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

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Abstract

Research on cryptic species complexes has reached a consensus on the necessity of integrating multiple sources of evidence. Low-coverage genomic scan techniques like 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 in a handful of Sanger-sequenced markers. Marchán et al. (submitted) delimited three well supported species-level clades on the basis of a genomewide 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.

Keywords

Genotyping-by-Sequencing; integrative taxonomy; earthworms; cryptic species; 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 & Fišer, 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; Z. Yang & Rannala, 2010; Puillandre, Lambert, Brouillet, & Achaz, 2012; Zhang, Kapli, Pavlidis, & Stamatakis, 2013; Ziheng 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 with proper cryptic species (Dupont, Porco, Symondson, & Roy, 2016).

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). The first work failed to detect differentiation between sympatric cryptic lineages, which was interpreted as these lineages not corresponding to biological species. The second work,

performed on different lineages of the same complex, found strong differentiation between them using similar analyses.

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; Shekhovtsov, Golovanova, & Peltek, 2013; Porco et al., 2018; Hormogastridae - Novo, Almodóvar, Fernández, Trigo, & Díaz Cosín, 2010; Megascolecidae - Chang, Lin, & Chen, 2008; Buckley et al., 2011, Moniligastridae - Ganin & Atopkin, 2018). Integrative taxonomy has yet to be widely employed in these complexes (Taheri et al., 2018).

One of the most studied cryptic species groups among these animals is the former *Hormogaster elisae* Álvarez, 1977, recognized as the genus *Carpetania* after Daniel Fernández Marchán et al., (2018). Six highly divergent cryptic lineages were identified using Sanger-sequenced mitochondrial and nuclear markers (Daniel F. 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 (Daniel F. Marchán, Sánchez, 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 rich genome-wide SNP dataset was obtained through GBS for seventeen populations and 85 individuals of *Carpetania* by Marchán et al. (submitted), 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 together with geometric morphometrics analysis (Fig. 1), finding congruent support for three species-level genetic clusters (hereafter cluster A, B and C), which comprised one or more of the six previously identified lineages as described in Daniel F. Marchán et al., (2017).

Figure 1.

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). To this end, this work will build upon the framework provided by Marchán et al. (submitted), with the following objectives: i) identify genome-wide nucleotidic 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

SNP data comprising the geographical distribution and internal lineages of *Carpetania* was generated in Marchán et al. (submitted). In brief, GBS libraries were generated and sequenced for seventeen populations with five individuals from each totalling 85 individuals. Different datasets were generated with STACKS2 (Rochette, Rivera-Colón, & Catchen, n.d.) based on: *de novo* assembly/mapping to a reference transcriptome of *C. elisae* ('reference-all SNPs' dataset) and inclusion of all SNPs/one random SNP per locus ('de novo-one SNP dataset').

'de novo-one SNP' and 'reference-all SNPs' datasets were further analysed in this work to estimate genetic diversity and to identify 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 (F_{ST}). These parameters were obtained from the *populations* function (STACKS2 package) summary files. Correlation between SNP-based genetic distances and F_{ST} and the same parameters obtained from cytochrome C oxidase 1 sequences (Daniel F. 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 nucleotide positions were also identified in the molecular markers COI, 16S-tRNAs, 28S and H3 retrieved from (Daniel F. Marchán et al., 2017) following the same method.

Morphological data

-External anatomy and morphometric characters

External anatomy characters commonly used in earthworm taxonomy were studied, with special attention to average weight and number of segments (found to have strong phylogenetic signal in Hormogastridae -Daniel F. 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, with special attention to the only variable internal character in the *Carpetania* species complex, relative position of septum 9/10 and spermathecae. In this genus, septum 9/10 can appear displaced backwards (to 10/11) in its dorsal insertion, resulting in the spermathecae 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 F_{ST} values) are represented in Fig. 2 (values can be found in suppl. Table 1). 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.

F_{ST} 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). F_{ST} 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 F_{ST} 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 F_{ST} 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 biogeographical evidence (Rougerie et al., 2009).

Figure 2.

Several diagnostic SNP positions were identified: twenty-three for Cluster A, four for Cluster B and eight for Cluster C (table 1). Twenty-eight diagnostic SNP positions were assigned to the putative protein coded by the surrounding region of the variant nucleotide (table 1). 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 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 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 celomic 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, & 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.

Figure 3.

-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

Description:

External characters- Average number of segments from 203 to 323. Average weight from 1.22 g to 7.6 grams. Clitellum in segments (12)13-27. Tubercula pubertatis in segments 22-25. Pigmentation absent, color: greyish-fleshy. Cephalic keels present, moderately developed. No lateral expansions of the clitellum. Chaetae disposition geminate. No genital papillae in cd. Posterior genital papillae constrained within 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 grooves absent. First nephridia with caeca between segments 10 and 12

***Names of the species are placeholders to avoid taxonomic conflicts once the manuscript is published.**

Carpetania **species B*** Álvarez 1977

Type material: Holotype and paratypes - 12 adult and subadult individuals collected in Siguero, 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 Siguero, Segovia (Spain), collectors Darío J. Díaz Cosín, Marta Novo, Dolores Trigo.

Distribution: Northernmost Community of Madrid, Southern Segovia, Southern Soria (Fig. 4). Full list of known localities are shown in Suppl. table 2.

Phylogenetic definition: includes the common ancestor of all the populations assigned in Marchán et al. (submitted) to the cluster B, and all its descendants. Known populations are shown in Suppl. table 2.

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

Description:

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

Average weight from 1.87 to 7.44 (mean= 4.98). Average number of segments from 231 to 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 1.

Remarks: this species includes specimens from Siguero, 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 **species A*** Marchán, Novo, Fernández and Díaz Cosín 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, Darío J. Díaz Cosín. 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 Suppl. table 2.

Phylogenetic definition: includes the common ancestor of all the populations assigned in Marchán et al. (submitted) to the cluster A, and all its descendants. Known populations are shown in Suppl. table 2.

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 to 4.66 (mean= 3.10). Average number of segments from 203 to 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 1.

Carpetania **species C*** Marchán, Novo, Fernández and Díaz Cosín 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, Darío J.

Díaz Cosín. Paratypes. 9 adults (UCMLT 00377-00385) with the same collection data of the holotype

Distribution: Southern and Central Community of Madrid, Northeastern Segovia, Southwestern Soria, Northwestern Guadalajara (Fig. 4). Full list of known localities are shown in Suppl. table 2.

Phylogenetic definition: includes the common ancestor of all the populations assigned in Marchán et al. (submitted) to the cluster C, and all its descendants. Known populations are shown in Suppl. table 2.

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 to 5.60 (mean= 3.9). Average number of segments from 204 to 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 1.

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

Figure 4.

Figure 5.

Table 1.

-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 on 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.

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References

- Anderson, C., Cunha, L., Sechi, P., Kille, P., & Spurgeon, D. (2017). Genetic variation in populations of the earthworm, *Lumbricus rubellus*, across contaminated mine sites. *BMC Genetics*, 18(1), 97.
- Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., ... Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, 22(3), 148–155.
- Brown, S. D. J., Collins, R. A., Boyer, S., Lefort, M.-C., Malumbres-Olarte, J., Vink, C. J., & Cruickshank, R. H. (2012). Spider: An R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. *Molecular Ecology Resources*, Vol. 12, pp. 562–565. <https://doi.org/10.1111/j.1755-0998.2011.03108.x>
- Brunet, B. M. T., Blackburn, G. S., Muirhead, K., Lumley, L. M., Boyle, B., Lévesque, R. C., ... Sperling, F. A. H. (2017). Two's company, three's a crowd: new insights on spruce budworm

species boundaries using genotyping-by-sequencing in an integrative species assessment

(Lepidoptera: Tortricidae). *Systematic Entomology*, Vol. 42, pp. 317–328.

<https://doi.org/10.1111/syen.12211>

Buckley, T. R., James, S., Allwood, J., Bartlam, S., Howitt, R., & Prada, D. (2011). Phylogenetic analysis of New Zealand earthworms (Oligochaeta: Megascolecidae) reveals ancient clades and cryptic taxonomic diversity. *Molecular Phylogenetics and Evolution*, 58(1), 85–96.

Chang, C.-H., Lin, S.-M., & Chen, J.-H. (2008). Molecular systematics and phylogeography of the gigantic earthworms of the Metaphire formosae species group (Clitellata, Megascolecidae). *Molecular Phylogenetics and Evolution*, 49(3), 958–968.

Cunningham, C. I., Cooke, J. E. K., Dang, S., & Coltman, D. W. (2013). A species-diagnostic SNP panel for discriminating lodgepole pine, jack pine, and their interspecific hybrids. *Tree Genetics & Genomes*, Vol. 9, pp. 1119–1127. <https://doi.org/10.1007/s11295-013-0608-x>

Decaëns, T., Porco, D., Rougerie, R., Brown, G. G., & James, S. W. (2013). Potential of DNA barcoding for earthworm research in taxonomy and ecology. *Applied Soil Ecology*, Vol. 65, pp. 35–42. <https://doi.org/10.1016/j.apsoil.2013.01.001>

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science*, Vol. 14, p. 927. [https://doi.org/10.1658/1100-9233\(2003\)014\[0927:vaporf\]2.0.co;2](https://doi.org/10.1658/1100-9233(2003)014[0927:vaporf]2.0.co;2)

Dupont, L., Porco, D., Symondson, W. O. C., & Roy, V. (2016). Hybridization relics complicate barcode-based identification of species in earthworms. *Molecular Ecology Resources*, 16(4), 883–894.

Fernández, R., Almodóvar, A., Novo, M., Simancas, B., & Díaz Cosín, D. J. (2012). Adding complexity to the complex: new insights into the phylogeny, diversification and origin of parthenogenesis in the Aporectodea caliginosa species complex (Oligochaeta, Lumbricidae). *Molecular Phylogenetics and Evolution*, 64(2), 368–379.

Ganin, G. N., & Atopkin, D. M. (2018). Molecular differentiation of epigeic and anecic forms of *Drawida ghilarovi* Gates, 1969 (Moniligastridae, Clitellata) in the Russian Far East: Sequence data of two mitochondrial genes. *European Journal of Soil Biology*, Vol. 86, pp. 1–7. <https://doi.org/10.1016/j.ejsobi.2018.02.004>

- Garg, K. M., Tizard, R., Ng, N. S. R., Cros, E., Dejtaradol, A., Chattopadhyay, B., ... Rheindt, F. E. (2016). Genome-wide data help identify an avian species-level lineage that is morphologically and vocally cryptic. *Molecular Phylogenetics and Evolution*, 102, 97–103.
- Giska, I., Sechi, P., & Babik, W. (2015). Deeply divergent sympatric mitochondrial lineages of the earthworm *Lumbricus rubellus* are not reproductively isolated. *BMC Evolutionary Biology*, Vol. 15. <https://doi.org/10.1186/s12862-015-0488-9>
- Hand, B. K., Hether, T. D., Kovach, R. P., Muhlfeld, C. C., Amish, S. J., Boyer, M. C., ... Luikart, G. (2015). Genomics and introgression: Discovery and mapping of thousands of species-diagnostic SNPs using RAD sequencing. *Current Zoology*, Vol. 61, pp. 146–154. <https://doi.org/10.1093/czoolo/61.1.146>
- Hebert, P. D. N., Ratnasingham, S., & de Waard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, Vol. 270. <https://doi.org/10.1098/rsbl.2003.0025>
- Huerta-Cepas, J., Forslund, K., Coelho, L. P., Szklarczyk, D., Jensen, L. J., von Mering, C., & Bork, P. (2017). Fast Genome-Wide Functional Annotation through Orthology Assignment by eggNOG-Mapper. *Molecular Biology and Evolution*, 34(8), 2115–2122.
- James, S. W., Porco, D., Decaëns, T., Richard, B., Rougerie, R., & Erséus, C. (2010). DNA barcoding reveals cryptic diversity in *Lumbricus terrestris* L., 1758 (Clitellata): resurrection of *L. herculeus* (Savigny, 1826). *PloS One*, 5(12), e15629.
- Jörger, K. M., & Schrödl, M. (2013). How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology*, 10(1), 59.
- King, R. A., Andrew King, R., Tibble, A. L., & Symondson, W. O. C. (2008). Opening a can of worms: unprecedented sympatric cryptic diversity within British lumbricid earthworms. *Molecular Ecology*, Vol. 17, pp. 4684–4698. <https://doi.org/10.1111/j.1365-294x.2008.03931.x>
- Knowlton, N. (1993). Sibling Species in the Sea. *Annual Review of Ecology and Systematics*, Vol. 24, pp. 189–216. <https://doi.org/10.1146/annurev.es.24.110193.001201>
- Koene, J. M., Pfortner, T., & Michiels, N. K. (2005). Piercing the partner's skin influences sperm uptake in the earthworm *Lumbricus terrestris*. *Behavioral Ecology and Sociobiology*, Vol. 59, pp.

243–249. <https://doi.org/10.1007/s00265-005-0030-y>

Lajus, D., Sukhikh, N., & Alekseev, V. (2015). Cryptic or pseudocryptic: can morphological methods inform copepod taxonomy? An analysis of publications and a case study of the *Eurytemora affinis* species complex. *Ecology and Evolution*, Vol. 5, pp. 2374–2385.

<https://doi.org/10.1002/ece3.1521>

León, G. P.-P. de, de León, G. P.-P., & Nadler, S. A. (2010). What We Don't Recognize Can Hurt Us: A Plea for Awareness About Cryptic Species. *Journal of Parasitology*, Vol. 96, pp. 453–464.

<https://doi.org/10.1645/ge-2260.1>

Marchán, D. F., Fernández, R., de Sosa, I., Díaz Cosín, D. J., & Novo, M. (2017). Pinpointing cryptic borders: Fine-scale phylogeography and genetic landscape analysis of the *Hormogaster elisae* complex (Oligochaeta, Hormogastridae). *Molecular Phylogenetics and Evolution*, 112, 185–193.

Marchán, D. F., Fernández, R., de Sosa, I., Sánchez, N., Díaz Cosín, D. J., & Novo, M. (2018).

Integrative systematic revision of a Mediterranean earthworm family: Hormogastridae (Annelida, Oligochaeta). *Invertebrate Systematics*, Vol. 32, p. 652.

<https://doi.org/10.1071/is17048>

Marchán, D. F., Novo, M., Fernández, R., de Sosa, I., Trigo, D., & Díaz Cosín, D. J. (2016).

Evaluating evolutionary pressures and phylogenetic signal in earthworms: a case study - the number of typhlosole lamellae in Hormogastridae (Annelida, Oligochaeta). *Zoological Journal of the Linnean Society*, Vol. 178, pp. 4–14. <https://doi.org/10.1111/zoj.12410>

Marchán, D. F., Sánchez, N., Novo, M., Fernández, R., Pardos, F., & Díaz Cosín, D. J. (2016).

Cryptic characters for cryptic taxa: On the taxonomic utility of the genital chaetae in earthworms (Oligochaeta, Hormogastridae). *Zoologischer Anzeiger - A Journal of Comparative Zoology*, Vol. 264, pp. 17–28. <https://doi.org/10.1016/j.jcz.2016.06.008>

Novo, M., Almodóvar, A., Fernández, R. M., Gutiérrez, M., & Díaz Cosín, D. J. (2010). Mate choice of an endogamic earthworm revealed by microsatellite markers. *Pedobiologia*, Vol. 53, pp. 375–379. <https://doi.org/10.1016/j.pedobi.2010.07.002>

Novo, M., Almodóvar, A., Fernández, R., Trigo, D., & Díaz Cosín, D. J. (2010). Cryptic speciation of hormogastrid earthworms revealed by mitochondrial and nuclear data. *Molecular Phylogenetics*

- and Evolution*, Vol. 56, pp. 507–512. <https://doi.org/10.1016/j.ympev.2010.04.010>
- Nygren, A. (2014). Cryptic polychaete diversity: a review. *Zoologica Scripta*, Vol. 43, pp. 172–183. <https://doi.org/10.1111/zsc.12044>
- Pfenninger, M., & Schwenk, K. (2007). Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. *BMC Evolutionary Biology*, 7, 121.
- Pons, J., Barraclough, T. G., Gomez-Zurita, J., Cardoso, A., Duran, D. P., Hazell, S., ... Vogler, A. P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, 55(4), 595–609.
- Porco, D., Chang, C.-H., Dupont, L., James, S., Richard, B., & Decaëns, T. (2018). A reference library of DNA barcodes for the earthworms from Upper Normandy: Biodiversity assessment, new records, potential cases of cryptic diversity and ongoing speciation. *Applied Soil Ecology*, Vol. 124, pp. 362–371. <https://doi.org/10.1016/j.apsoil.2017.11.001>
- Porto, P. G. (2014). *La selección sexual post-cópula en la lombriz roja (Eisenia andrei Bouché, 1972)* (Doctoral dissertation, Universidade de Vigo).
- Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology*, 21(8), 1864–1877.
- Queiroz, K. D., & De Queiroz, K. (2007). Species Concepts and Species Delimitation. *Systematic Biology*, Vol. 56, pp. 879–886. <https://doi.org/10.1080/10635150701701083>
- Rancilhac, L., Goudarzi, F., Gehara, M., Hemami, M.-R., Elmer, K. R., Vences, M., & Steinfarz, S. (2019). Phylogeny and species delimitation of near Eastern Neurergus newts (Salamandridae) based on genome-wide RADseq data analysis. *Molecular Phylogenetics and Evolution*, 133, 189–197.
- Rochette, N. C., Rivera-Colón, A. G., & Catchen, J. M. (n.d.). *Stacks 2: Analytical Methods for Paired-end Sequencing Improve RADseq-based Population Genomics*. <https://doi.org/10.1101/615385>
- Rougerie, R., Decaëns, T., Deharveng, L., Porco, D., James, S. W., Chang, C.-H., ... Hebert, P. D. N. (2009). DNA barcodes for soil animal taxonomy. *Pesquisa Agropecuária Brasileira*, Vol. 44, pp. 789–802. <https://doi.org/10.1590/s0100-204x2009000800002>

- Shekhovtsov, S. V., Golovanova, E. V., & Peltek, S. E. (2013). Cryptic diversity within the Nordenskiöld's earthworm, *Eisenia nordenskioldi* subsp. *nordenskioldi* (Lumbricidae, Annelida). *European Journal of Soil Biology*, Vol. 58, pp. 13–18.
<https://doi.org/10.1016/j.ejsobi.2013.05.004>
- Taheri, S., James, S., Roy, V., Decaëns, T., Williams, B. W., Anderson, F., ... Dupont, L. (2018). Complex taxonomy of the “brush tail” peregrine earthworm *Pontoscolex corethrurus*. *Molecular Phylogenetics and Evolution*, 124, 60–70.
- Trontelj, P., & Fišer, C. (2009). Perspectives: Cryptic species diversity should not be trivialised. *Systematics and Biodiversity*, Vol. 7, pp. 1–3. <https://doi.org/10.1017/s1477200008002909>
- Wang, Y., Zhou, Q.-S., Qiao, H.-J., Zhang, A.-B., Yu, F., Wang, X.-B., ... Zhang, Y.-Z. (2016). Formal nomenclature and description of cryptic species of the *Encyrtus sasakii* complex (Hymenoptera: Encyrtidae). *Scientific Reports*, 6, 34372.
- Yang, Z. (2015). The BPP program for species tree estimation and species delimitation. *Current Zoology*, Vol. 61, pp. 854–865. <https://doi.org/10.1093/czoolo/61.5.854>
- Yang, Z., & Rannala, B. (2010). Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences*, Vol. 107, pp. 9264–9269.
<https://doi.org/10.1073/pnas.0913022107>
- Zhang, J., Kapli, P., Pavlidis, P., & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics*, 29(22), 2869–2876.

Tables

SNP	Scaffold		Base	Protein
Cluster A				
344	COMP190067_C0_SEQ1	395	T/C	
727	COMP206745_C0_SEQ1	527	G/A	Retinol binding protein
784	COMP207582_C0_SEQ2	792	G/C	SWAP switching B-cell complex 70kDa subunit
949	COMP209848_C1_SEQ1	269	T/G	
1009	COMP210899_C0_SEQ5	908	A/T	Zinc finger, FYVE domain containing 20
1113	COMP211847_C2_SEQ1	3393	A/G	ATP-binding cassette, subfamily B (MDR TAP), member
1291	COMP213230_C0_SEQ4	658	T/C	
1409	COMP213929_C0_SEQ2	1505	A/G	nuclear respiratory factor 1
1425	COMP214029_C0_SEQ1	1951	T/C	NADP-dependent oxidoreductase domain containing 1
1624	COMP215394_C0_SEQ1	978	T/C	
1784	COMP216453_C0_SEQ4	1281	G/C	
1814	COMP216630_C0_SEQ7	1725	C/T	leucine rich repeat and Ig domain containing 4
1842	COMP216828_C1_SEQ13	4668	G/C	Protein phosphatase 1 regulatory subunit 37
2127	COMP218319_C1_SEQ44	2385	T/C	Armadillo repeat containing 4
2169	COMP218535_C0_SEQ2	1141	C/G	Enzyme required for electron transfer from NADP to cytochrome P450
2267	COMP219108_C2_SEQ1	3021	A/G	SH3-domain binding protein 5 (BTK-associated)
2417	COMP219800_C0_SEQ6	2892	A/C	CREB regulated transcription coactivator 1
2609	COMP220400_C0_SEQ6	2547	A/C	Nucleoporin 54kDa
2639	COMP220522_C0_SEQ13	4055	G/T	ubiquitin- specific peptidase
2652	COMP220555_C0_SEQ8	3693	T/C	integrator complex subunit 1
2712	COMP220746_C1_SEQ15	359	T/C	crumbs homolog 2 (Drosophila)
2800	COMP220908_C0_SEQ3	6612	A/T	small nuclear RNA activating complex, polypeptide 4, 190kDa
3071	COMP62959_C0_SEQ1	45	A/G	
Cluster B				
195	COMP1730661_C0_SEQ1	182	T/G	

1410	COMP213937_C2_SEQ1	662	T/C	Solute carrier family 25 member
1512	COMP214730_C0_SEQ1	1067	T/G	Peptidase family M41
2800	COMP220908_C0_SEQ3	6612	T/A	small nuclear RNA activating complex, polypeptide 4, 190kDa
Cluster C				
491	COMP200542_C1_SEQ2	499	A/C	Galactose binding lectin domain
782	COMP207582_C0_SEQ2	710	G/T	SWAP switching B-cell complex 70kDa subunit
893	COMP209092_C1_SEQ1	734	A/T	phosphodiesterase PDE8B
1277	COMP213187_C0_SEQ1	867	G/A	Organic Anion Transporter Polypeptide (OATP) family
2169	COMP218535_C0_SEQ2	1141	G/C	Enzyme required for electron transfer from NADP to cytochrome P450
2397	COMP219681_C0_SEQ1	375	G/A	PRP40 pre-mRNA processing factor 40 homolog
2430	COMP219875_C0_SEQ3	3810	C/T	collagen, type XII, alpha 1
2776	COMP220864_C4_SEQ4	4882	G/A	kiaa2026

Table 1. Diagnostic nucleotidic positions for the three species of the genus *Carpetania*. First nucleotide is the character state found on the species and the second nucleotide is the state for the rest of the species. Putative protein coded by the contig in where diagnostic positions are located is indicated when annotation is available.

Figure captions

Figure 1. Different approaches to genetic structure identification and species delimitation in the *Carpetania* complex (modified from Marchán et al. submitted). Previously defined cryptic lineages (Daniel F. Marchán et al., 2017) are represented by a colour and roman number. Color codes and nomenclature are kept throughout the manuscript. a) Maximum likelihood inference of the phylogenetic relationships of the studied populations of *Carpetania*. Solid outlines indicate the species-level genetic clusters (A, B, C) found by the other analyses b) Principal Component Analysis (PCA) c) Barplot of STRUCTURE analysis. Each color shows percentage of assignment to a cluster or ancestral population. d) Canonical variate analysis of the shape of the genital chaetae of *Carpetania* main genetic clusters.

Figure 2. Heatmap and clustering graphs displaying F_{ST} values (left) and Identity by state (IBS) distances (right) between populations of *Carpetania* obtained from genome-wide SNPs.

Figure 3. Relative position of spermathecae and septum 9/10 for the populations included in the phylogenetic analysis. A hypothetical position of the sister genus *Diazcossinia* (not included in the phylogenetic reconstruction) and its character state are shown by a dashed line. Lineages from (Daniel F. Marchán et al., 2017) are shown with the same code and colors (roman numbers, dashed outlines). Species-level clusters are represented by solid outlines and letters A, B, C.

Figure 4. Known distribution of the three species of *Carpetania*.

Figure 5. Genital chaetae distal end from representatives of the three species in *Carpetania*: a) *Carpetania* **species B**, b) *Carpetania* **species A**, c) *Carpetania* **species C**. Black and white dots show the landmarks used in geometric morphometrics analyses for reference. Scale bar: 10 μ m.

a

b

c