Research Article

Genome-informed integrative taxonomic description of three cryptic species in the earthworm genus *Carpetania* (Oligochaeta, Hormogastridae)

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Research on cryptic species complexes has reached a consensus on the necessity of integrating multiple sources of evidence. Reduced representation library techniques such as Genotyping-by-Sequencing (GBS) have proven useful to study these groups. Both integrative taxonomy and genome-wide single nucleotide polymorphism (SNP) data remain to be widely applied to earthworms, an animal group with widespread presence of cryptic diversity. The genus *Carpetania* (formerly the *Hormogaster elisae* species complex) was found to contain six deeply divergent genetic lineages and some inconspicuous morphological differentiation based on a handful of Sanger-sequenced markers. Marchán et al. (submitted) delimited three well-supported species-level clades on the basis of a genome-wide SNP dataset and geometric morphometric analyses, highlighting the necessity of a formal taxonomic description of these taxa. In this work, further analyses are applied to the SNP data and a thorough morphological study is performed in order to provide an integrative description of two new species and to redescribe *Carpetania elisae*. Species-specific SNPs are identified and used as diagnostic characters, and genome-wide and cytochrome oxidase C subunit 1 (COI) genetic distances are compared finding a strong correlation between them. The taxonomic description of these three cryptic species provides a useful tool to include them effectively in ecological studies and biodiversity conservation actions.

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Key words: cryptic species, earthworms, Genotyping By Sequencing, integrative taxonomy, soil fauna

Introduction

Since the advent of molecular phylogenetic techniques, discovery of cryptic species complexes showed a surge in scientific interest (Bickford et al., 2007; Pfenninger & Schwenk, 2007; Trontelj & Fisër, 2009; León, de León, & Nadler, 2010; Nygren, 2014), followed by a stage of novelties in species delimitation methodologies including different algorithms, genetic markers, and integrative approaches (Pons et al., 2006; Yang & Rannala, 2010; Puillandre, Lambert, Brouillet, & Achaz, 2012; Zhang, Kapli, Pavlidis, & Stamatakis, 2013; Yang, 2015). Two main lessons can be gathered from those studies: to obtain robust species delimitation hypotheses, several information sources (molecular, morphological, ecological, ethological) must be integrated (Queiroz & De Queiroz, 2007); and nuclear molecular markers are necessary to rule out the possibility of confounding deep mitochondrial lineages (and the effect of Incomplete Lineage Sorting) with proper cryptic species (Dupont, Porco, Symondson, & Roy, 2016). Recent debate has brought into the spotlight the necessity of refining the terminology related to the cryptic speciation phenomenon, as well as the validity of the concept itself (Heethoff et al., 2018; Struck et al.,...
Some works have already shown the potential of genome-wide single nucleotide polymorphism datasets (either generated by RADseq or GBS – Genotyping By Sequencing) to provide rich phylogeographic and species delimitation information for cryptic species complexes (Garg et al., 2016; Brunet et al., 2017; Rancilhac et al., 2019), but have only been applied to earthworms in the Lumbricus rubellus complex (Giska, Sechi, & Babik, 2015; Anderson, Cunha, Sechi, Kille, & Spurgeon, 2017). Giska et al. (2015) found no differentiation between sympatric cryptic lineages based on RAD-seq data, which was interpreted as these lineages not corresponding to biological species. However, Anderson et al. (2017) performed equivalent analyses on different lineages of the same cryptic complex and found strong differentiation between them.

Pervasive cryptic diversity has been found in earthworms across different families (Lumbricidae – King, Andrew King, Tibble, & Symondson, 2008; Fernández, Almodóvar, Novo, Simancas, & Díaz Cosín, 2012; Shekhetsov, Golovanova, & Peltek, 2013; Porco et al., 2018; Hormogastridae – Novo, Almodóvar, Fernández, Trigo, & Díaz Cosín, 2010; Megascoleidae – Chang, Lin, & Chen, 2008; Buckley et al., 2011, Moniligastridae – Ganin & Atopkin, 2018). Integrative taxonomy has yet to be widely employed in these complexes (but see Taheri et al., 2018).

One of the most studied cryptic species groups among these animals is the former Hormogaster elisa Alvarez, 1977, recognized as the genus Carpetania after Marchán et al. (2018). Six highly divergent cryptic lineages were identified using Sanger-sequenced mitochondrial and nuclear markers (Marchán, Fernández, de Sosa, Díaz Cosín, & Novo, 2017), but their description was precluded by the absence of clear-cut limits between the putative species. Quantitative differences in the distal end of genital chaetae were discovered between those cryptic lineages through geometric morphometrics (Marchán, Novo, et al., 2016), hinting a possible pseudocryptic status for the identified lineages. Pseudocryptic species are those classified as cryptic due to the ‘inadequacy of the morphological analysis’ (Knowlton, 1993) and can usually be distinguished after careful morphological analysis together with molecular data (Lajus, Sukhikh, & Alekseev, 2015).

Recently, a genome-wide SNP dataset was obtained through GBS for 17 populations and 85 individuals of Carpetania by Marchán et al. (2020), with the main objective of studying selection signatures and local adaptation in the cryptic complex. The authors applied different approaches to genetic structure identification and species delimitation (see Materials and methods) together with geometric morphometrics analysis (Fig. 1), finding congruent support for three species-level genetic clusters (hereafter clusters A, B and C), which comprised one or more of the six previously identified lineages as described in Marchán et al. (2017).

The well-supported putative species identified in Carpetania demanded a full, detailed taxonomic description: several authors have stressed the necessity of going a step further from cryptic species identification (Jörger & Schrödl, 2013; Wang et al., 2016; Fiser, Robinson & Malard, 2018; Chenuil, Cahill, Délemontey, Du Luc, & Fanton, 2019). To this end, this work will build upon the framework provided by Marchán et al. (2020), with the following objectives: (i) identify genome-wide nucleotide positions to be used as diagnostic characters in species description (molecular taxonomy); (ii) explore further morphometric and internal anatomic characters to reinforce species description; (iii) formally describe the species within Carpetania; (iv) compare genomic and barcode genetic distances to validate the widespread use of the latter.

Materials and methods
Molecular data
Background: SNP data comprising the geographic distribution and internal lineages of Carpetania was generated in Marchán et al. (2020). A brief summary of the methodology used to generate and analyse said data is presented below.

GBS libraries were generated using the restriction enzyme PstI following the GBS protocol from Elshire et al. (2011) and sequenced (in NextSeq500 Illumina platform) for 17 populations with five individuals from each totalling 85 individuals. Different datasets were generated with STACKS2 (Rochette, Rivera-Colón, & Catchen, n.d.), in order to compare the performance of the different analyses and due to the specific requirements of some of the analyses.

- ‘de novo-all SNPs’, obtained by de novo assembly. This dataset contained the highest number of SNPS (26,240).
- ‘de novo-one SNP’, obtained after selecting a random SNP per locus through the function populations in STACKS2. This dataset contained 4,767 SNPs, and allowed the use of Bayesian clustering in STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). Another added advantage of the removal of linked SNPs within the same loci was to avoid the inflation of outlier loci in selection analyses (PCAadapt – Duforet-Frebourg, Bazin, & Blum, 2014; Fsthet – Flanagan & Jones, 2017).
- ‘reference-one SNP’, 3,181 SNPs, obtained by mapping the reads against a reference transcriptome of Carpetania elisa lacine 1. Transcriptome reads can be accessed at NCBI Short Read Archive project: PRJNA196484, and the assembly (obtained with Trinity v. r2013-08-14 (Haas et al.,
(2013)) can be accessed in the Harvard Dataverse repository https://doi.org/10.7910/DVN/RVMQND.

Three different methodologies were applied to the SNP datasets to recover the genetic structure and phylogenomic relationships: Maximum likelihood (ML) phylogenetic inference, Principal component analysis (PCA), and Bayesian clustering.

A ML approach was applied to the concatenated sequences of SNPs datasets as implemented in RAXML-HPC v8 (Stamatakis, 2014) in Cipres Science Gateway (https://www.phylo.org/) with default parameters (GTRCAT model, ascertainment bias correction (Lewis, 2001), 1,000 rapid bootstrap inferences). PCA was performed (function glPCA, R package adegenet v2.0.1 (Jombart, 2008; Jombart & Ahmed, 2011) for the ‘de novo-one SNP’ dataset. Bayesian clustering was performed in STRUCTURE 2.3.4 (Pritchard et al., 2000). Ten separate runs were performed for each number of genetic clusters K (1–7). The Evanno’s method (ΔK criterion) (Evanno, Regnaut, & Goudet, 2005)
implemented in Structure Harvester (Earl & vonHoldt, 2012) identified K = 3 as the optimal number of clusters for the ‘de novo-one SNP’ dataset.

New molecular analyses: The datasets ‘de novo-all SNPs’ and ‘reference-one SNP’ were further analysed in this work to estimate genetic diversity and to identify species-specific diagnostic positions, respectively.

Genetic diversity of the studied populations was described through identity by state (IBS) genetic distance within and between populations and fixation index (FST). These parameters were obtained from the populations function (STACKS2 package) summary files. Correlation between SNP-based genetic distances and FST and the same parameters obtained from cytochrome C oxidase 1 sequences (Marchán et al., 2017) was tested through a Mantel test in the R package vegan (Dixon, 2003).

Diagnostic SNP positions were identified using function nucDiag in the R package spider (Brown et al., 2012) and parsed to the corresponding contig using the Carpetania elisae transcriptome after it was functionally annotated with eggNOG-mapper (Huerta-Cepas et al., 2017). Diagnostic nucleotidic positions were also identified in the molecular markers COI, 16S-tRNAs, 28S and H3 (retrieved from Marchán et al., 2017) following the same method.

Morphological data

Background: Geometric morphometric data of the genital chaetae of Carpetania comprising its geographic distribution and internal lineages was generated in Marchán et al. (2020). A brief summary of the methodology used to generate and analyse said data is presented below.

Thirteen populations (from a total of 17 analysed for GBS) were chosen for genital chaetae extraction and preparation for scanning electron microscopy. Geometric morphometrics analyses included acquisition of landmarks, Canonical Variate Analysis (CVA) and Discriminant Function Analysis (DFA). Landmarks established in Marchán, Novo, et al. (2016) were chosen for the analysis. Genital chaetae were grouped by population and populations were subsequently grouped in the three species-level clusters recovered by the molecular analyses.

External anatomy and morphometric characters. External anatomy characters commonly used in earthworm taxonomy were studied (type of prostomium, chaetae disposition, position of female and male pores, position of clitellum and tubercula pubertatis), with special attention to average weight and number of segments (found to have strong phylogenetic signal in Hormogastridae Marchán, Novo, et al., 2016). These were measured in at least five mature individuals per population in a total of 22 populations: 9 populations from Cluster A, 6 populations from Cluster B, and 7 populations from Cluster C. Statistical significance of differences was evaluated using ANOVA, Fisher’s LSD and Kruskal–Wallis tests in Statgraphics 18.

Internal anatomy. Internal anatomy characters commonly used in earthworm taxonomy were studied (number and position of gizzards, number of typhlosole lamellae, number and position of spermathecae, type of nephridial bladders), with special attention to the only variable internal character in the Carpetania species complex, relative position of septum 9/10 and spermatheca. In this genus, septum 9/10 can appear displaced backwards (to 10/11) in its dorsal insertion, resulting in the spermatheca of segments 9 and 10 belonging functionally to the same segment, or show an unmodified disposition separating both pairs of spermathecae. This character was studied in 22 populations as well.

Results and discussion

Genomic divergence and diagnostic positions

Genetic divergence parameters (average IBS distances and FST values) are presented in Fig. 2 (values can be found in Table S2). IBS distances between populations within the main clusters ranged from 0.09 to 0.15 (mean = 0.12) in both clusters A and B, while they ranged from 0.07 to 0.22 (mean = 0.17) in cluster C. IBS distances between populations of different clusters ranged between 0.15–0.24 (mean = 0.20) for clusters A and B, while they ranged between 0.18–0.24 (mean = 0.21) for cluster C vs A and B.

FST values between populations within the main clusters were lower for cluster A (0.30–0.60, mean = 0.46) than for the other two clusters (B: 0.31–0.76, mean = 0.59; C: 0.30–0.75, mean = 0.63). FST values between populations of different clusters showed similar ranges for all of them (A: 0.51–0.82, mean = 0.65; B: 0.51–0.84, mean = 0.68; C: 0.55–0.79, mean = 0.67). Clustering analysis of both IBS distances and FST values (Fig. 2) recovered the same clusters as the phylogenetic inference and subsequent analyses.

Mantel test for genomic IBS distances and uncorrected pairwise COI distances showed a strong, statistically significant correlation between both sets of values (r = 0.7956, P = 0.001). Mantel test for genomic and COI-based FST values showed a weaker, statistically significant correlation between them (r = 0.4801, P = 0.002).

These results suggest that divergence in COI sequence in the Carpetania species complex reflects to a
significant extent genetic divergence across the whole genome, supporting the use of this molecular marker as a proxy for the identification of the cryptic species. COI barcoding (Hebert, Ratnasingham, & de Waard, 2003) has been widely accepted by the scientific community as a fast, simple, and standardized method to identify, classify, and delimit species (Decaëns, Porco, Rougerie, Brown, & James, 2013). It is worth noting that lineages identified on the basis of COI distance alone must be supported with additional molecular (nuclear markers), morphological, ecological, ethological, and biogeographic evidence (Rougerie et al., 2013).

Several diagnostic SNP positions were identified: 23 for Cluster A, four for Cluster B, and eight for Cluster C (Table S1). Twenty-eight diagnostic SNP positions were assigned to the putative protein coded by the surrounding region of the variant nucleotide (Table S1). Diagnostic SNP positions were found in genes with different biological functions, as regulation, transport, response to stimuli, developmental processes, cellular processes, multicellular organismal processes, and metabolism among others.

A single diagnostic nucleotidic position was found for the 28S molecular marker, distinguishing Cluster C from the other two. No diagnostic positions were identified for the rest of the Sanger-sequenced molecular markers.

Species-diagnostic SNPs have been successfully identified in plants (Cullingham, Cooke, Dang, & Coltman, 2013) and fishes (Hand et al., 2015). Even though they may appear more difficult to use for species identification than diagnostic positions in traditional Sanger-sequenced molecular markers, the cost of GBS and RAD-sequencing has lowered significantly making their use in taxonomic studies more viable. Another alternative is the development of SNP arrays based on the previously established (through GBS/RADseq) diagnostic SNPs, as an inexpensive, automatized approach.

**Morphological analyses**

Studied Cluster A and Cluster B populations showed significantly different average body weights (3.03 grams vs 4.98 grams) according to the different statistical tests. However, Cluster C average body weight (3.89 grams) was statistically indistinguishable from the other two.

No significant differences were found between the average number of segments of the studied populations of Clusters A, B, and C, even though Cluster B showed the smallest average (248 segments) and C showed the highest (273 segments).

The difference in average values of morphometric characters is reminiscent of the ones found between *Lumbricus terrestris* and its sibling species *Lumbricus herculeus* (James et al., 2010). As found here, differences in weight and number of segments were not clear-cut, with overlapping distributions. This precludes the use of these characters in the diagnosis of the cryptic species, yet they are valuable for preliminary assignment in the field.

Character states for the relative position of spermathecae and septum 9/10 (separated/not separated by septum 9/10) showed high consistency for individuals from each population. However, those character states were not shared by all populations within the clusters.
(Fig. 3), with the exception of cluster B – where all studied populations showed spermathecae not separated by septum 9/10. Cluster A populations showed separated spermathecae, but the most basal populations within the clade showed not separated spermathecae. For cluster C this character showed no clear pattern, with two of its internal lineages showing constant states and the third containing populations with either character state. Both described species in the sister genus *Diazcosinia* possess spermathecae not separated by septum 9/10, suggesting this could be the ancestral character state for *Carpetania*. The complex evolution of this trait disallows its use as a diagnostic taxonomic character. However, it is an interesting example of multiple independent events of regression of a character state. Spermathecae not separated by septum 9/10 due to the backward displacement of the septum appears as a derived character state present in the common ancestor of *Carpetania* and *Diazcosinia*, while spermathecae separated by septum 9/10 is the most common disposition in other species of Hormogastridae and Lumbricidae. In order to disentangle the evolutionary pressures behind these changes, it would be necessary to understand the biological advantage of both character states. Even when the mechanisms of filling and release of sperm in earthworm spermathecae is not fully known, it is likely that contraction (and subsequent increased coelomic pressure) of each segment should have a role in the process. Under this assumption, spermathecae separated by a septum would function independently, while spermathecae belonging functionally to the same segment would work coordinately. This would be relevant in the context of sexual selection and hermaphroditic sexual conflict: the capability of controlling which spermathecae stores sperm from a different mate is favourable to the female part, while diminished control over this uptake would be favourable to the male part. Novo, Almodóvar, Fernández, Gutiérrez, and Díaz Cosín (2010) found no evidence of differential sperm storage from different partners in each of the four spermathecae in *Carpetania* specimens from El Molar, which according to our hypothesis should have the ability to control front and back spermathecae separately. On the other hand, different sperm storage in front and back spermathecae has been observed in other earthworms (*Lumbricus terrestris* – Koene, Pförtner, & Michiels, 2005; *Eisenia andrei* – Porto, 2014). Further research would be needed to test this hypothesis and to propose other alternatives.

**Taxonomic implications**

The genetic structuring of *Carpetania* populations into three clearly separated clusters, characterized by significant genome-wide divergence, inconspicuous but detectable morphological differences and with no admixture between them calls for the formal taxonomic description of those clusters as species. Cluster B includes the type locality of *Hormogaster (Carpetania) elisae*, hence the original description is assigned to this clade. Meanwhile, Clusters A and C are described below as new species.

**Phylum Annelida Lamarck, 1802**

**Subphylum Clitellata Michaelsen, 1919**

**Class Oligochaeta Grube, 1850**

**Superorder Megadrili Benham, 1890**

**Order Haplotaxida Michaelsen, 1900**

**Family Hormogastridae Michaelsen, 1900**

**Genus Carpetania Marchán, Fernández, Díaz Cosín & Novo, 2018**

the extension of the clitellum. Cephalic segments not imbricated.

Internal characters – First thickened septum in 6/7. Last thickened septum in 9/10. Backward displacement of dorsal insertion of septum 9/10, one or two segments. No forward displacement of dorsal insertion of septa 7/8, 8/9. Spermathecal pores in intersegments 9/10, 10/11. Spermathecae tubular, the first pair smaller, with no repetition. Seven pairs of clearly developed hearts. Three oesophageal gizzards in segments 6, 7, and 8. Five typhlosole lamellae. Genital chaetae lanceolate, with strong dorsoventral differentiation and tip ornamentation; pore present, teeth present, dorsal depression absent, ventral groves absent. First nephridia with caeca between segments 10 and 12.

_Carpetania elisae_ (Álvarez, 1977)

**Type material:** Holotype and paratypes – 12 adult and subadult individuals collected in Sigüero, Segovia, deposited by J. Álvarez in the Spanish Entomology Institute collection with numbers 4661–4669 and 46610–46612. Topotypes: 8 adults (UCMLT 00368-00375), 41.185–3.6186, from a meadow in the outskirts of the village of Sigüero, Segovia (Spain), collectors Dario J. Díaz Cosín, Marta Novo, Dolores Trigo.

**Distribution:** Northernmost community of Madrid, Southern Segovia, Southern Soria (Fig. 4). Full list of known localities is shown in Table S3.

**Phylogenetic definition:** Includes the common ancestor of all the populations assigned in Marchán et al. (2020) to the cluster B, and all its descendants. Known populations are shown in Table S3.

**Reference sequences:** COI – accession EF653893.1; 16S-tRNAs – accession GQ409710.1; 28S – accession GQ409654.1; H3 – accession HQ622033.1.

**Amended description:** External and internal morphological characters match the description of the genus _Carpetania_ in all aspects but the following.

Average weight from 1.87–7.44 (mean = 4.98). Average number of segments from 231–288
(mean = 248). Generally heavier and shorter than the other species: 'stout’ appearance after fixation. Both pairs of spermathecae not separated by septum 9/10.

**Diagnosis:** Genital chaetae with an elongated, wide tip (Fig. 5). Diagnostic nucleotidic positions shown in Table S1.

**Remarks:** This species includes specimens from Sigüero, topotypes of the holotype of *Hormogaster elisae*. Thus, the whole species must carry this name and be the type species of the genus *Carpetania*.

*Carpetania matritensis* sp. nov.

**Type material:** Holotype. Adult (UCMLT 00386), 40.7394 / -3.5647, from a meadow in the outskirts of the village of El Molar, Madrid (Spain), collectors Marta Novo, Dario J. Diaz Cosin. Paratypes. 7 adults (UCMLT 00387-00393), with the same collection data of the holotype.

**Distribution:** Northern and Central Community of Madrid, Madrid-Guadalajara border (Fig. 4). Full list of known localities are shown in Table S3.

**Phylogenetic definition:** Includes the common ancestor of all the populations assigned in Marchán et al. (2020) to the cluster A, and all its descendants. Known populations are shown in Table S3.

**Reference sequences:** COI – accession EF653876.1; 16S-tRNAs – accession JN209295.1; 28S – accession GQ409653.1; H3 – accession JN209636.1.

**Description:** External and internal morphological characters match the description of the genus *Carpetania* in all aspects but the following.

Average weight from 1.22–4.66 (mean = 3.10). Average number of segments from 203–285 (mean = 253). 'Slender' appearance after fixation. Both pairs of spermathecae separated by septum 9/10 in most populations (except in basal clades).

**Diagnosis:** Genital chaetae with a shortened tip and serrated lateral ridges (Fig. 5). Diagnostic nucleotidic positions shown in Table S1.

**Etymology:** The name of this species refers to Community of Madrid, the region to which known populations of *C. matritensis* are restricted.

*Carpetania complutensis* sp. nov.

**Type material:** Holotype. Adult (UCMLT 00376), 40.4306 – 3.925, from a open holm oak woodland in the...
outskirts of the village of Boadilla del Monte, Madrid (Spain), collectors Marta Novo, Dario J. Díaz Cosín. Paratypes. 9 adults (UCMLT 00377-00385) with the same collection data of the holotype.

**Distribution:** Southern and Central Community of Madrid, North-eastern Segovia, South-western Soria, North-western Guadalajara (Fig. 4). Full list of known localities are shown in Table S3.

**Phylogenetic definition:** Includes the common ancestor of all the populations assigned in Marchán et al. (2020) to the cluster C, and all its descendants. Known populations are shown in Table S3.

**Reference sequences:** COI – accession GQ409664.1; 16S-tRNAs – accession GQ409704.1; 28S – accession GQ409656.1; H3 – accession HQ622004.1.

**Description:** External and internal morphological characters match the description of the genus *Carpetania* in all aspects but the following.

Average weight from 1.41–5.60 (mean = 3.9). Average number of segments from 204–323 (mean = 273). Generally longer than the other species: ‘slender’ appearance after fixation. Both pairs of spermathecae separated (or not) by septum 9/10, with variation between its internal lineages.

**Diagnosis:** Genital chaetae with elongated, narrow tip (Fig. 5). Diagnostic nucleotidic positions shown in Table S1.

**Etymology:** The name of this species refers to the Universidad Complutense de Madrid, the university in which the genus *Carpetania* was studied for close to three decades.

**Remarks:** This species includes three deeply divergent lineages. Further research on their range, ecology, and other characters could merit their recognition as subspecies.

**Recommendations for species identification within Carpetania**

Morphological study of traits and character states included in the descriptions and diagnoses of the different *Carpetania* species allow a preliminary approximation to assignment of individuals. However, there is overlap between the range of morphometric characters, and scanning electron microscopy imaging of genital chaetae is demanding for untrained researchers.

The most straightforward and effective approach to species identification would consist of sequencing one or more of the proposed reference molecular markers (COI, 16S-tRNAs, 28S, H3) and their comparison with the reference sequences provided in the descriptions. This would provide a reliable assignment to one of the species.

This method does not require expertise in earthworm taxonomy and should facilitate the inclusion of these species into community and soil ecology studies. Considering existing evidence of different ecological preferences between *Carpetania* species (personal communication), it is necessary that they are not bunched together in such analyses. It is also a requisite to evaluate the conservation status of the pseudocryptic species and to preserve the diversity of this endemic genus through conservation actions.

### Carpetania within the context of the current ‘cryptic species’ debate

Several aspects of the accumulated knowledge on the *Carpetania* species complex can be discussed in the light of the recent works delving deeper in the evolutionary and conceptual meaning of cryptic species.

Fiser, Robinson, and Malard (2018) explored the relevance of recent divergence between cryptic species, finding it could explain only a small percentage of studied morphologically similar species. In the case of *Carpetania*, the three currently described species were estimated to have diverged more than 35 mya by Marchán et al. (2017): this can be considered a remarkably ancient divergence when compared with other species in the same family (most of them falling within the 10–15 mya range).

Struck et al. (2018) proposed the comparison of evolutionary rates between different species pairs within a lineage as a test for higher degrees of phenotypic similarity: in the case of the family Hormogastridae this was already explored in Novo et al. (2012) and Marchán, Novo, et al. (Marchán et al., 2016). Morphological disparity in some of the most frequently used characters in earthworm taxonomy (position of clitellum and tubercula pubertatis; position, number and type of spermathecae, number of typhlosole lamellae) was very high for other clades/genera (with a similar divergence time) when compared with *Carpetania*. This would lead to the acceptance of the species within *Carpetania* as cryptic according to the criterium proposed by Struck et al. (2018), even in the presence of some statistical morphological differences and scarce morphological diagnostic characters.

Korshunova et al. (2019) suggested that cryptic species would be expected to show high genetic similarity across the genome, with significant similarity in developmental genes explaining morphological similarity. Even though...
this statement concerned recently diverged species, the evidence from \textit{Carpetania} (with ancient diversification) contradicts that prediction according to Marchán et al. (2020) and this work. Widespread genomic divergence was found between its species, including more than 800 single nucleotide polymorphisms (several of them within developmental genes) with selection signatures and 35 diagnostic mutations of genes of diverse functions.

Another interesting conundrum presented by Korshunova et al. (2019) is the misuse or vogue use of the cryptic species term for different degrees of morphological similarity vs genetic distinctiveness. In their study case (\textit{Trinchesia caerulea} complex) morphological differences were identified \textit{a priori} to molecular data (contradicting the concept of cryptic species being morphologically indistinguishable), but technical limitations and taxonomic framework ("lumper" mindset) at the time of original description precluded their identification as non-cryptic species. In \textit{Carpetania}, its status as a cryptic/pseudo-cryptic/non-cryptic is also complex. The first work on \textit{Carpetania} genetic diversity (Novo, Almodóvar, & Díaz Cosín, 2009) followed the \textit{a priori} observation of differences in body size between the different populations. Due to incomplete sampling across the range and limited molecular marker data, these statistical differences could not be unequivocally assigned to putative species. At the time, another \textit{a priori} hint of morphological differentiation was already available: the relative position of spermathecae and septum 9/10, but it was overlooked. It was after the implementation of scanning electron microscopy to the study of genital chaetae (Marchán, Novo, et al., 2016) that a truly diagnostic morphological character could be identified (Marchán et al., 2020).

To help with the inconsistent concept and terminology of cryptic speciation, Chenuil et al. (2019) proposed a framework for identification of cryptic species together with a practical and straightforward classification. This classification relies on the crossing of two criteria: genetic isolation and morphological differentiation.

In the case of \textit{Carpetania}, genetic isolation was strongly supported by high genomic divergence and $F_{ST}$. Bayesian clustering and different phylogenetic methods. The next stage, biological species status, has not been formally demonstrated, but Marchán et al. (2017) performed a preliminary cross-breeding experiment between \textit{Carpetania elisae} and \textit{Carpetania matritensis} and found strong hints of pre- and post-copulatory reproductive isolation.

Regarding morphological differentiation, both statistical (average weight and number of segments) and diagnostic (shape of distal tip of genital chaetae) morphological differences have been described for the three species of \textit{Carpetania}.

Crossing both criteria, \textit{Carpetania} species would be considered cryptic species \textit{sensu lato}, in need of taxonomic revision as new species. Thus, this work is in agreement with the framework of Chenuil et al. (2019), which appears as a promising guide for the systematized research and evaluation of putative cryptic diversity that has been shown to be pervasive across the animal kingdom.

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**Supplemental data**

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**References**


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