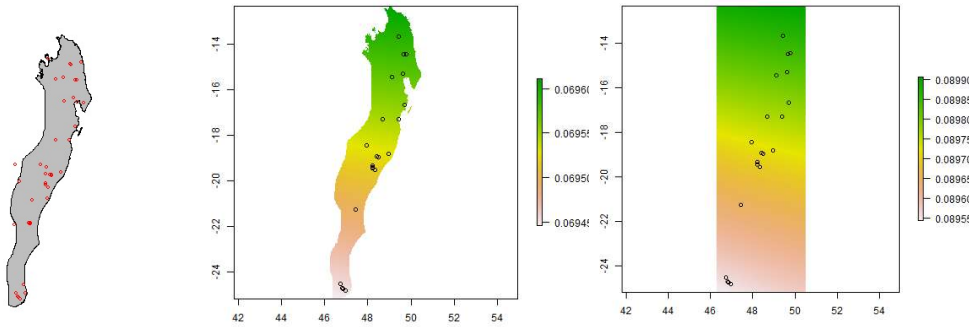
C)



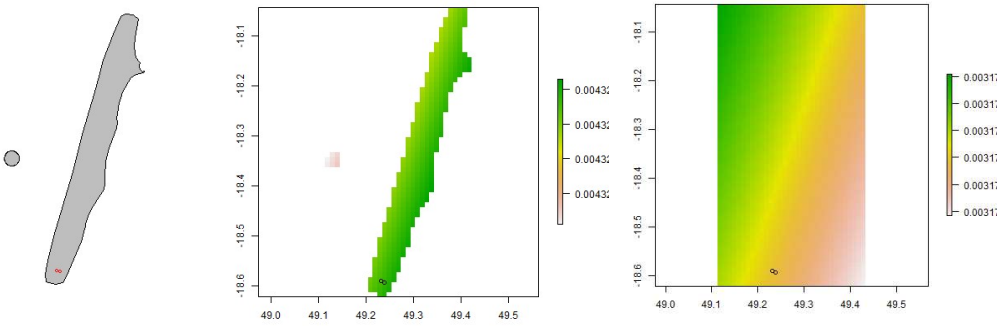
D)


-13.7
-13.8
-13.9
-14.0
-14.1
-14.2
48.6
48.7
48.8
48.9
49.0
49.1
0.0100
0.0100
0.0100
0.0100
0.0100
0.0100
0.0115
0.0115
0.0115
0.0115

E)


-14
-16
-18
-20
-22
-24
42
44
46
48
50
52
54
0.0696
0.0695
0.0695
0.0694
0.0899
0.0898
0.0898
0.0897
0.0897
0.0896
0.0896
0.0895

F)


-18.1
-18.2
-18.3
-18.4
-18.5
-18.6
49.0
49.1
49.2
49.3
49.4
49.5
0.0043
0.0043
0.0043
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For *Furcifer lateralis* we observe an inferred higher genetic diversity in the south (Fig. 5A).

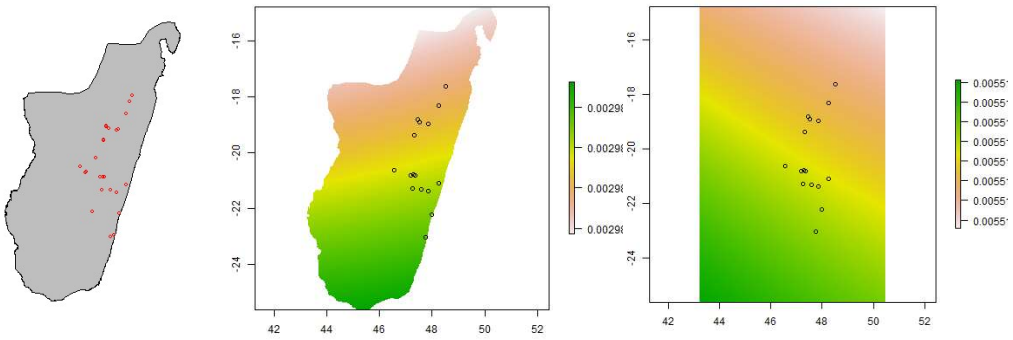
In the case of *Furcifer major* the inferred genetic diversity increases from east to west and the sampling localities of the used ND2 sequences cover a good portion of the species range (Fig. 5B).

The inferred genetic diversity of *Furcifer viridis*, is highest in the south and lowest in the north and sampling localities corresponding the ND2 sequences that we used have a good overlap with the obtained range.

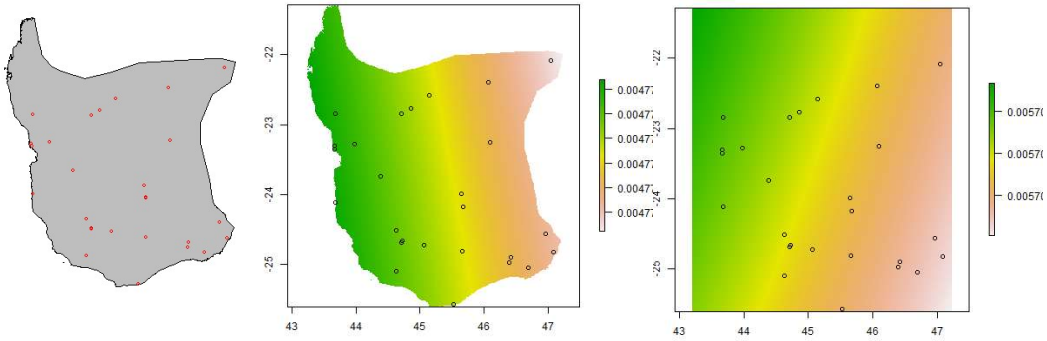
Lastly, the two species *Furcifer oustaleti* and *Furcifer verrucosus* bared no results, since after the sequence alignment, there were too few sequences (only one in each case) to calculate a minimum distance between two points, thus we weren't able to compute a genetic diversity map.

Figure 5. Maps showing the spatial distribution of the inferred nucleotide diversity for each species of the genus *Furcifer* analyzed in this study: A) *Furcifer lateralis*, B) *Furcifer major*, C) *Furcifer viridis*, D) *Furcifer oustaleti* and E) *Furcifer verrucosus.*

A)



B)


-22
-23
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-25
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46
47
0.0047
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0.0047
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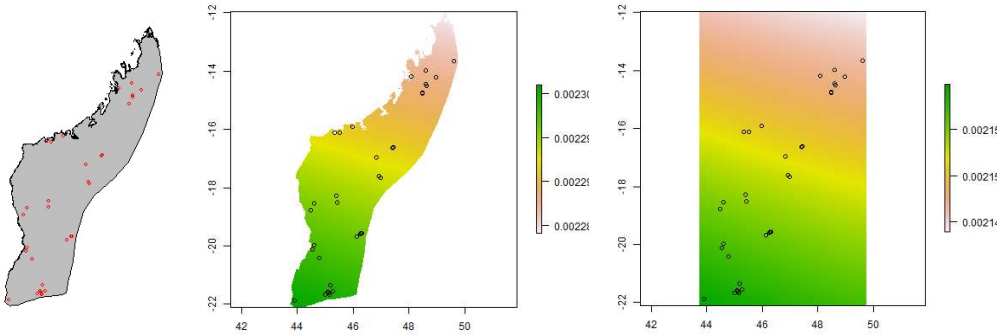
0.0057
0.0057

C)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-18
-20
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4. Discussion

Characterizing the distribution of different species is extremely important, as it allows to better tune conservation actions, especially when they are found in fragmented habitats or ecosystems (Tremlova & Münzbergová, 2007), as it is the case of Madagascar (Ganzhorn et al., 2001). In addition, being the distribution of genetic diversity unevenly distributed, it is important to better understand it, so that the population holding the majority of a species' genetic diversity can be prioritized for conservation (Rauch & Bar-Yam, 2004). Therefore, if any negative ecological phenomenon were to occur, for example climate change (Carvalho et al., 2019), this population and the genetic diversity it holds would be more protected.

The biggest problems observed in obtained results is, in some instances, the poor match between the distribution of the available ND2 sequences and the known species distribution (this being true especially for species of the genus *Calumma*).

Prötzel et al. (2020) carried out a systematic review of the C. nasutum group and formally described or resurrected several species of Calumma from this species group. As a result, the taxonomic concept that is applied in the database obtained from Roll et al. (2017) no longer corresponds to the current understanding of species diversity and distribution. The sequences that were analysed here for C. nasutum and the species distribution obtained correspond to a large number of species that were previously hidden under the name Calumma nasutum: Calumma emelinae, Calumma ratnasariae, Calumma radamanus, Calumma tjiasmantoi (Prötzel et al., 2020).

It was also observed that a number of inconsistencies or minor issues associated with the code that builds the genetic diversity maps that should be solved. For example, when calculating the two inferred genetic diversity distribution maps (the one with or without the obtained species range), the same scale should be used to enhance comparison between the maps. In addition, although it was confirmed that this is just a plotting problem, we have observed that in some cases some sequence sampling localities are not plotted on the maps shown on the results (e.g., Figure 4A, C, E and 5A). Furthermore, there seems to be an inconsistency with the inference of the distribution of the genetic diversity between the distribution of the inferred genetic diversity when the distribution

range is used or when all the sampling points are considered (e.g., Figure 4F and 5). Last but not least, the images were partially cropped, and the scale values were too similar. More time should be invested in solving these issues so that this approach can more easily be expanded to the study of all reptiles of Madagascar.

This study served as a pilot case to test a semi-automated pipeline for mapping genetic diversity inference for numerous species. This same methodology can be used for all species, thus providing considerable potential for mapping intraspecific diversity at macro-scales for several taxa. This has the potential to generate interesting opportunities for optimising the conservation of specific genetic diversity, a parameter that is currently specifically aimed at in the recently ratified Kunming-Montreal Global Biodiversity Framework. In addition, these data can also be used to further test various biogeographic hypotheses in order to better understand the source of phylogeographic patterns and the respective factors that mould them.

Although this research was setup to obtain a more current overview of the distribution of the genetic diversity of the two genera that were studied, the resulting data, while reasonably similar to the one available at the time this report was written, is based on information collected in 2017. However, bearing in mind that the present study was carried out in the context of a curricular internship, the main aim of which is to bring students into contact with the context of research and the production of scientific knowledge, it is possible to say that the main didactic purpose of this internship program has certainly been achieved.

5. Conclusion

There is a large information gap on the taxonomy, species distribution and genetic diversity of the reptiles of Madagascar. There is the need to continue collecting data on all these aspects of the biodiversity research, and it is crucial to increase efforts in characterizing the level of intraspecific genetic divergence, so that genetic diversity can also be considered in future possible optimisation strategies of protected area prioritisation. As for the problems relating to the methodology, this work suggests that there is a need to optimise the data processing pipeline, especially when it comes to compiling the genetic diversity maps, especially for species with very restricted distribution areas or very irregular sampling. Biodiversity and all its components have suffered great damage around the world, mainly due to anthropogenic activity (Prakash & Verma, 2022). We believe that responsibility must be taken to propose solutions for the recovery of habitats and diversity. With these efforts being adopted worldwide, there will be a chance for improving ecosystem services and human well-being.

References

Amos, W., & Harwood, J. (1998). Factors affecting levels of genetic diversity in natural populations. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *353*(1366), 177–186. https://doi.org/10.1098/rstb.1998.0200

Antonelli, A., Smith, R., Perrigo, A., Crottini, A., Hackel, J., Testo, W., Farooq, H., Torres Jiménez, M., Andela, N., Andermann, T., Andriamanohera, A., Andriambololonera, S., Bachman, S., Bacon, C., Baker, W., Belluardo, F., Birkinshaw, C., Borrell, J., Cable, S., Canales, N., Carrillo, J., Clegg, R., Clubbe, C., Cooke, R., Damasco, G., Dhanda, S., Edler, D., Faurby, S., Ferreira, P., Fisher, B., Forest, F., Gardiner, L., Goodman, S., Grace, O., Guedes, T., Henniges, M., Hill, R., Lehmann, C., Lowry, P., Marline, L., Matos-Maraví, P., Moat, J., Neves, B., Nogueira, M., Onstein, R., Papadopulos, A., Perez-Escobar, O., Phelps, L., Phillipson, P., Pironon, S., Przelomska, N., Rabarimanarivo, M., Rabehevitra, D., Raharimampionona, J., Rajaonah, M., Rajaovelona, L., Rakotoarinivo, M., Rakotoarisoa, A., Rakotoarisoa, S., Rakotomalala, H., Rakotonasolo, F., Ralaiveloarisoa, B., Ramirez-Herranz, M., Randriamamonjy, J., Randriamboavonjy, T., Randrianasolo, V., Rasolohery, A., Ratsifandrihamanana, A., Ravololomanana, N., Razafiniary, V., Razanajatovo, H., Razanatsoa, E., Rivers, M., Sayol, F., Silvestro, D., Vorontsova, M., Walker, K., Walker, B., Wilkin, P., Williams, J., Ziegler, T., Zizka, A., Ralimanana, H. (2022). Madagascar's extraordinary biodiversity: Evolution, distribution, and use. *Science*, *378*(6623). https://doi.org/10.1126/science.abf0869

Brown, J., Sillero, N., Glaw, F., Bora, P., Vieites, D., Vences, M. (2016). Spatial Biodiversity Patterns of Madagascar's Amphibians and Reptiles. *PLOS ONE*, *11*(1). https://doi.org/10.1371/journal.pone.0144076

Carvalho, S. B., Torres, J., Tarroso, P., Velo-Antón, G. (2019). Genes on the edge: A framework to detect genetic diversity imperiled by climate change. *Global Change Biology*, *25*(12), 4034–4047. https://doi.org/10.1111/gcb.14740

Crottini, A., Chiari, Y., Mercurio, V., Meyer, A., Vences, M., Andreone, F. (2008). Into the canyons: The phylogeography of the Malagasy frogs *Mantella expectata* and *Scaphiophryne gottlebei* in the arid Isalo Massif, and its significance for conservation

(Amphibia: Mantellidae and Microhylidae). *Organisms Diversity & Evolution*, *8*(5), 368–377. https://doi.org/10.1016/j.ode.2008.08.001

Florio, A., Ingram, C., Rakotondravony, H., Louis, E., Raxworthy, C. (2012). Detecting cryptic speciation in the widespread and morphologically conservative carpet chameleon (*Furcifer lateralis*) of Madagascar. *Journal of Evolutionary Biology*, *25*(7), 1399-1414. https://doi.org/10.1111/j.1420-9101.2012.02528.x

Florio, A., & Raxworthy, C. (2016). A Phylogeographic Assessment of the Malagasy Giant Chameleons (*Furcifer verrucosus* and *Furcifer oustaleti*). *PLOS ONE*, *11*(6). https://doi.org/10.1371/journal.pone.0154144

Ganzhorn, J., Lowry, P., Schatz, G., Sommer, S. (2001). The biodiversity of Madagascar: One of the world's hottest hotspots on its way out. *Oryx*, *35*(4), 346–348. https://doi.org/10.1046/j.1365-3008.2001.00201.x

Gaston, K., & Spicer, J. (2004). *Biodiversity: An introduction*, 2ND Ed.

Gehring, P.-S., Ratsoavina, F., Vences, M., Glaw, F. (2011). *Calumma vohibola* , a new chameleon species (Squamata: Chamaeleonidae) from the littoral forests of eastern Madagascar. *African Journal of Herpetology*, *60*(2), 130–154. https://doi.org/10.1080/21564574.2011.628412

Glaw, F., & Vences, M. (2007). *A field guide to the amphibians and reptiles of Madagascar*.

Glaw, F., Köhler, J., Vences, M. (2009). A distinctive new species of chameleon of the genus *Furcifer* (Squamata: Chamaeleonidae) from the Montagne d'Ambre rainforest of northern Madagascar. *Zootaxa*, *2269*(1), 32–42. https://doi.org/10.11646/zootaxa.2269.1.2

Goodman, S. M., Raherilalao, M. J., & Wohlhauser, S. (2019). *Les Aires Protégées Terrestres de Madagascar: Leur Histoire, Description Et Biote*. Association Vahatra in Antananarivo.

Hoban, S., Bruford, M., Funk, W., Galbusera, P., Griffith, M., Grueber, C., Heuertz, M., Hunter, M., Hvilsom, C., Stroil, B., Kershaw, F., Khoury, C., Laikre, L., Lopes-Fernandes, M.,

MacDonald, A., Mergeay, J., Meek, M., Mittan, C., Mukassabi, T., O'Brien, D., Ogden, R., Palma-Silva, C., Ramakrishnan, U., Segelbacher, G., Shaw, R., Sjögren-Gulve, P., Velickovic, N., Vernesi, C. (2021). Global Commitments to Conserving and Monitoring Genetic Diversity Are Now Necessary and Feasible. *BioScience*, *71*(9), 964–976. https://doi.org/10.1093/biosci/biab054

Hughes, A., Inouye, B., Johnson, M., Underwood, N., Vellend, M. (2008). Ecological consequences of genetic diversity. *Ecology Letters*, *11*(6), 609-623. https://doi.org/10.1111/j.1461-0248.2008.01179.x

IUCN (2011a). *Calumma hafahafa:* Jenkins, R., Andreone, F., Andriamazava, A., Anjeriniaina, M., Brady, L., Glaw, F., Griffiths, R., Rabibisoa, N., Rakotomalala, D., Randrianantoandro, J., Randrianiriana, J., Randrianizahana , H., Ratsoavina, F., Raxworthy, C., Robsomanitrandrasana, E., Vences, M.: The IUCN Red List of Threatened Species 2011 E.T172796A6920084. https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T172796A6920084.en

IUCN (2011b). *Furcifer tuzetae* : Jenkins, R., Andreone, F., Andriamazava, A., Anjeriniaina, M., Brady, L., Glaw, F., Griffiths, R., Rabibisoa, N., Rakotomalala, D., Randrianantoandro, J., Randrianiriana, J., Randrianizahana , H., Ratsoavina, F., Raxworthy, C., Robsomanitrandrasana, E., Tolley, K.: The IUCN Red List of Threatened Species 2011 E.T176320A7217945. https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T176320A7217945.en

IUCN (2011c). *Calumma fallax:* Jenkins, R., Andreone, F., Andriamazava, A., Anjeriniaina, M., Brady, L., Glaw, F., Griffiths, R., Rabibisoa, N., Rakotomalala, D., Randrianantoandro, J., Randrianiriana, J., Randrianizahana , H., Ratsoavina, F., Raxworthy, C., Robsomanitrandrasana, E., Carpenter, A.: The IUCN Red List of Threatened Species 2011 E.T176302A7213659. https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T176302A7213659.en

IUCN (2011d). *Calumma gallus:* Jenkins, R., Andreone, F., Andriamazava, A., Anjeriniaina, M., Brady, L., Glaw, F., Griffiths, R., Rabibisoa, N., Rakotomalala, D., Randrianantoandro, J., Randrianiriana, J., Randrianizahana , H., Ratsoavina, F., Robsomanitrandrasana, E.,

Carpenter, A.: The IUCN Red List of Threatened Species 2011 E.T176303A7213908. https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T176303A7213908.en

IUCN (2011e). *Calumma guibei:* Jenkins, R., Andreone, F., Andriamazava, A., Anjeriniaina, M., Glaw, F., Rabibisoa, N., Rakotomalala, D., Randrianantoandro, J., Randrianiriana, J., Randrianizahana , H., Ratsoavina, F., Robsomanitrandrasana, E.: The IUCN Red List of Threatened Species 2011 E.T172778A6916040. https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T172778A6916040.en

IUCN (2011f). *Calumma nasutum:* Jenkins, R., Andreone, F., Andriamazava, A., Anjeriniaina, M., Brady, L., Glaw, F., Griffiths, R., Rabibisoa, N., Rakotomalala, D., Randrianantoandro, J., Randrianiriana, J., Randrianizahana , H., Ratsoavina, F., Robsomanitrandrasana, E.: The IUCN Red List of Threatened Species 2011 E.T172861A6931331. https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T172861A6931331.en

IUCN (2011g). *Furcifer oustaleti:* Jenkins, R., Andreone, F., Andriamazava, A., Anjeriniaina, M., Brady, L., Glaw, F., Griffiths, R., Rabibisoa, N., Rakotomalala, D., Randrianantoandro, J., Randrianiriana, J., Randrianizahana , H., Ratsoavina, F., Robsomanitrandrasana, E.: The IUCN Red List of Threatened Species 2011: E.T172866A6932058. https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T172866A6932058.en

IUCN (2011h). *Furcifer verrucosus:* Jenkins, R., Andreone, F., Andriamazava, A., Anjeriniaina, M., Brady, L., Glaw, F., Griffiths, R., Rabibisoa, N., Rakotomalala, D., Randrianantoandro, J., Randrianiriana, J., Randrianizahana , H., Ratsoavina, F., Robsomanitrandrasana, E. 2011. The IUCN Red List of Threatened Species 2011: E.T172749A6910799. [dataset]. https://dx.doi.org/10.2305/IUCN.UK.2011-2.RLTS.T172749A6910799.en. Accessed on 26 August 2023.

IUCN (2014a). *Calumma vohibola:* Jenkins, R.: The IUCN Red List of Threatened Species 2020 E.T42689609A176117232. https://doi.org/10.2305/IUCN.UK.2020-3.RLTS.T42689609A176117232.en

IUCN (2014b). *Furcifer lateralis:* Raxworthy, C. & Jenkins, R.: The IUCN Red List of Threatened Species 2014 E.T42696174A42695999. https://doi.org/10.2305/IUCN.UK.2014-3.RLTS.T42696174A42695999.en

IUCN (2014c). *Furcifer major:* Jenkins, R. & Raxworthy, C.: The IUCN Red List of Threatened Species 2014 E.T42695944A42695956. https://doi.org/10.2305/IUCN.UK.2014-3.RLTS.T42695944A42695956.en

IUCN (2015). *Calumma boettgeri:* Glaw, F., Jenkins, R., Proetzel, D., Tolley, K.: The IUCN Red List of Threatened Species 2015 E.T176301A141979063. https://doi.org/10.2305/IUCN.UK.2015-4.RLTS.T176301A141979063.en

Jenkins, R., Tognelli, M., Bowles, P., Cox, N., Brown, J., Chan, L., Andreone, F., Andriamazava, A., Andriantsimanarilafy, R., Anjeriniaina, M., Bora, P., Brady, L., Hantalalaina, E., Glaw, F., Griffiths, R., Hilton-Taylor, C., Hoffmann, M., Katariya, V., Rabibisoa, N., Rafanomezantsoa, J., Rakotomalala, D., Rakotondravony, H., Rakotondrazafy, N., Ralambonirainy, J., Ramanamanjato, J.-B., Randriamahazo, H., Randrianantoandro, J., Randrianasolo, H., Randrianirina, J., Randrianizahana, H., Raselimanana, A., Rasolohery, A., Ratsoavina, F., Raxworthy, C., Robsomanitrandrasana, E., Rollande, F., Djik, P., Yoder, A., Vences, M. (2014). Extinction Risks and the Conservation of Madagascar's Reptiles. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0100173

Myers, N., Mittermeier, R., Mittermeier, C., Da Fonseca, G., Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, *403*(6772), 853–858. https://doi.org/10.1038/35002501

Nei, M., & Li, W. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(10), 5269-5273. https://doi.org/10.1073/pnas.76.10.5269

Portik, D. M., & Wiens, J. J. (2020). SuperCRUNCH: A bioinformatics toolkit for creating and manipulating supermatrices and other large phylogenetic datasets. *Methods in Ecology and Evolution*, *11*(6), 763–772. https://doi.org/10.1111/2041-210x.13392

Prakash, S., & Verma, A. K. (2022). ANTHROPOGENIC ACTIVITIES AND BIODIVERSITY THREATS. *International Journal of Biological Innovations*, *04*(01), 94–103. https://doi.org/10.46505/IJBI.2022.4110

Prötzel, D., Ruthensteiner, B., Scherz, M. D., & Glaw, F. (2015). Systematic revision of the Malagasy chameleons Calumma boettgeri and C. linotum (Squamata: Chamaeleonidae). *Zootaxa*, *4048*(2), 211. https://doi.org/10.11646/zootaxa.4048.2.4

Prötzel, D., Scherz, M., Ratsoavina, F., Vences, M., Glaw, F. (2020). Untangling the trees: Revision of the *Calumma nasutum* complex (Squamata: Chamaeleonidae). *VERTEBRATE ZOOLOGY, 70*(1), 23 –59. https://doi.org/10.26049/vz70-1-2020-03

Prötzel, D., Vences, M., Scherz, M., Vieites, D., Glaw, F. (2017). Splitting and lumping: An integrative taxonomic assessment of Malagasy chameleons in the *Calumma guibei* complex results in the new species *C. gehringi* sp. nov. *Vertebrate Zoology*, *67*(2), 231-249.https://doi.org/10.3897/vz.67.e31591

Ralimanana, H., Perrigo, A., Smith, R., Borrell, J., Faurby, S., Rajaonah, M., Randriamboavonjy, T., Vorontsova, M., Cooke, R., Phelps, L., Sayol, F., Andela, N., Andermann, T., Andriamanohera, A., Andriambololonera, S., Bachman, S., Bacon, C., Baker, W., Belluardo, F., … Antonelli, A. (2022). Madagascar's extraordinary biodiversity: Threats and opportunities. *Science*, *378*(6623), eadf1466. https://doi.org/10.1126/science.adf1466

Rauch, E., & Bar-Yam, Y. (2004). Theory predicts the uneven distribution of genetic diversity within species. *Nature*, *431*(7007), 449–452. https://doi.org/10.1038/nature02745

Reid, W. (1998). Biodiversity hotspots. Trends in Ecology and Evolution, 13(7), 275–280. https://doi.org/10.1016/s0169-5347(98)01363-9

Roll, U., Feldman, A., Novosolov, M., Allison, A., Bauer, A., Bernard, R., Böhm, M., Castro-Herrera, F., Chirio, L., Collen, B., Colli, G., Dabool, L., Das, I., Doan, T., Grismer, L., Hoogmoed, M., Itescu, Y., Kraus, F., LeBreton, M., Lewin, A., Martins, M., Maza, E., Meirte, D., Nagy, Z., Nogueira, C., Pauwels, O., Pincheira-Donoso, D., Powney, G., Sindaco, R., Tallowin, O., Torres-Carvajal, O., Trape, J.-F., Vidan, E., Uetz, P., Wagner, P.,

Wang, Y., Orme, C., Greyner, R., Meiri, S. (2017). The global distribution of tetrapods reveals a need for targeted reptile conservation. *Nature Ecology & Evolution*, *1*(11), 1677–1682. https://doi.org/10.1038/s41559-017-0332-2

Rosauer, D., Blom, M., Bourke, G., Catalano, S., Donnellan, S., Gillespie, G., Mulder, E., Oliver, P., Potter, S., Pratt, R., Rabosky, D., Skipwith, P., Moritz, C. (2016). Phylogeography, hotspots and conservation priorities: an example from the Top End of Australia. *Biological Conservation*, *204*, 83–93. https://doi.org/10.1016/j.biocon.2016.05.002

Singh, J. (2002). The biodiversity crisis: A multifaceted review. *Current Science*, *82*(6), 638-647. https://www.jstor.org/stable/24106689.

Tarroso, P., Velo-Antón, G., Carvalho, S. (2014). phylin: an r package for phylogeographic interpolation. *Molecular Ecology Resources*, *15*(2), 349-357. https://doi.org/10.1111/1755-0998.12312

Tremlová, K., & Münzbergová, Z. (2007). IMPORTANCE OF SPECIES TRAITS FOR SPECIES DISTRIBUTION IN FRAGMENTED LANDSCAPES. *Ecology*, *88*(4), 965–977. https://doi.org/10.1890/06-0924

Vences, M., Wollenberg, K., Vieites, D., Lees, D. (2009). Madagascar as a model region of species diversification. *Trends in Ecology & Evolution*, *24*(8), 456-465. https://doi.org/10.1016/j.tree.2009.03.011

Winter, D. (2017). rentrez: An R package for the NCBI eUtils API. *PeerJ Preprints,* 5:e3179v2. https://doi.org/10.7287/peerj.preprints.3179v2