ZOOLOGICAL RESEARCH

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. alejandroi*, *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. Réveillion 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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Table 1 Genetic diversity parameters within three *Heterophrynus* morphospecies

Taxa	*N*	ISO	BIN	BS	Hap	AveD	MaxD	DNN (BIN)
H. alces	5	GF	ADC2276	0.883	1	0	0	4.91 (ACH7023)
	1	GF	ACH7023	0.883	1	N/A	N/A	4.91 (ADC2276)
H. batesii	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*)
H. longicornis	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	BRCeará	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.

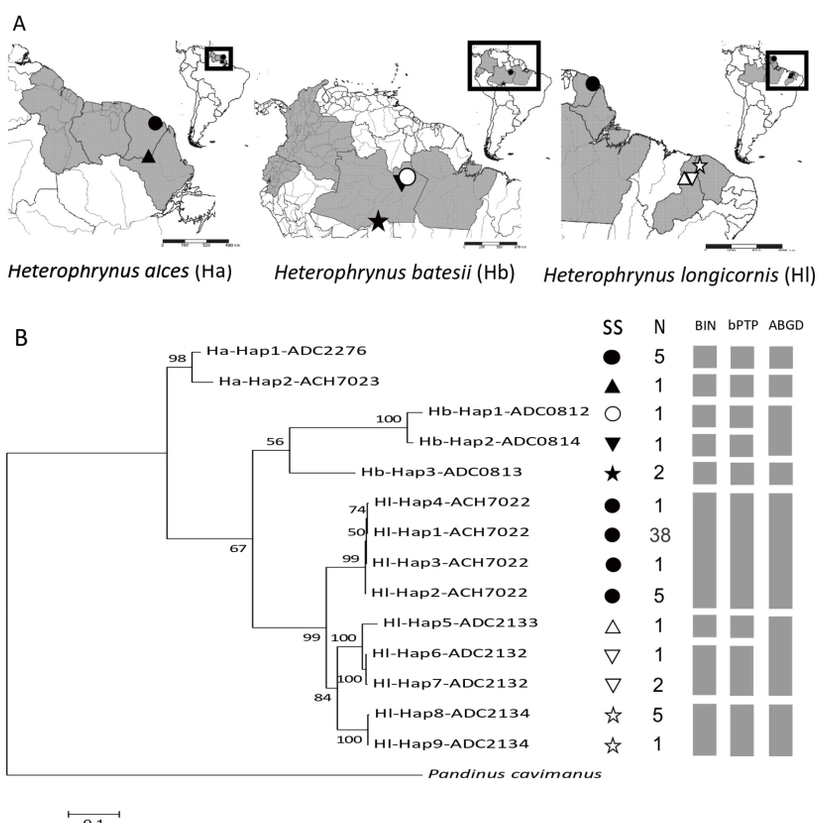

Figure 1 Collection site and phylogenetic position of three Amblypygi species

A: Distribution map and eight sampling sites for three Amblypygi nominal species: *H. alces* (Ha), *H. batesii* (Hb), and *H. longicornis* (Hl). 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 that 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 Piaui, 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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