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► **To cite this version:**

Florian Réveillion, Rémi Wattier, Sophie Montuire, Leonardo Sousa Carvalho, Loïc Bollache. Cryptic diversity within three South American whip spider species (Arachnida, Amblypygi).. *Zoological Research*, 2020, 41 (5), pp.595-598. 10.24272/j.issn.2095-8137.2020.068 . hal-02956293

HAL Id: hal-02956293

<https://hal.science/hal-02956293v1>

Submitted on 24 Sep 2024

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Cryptic diversity within three South American whip spider species (Arachnida, Amblypygi)

DEAR EDITOR,

Cryptic diversity (CD), the presence of highly divergent phylogenetic lineages within closed morphological species, has been documented for many taxa. Great arachnid orders such as Araneae or Scorpiones are well studied and many cases of CD have been described therein; to date, however, related research on smaller arachnid orders, such as whip spiders (Amblypygi), remains lacking. In the current study, we investigated CD based on cytochrome oxidase 1 (*COI*) in three nominal species of the genus *Heterophrynus* (*H. alces*, *H. batesii*, and *H. longicornis*), represented by 65 specimens. The sequences were compared using three different methods. All three species showed geographically structured CD. Thus, given its existence in this genus, it is important that CD and its spatial distribution be considered in future studies and possible conservation projects.

Cryptic diversity (i.e., a nominal species that includes two or more highly divergent lineages at the DNA level) has been detected in many biomes and taxa (Pfenninger & Schwenk, 2007), and is now a key element in conservation biology (Bickford et al., 2007). Indeed, CD and its geographical patterns are seminal for identifying the true extent of biodiversity, including biodiversity hotspots and taxa that warrant special conservation status (Funk et al., 2011). This is particularly true for invertebrates in tropical regions, which exhibit low mobility and are often used as predictors of conservation priorities (Moritz et al., 2001).

Whip spiders are a small arachnid order (Weygoldt, 2000) characterized by spectacular appendages such as raptorial pedipalps and extremely elongated antenniform first legs. Currently, over 200 species are described within Amblypygi (Miranda et al., 2016), most of which are found in tropical regions of the world. Although studies targeting the phylogeography of Amblypygi are scarce, Prendini et al.

(2005) on *Damon variegatus* (Amblypygi: Phrynichidae) and Esposito et al. (2015) on *Phrynus longipes*, *P. alexandroi*, *P. eucharis* (Amblypygi: Phrynidae) reported at least two highly molecularly divergent lineages within each nominal species, thus suggesting CD. The above studies also showed that such lineages were geographically structured, which has implications for conservation strategies.

The *Heterophrynus* (Amblypygi: Phrynidae) genus of whip spiders includes 16 nominal species endemic to South America (Weygoldt, 2000). The distribution and ecology of each species remain poorly documented except for *H. longicornis* (see Carvalho et al., 2011, 2012; Dias & Machado, 2006) and *H. batesii* (see Chapin, 2014). Like most whip spiders, *Heterophrynus* species are nocturnal and can be found in diverse microhabitats including rocks, caves, large trees with buttresses, burrows, and termite nests (Carvalho et al., 2012; Dias & Machado, 2006). Recently, Lehmann & Friedrich (2018) showed the absence of CD to be associated with very low (average 0.2%) genetic divergence for the *COI* DNA barcode sequence (i.e., 5' part of the mtDNA *COI* gene) among six sympatric specimens of *H. elaphus* from Peru. However, our knowledge of CD in other *Heterophrynus* species remains lacking.

Here, we explored the level of CD in three nominal species of the genus *Heterophrynus* (i.e., *H. alces*, *H. batesii*, and *H. longicornis*) from five populations collected from five geographical areas in South America. Material originated from eight sites in French Guiana and Brazil (Table 1, Figure 1), totaling 65 individuals ascribed to *H. alces* (6), *H. batesii* (4), and *H. longicornis* (55). Specimens were identified by F. Réveillon or L. Sousa Carvalho. All identifications were done using the morphological diagnostic characters proposed by Quintero (1981) and Weygoldt (2000), including pedipalp spination and shape of genitalia. The *COI* DNA barcode sequence (i.e., 5' part of the mtDNA *COI* gene) was used to assess the extent of CD as this marker has been successfully used in insects (Low et al., 2016). Following Kekkonen & Hebert (2014), *COI* was used as a quantifier of diversity and an "efficient start for taxonomic workflow", targeting the delineation of molecular operational taxonomic units (MOTUs) as a way to propose a testable "species hypotheses" (e.g., Fišer et al., 2018). Detailed methods are available in the

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Received: 03 April 2020; Accepted: 21 July 2020; Online: 30 July 2020
DOI: 10.24272/zj.zr.2019-8137.2020.068

Table 1 Genetic diversity parameters within three *Heterophrynus* morphospecies

Taxa	N	ISO	BIN	BS	Hap	AveD	MaxD	DNN (BIN)
<i>H. alces</i>	5	GF	ADC2276	0.883	1	0	0	4.91 (ACH7023)
	1	GF	ACH7023	0.883	1	N/A	N/A	4.91 (ADC2276)
<i>H. batesii</i>	1	BR Amazonas	ADC0812	0.794	1	N/A	N/A	3.68 (ADC0814)
	1	BR Amazonas	ADC0814	0.794	1	N/A	N/A	3.68 (ADC0812)
	2	BR Amazonas	ADC0813	1.000	1	0	0	17.19 (ACA0762')
<i>H. longicornis</i>	3	BR Piauí	ADC2132	0.742	2	0.12	0.18	3.33 (ADC2133)
	1	BR Piauí	ADC2133	0.783	1	N/A	N/A	3.33 (ADC2132)
	6	BR Ceará	ADC2134	0.911	2	0.06	0.18	8.77 (ADC2132)
	45	GF	ACH7022	0.914	4	0.08	0.53	9.31 (ADF0920')

N: Number of sampled individuals; ISO code for country (BR, Brazil; GF, Guiana French); BIN: Barcode Index Number; BS: Bayesian support in bPTP analysis for same MOTU as BIN; Hap: Number of haplotypes; AveD: average *P* (%) distance within BIN members; MaxD: Maximum *P* (%) distance within BIN members; DNN (BIN): Distance to nearest neighbor and (associated BIN); N/A: Not available; ': Records of *Heterophrynus vesanicus* from Brazil can be found in BOLD, but raw sequence data are not publicly available in BOLD and have not been deposited in GenBank.

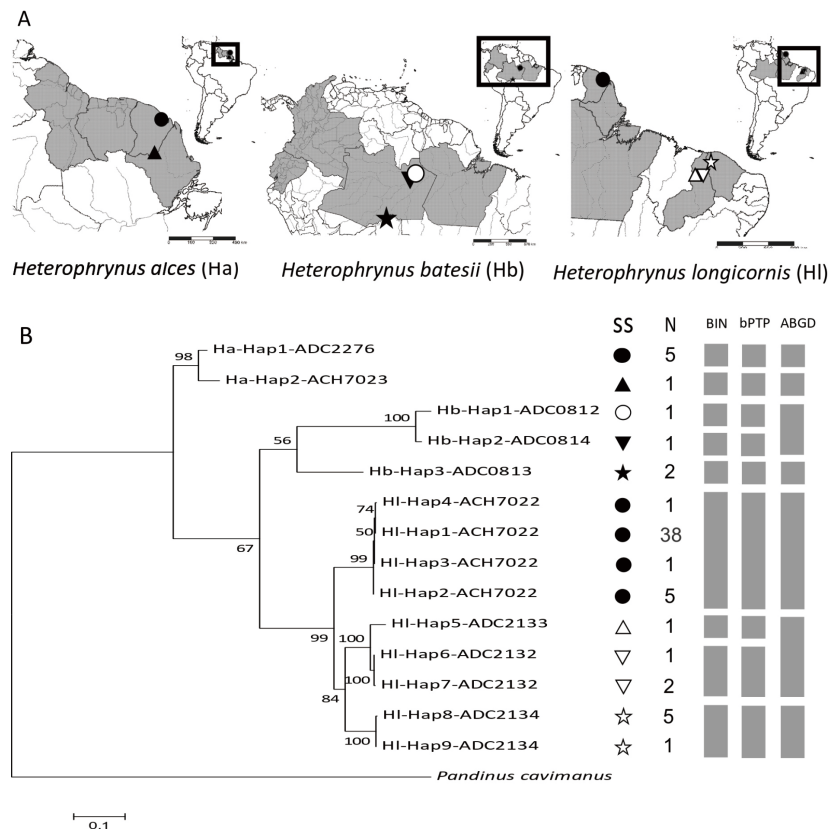


Figure 1 Collection site and phylogenetic position of three Amblypygi species

A: Distribution map and eight sampling sites for three Amblypygi nominal species: *H. alces* (Ha), *H. batesii* (Hb), and *H. longicornis* (HI). States with recorded presence of a given species are in gray. B: Maximum-likelihood (ML) phylogenetic tree based on haplotypes (Hap) of *COI* sequences (570 nucleotides). Code associated with each haplotype is BOLD BIN code. Bootstrap values are shown at nodes. Scale bar represents 0.1 substitutions per nucleotide position. Columns beside tree represent sampling sites (SS), sampling sizes (N), and clusters recognized by three MOTU delimitation methods (BIN, bPTP, and ABGD). Discrepancies among delineation methods are observed for MOTUs with low divergence (*P*-distance divergence of 3.5%) i.e., ADC0812 and ADC0814; and ADC2132 and ADC2133, respectively.

Supplementary Materials and Methods.

The *COI* barcodes of the 65 individuals of *H. alces* (six sequences), *H. batesii* (four), and *H. longicornis* (55) were

associated with two, three, and nine haplotypes, respectively (Figure 1). The maximum-likelihood (ML) phylogenetic tree (Figure 1) showed that the haplotypes associated with each

nominal species represented monophyletic groups. However, all three MOTU delimitation methods (i.e., Barcode Index Number (BIN), Bayesian implementation of Poisson Tree Processes (bPTP), and Automatic Barcode Gap Discovery (ABGD)) depicted CD in each nominal species.

For *H. alces*, all three methods were congruent at identifying two MOTUs, each being geographically restricted to northern and southern French Guiana, respectively (Figure 1). The two identified BINs (ADC2276 and ACH7023) presented 4.91% of genetic divergence (*P*-distance, i.e., distance to nearest neighbor, Table 1).

For *H. batesii* and *H. longicornis*, the methods were only partially congruent. Both the BIN and bPTP methods identified three and four MOTUs, respectively, but ABGD identified one MOTU less than the two other methods for each nominal species (Figure 1). It should be noted that each MOTU defined by bPTP and recognized as only one MOTU by ABGD had the lowest Bayesian support (BS) values in bPTP analyses (Table 1). Even based on the most conservative method in the present study (ABGD), CD was still present in both *H. batesii* and *H. longicornis*.

For *H. batesii* and *H. longicornis* nominal species, the two BINs (ADC0812 and ADC0814 and ADC2132 and ADC2133, respectively) shared a similar pattern: i.e., (i) *P*-distance divergence of 3.5% (Table 1) and (ii) association to a single known and very close (≈ 100 km apart) locality in the same state in Brazil (Amazonas and Piauí, respectively) (Figure 1).

In addition, BINs ADC0813 and ACH7022 shared a similar pattern relative to other BINs within their respective nominal species: (i) more distantly related in ML analysis (Figure 1) and (ii) more geographically distant (i.e., ADC0813 is 900 km southward in the same Brazilian state (Amazonas) and ACH7022 is 1 500 km north-eastward in French Guiana) (Figure 1). These two BINs (ADC0813 and ACH7022) shared a third surprising feature. Although one would expect to find the closest BIN to be a BIN of the same nominal species, this was not the case here, as the closest BIN was associated with a fourth *Heterophrynus* nominal species, *Heterophrynus vesanicus* Mello Leitão, 1931 from Brazil (BINs ACA0762 and ADF0920, respectively) (Table 1). Unfortunately, the sequences associated with these BINs are not publicly available in the barcode of life data (BOLD) nor have the associated sequences been deposited in GenBank. While BOLD analysis results of private data are available, the raw data are not, which prevented the inclusion of *H. vesanicus* in the ML tree in Figure 1B.

In addition, for *H. longicornis*, a third Brazilian BIN (ADC2134; from Ubajara, state of Ceará) showed 8.77% genetic divergence from ADC2132 from Castelo do Piauí, state of Piauí (Table 1). Although located in different states in Brazil, both localities are only ~ 170 km apart. This pattern illustrates that CD can increase even at a moderate geographic scale.

Our analysis of 65 individuals ascribed to three nominal species of *Heterophrynus* showed CD even with the most

conservative method of MOTU delimitation (ABGD). It should be noted that even in close localities, moderate genetic divergence was observed, unravelling unexpected CD in both *H. batesii* and *H. longicornis*. Providing a time frame or a full biogeographical scenario explaining the observed pattern are not within the scope of the present paper and are unachievable given current sampling. However, our study identified high intramorphospecific genetic divergence (ca $>10\%$) in the examined *Heterophrynus* specimens. All three nominal species have large geographical distribution areas, spanning many thousands of kilometers (Carvalho et al., 2011), but low dispersal capabilities. Many DNA barcoding analyses have revealed that such nominal species initially considered as widespread should instead be considered as an assemblage of short-range endemics (Magalhães et al., 2014), and should be re-evaluated taxonomically and formally described (e.g., Magalhães et al., 2017). At present, only two Amblypygi species are classified as vulnerable on the red list of endangered species of the IUCN (International Union for the Conservation of Nature), i.e., Seychelles small whip spider, *Charinus seychellarum* (Gerlach, 2014a) and Indian Ocean whip spider, *Phrynichus scaber* (Gerlach, 2014b). Their vulnerable status is partly due to their restricted geographic distribution and habitat degradation.

Our study highlights new elements of an understudied group and shows that the taxonomy of the genus *Heterophrynus* is complex. The fact that the closest BIN for those of *H. batesii* and *H. longicornis* was from *H. vesanicus* and not, as one would expect, one of the BINs already identified in their respective nominal species, is puzzling. Although only based on phenetic distance and not on refined phylogenetic analyses, our results thus challenge the monophyly of both taxa. Alternatively, one could consider *H. vesanicus* sequences as based on a misidentified specimen, possibly the most parsimonious hypothesis. The MOTUs identified in the present study can be considered as a way in which to propose a testable "species hypothesis" (e.g., Fišer et al., 2018). The observed MOTUs still require further evaluation by integrating morphological and molecular data of specimens from other localities throughout their wide geographic distribution. In addition, *Heterophrynus* species are known to use many habitats, and present highly complex microhabitat selection and interaction among individuals (Carvalho et al., 2012; Dias & Machado, 2006). Thus, with the confirmation of the existence of cryptic species, their taxonomy, ecology, and behavior should be re-assessed. Further studies on genetic and morphological discrepancies with integration of geometric morphometry, as exemplified for whip spiders (*Phrynus barbadensis*) by Torres et al. (2018), could provide valuable information for species delimitation and description of new species.

More broadly, the existence of CD in this genus highlights the importance of considering MOTUs and geographical distribution and not species based on morphology in future studies and conservation projects, especially for invertebrates with limited dispersal capacity.

SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Specimen collection protocols were approved by the Institutional Ethical Committee of Animal Experimentation of the University of Burgundy.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

F.R. and L.B. designed the study. F.R., L.B., and R.W. supervised the analyses. R.W. extracted genomic DNA. F.R. and L.B. wrote the manuscript with the other authors' input. R.W., L.B., and R.W. revised the manuscript. All authors read and approved the final version of the manuscript.

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Supplementary Materials and Methods

The material analyzed in this study originated from eight sites in French Guiana and Brazil (Table 1, Figure 1), totaling 65 individuals ascribed to three nominal morphospecies, i.e., *H. alces* (6), *H. batesii* (4), and *H. longicornis* (55) (Table 1, Figure 1). Samples were either newly collected material as part of the present study (63 individuals) or were already available from GenBank (two sequences) as part of research conducted by Arabi et al. (2012) on Amblypygi phylogeny. Newly captured animals were ascribed to a given nominal species based on the key from Quintero (1981) and Weygoldt (2002). The three studied species use the same habitats. During the day they can be found under rocks, in caves, in large trees with buttresses, and in burrows, where they hide and predate on arthropods, and during the night can be found on rocks and large trees from the floor to 2 m high (Dias & Machado, 2006; Weygoldt, 2000). In French Guiana, to limit our impact on population dynamics in this area known for harboring low density, the terminal parts (1 to 1.5 cm) of the antenniform leg tarsus were removed from live captured individuals before releasing the specimens at the same site, with a complete specimen of each species conserved in the collections at the University of Burgundy. The leg part, which was sufficient for DNA analysis, was situated after the autotomy point on the leg and can regenerate after molting (Weygoldt, 2000). As individuals were released at the same site of capture, they were marked by a small spot of Tipp-Ex® (type correction fluid) on the cuticle before release to avoid sampling them twice. This mark resists the high

hygrometry of the habitats, does not injure the animals, and is removed after molting.

The samples were stored on site in 96% ethanol.

In Brazil, we used entire specimens conserved in the collections of the Universidad Federal do Piauí and the Universidade Federal do Mato Grosso. The specimens were stored in 96% ethanol. The terminal part of an antenniform leg (1 cm) was used for DNA analysis.

Total genomic DNA was extracted following a phenol-chloroform protocol modified from Hillis et al. (1996) using three to six apical segments of an antenniform leg tarsus. Polymerase chain reaction (PCR) DNA amplification targeting the mitochondrial-encoded *COI* gene was performed using a final volume of 30 μ L of 1X buffer (Eppendorf Inc. France), including ca 5 ng genomic DNA, 0.15 U Hotstart DNA polymerase (Eppendorf Inc. France), 200 μ mol/L dNTPs, and 200 nmol/L forward LCO1490 and reverse HCO2198 primers (Folmer et al., 1994). PCR cycling parameters were as follows: initial denaturation for 3 min at 95 °C, followed by 35 cycles of 20 s at 95 °C, 45 s at 40 °C, and 1 min at 65 °C, terminating with a final elongation at 65 °C for 2 min. PCR amplifications were performed with a Dyal DNA Engine BioRad thermocycler (Biorad, France). A 2 μ L aliquot of each PCR product was checked to ensure it was the expected size (ca 710 bp) prior to purification with ExoSAP-IT Express (Thermo Fisher Scientific, France) according to the manufacturer's recommendations. Purified PCR products were sent to Macrogen Europe for sequencing using a BigDye Terminator 3.1 Sequencing kit and electrophoresed using an ABI 3730XL Genetic Analyzer (Applied Biosystems Inc.,

USA).

Newly produced sequences were edited with Genious R10.2.2 (Kearse et al., 2012) and all 63 sequences were deposited in GenBank (accession Nos.: MT899086–MT899148) and BOLD SYSTEMS (project AMBLY) (Ratnasingham & Hebert, 2007). All sequences (including two sequences already published by Arabi et al. (2012) *H. alces* JN018118 and *H. longicornis* JN018119) were aligned using the MAFFT V7 algorithm (Kato & Standley, 2013) and trimmed to a final dataset of 570 bp sequences for Automatic Barcode Gap Discovery (ABGD), Bayesian implementation of Poisson Tree Processes (bPTP), and maximum-likelihood (ML) analyses (see below).

To explore the number of MOTUs that could represent putative cryptic species within the morphospecies, we applied three different approaches, two based on genetic distance, i.e., ABGD (Puillandre et al., 2012) and Barcode Index Number (BIN) (Ratnasingham & Hebert, 2013), and one based on phylogenetic tree analysis using bPTP (Zhang et al., 2013).

Both BIN and ABGD share the principle of clustering sequences into MOTUs according to their molecular divergence using algorithms to find discontinuities, i.e., barcoding gaps, between clusters. For the ABGD method, we used primary partitions as a basis for group definition, *P*-distance model, and the default value of 0.001 the minimum intraspecific distance. The BIN algorithm is implemented in the Barcode of Life Data system (BOLD; Ratnasingham & Hebert, 2007). Sequences deposited in BOLD or mined by BOLD from GenBank are clustered and each cluster is ascribed a

globally unique and specific identifier (aka BIN). In addition, BINs may be registered and made publicly available through BOLD, allowing tractability and have similar properties as classic taxonomy descriptors (e.g., can be split or synonymized).

The tree-based bPTP method assumes the branch lengths of a non-ultrametric phylogenetic tree to be generated by two independent classes of Poisson events, one corresponding to speciation and one to coalescence (Zhang et al., 2013). Additionally, the Bayesian version of the PTP method adds Bayesian support values (BS) for the delimited “species” (Zhang et al., 2013). For the input tree, we used an ML tree obtained using Mega-X (Kumar et al., 2018) from the haplotype data. The ML analysis was run under a thorough tree search with the HKY+G+I model of substitution i.e., Hasegawa-Kishino-Yano model (HKY, Hasegawa et al., 1985), with a discrete Gamma rate difference among sites (+G) and some sites being evolutionarily invariable (+I). Scorpionidae *Pandinus cavimanus* (GenBank accession number AY156580) was used as an outgroup. The bPTP analysis was performed on the bPTP web server (available at: <http://www.species.h-its.org/ptp/>) with 250 000 MCMC iterations, 10% burn-in, and outgroup exclusion.

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