SPIXIANA	47	1	95–104	München, Dezember 2024	ISSN 0341-8391

Notable genetic distance in critically endangered lizard *Homonota rupicola* Cacciali, Ávila & Bauer, 2007 identified through DNA barcodes

(Squamata, Phyllodactylidae)

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Cacciali, P., Marecos, J. & Cardozo, J. 2024. Notable genetic distance in critically endangered lizard *Homonota rupicola* Cacciali, Ávila & Bauer, 2007 identified through DNA barcodes (Squamata, Phyllodactylidae). Spixiana 47(1): 95–104.

Among modern taxonomy tools genetic barcoding is a widely used method for species identification, although additional molecular inferences can be made, such as information about genetic distances and phylogenies. In Paraguay, there is a large mtDNA library based on the 16S gene. Using samples from newly described localities for the critically endangered and Paraguayan endemic, *Homonota rupicola*, we generated valuable information. Results show a concordance with previous phylogenies. Additionally, based on the high genetic distance between samples, it is possible to infer that the two analyzed populations are isolated, and it is likely that the genetic interchange is somewhat reduced. This situation is worsened by the fact that there is a highway crossing between the rocky outcrops. Conservation attention and further research are crucial to understanding the genetic status of this micro-endemic lizard.

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Introduction

Genetic barcoding is a method of species identification that uses a short section of DNA from a specific gene or genes such as a specific DNA marker useful for taxonomic assignment (Antil et al. 2023). Barcoding DNA genes have certain characteristics. For example, they have a variable region flanked by highly conserved gene regions and identifications of these gene regions constitute a "barcoding gap", where the intraspecific variation is lower than interspecific variation (Bystrykh et al. 2014, Thielecke et al. 2017). Different genes are employed based on the purpose, and in this respect, for Squamata identification, the most used genetic mitochondrial markers are the 16S and Cytochrome Oxydase I (COI), and in particular for Paraguay there is already a large library of genetic barcodes of the first one (Cacciali et al. 2019). This gene, in addition to providing valuable data for species identification, also proved to have a high degree of confidence in identifying shallow phylogenetic relationships, i. e., it is better for closely related taxa and weaker for deeper phylogenetic signals among major groups (Hertwig et al. 2004).

Genetic barcodes not only provide a first clue in the identification of taxonomic questions (Cacciali et al. 2017), but also provide information about genetic diversity, an important component of the biodiversity; since it improves the resilience of an organism by allowing crossbreeding, thereby increasing disease resistance and adaptation to environmental changes. And, from an anthropogenic point of view, it also provides an economic value for society (Bonneuil & Boucekkine 2020, Nonić & Šijačić-Nikolić 2021). The molecular genetics of the genus *Homonota* were thoroughly analyzed by Morando et al. (2014), through the study of mitochondrial (Cyt-b and 12S), and several nuclear protein-coding genes (RBMX, DMLX, NKTR, PLRL, SINCAIP, MXRA5, ACA4) from representatives of all described species to date, and some years later the genus was also analyzed from the perspective of the mtDNA 16S barcoding gene, without the inclusion of *H. rupicola* (Cacciali et al. 2017, 2018a, Cabral & Cacciali 2021). Genetic trees strongly support three groups within *Homonota*: *whitii*, *borellii*, *horrida* (Morando et al. 2014, Cacciali et al. 2017). Based on the results of molecular dating, a notably marine transgression during the Middle and Late Miocene, this likely isolated the ancestors of these clades in different regions, shaping their genetic diversity and distribution (Morando et al. 2014).

Homonota rupicola belongs to the *borellii* group (Morando et al. 2014), and it was described from a single locality: a rocky outcrop in a hilly landscape in eastern Paraguay (Cacciali et al. 2007), and for several years that was the only locality known for the species (Cacciali et al. 2015). Recently, a few additional records were published, although all were from the same rocky hill area in a geographic range of about 12–13 km (Cacciali et al. 2024). This species is critically endangered (Cacciali 2017), absent in conservation units (Cacciali et al. 2024), and the isolation of this lineage likely occurred between 4 and 2.5 million years ago (Morando et al. 2014). In this

Table 1. Details of specimens used for genetic analysis, including GenBank accession numbers (GBAN). Numbers in bold indicate sequence data generated in this study. Museum codes following Sabaj (2023). Localities shown in Figure 1.

Species	Voucher	Locality	Coordinates	GBAN
Homonota horrida	BYU 47941	RP 190, Mendoza, Argentina	35°26'45.7"S 67°49'48.9"W	MF278828
	LJAMM-CNP 10495	RP 190, Mendoza, Argentina	35°26'45.7"S 7°49'48.9"W	MF278829
	LJAMM-CNP 10576	2.3 km S from Punta del Agua, Mendoza, Argentina	35°32'50.3"S 68°05'01.6"W	MF278830
Homonota itambere	IIBP-H 4437	Estancia Guajho, Paraguarí, Paraguay	26°14'55.8"S 57°17'48.0"W	MZ098621
Homonota marthae	SMF 101438	Filadelfia, Boquerón, Paraguay	22°20'49.2"S 60°02'56.0"W	MG947388
Homonota rupicola	PCS 753	Piraretá, Cordillera, Paraguay	25°30'20.8"S 56°55'29.4"W	**PP920678**
	PCS 754	Piraretá, Cordillera, Paraguay	25°30'20.8"S 56°55'29.4"W	**PP920679**
	PCS 758	Itá Moroti, Cordillera, Paraguay	25°30'58.6"S 57°01'41.8"W	**PP920680**
Homonota septentrionalis	MNHNP 12238	Infante Rivarola, Boquerón, Paraguay	21°40'44.4"S 62°24' 3.6"W	MF278832
	SMF 101984	Infante Rivarola, Boquerón, Paraguay	21°40'44.4"S 62°24' 3.6"W	MF278833
Phyllopezus heuteri	MNHNP-TH 2-39	Cerro de Tobatí, Cordillera, Paraguay	25°16'46.6"S 57°05'32.1"W	MH397468
	MNHNP 12238	Infante Rivarola, Boquerón, Paraguay	21°40'44.4"S 62°24' 3.6"W	MF278832
	SMF 101984	Infante Rivarola, Boquerón, Paraguay	21°40'44.4"S 62°24' 3.6"W	MF278833
Phyllopezus heuteri	MNHNP-TH 2-39	Cerro de Tobatí, Cordillera, Paraguay	25°16'46.6"S 57°05'32.1"W	MH397468

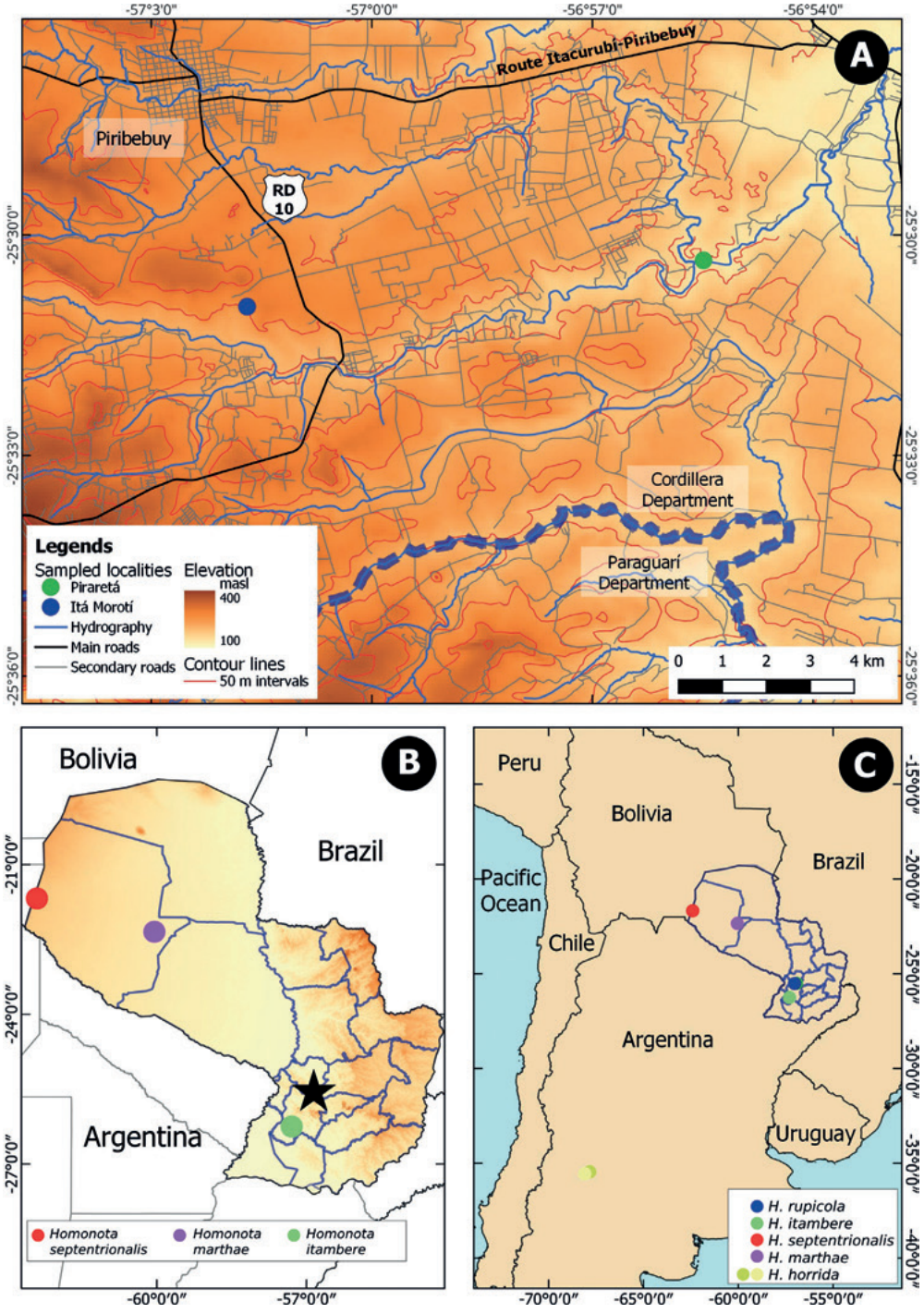

Fig. 1. Sampled localities used for molecular analyses. **A.** Detail of genetic localities for *H. rupicola*: Piraretá (PCS 753–754) and Itá Morotí (PCS 758), showing the main routes. RD 10: Departmental Route N°10. **B.** Location of samples inside Paraguay. Black star represents the area of *H. rupicola*. **C.** Central and southern region of South America, showing the Paraguayan and Argentinean localities used for genetic analyses.

work we provide the first genetic barcodes of 16S, and for the first time they are sequenced data from two different localities, providing critical information about their conservation.

Materials and methods

Data collection

The area where samples were collected consists mainly of thick to medium sandstones with weathering processes in rocky outcrops. The surface is rough with cracks and gaps, covered with lichens; and the vegetation is mainly shrubby, covered with Bromeliads, *Cereus* sp. (Cactaceae), *Polycarpaea* sp. (Caryophillaceae) and other plants, with xerophytic characteristics ranging from 4 to 20 meters high (Cacciali et al. 2015). The area has climatological traits typical of humid forests due to the presence of aquatic springs in the region. The weather shows a seasonality with cooler mean temperatures in June and July (Min $\bar{x}$: 12–14°C, Max $\bar{x}$: 23–24°C) and the driest months in July (62 mm of rain) and August (53 mm), with higher temperatures in December and January (Min $\bar{x}$: 21°C, Max $\bar{x}$: 32°C), and with maximum precipitation occurring during April (163 mm). Climatic data was obtained from the WorldClim database (Global Climate Data) based on Hijmans et al. (2005).

The sampled taxon was *Homonota rupicola*. Specimens of this species were captured, their tails clipped and stored in vials with ethanol alcohol (96 %), and then released at their exact point of capture. Permits were issued by Ministerio del Ambiente y Desarrollo Sostenible (MADES N° 024/2020 and 004/2022). Three specimens were collected, two samples from Piraretá, in the surroundings of 25°30'20.8''S, 56°55'29.4''W (Field numbers: PCS 753–754) and one sample from Itá Morotí near 25°30'58.6''S, 57°01'41.8''W (Field number: PCS 758) (Fig. 1), for additional details see Table 1. Museum acronyms follow Sabaj (2023). Tail clippings were deposited in the herpetological collection of the Museo Nacional de Historia Natural del Paraguay (MNHNP).

Molecular protocol

Samples were initially washed in a 50 µL solution of 1× TE Buffer for ~20 h to remove ethanol, and then were digested with a solution of 50 µL of Vertebrate lysis Buffer and proteinase K (10:1) and incubated in a rocking platform at 56°C for ~24 h. Concentrations and proportions of reagents are detailed in Appendix 1. DNA extraction follows Ivanova et al. (2006), for which 100 µl of Binding Buffer was added to each sample, and these products were transferred to a Pall® (Cortland, NY, USA) AcroPrep® filter plate, and vacuumed for ~8 min while adding 180 µl of Washing Buffer 1 and 750 µL of Washing Buffer 2. Amplification was made using fragments of the mtDNA 16S gene with primers for forward (L2510: 5′-CGC CTG TTT AAC AAA AAC AT-3′) and reverse (H3056: 5′-CGG TCT GAA CTC AGA TCA CGT-3′) reactions according to Palumbi et al. (1991). Thermocycling conditions were 94°C (2 min), 40× [94°C (35 s) – 48.5°C (35 s) – 72°C (60 s)], 72°C (10 min).

Data analysis

Trace files of forward and reverse sequences were visualized and assessed as chromatograms in SeqTrace 0.9.0 (Stucky 2012). To assess the relationships of the new samples of *H. rupicola* with other species of the genus, sequences from GenBank were downloaded (Table 1). A sample of *Phyllopezus heuteri* was used to root the tree. Sequences alignment was performed in MAFFT2 (Katoh et al. 2002, Katoh & Standley 2013) using the webserver (Katoh et al. 2017), including a special search strategy (Q-INS-i) for the secondary structure of the rRNA 16S (Katoh & Toh 2008). MSA viewer was used for visualization of alignments and export in fasta format (Yachdav et al. 2016).

Table 2. Genetic (below left) and geographic (above right) distances between analyzed samples of *Homonota*. Genetic distances are represented by the number of base substitutions per site between sequences. Geographic distances are provided in km and estimated in straight airline.

		H. septentrionalis SMF 101984	*H. septentrionalis* MNHNP 12238	*H. marthae* SMF 101438	*H. itambere* IIBP-H 4437
H. septentrionalis	SMF 101984		~0.000	253.85	725.12
H. septentrionalis	MNHNP 12238	~0.000		253.85	725.12
H. marthae	SMF 101438	0.006	0.006		514.55
H. itambere	IIBP-H 4437	0.049	0.049	0.046	
H. horrida	LJAMM-CNP 10576	0.033	0.033	0.030	0.052
H. horrida	LJAMM-CNP 10495	0.024	0.024	0.021	0.042
H. horrida	BYU 47941	0.024	0.024	0.021	0.042
H. rupicola	PCS 754	0.154	0.154	0.150	0.169
H. rupicola	PCS 753	0.154	0.154	0.150	0.169
H. rupicola	PCS 758	0.157	0.157	0.153	0.164

The best substitution model scheme was selected using the AICc (Burnham & Anderson 2002). According to Yang et al. (2016), it is not recommended to use both +I (invariable sites) and +G (gamma distribution) in the same substitution model, thus, in case of a suggestion of +I+G in a model, the next best model containing only +I or +G was selected.

For analysis of clusters and relations of sequences phylogenetic hypothesis was performed using a Maximum Likelihood (ML) method in IQ-Tree (Nguyen et al. 2015) webserver platform (Trifinopoulos et al. 2016), under the following settings: 10000 non-parametric bootstrap replicates adding 10000 replicates of Shimodaira-Hasegawa approximate likelihood ratio (SH-aLRT) (Anisimova et al. 2011) and 10000 approximation replicates of ultrafast bootstrap (UFBoot) (Minh et al. 2013). FigTree 1.4.3 (Bogaardt et al. 2018) was used for tree visualization. Sequences are stored in GenBank under accession numbers (PP920678–PP920680), and final alignment with associated tree stored in TreeBASE repository (Submission ID 31492).

Uncorrected pairwise distance (p-distance) was estimated (Brown et al. 1979, Lopez et al. 1997) in MEGA 11 (Tamura et al. 2021) with 10000 bootstrap replicates and excluding the outgroup, using the Maximum Composite Likelihood model (Tamura et al. 2004), to assess the evolutionary divergence between sequences, followed by a comparison of the genetic distance with the geographic distance. Maps generated in QGIS 3.22.7, using high resolution elevation SRTM30 (30 seconds resolution) datasets taken from Consortium for Spatial Information (CGIAR-CSI) available on http://www.diva-gis.org/gdata (Jarvis et al. 2008). Finally, genetic distances were overlapped with geographic distance in Barrier 2.2 (Manni et al. 2004) to visualize the most significant barriers.

Results

The final alignment length was 530 bp, and the selected substitution model GTR+G. Results show samples of *H. rupicola* (*borellii* group) as a sister group to the remaining *Homonota* (*horrida* group), where samples from Piraretá (PCS 753 and 754) are different from the specimen from Itá Morotí (PCS 758) (Fig. 2). Within the *horrida* group, *H. horrida* (from Argentina) is nested as the basal clade (yellow clade), and sister to the Paraguayan specimens, and within the Paraguayan samples, *H. itambere*, the only species east of the Paraguay River, is the most basal lineage (blue clade), and sister to the species distributed west of the Paraguay River (*H. marthae* and *H. septentrionalis*) (Figs 1–2). The weakest support in the branches is found within *H. horrida*, and the highest value is present in the support of the *horrida* group.

The analysis of genetic distances shows a divergence of 0.02 base substitutions per site between samples of *H. rupicola* from Itá Moroti and Piraretá (Table 2), where the geographic distance is about 10 km. This genetic distance is higher than the distance between *H. septentrionalis* and *H. marthae* (0.05) which are separated by 260 km, and similar to the distance between *H. septentrionalis* and *H. horrida* (0.02–0.03) separated by about 1600 km (Table 2). Within the *horrida* group, *H. itambere* has the highest genetic distance from the remaining members (0.04–0.05), and *H. rupicola* (*borellii* group) shows a genetic distance of 0.14–0.16 base substitutions per site from the *horrida* group. Overall, the estimation of genetic distance ratio per kilometer between the samples of *H. rupicola* is 0.0022, whereas in *H. horrida* it is 0.00035, and even lower (< 0.0001) between the other species within the *horrida* group.

The identification of barriers categorizes as the most important (barrier "a") a difference between the species *H. itambere* and *H. horrida*, and the two Chaco species (*H. marthae* and *H. septentrionalis*) and the only member of the *borelli* group (*H. rupicola*) (Fig. 3). The second barrier (b) separates *H. rupicola* from *H. marthae* and *H. septentrionalis* and the third barrier (c) isolates the southernmost population (*H. horrida*) (Fig. 3). Notably, the fourth division (d) is the first intraspecific barrier located between the two populations of *H. rupicola*, and the following

H. horrida LJAMM-CNP 10576	*H. horrida* LJAMM-CNP 10495	*H. horrida* BYU 47941	*H. rupicola* PCS 754	*H. rupicola* PCS 753	*H. rupicola* PCS 758
1632.73	1632.46	1632.46	701.30	701.30	693.61
1632.73	1632.46	1632.46	701.30	701.30	693.61
1657.12	1656.88	1656.88	472.54	472.54	466.61
1455.81	1454.90	1454.90	90.35	90.35	85.49
	25.00	25.00	1541.69	1541.69	1533.97
0.009		~0.00	1541.54	1541.54	1533.82
0.009	~0.000		1541.54	1541.54	1533.82
0.138	0.131	0.131		0.00	10.47
0.138	0.131	0.131	~0.000		10.47
0.148	0.141	0.141	0.024	0.024	

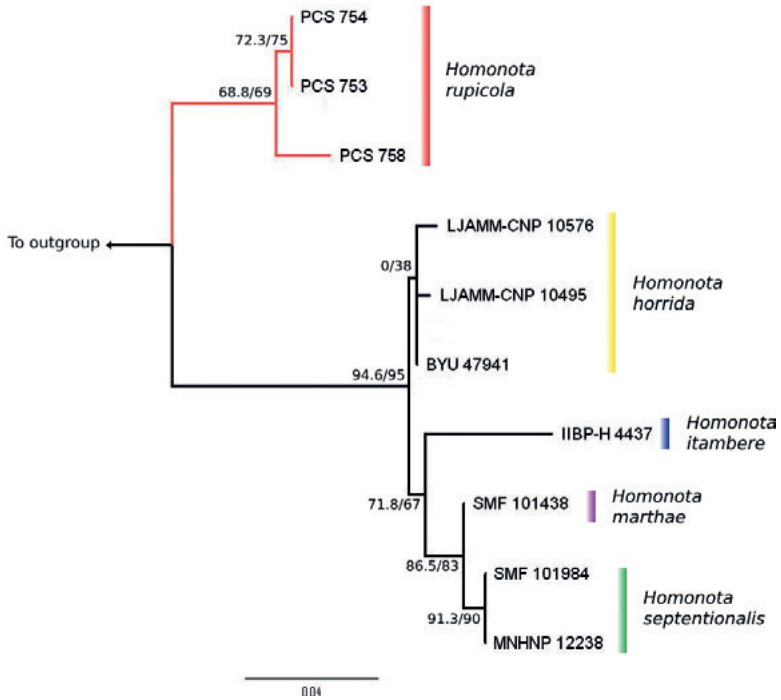

Fig. 2. Maximum likelihood relationships based on mtDNA gene 16S, showing in red the new genetic data for *H. rupicola*. Scale bar represents rate of substitution/site.

barrier (e), also intraspecific, divides two populations of *H. horrida* in the south (Fig. 3). Following, there is an interspecific barrier (f) between *H. marthae* and *H. septentrionlis*.

Discussion

This contribution improves the database of genetic barcodes of Squamata from Paraguay by adding mtDNA 16S sequence data for *Homonota rupicola* from two localities, one of which (Itá Morotí) is located less than 2 km from the type locality of the species. These are the first 16S genetic barcodes for this critically endangered species. The topology of the inferred tree represents relationships that match previous topologies of species phylogenies based on multigene data (Cacciali et al. 2017, 2018a), where *H. horrida* is the sister lineage to the *H. marthae* and *H. septentrionalis*. According to Cabral & Cacciali (2021), *H. itambere* is sister to *H. horrida* from Argentina, but in our tree all the Paraguayan species are nested together (within the *horrida* group), and *H. itambere* as the basal clade within the group. As expected, *H. rupicola* is nested in a different clade confirming the monophyly of the *horrida* group.

Regarding the genetic distance, the base substitutions per site between the samples of *H. rupicola* from the two analyzed localities, shows a difference of 0.02 (2 %), which is rather high for a distance of ~10 km. According to other species of the *Homonota horrida* group, the intraspecific 16S genetic distance goes only up to 1 % (Cacciali et al. 2017). For other Phyllodactylidae, such as those in the genus *Phyllopezus*, it is usually less than 0.5 %, but in some clades, it can reach up to 4.4 or 6.6 % (Cacciali et al. 2018b), and for *Lygodactylus* it ranges from 0 to 9 % with a mean of 1.8 % (Castiglia & Annesi 2011). In a comparison between genetic and geographic distances, Cacciali & Köhler (2018) found in the lizard genus *Tropidurus* a genetic distance of 0.4 % in specimens separated by ~450 km. Thus, the genetic distance of 2 % found between samples of *H. rupicola* separated by ~10 km is rather high. This elevated genetic distance typically signifies substantial genetic differentiation between populations or species. This can be interpreted as evidence of long-term evolutionary separation where populations have evolved independently over extended periods, accumulating distinct genetic mutations (Mueller & Ayala 1982, Aguillon et al. 2017). The analysis of barriers shows a high degree of differentiation between the popula-

tions of *H. rupicola* when genetic distance and geographic closeness are considered, being even more differentiated than *H. marthae* and *H. septentrionalis*, suggesting a high level of isolation.

Here it is important to highlight that it is not known if *H. rupicola* is strongly associated with rocky habitat and uses the forests to move between outcrops. Deeper genetic analyses are required to understand the actual genetic diversity and the mechanism used by the species to overcome genetic depression. When isolated populations have sufficient genetic variation, this can lead to speciation (Wu 2001, Fitzpatrick et al. 2009). On the other hand, when genetic diversity is not adequately high, the lack of gene flow may lead to the reduction of genetic diversity and inbreeding depression (Amos & Harwood 1998, Booy et al. 2000), and this is especially remarkable in mountain organisms (Prieto-Benítez et al. 2021). In addition to the natural barriers between rocky hills, in the distribution area of *H. rupicola* there is an important highway (Departmental Route N° 10) going through the two sampled populations, which further reduces the capability of gene flow interchange. Some authors suggest that due to the high anthropogenic alterations to the environment, some species, such as those inhabiting mountains, must be genetically "assisted" (Aitken & Whitlock 2013, Whiteley et al. 2015).

Despite the need for robust knowledge, the scope of lizard sampling was constrained by species conservation reasons, which severely limit the conclusions. Thus, although limited, our findings provide a snapshot into the ecological dynamics of *H. rupicola* populations, offering a foundation for subsequent research endeavours. This is a species that needs strong attention, and perhaps some assistance as well, to guarantee its conservation over time. This is a first contribution that seeks to add to the knowledge of this critically endangered and still poorly known species.

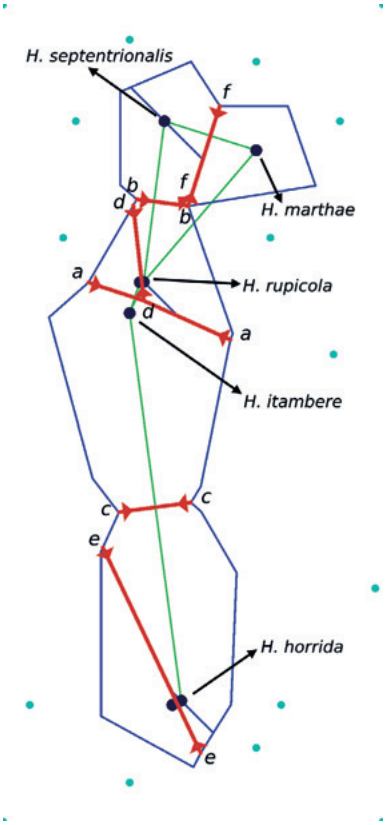

Fig. 3. Barriers identification (red arrows) among samples of *Homonota* species (black dots), categorized from most (a) to less (f) important. Blue polygons: Voronoï tessellation; green lines: Delaunay triangulation; light blue dots: virtual points for triangulation.

Acknowledgements

We thank Marcela Ferreira, Sara Escobar, Mario Bóveda, Lucas Cañete, Nicolás Cantero, Alexandra Spangenberg, José Maciel, Lorena Sforza, and Natalia Justiniano for their help during fieldwork, and Martha Motte and Nicolás Martínez (Museo Nacional de Historia Natural del Paraguay) for assistance during specimens' storage. PC acknowledges CONACYT (through PRONII program) and Zoological Society of London (through EDGE program) for financial support (for fieldwork, CONACYT (research fellowship BINV02-39) for financial aid for molecular analyses), and Guyra Paraguay and Senckenberg Forschungsinstitut and Naturmuseum Frankfurt (SMF) for endorse this study. Special thanks to Gunther Köhler, Hike Kappes, and Linda Mogk (SMF) for assistance during laboratory procedures. Thanks to Paul Freed and Calandra Stanley for language review. This study was based on regenerative tissue tails, harm was minimum, and bioethical considerations were approved by Ministerio del Ambiente y Desarrollo Sostenible (MADES N° 024/2020 and 004/2022).

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Appendix 1. Reagents and buffer ingredients used for molecular protocols, indicating volumes and concentrations.

Reagents	Volume	Concentration
TE Buffer	100 ml	
Tris-HCl	90 ml	10.0 mM
EDTA	10 ml	1.0 mM
Vertebrate Lysis Buffer	100 ml	
NaCl	4 ml	100.0 M
Tris-HCl	10 ml	1.0 M
EDTA	4 ml	0.5 M
SDS	5 ml	20 %
Water	77 ml	
Proteinase K solution		20 mg/ml
Binding Buffer	14 ml	
GuSCN	7 ml	4.0 M
Ethanol	7 ml	96 %
Washing Buffer 1	20 ml	
GuSCN	5.2 ml	4.0 M
Ethanol	14.8 ml	96 %
Washing Buffer 2	475 ml	
Ethanol	300 ml	60 %
NaCl	4.75 ml	50.0 mM
Tris-HCl	4.75 ml	10.0 mM
EDTA	0.475 ml	0.5 mM
Taq-DNA Polymerase		5 U/µl
Reaction buffer Y		2.5 mM
MgCl2		25.0 mM
dNTPs		2.5 mM
EasyLadder I		100 lanes
peqGOLD Universal Agarose		
Loading Buffer	10 ml	
Bromophenol blue	0.25 g	
Sucrose	4 g	
Water	5 ml	
TE Buffer	5 ml	1×
HD-Green Plus™ (DNA stain)	6 µl	10000×