Complex taxonomy of the 'brush tail' peregrine earthworm *Pontoscolex corethrurus*

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ABSTRACT

Pontoscolex corethrurus is the most widespread earthworm species in tropical and sub-tropical zones and one of the most studied in soil science. Although, ecological interactions of *P. corethrurus* with its environment are well documented, the taxonomic status of the species remains unclear. In this study, we investigated phylogenetic relationships within the genus *Pontoscolex*, in particular focusing on morphologically indistinguishable (i.e., cryptic) lineages. A total of 792 specimens collected from 25 different countries and islands all over the world were analyzed using two mitochondrial (COI and 16S rDNA) and two nuclear (internal transcribed spacers 2 and 28S rDNA) markers, and a total of 11 morphological characters both internal and external were investigated in all genetically characterized lineages. A large-scale multilocus sequence data matrix was also obtained for *Pontoscolex* spp. specimens using the Anchored Hybrid Enrichment (AHE) method. Multilocus phylogenetic and phylogenomic analyses, combined with species delimitation methods; including single locus (mPTP, ABGD) and multilocus (BPP) approaches, revealed congruent results. Four cryptic species were supported within the *P. corethrurus* species complex, and four potentially new species within the genus *Pontoscolex*. One widespread lineage (L1), within *P. corethrurus* complex was observed in the current population of Fritz Müller's garden where *P. corethrurus* was first described in 1856. Cryptic lineages were observed in sympatry at several localities. This, in combination with observed heteroplasmy in COI gene in one population raises an important question of reproductive isolation between these species.

1. Introduction

Speciation not accompanied by morphological changes results in

formation of cryptic species (Bickford et al., 2007). Although this phenomenon has been recognized for centuries (Winker, 2005), application of molecular approaches over the last few decades has provided a

rapid and increasingly inexpensive means for unraveling these taxonomic challenges. Bickford et al. (2007) emphasized that strong environmental constraints may drive morphological stasis through stabilizing selection or adaptation to specific hosts (i.e., strong selection on behavioral or physiological characters). An alternative hypothesis is that cryptic species might be common in environments that hamper transmission of visual signals. In soil for instance, chemical signaling may play a more crucial role than morphological traits in sexual recognition (Novo et al., 2013).

Over the past decade, several genetically distinct, yet morphologically indistinguishable lineages have been identified within several earthworm morphospecies, including; *Hormogaster elisae* Alvarez, 1977 (Marchán et al., 2017; Novo et al., 2010, 2009), *Metaphire paiwanna* Tsai et al., 2000 (Chang et al., 2008), *Allolobophora chlorotica* Savigny, 1826 (Dupont et al., 2011; King et al., 2008), *Aporrectodea caliginosa* Savigny, 1826 (Pérez-Losada et al., 2009), *Lumbricus terrestris* Linnaeus, 1758 (James et al., 2010), *Lumbricus rubellus* Hoffmeister, 1843 (Donnelly et al., 2013), *Pontoscolex corethrurus* Müller, 1856 (Cunha et al., 2014), *Eisenia nordenskioldi* Eisen, 1879 (Shekhovtsov et al., 2016a, 2013), and *Eisenia nordenskioldi pallida* Malevick, 1956 (Shekhovtsov et al., 2016b). These cryptic species were rarely observed in sympatry or occupying the same ecological niche within the same habitat (King et al., 2008). Competition theory predicts that morphologically similar species should not coexist (Chesson, 1991), even though coexistence could be facilitated by differences in their biology (Zhang et al., 2004) and ecology. For instance, some of *A. chlorotica* lineages overlap in geographic distribution (i.e., sympatry) but are allotopic (i.e., do not occupy the same macrohabitat; Dupont et al., 2016) and have contrasting ecological preferences (Lowe and Butt, 2007).

Even in the rare cases where cryptic earthworm species have been found in sympatry, hybridization is uncommon. Limited hybridization has been, however, documented between *Lumbricus terrestris* and *L. herculeus* (Martinsson and Erséus, 2017), which were considered as one for a long time (James et al., 2010). Martinsson and Erséus (2017) have also detected limited hybridization among *L. rubellus* cryptic lineages using one nuclear and one mitochondrial gene. Similarly, Dupont et al. (2016) demonstrated that hybridization events were rare between lineages within the *A. chlorotica* species complex, but that historical hybridization can be inferred from patterns of mitochondrial introgression.

These complexes of cryptic earthworm species are mostly known from temperate and subtropical regions whereas a few cases of cryptic diversity were reported in tropical zone (Chang et al., 2008, 2007; Chang and Chen, 2005). Here, we investigated the genetic diversity within specimens that are commonly attributed to *Pontoscolex corethrurus* (Müller, 1856), a pantropical earthworm that is tolerant to a wide range of biotic and abiotic environmental conditions, traits that make it a successful colonizer (Fragoso et al., 1999; Lavelle et al., 1987). It is believed that *P. corethrurus* reproduces by parthenogenesis, however sexual reproduction is also possible (review in Taheri et al., 2018). *P. corethrurus* belongs to the family Rhinodrilidae (James, 2012), and it is believed to have originated from the Guayana shield area (Righi, 1984). A total of 20 nominal species have been formally described in the genus *Pontoscolex* on the basis of morphological diagnoses (Feijoo and Celis, 2012; Moreno, 2004). They were clustered in three distinct subgenera: *Pontoscolex*, *Meroscolex* and *Mesoscolex* (Borges, 1992). *P. (Pontoscolex) corethrurus* is the most widespread earthworm in the tropics (Plisko, 2001; Römbke et al., 2009) and subtropics (Taheri et al., 2018). It was described for the first time in 1856 by Fritz Müller from individuals collected in Itajaí (currently Blumenau), in Santa Catarina state, southern Brazil (Müller, 1856). The quincunx formation of setae on the last quarter of the body is the most commonly used character for the diagnosis of this morphospecies (Fig. 1A).

Pontoscolex corethrurus has been extensively used as a biological model in soil science (Duarte et al., 2012; Fragoso et al., 1997; Ganihar, 2003; García and Fragoso, 2002; Henrot and Brussaard, 1997; Jusselme et al., 2012; Liang et al., 2011; Moreno, 2004; Nath and Chaudhuri, 2012; Plisko, 2001; Senapati et al., 1999; Topoliantz et al., 2002). Because of their major roles in many aspects of soil fertility, food web ecology and ecosystem functioning, earthworms are common subjects of ecological and toxicological researches (Edwards, 2004; King et al., 2008). A comprehensive knowledge of boundaries between species is then crucial when using earthworms as biological models, as different species may have different impacts on their environments and respond differently to environmental stresses (Domínguez et al., 2005; Donnelly et al., 2013; Kille et al., 2013; Römbke et al., 2016; Voua Otomo et al., 2013). Despite the widespread interest by scientists in *P. corethrurus*, only three studies investigated the genetic characteristics of this morphospecies (Conrado et al., 2017; Cunha et al., 2014; Dupont et al., 2012). Interestingly, Cunha et al. (2014) found two genetically divergent lineages within *P. corethrurus* samples from the island of São Miguel (Azores) suggesting for the first time the existence of cryptic species in this morphospecies. They showed that the lineage found in the Furnas volcano could cope with extreme conditions found in the caldera, where it tolerates a mixture of non-anthropogenic chemical and physical stresses.

The objectives of the present study were to characterize the genetic structure and diversity within the *P. corethrurus* complex at a global scale, and to use these data to infer phylogenetic placement of the type material of *P. corethrurus* described by Fritz Müller. For this purpose, we reconstructed phylogenetic relationships among lineages belonging to the genus *Pontoscolex* using two mitochondrial and two nuclear genes and checked our results using a large-scale multilocus sequence data matrix obtained for a restricted number of samples. Moreover, we examined morphological traits generally used to identify *P. corethrurus* to assess their taxonomic value.

2. Material and methods

2.1. Phylogenetic approach

2.1.1. Samples

A total of 792 earthworms morphologically close to *P. corethrurus* were collected by soil scientists and biologists from 25 countries and islands in tropical, subtropical and temperate zones (Table 1). These countries and islands included Fiji, Gabon, Madagascar, Brazil, Cuba, Dominican, French Guiana, Jamaica, Mexico, Peru, St. Croix, St. Kitts, St. Lucia, St. Vincent, Trinidad and Tobago, Martinique, Hawaii, India, Malaysia, Philippines, Taiwan, Thailand, and the Azores, which in total represent 6 of the biogeographic realms used in Hendrix et al. (2008). Specimens from the two localities of Azores studied by Cunha et al. (2014) were added to our samples. Between 1 and 22 sites were sampled in each country or island (Fig. 2 and Table A.1 of Supplementary data). One of the sampling sites was Fritz Müller's garden, the probable type locality from where this species was first described in 1856 (Müller, 1856). Individuals from this site were considered as topotypes (i.e., a specimen originating from the type locality of the species or subspecies to which it is thought to belong) for *P. corethrurus*. GenBank sequences for mitochondrial and nuclear markers corresponding to *P. corethrurus* were also added to our data, including cytochrome oxidase I (COI) (JN036370, JQ279700, AB543229, JN887898, JN185607, JQ279698, JQ279699, JN887896, JN887897), 16S rDNA gene (JN793524, AB543235, JN887906, JN887905), and the 28S rDNA gene (AY101571, KJ912259). These sequences were from Australia, Brazil, French Guiana, India and Japan. Two specimens of *P. (Pontoscolex) spiralis* (only known from a few locations in the Caribbean; Borges and Moreno, 1990; James and Gamiette, 2016) from Guadeloupe and Puerto Rico were added. This species has a distinct morphology making it easy to recognize and was used as a reference point in the genus *Pontoscolex*. After collection, specimens were washed in distilled water, and preserved in 100% ethanol.
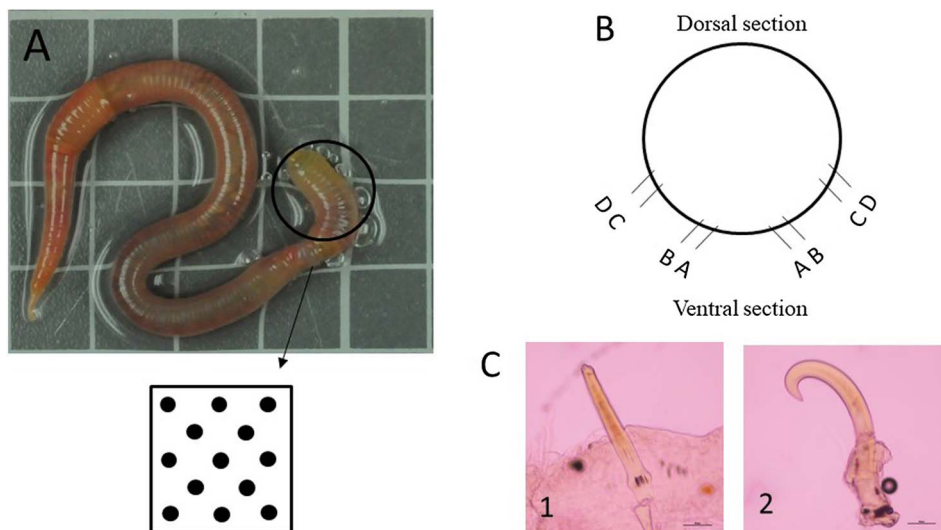
Fig. 1. Morphological characteristics of *Pontoscolex* spp.: (A) Quincunx formation of setae observed on the last quarter of some species within *Pontoscolex* genus. A characteristic used to diagnose. *P. corethrurus*; (B) A, B, C, and D setae positions, on top is the dorsal and on down the ventral part of an earthworm and (C) Hooked-setae in comparison with non-hooked-setae. Both pictures represent lateral views. (1) setae of an individual from the Philippines (Tawi Tawi island, L1). (2) setae of an individual from French Guiana (Pararé C, *P.* sp. 1).

2.1.2. DNA extraction, amplification, and sequencing

A 20-mg piece of dorsal tegument was cut between the tail and the clitellum. DNA extraction was performed using the Nucleospin tissue kit (Macherey Nagel, France). Two mitochondrial regions; fragments of the cytochrome oxidase I (COI) and the 16S-rDNA genes, and two nuclear sequences; a fragment of the 28S-rDNA gene and internal transcribed spacer 2 (ITS2), were amplified. The COI fragment corresponds to the standard DNA barcode for animals (Hebert et al., 2003) and was amplified using primers LCO1490 (5′-GGTCAACAAATCATAAAGATAT TGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). The PCR reaction mixture contained 10 ng of DNA template, 0.2 µM of each primer, 0.5 mM of dNTPs, 1 mg/ml of BSA, 2.5 mM of MgCl$_2$, 1U of Flexi*Taq* polymerase (Promega, France) and 1X of *Taq* buffer, in a total volume of 25 µl. PCR conditions were: an initial denaturation step at 94 °C for 3 min and 10 amplification cycles at 94 °C for 30 s, 44 °C for 45 s, 72 °C for 60 s; another 30 cycles of amplification at 94 °C for 30 s, 50 °C for 45 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. For specimens showing poor PCR success, two alternate COI primer pairs were used; the first one was LepF (5′-ATTCAAC CAATCATAAAGATATTGG-3′) and LepR (5′-TAAACTTCTGGATGTCCA AAAAATCA-3′) (Hajibabaei et al., 2006), and the second one, designed from this study for the genus *Pontoscolex* using Primer3 (http://www. simgene.com/Primer3), was *Ponto*F (5′-CTAGGAGTGTGGGCTGG AAT-3′) and *Ponto*R (5′-AGCAGGATCAAAGAAGGAGGT-3′). The 16S rDNA gene fragment was amplified using primers LR-J-12887 (5′-CCG GTCTGAACTCAGATCACGT-3′) (Palumbi et al., 2002) and LR-N-13398 (5′-CGCCTGTTTAACAAAAACAT-3′) (Simon et al., 1994). The ITS2 fragment was amplified using primers 5.8Smuss-F (5′-CGCAGCCAGCT GCGTGAATTAATGT-3′) (Kallersjo et al., 2014) and ETTS1-R (5′-GCTT AAGTTCAGCGGGT-3′) (Vilgalys and Hester, 1990) and the 28S rDNA fragment was amplified using primers F1 (5′-GAGTACGTGAAACCGTC TAG-3′) and R1 (5′-CGTTTCGTCCCCAAGGCCTC-3′) (Pérez-Losada et al., 2009). For all primer pairs, PCR conditions were: an initial denaturation step at 94 °C for 3 min and 40 amplification cycles with 94 °C for 30 s, annealing temperature differed between primers; i.e., COI Lep at 49 °C, 16S and ITS2 at 50 °C, 28S at 54 °C and COI *Ponto* at 55 °C for 45 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were purified, and forward and reverse strands sequenced by Eurofins (https://www.eurofinsgenomics.eu/). Sequences were aligned and edited manually using BioEdit v.7.1.9 (Hall, 1999) and Seaview v.4.5.4 (Gouy et al., 2010). For the protein-coding gene COI, alignment was translated to amino acids using MEGA 6 (Tamura et al., 2013) to detect frame-shift mutations or stop codons; which may indicate the presence of pseudogenes. In cases of ambiguous sequencing results (e.g., ambiguous base calls, unexpected position of a sequence in the

reconstructed tree), DNA was re-extracted and re-sequenced. Heterozygous positions were either treated as missing data (N) for phylogenetic reconstructions or considered as informative polymorphism (used for phasing, see below) for haplotype network reconstructions and heteroplasmy analyses.

2.1.3. Analyses of molecular data
2.1.3.1. Phasing and haplotype networks.
Heterozygous sites were observed for some sequences of nuclear markers. In order to derive information from these sites, phasing was performed on these sequences. Haplotypes were inferred using the PHASE program implemented in the software DnaSP v.5, based on alignments containing available unphased sequences from the present study. A haplotype network was constructed based on derived haplotypes after phasing, in Network v. 5.0.0.1 with median joining calculations (Bandelt et al., 1999).

2.1.3.2. Phylogenetic trees.
The 16S rDNA, COI, 28S rDNA and ITS2 alignments were concatenated using Seaview v.4.5.4 and haplotypes were generated using DnaSP v.5 (Librado and Rozas, 2009). Topological and branch-length congruence of the mitochondrial (COI and 16S rDNA) and nuclear (ITS2 and 28S rDNA) datasets were tested using Concaterpillar 1.8a (Leigh et al., 2008). Concaterpillar uses the likelihood ratio test for congruence developed by Huelsenbeck et al. (1996) in a hierarchical framework to identify congruent subsets of genes. DNA sequences were analyzed under the GTR model proposed by the software. The mitochondrial and nuclear datasets were found to be topologically congruent (raw P-value = 0.17, Weibull-smoothed P-value = 0.16). They were also congruent with each other according to the branch length congruence test (P = 0.16). Absence of significant incongruence between partitions (p = 0.72) was also showed by the incongruence length difference (ILD) test of Farris et al. (1995), also known as the partition homogeneity test, in PAUP v. 4 (Swofford, 2002). Heuristic searches were performed on 500 pseudoreplicates with ten random additions of taxa, holding one tree per iteration to a maximum of 100 trees. Phylogenetic analyses were thus performed on the concatenated sequences.

The best fitting evolutionary model was selected with MrModelTest (Nylander, 2016) using the Akaike Information Criterion for each individual partition and for the total concatenated dataset. For Bayesian Inference, appropriate models of substitution per gene was: GTR + G, HKY + I + G, GTR + I and GTR + G for the 16S rDNA, COI, 28S rDNA and ITS2 datasets, respectively. The Bayesian search was carried out with MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) using four simultaneous Markov chains, 2 million generations and sampling every

Table 1
Samples origins, number of sample sites, cryptic lineages found and the number of mitochondrial (COI and 16S) and nuclear (ITS2 and 28S) sequences used for Pontoscolex specimens collected in each country. Missing data where the amplification was not performed are shown with a dash symbol (–).

Biogeographic realm	Country or Island	Nb of Sites	COI	16 s	ITS2	28 s	Cryptic lineage	Other species
Australian	Fiji	1	–	1	–	–	1	
Ethiopian	Gabon	1	20	13	15	1	1	
Ethiopian	Madagascar	2	15	14	1	1	1	
Neotropical	Brazil	14	139	109	70	11	1, 3, 4	*P.* sp. 4
Neotropical	Cuba	1	3	3	1	1	3	
Neotropical	Dominican	1	–	2	–	–	1	
Neotropical	French Guiana	22	237	169	61	26	1, 2	*P.* sp. 1 and *P.* sp. 2
Neotropical	Jamaica	1	–	2	–	–	1, 3	
Neotropical	Mexico	3	38	39	38	5	1, 3	*P.* sp. 3
Neotropical	Peru	16	19	15	5	5	1	
Neotropical	St. Croix	1	–	1	–	–	1	
Neotropical	St. Kitts	1	–	1	–	–	1	
Neotropical	St. Lucia	1	1	2	1	1	1	
Neotropical	St. Vincent	1	1	2	1	1	1	
Neotropical	Trinidad & Tobago	1	1	1	1	–	1	
Neotropical	Martinique	3	15	4	4	4	1	
Neotropical	Guadeloupe	1	1	1	1	–	–	*P. spiralis*
Neotropical	Peurto Rico	1	1	1	1	1	–	*P. spiralis*
Oceanic	Hawai	1	1	7	1	1	1	
Oriental	India	2	15	4	4	2	4	
Oriental	Malaysia	1	1	3	–	1	1	
Oriental	Philippeans	10	10	57	2	2	1	
Oriental	Taiwan	21	61	15	5	5	1, 3, 4	
Oriental	Thailand	7	64	65	1	1	1	
Palearctic	Azores	2	8	6	4	4	1, 4	
Total	25	116	653	537	217	73		
Genbank	Australia, Brazil, Fr. Guiana, India, Japan	–	JQ279700-L1-Fr.GUI* JQ279698-L1-Fr.GUI JQ279699-L1-Fr.GUI AB543229-L1-JPN* JN887898-L1-IND* JN036370-L1-IND JN185607-L1-IND JN887896-*P.*sp.3-IND JN887897-*P.*sp.3-IND	JN793524-L1-IND AB543235-L1-JPN JN887905-*P.*sp.3-IND JN887906-*P.*sp.3-IND	–	AY101571-L1-AUS* KJ912259-L1-BRA*	L1	*P.* sp. 3, IND

Fr. GUI*: French Guiana.
JPN*: Japan.
IND*: India.
AUS*: Australia.
BRA*: Brazil.

100 generations, with a burn-in of 2000. Resulting p-files were examined in Tracer v.1.6 (Rambaut et al., 2014) to evaluate convergence and to ensure sufficient burn-in for the trees. Maximum likelihood (ML) analysis was performed with PhyMl online analysis (http://www.atgc-montpellier.fr/phyml/, Guindon et al., 2010) for the concatenated dataset using the global GTR + I + G model of substitution using default parameters. Clade support was assessed using bootstrap with 1000 pseudoreplicates. Trees were visualized and edited using FigTree v. 1.4.2. (Rambaut, 2014).

2.1.3.3. Molecular species delimitation analyses. For species delimitation, we followed a two-step procedure: we first used single locus methods applied to the complete COI haplotype dataset (79 haplotypes) in order to infer species hypotheses, which were tested in a second step using multilocus analyses applied to the concatenated dataset from the 4 genes (63 sequences out of 1474; i.e., those specimens for which we had the 4 genes sequences) in order to confirm putative species.

For single locus analysis, the Automatic Barcode Gap Discovery (ABGD) method developed by Puillandre et al. (2012) was performed. 79 COI haplotypes were used in PAUP v. 4 software to have the distance matrix under the appropriate model for this gene i.e., HKY + I + G as an input on the ABGD webpage http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. Initial partitions were selected as they are typically more stable on a wide range of prior values and are generally closer to the number of groups described by taxonomists than recursive partitions (i.e., primary partitions assume a single gap for the entire data set, while the recursive partition splits the primary partitions into secondary partitions, and so on until no further splittings occur) (Puillandre et al., 2012).

Another single locus analysis was the multi-rate Poisson Tree Process (mPTP) analysis which is a model that accommodates different rates of coalescence within clades (Kapli et al., 2017). We performed the mPTP analysis on the COI ML tree reconstructed by PhyMl, using the online mPTP implementation http://mptp.h-its.org/#/tree.

We then tested the species hypotheses inferred from ABGD and mPTP using the multilocus Bayesian Phylogenetics and Phylogeography (BPP) v.3.1 analysis applied to a dataset combining all four genes
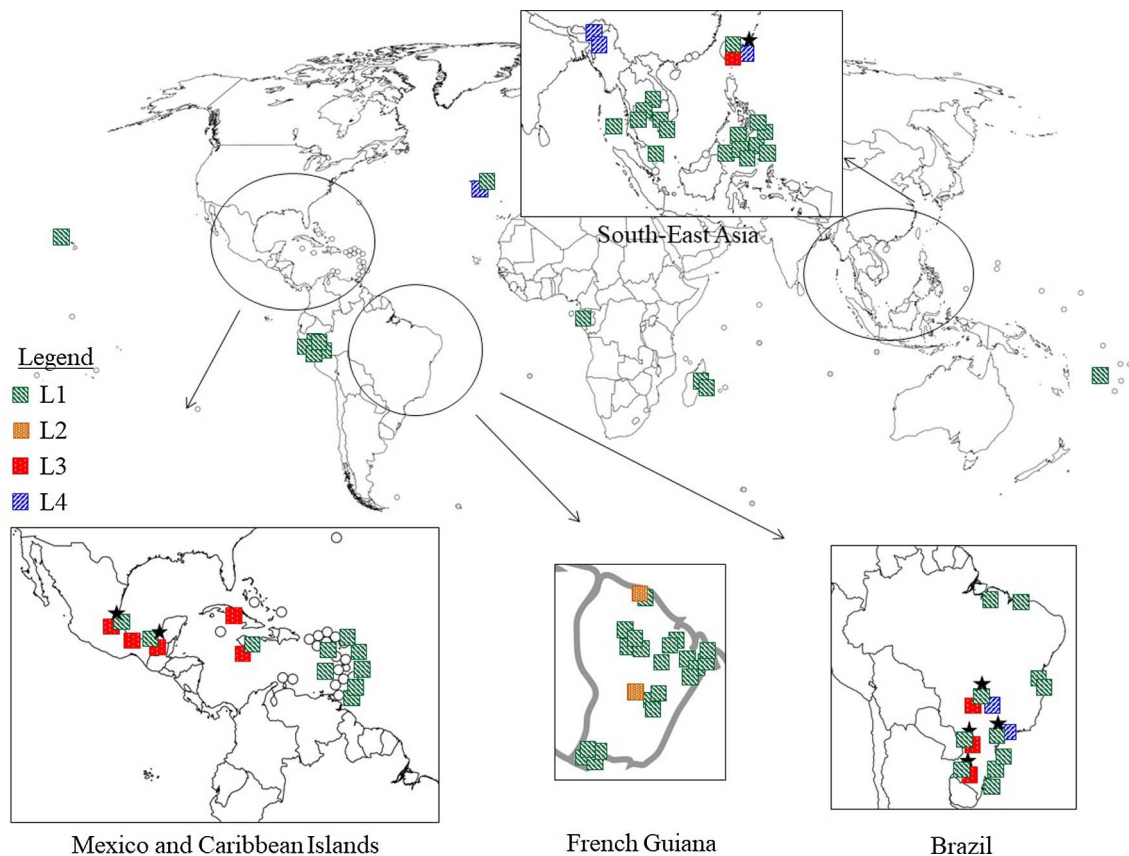
Fig. 2. Cryptic lineages in *Pontoscolex corethrurus*. Mexico and Caribbean islands, French Guiana, Brazil and South-East Asia are magnified in the squares. Cryptic lineages are shown by different colors: L1 (green lineage), L2 (orange), L3 (red) and L4 (blue). L1 is the most widespread lineage. Sites with sympatry of cryptic lineages are marked by a star. Refer to Appendix for more information on the sampled sites within each country. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sequenced: 16S rDNA, COI, 28S rDNA and ITS2. BPP uses the multi-species coalescent to analyze DNA sequence data, and can accommodate incomplete lineage sorting and uncertainty in the topology of the species tree (Rannala and Yang, 2003; Yang, 2002). We used the joint species delimitation and species-tree inference (unguided species delimitation), for which the topology from the concatenated Bayesian reconstruction was used as a guide tree, and species numbers were used based on the ABGD and mPTP approaches. As different parameters in this approach could influence the results, we used the parameters proposed by Martinsson and Erséus (2017). Two analyses, A and B, were run to ensure robust results within analyses, with different parameters as follows: in analysis A the population size parameter (θs) was assigned the gamma prior G (2, 100), with mean of 2/100 = 0.02 and the divergence time at the root of the species tree (τ0) was assigned the gamma prior G (2, 20); for analysis B, the population size parameter (θs) was assigned the gamma prior G (2, 400), with mean of 2/400 = 0.005, and (τ0) was assigned the gamma prior G (2, 40). The analyses were run for 200,000 generations with a burn-in of 10,000 generations and a sample frequency of 5. A and B each were run three times to confirm consistency between runs. Lineages delimited with a posterior probability of > 0.95 in all analyses are considered as well supported.

At the end, we calculated the mean genetic differentiation between putative species based on ABGD, mPTP and BPP species delimitations. This analysis was done based on 79 haplotypes of COI using Kimura's 2-parameter (Kimura, 1980) model of sequence evolution by MEGA v.6. (Tamura et al., 2013). This model was chosen for facilitating comparisons with other studies focusing on genetic divergence between earthworm species (Chang and James, 2011; Porco et al., 2013; Römbke et al., 2016; Voua Otomo et al., 2013).

2.2. Phylogenomic approach

We also produced a genome wide multilocus sequence dataset for eight of the *Pontoscolex* species sampled for the phylogenetic analysis described above. Using the results of the latter, one specimen per lineage was selected for phylogenomic study, plus the Rhinodrilidae outgroup taxa *Andiorrhinus* sp. and *Urobenus* sp. For this analysis, Cunha et al. (2014) lineage samples were not available.

We used Anchored Hybrid Enrichment (AHE) following Lemmon et al. (2012) and Lemmon and Lemmon (2013), and the workflow as described here: http://anchoredphylogeny.com/workflow/ (accessed 22 September 2017). Data were collected in the Center for Anchored Phylogenomics at Florida State University. A custom probe kit was prepared following Hamilton et al. (2016) by analyzing 2 Clitellate genomes (*Helobdella robusta* and *Lumbricus rubellus*) and one Polychaete genome (*Capitella teleta*), and 130 transcriptomes derived from broad taxon sampling of the Annelida. These transcriptomes were obtained for and used in Anderson et al. (2017) and by the Halanych Lab at Auburn University (unpublished data). In total, 594 anchor loci were targeted in the probe kit. After merging the overlapping read pairs (Rokyta et al., 2012), reads were mapped to the target regions using *Helobdella*, *Dendrobaena*, and *Mesenchytraeus* as references (for details of the assembly, and orthology methods, see Hamilton et al., 2016). In order to avoid contamination caused by indexing error, consensus sequences resulting from the assembly were screened to remove those originating from fewer than 650 loci. Orthology was assessed on the resulting consensus sequences using a neighbor-joining approach based on alignment-free pairwise sequence distances. Finally, alignments were performed in MAFFT (v7.023b, with –genafpair and –maxiterate 1000 flags, Katoh and Standley, 2013) and trimmed to remove poorly aligned regions.

After read assembly, quality control, alignment and alignment

trimming, sequence data were used to derive phylogenetic trees using ASTRAL (Mirarab et al., 2014; Mirarab and Warnow, 2015), which calculates individual gene trees and assembles them into a supertree; and PhyMl online (http://www.atgc-montpellier.fr/phyml/, Guindon et al., 2010) analysis in which concatenated alignments were used as input data for ML estimation and run under the GTR + G + I model selected by SMS (Lefort et al., 2017) using the AIC criterion. Clade support was assessed using bootstrap with 1000 pseudoreplicates.

2.3. Morphological analyses

Based on phylogenetic, phylogenomic and delimitation results, at least three specimens per lineage were chosen for morphological and anatomical study. Some key external characteristics of *Pontoscolex*, such as the positions of the clitellum and tubercula pubertatis, the shape and (ir)regularity of setae (A, B, C, and D) on the body (Fig. 1B), and presence or absence of genital markings, were carefully examined. Quincunx formation of setae in the last quarter of the body was also verified for each lineage. Some key internal characteristics of the genus were also observed, including presence or absence of seminal vesicles, shape and positions of calciferous glands, gizzard and hearts.

3. Results

3.1. Phylogenetic relationships and species delimitation within the genus Pontoscolex

A total of 1474 sequences were acquired in this study and 15 sequences were obtained in GenBank. The lengths of each marker after alignment were: 492 bp for 16S rDNA (32 haplotypes/ 540 sequences), 634 bp for COI (79 haplotypes/659 sequences), 712 bp for 28S rDNA (27 haplotypes/73 sequences), 409 bp for ITS2 (48 haplotypes/217 sequences). The DNA sequences were deposited in GenBank; the Accession Numbers are shown in Supplementary data (Table A.2). Our concatenated 4-genes dataset (47 haplotypes/63 specimens; see Table 1) had a total length of 2247 bp. The trees obtained from the analyses of the concatenated dataset using Bayesian inference (BI) and Maximum Likelihood (ML) methods revealed congruent topologies (Fig. 3 for BI and Fig. A.1 of Supplementary data for ML). The estimated genealogies for the four genes based on BI showed good convergence and high ESS (estimated sample size) values (i.e., > 200).

The number of candidate species (9 species) recovered by ABGD (initial partitions) was very stable across all prior intraspecific divergences (P = 0.1 to P = 0.0017), except for the extreme value of P = 0.0010 (for which 40 species were recovered). The same results were observed for mPTP procedure of species delimitation (Fig. 3).

All three runs of the multilocus species delimitation analyses A and B in BPP produced congruent results with the single locus approach. Rannala and Yang (2003) suggested that putative species could be considered distinct if their posterior probability (PP) exceeded a threshold of 95–99%. In our results, except for L2 and L3 in analysis B (which had a PP of 99%), all the other lineages for both analyses A and B had a PP of 100%, suggesting that these lineages correspond to nine distinct species (Fig. 3).

Results of COI K2P genetic distances between the 9 putative species are shown in Table A.3 of Supplementary data. The lowest genetic divergence found in *P. corethrurus* complex is 14.1% between L3 and L4 and, the highest value is 20.7% between L1 and L2. For other *Pontoscolex* species, the lowest value was between *P.* sp. 2 and *P.* sp. 3 (17.3%) and the highest, between *P.* sp. 1 and *P.* sp. 4 (25.4%).

3.2. Phylogenomic data

For the 8 in-group *Pontoscolex* samples plus two Rhinodrilidae outgroup samples, an average of 577 loci > 125 bp, 575 loci > 250 bp, 554 loci > 500 bp and 115 loci > 1000 were captured. The

final data set consisted of 609 loci, comprising 238,549 sites, of which 63,124 were variable and 27,483 were informative (genomic data are available at: http://purl.org/phylo/treebase/phylows/study/ TB2:S22250?x-access-code=3cfa49fbd797d19078a0fe3adbd8525d& format=html. Missing data made up 25.12% of the matrix. ML tree topology was exactly the same to those obtained from four genes except that lineage L4 was absent in phylogenomic analyses (no samples provided). All nodal support values are 1000 except for the node connecting *P. spiralis* to the tree which is 888. The phylogenomic trees (Fig. A.2 of the Supplementary data for ML tree) were highly similar to the concatenated 4 gene trees.

3.3. Morphological traits of the recovered lineages

The results of the comparative analysis of key morphological traits from at least three specimens per putative species are shown in Table 2. All the individuals were consistent with respect to the morphological characters defining the genus *Pontoscolex*, which are the position of gizzard on the 6th segment, calciferous glands in 7th, 8th and 9th segments, and hearts in the 10th and 11th segments. Among the nine lineages recovered by ABGD/mPTP and confirmed by BPP approaches, five could be consistently distinguished morphologically: one matches the formally named species *P. spiralis,* and four other unnamed lineages are designated as *P.* sp. 1, *P.* sp. 2, *P.* sp. 3 and *P.* sp. 4 according to their position in the phylogenetic tree. The four remaining lineages could not be distinguished morphologically and were named L1, L2, L3 and L4. They constituted a monophyletic group in the phylogenetic tree (Fig. 3) and were separated from *P.* sp. 1, *P.* sp. 2, *P.* sp. 3 and *P.* sp. 4 by *P. spiralis*. Moreover, the topotypes clustered with lineage L1 and the samples from the Azores were grouped with both L1 and L4. Consequently, this clade was representative of the *P. corethrurus* complex. Sequences from GenBank were all grouped with L1, but four sequences from India (COI sequences JN887896 and JN887897, and 16S sequences JN887906 and JN887905) fell within *P.* sp. 3 lineage. All the lineages had quincunx formation on the tails, except *P. spiralis* and *P.* sp. 4 that had regular A, B, C, and D setae all over the body. The position of the clitellum was different between the *P. corethrurus* complex and *P. spiralis*, *P.* sp. 3, and *P.* sp. 4. This character could not be recorded for *P.* sp. 1 and *P.* sp 2 because all specimens were juveniles. *P.* sp. 1 was the only lineage where hooked setae were observed (Fig. 1C), with regular A setae row (Fig. 1B). Additionally, *P.* sp. 2 had regular A and C setae all over the body. Almost all lineages had seminal vesicles, but in the *P. corethrurus* complex it was not observed in all specimens. Genital markings were observed for *P.* sp. 3, *P.* sp. 4, and *P. spiralis* individuals at two positions.

3.4. Geographic distribution and relationship between cryptic lineages in the P. corethrurus complex

The geographic distributions of the *P. corethrurus* lineages are shown in Fig. 2. L1 was the most widespread lineage. In seven sites, different cryptic lineages were observed in sympatry: L1/L3 (2 sites) in Mexico, L1/L3/L4 in Taiwan, and in Brazil L1/L3 (2 sites), L1/L3/L4, and L1/ L4. Relationships among these lineages were further investigated using a haplotype network obtained with phased nuclear sequences. Because of very few polymorphisms and low resolution of the 28S rDNA marker (9 polymorphic sites on a total of 712 bp), we present only the results for ITS2 (Fig. 4). The ITS2 alignment comprised a total of 371 sites: 300 sites were invariable, 27 were polymorphic, 2 contained gaps and 42 contained missing data. The length of the alignment was shorter than for the ITS2 alignment mentioned earlier (371 vs. 409 bp), because only *P. corethrurus* complex sequences were used for this analysis and these contained no indels or gaps in the alignment. Heterozygous ITS2 sites were observed for 192 sequences from the *P. corethrurus* complex. The analysis was done on the 38 haplotypes used in the tree resulting from the analysis of the concatenated dataset. Each lineage of the *P.*
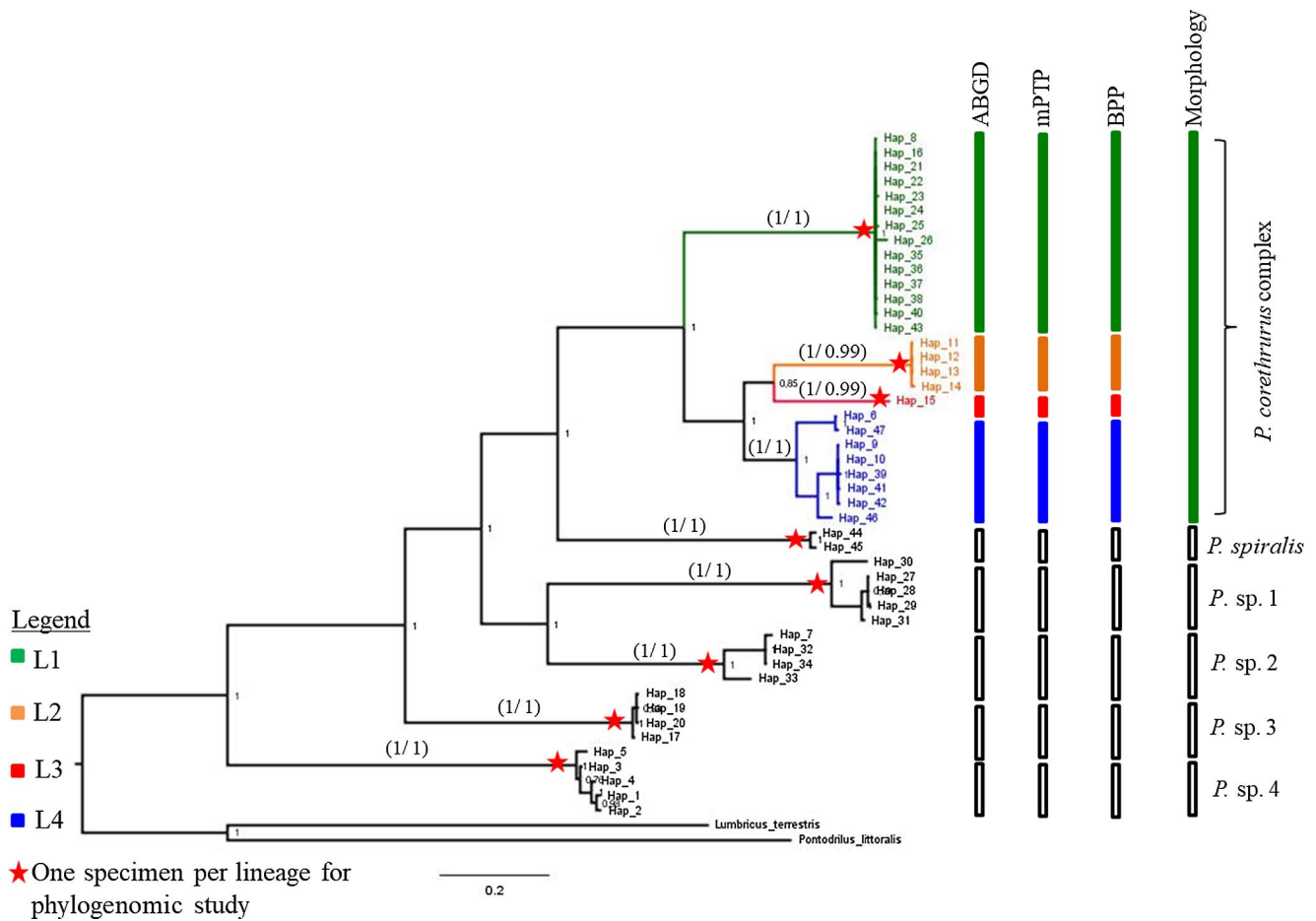
Fig. 3. Bayesian haplotype tree of the four genes (16S rDNA + COI + 28S rDNA + ITS2). Bayesian posterior probability values > 0.85 are disposed at the node of each cluster. Species delimitation analyses by ABGD, mPTP, BPP and morphological approaches are shown by bars. Posterior probability values based on BPP analysis for species delimitation are shown on each branch (Analysis A/Analysis B). Generally species with both PP > 0.95 are considered as well supported species. Outgroup species and species other than *P. corethrurus* (*P. spiralis*, *P.* sp. 1, *P.* sp. 2, *P.* sp. 3, and *P.* sp. 4) are shown in black text, without coloration. Color codes for lineages are the same as in Fig. 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

corethrurus complex was separated in the haplotype network except for haplotype 5 which was shared between L3 and L4 and was observed in the following locations; Furnas (Azores), Rabung and Hualtu (India), National Taiwan University (Taiwan) in L4, Mancha, Tlalcotlen and Hayas (Mexico), and Palmar de la Jarreta (Cuba) in L3.

Interestingly, three specimens from one site in Taiwan (National Taiwan University) showed heteroplasmy for COI mitochondrial gene. The DNA of these samples was extracted 2 times and sequenced 3 times in order to exclude the contamination hypothesis. We observed that for each heterozygous site, one possible case corresponded to the L3 lineage and the other to L4.

4. Discussion

4.1. New species within the genus Pontoscolex

Molecular analyses of 792 specimens belonging to the genus *Pontoscolex* representing a global sampling, from the tropics and subtropics, revealed the existence of nine genetically divergent lineages. One of these lineages corresponded to the species *P. spiralis* which is taxonomically well-known (Borges, 1996). Four lineages showed distinct morphological traits that allow us to differentiate them from the morphospecies *P. corethrurus* and from *P. spiralis*. These species were named *P.* sp. 1, *P.* sp. 2, *P.* sp. 3 and *P.* sp. 4. We were unable to assign these four species to previously established names and some of them could be new to science.

P. sp. 1 presented hooked setae and *Pontoscolex (Pontoscolex) kuneguara* is the only species within the genus reported to have hooked-setae on the body excluding the tail. Yet, hooked setae were observed over the entire body of *P.* sp. 1, including the tail, thereby differentiating these specimens from *P. kuneguara*. *Pontoscolex* sp. 2 was characterized by regular A and C setae, a characteristic also reported for *P. hingstoni* (Stephenson, 1931) and *P. cuasi* (Christoffersen, 2008; Righi, 1984). In the former, seminal vesicles extend up to the 31st segment while for the latter to the 25th. However, we were unable to determine the seminal vesicles position in our specimens of *P.* sp. 2 because they were juveniles. The presence of irregular setae observed on specimens representing the *P.* sp. 3 lineage indicate that this lineage could be assigned to either the *Pontoscolex* or *Mesocolex* subgenus. The subgenus *Pontoscolex* contains 15 species with and without regular setae; in *Mesoscolex* only one species is currently known, and it has irregular setae; and all four described species in the subgenus *Meroscolex,* have regular setae (Moreno, 2004). As *P.* sp. 3 was only represented by four juveniles from a single population in Mexico (i.e., Mancha), we were unable to compare the clitellum and tubercula pubertatis positions with those of described species of *Pontoscolex*. Despite small body size (in comparison with other specimens) and juvenile state, big seminal vesicles were remarkable during dissection of this lineage. It is noteworthy that two sequences from GenBank for COI (JN887896 and JN887897) and 16S (JN887906 and JN887905) genes, labeled as *P. corethrurus* from India, clustered with this lineage. This result highlights the risk of mistaking *P.* sp. 3 with *P. corethrurus* and

Table 2
Morphological (internal and external) characters of Pontosocolex specimens found in each lineage and species. Missing data or information are shown with a dash symbol (–).

Lineage	Clitellum position (segments)	Tubercula Pubertatis position (segments)	Setae shape	Regular setae	Genital markings (segments)	Quincunx formation on tail	Seminal Vesicles	Gizzard (segment)	Calciferous glands (segments)	Calciferous glands shape	Hearts (segments)
Pontoscolex corethrurus complex (L1, L2, L3, L4)	15–23 or 1/2 15–1/2 23	19–21	Non hooked	None	No	Yes	Yes/no	6	7, 8, 9	Tubular	10, 11
Pontoscolex spiralis	16–24	20–22	Non hooked	A, B, C, D	Yes, 8–12 and 19–23	No	Yes	6	7, 8, 9	No data	10, 11
P. sp. 1	16–24	20–22	Hooked	A	Yes, 8, 9 and 19–22	Yes	Yes	6	7, 8, 9	No data	10, 11
P. sp. 2	Juveniles	20–22 (intersegmental)	Non hooked	A, C	No	Yes	Yes	6	7, 8, 9	No data	10, 11
P. sp. 3	Juveniles	No data	Non hooked	None	No	Yes	Yes	6	7, 8, 9	Tubular	10, 11
P. sp. 4	13–23	19–1/2 22	Non hooked	A, B, C, D	Yes, 5, 6, 9 and 18–22AB	No	Yes	6	7, 8, 9	Oval parallel tubular duct from side	10, 11

reveals that *P.* sp. 3 is also present in India. Moreno (2004) emphasized that a source of misidentification could be the use of the quincunx formation of setae as diagnostic character for *P. corethrurus.* The quincunx formation indeed exist in *P.* sp. 1, *P.* sp. 2 and *P.* sp. 3 as well. Apart from *P. spiralis,* regular setae all over the body was also observed for *P.* sp. 4 which was found in Manaus (Brazil). We assume that *P.* sp. 4 belongs to either *Meroscolex* or *Pontoscolex* subgenus.

Additional investigations (molecularly and morphologically) are necessary in the future to disentangle the diversity within the genus *Pontoscolex.* Ideally, these questions should be addressed through genetic comparison with the actual type specimens of already named species, a very challenging task since in most cases the type specimens are lost, such as for *P. corethrurus,* or difficult to gain access to, and the molecular analysis of tissues is also almost impossible because of DNA preservation issues.

4.2. Single and multilocus methods revealed a complex of cryptic species in P. corethrurus

In the phylogenetic trees, the *P. corethrurus* complex of cryptic species was represented by a monophyletic clade composed of four morphologically indistinguishable lineages (L1, L2, L3 and L4) separated from the other species by *P. spiralis.* The haplotype network for ITS2 which is a fast-evolving nuclear sequence where signal of hybridization or mating with nonspecific relatives are often shown (Vollmer and Palumbi, 2004), confirmed the differentiation of the four lineages within the *P. corethrurus* complex (Fig. 4).

Single (ABGD/mPTP) and multilocus (BPP) delimitation methods produced congruent results. Single locus delimitation methods were often criticized, because when applied among closely related species, these approaches may result in an over- or under-estimation of species diversity (Meyer and Paulay, 2005; Roe et al., 2010; Roe and Sperling, 2007; Will and Rubinoff, 2004). Alternatively, multilocus analysis constitutes a valuable source of information for species delimitation because it lowers the risk of incorrect results based on single locus datasets (Dupuis et al., 2012; Roe et al., 2010). Our multilocus analysis with 4 genes was run with different values of the priors for the population size (θs) and the divergence time at the root (τ0) (Rannala, 2015) as it has been shown that different parameters (particularly θ priors) give different results i.e., smaller values give higher probabilities for species splits (Rannala, 2015). Our study confirms this prediction; as θ prior was smaller, higher probability was observed for L2 and L3 (PP = 1 compared to PP = 0.99). Other PP values for all the other lineages in both analyses were the same (PP = 1).

We recognize that low levels of locus sampling (i.e., 4 locus) invite criticisms that the data may not be adequate to address phylogenetic questions. Therefore, we included the AHE method to evaluate the validity of a result from lower sampling intensity. Here, we did not go into details of lineage sorting or conflicts among gene trees, yet the outcome of the AHE analysis was quite clear. Four genes results were obtained by conventional sequencing, and at a lower cost per sample than the AHE, but in spite of having about 1% as many sequence positions, produced similar results. We expected that recombination among lineages would have led to incongruent multilocus topologies. Our results suggested a lack or limited recombination among lineages and that low effective population sizes and long evolutionary periods of time, led to complete lineage sorting of multiple independent loci. Overall, our results from both single locus and multilocus analyses support the hypothesis that four cryptic species exist in this morphospecies.

4.3. Phylogenetic placement of the topotypes and cryptic species distribution

The lineage L1 matched the topotype samples from Fritz Müller's garden. Therefore, we suggest that this lineage corresponds to *Pontoscolex corethrurus* sensu stricto. Additionally, L1 is the most
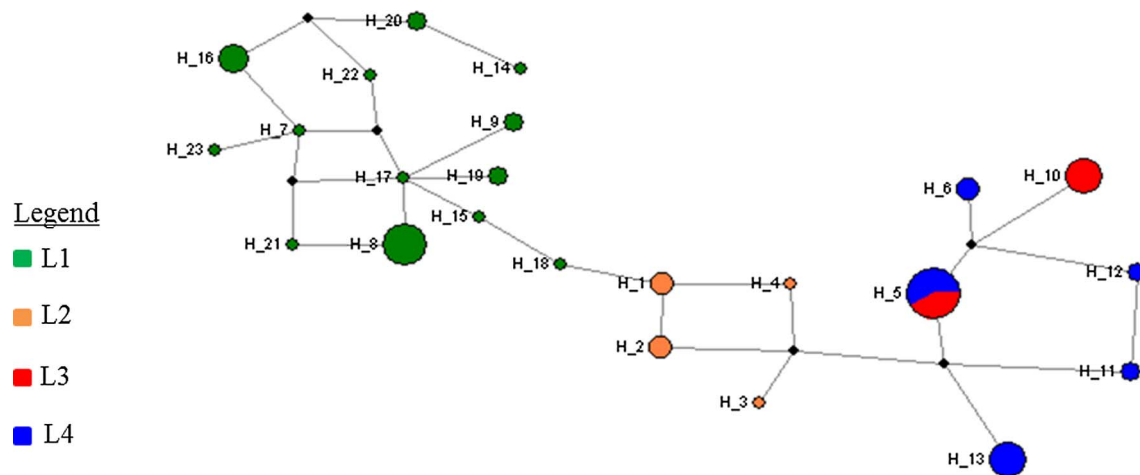
Fig. 4. ITS2 marker haplotype network of the four main *P. corethrurus* lineages after phasing Color codes for lineages are the same as in Fig. 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

widespread lineage, being recorded in 100 out of a total of 116 sampled sites. This suggests that most of the studies done on *P. corethrurus* were most probably on this lineage and not on other species in the complex.

L4 corresponds to the cryptic species already found by Cunha et al. (2014) for the Azores. We found this lineage in Taiwan, India and Brazil as well. Cunha et al. (2014) revealed that this species has a remarkable tolerance to hostile conditions (i.e., high soil temperature, high carbon dioxide and low levels of oxygen and elevated metal bioavailability) as it was found on Furnas volcano of the Azores. It is noteworthy that in the other countries (Brazil, Taiwan and India), L4 was found in non-hostile environments i.e., in gardens of National Taiwan University and Embrapa in Brazil, and in grass area of village center/grass lawn in front of a forest reserve in India. Further investigations to understand the geographical distribution of L4 are necessary.

Sympatry of lineages was observed in seven of 116 localities. It occurred in two sites in Mexico between lineages L1/L3, in four sites in Brazil between L1/L3/L4, L1/L4, and two sites L1/L3, and in one site in Taiwan between L1/L3/L4. Interestingly, in the latter population (National Taiwan University, Taipei, Taiwan), COI heteroplasmy was observed for three specimens, with all the heterozygous sites corresponding either to the L3 haplotype or to the L4 haplotype. The ITS2 network also showed that haplotype 5 is shared between L3 and L4 specimens (i.e., in the sites of Mexico, India, Azores, Taiwan and Cuba) (Fig. 4). This result suggests sexual reproduction for L3 and L4 and that reproductive isolation is not yet complete between these lineages. The observed heteroplasmy could be explained by paternal leakage (i.e., low-level paternal contribution of mtDNA to progeny) (Birky, 2001; Kmiec et al., 2006). Sequencing lacks the sensitivity to show mtDNA mutations that are present at low percentages (i.e., 0–20%) (Wong and Boles, 2005) which could explain why heteroplasmy was not detected with the 16S rDNA gene. The observed heteroplasmy cannot be due to contamination, as we sequenced the specimens from re-extracted DNA three times, even if true we would observe the heteroplasmy for 16s rDNA as well. Further population genetics analyses are necessary to investigate the reproductive isolation between L3 and L4 in the complex and to test whether retention of genetic polymorphisms due to past hybridizations could explain this phenomenon. Reproductive isolation among these species, could also be tested by laboratory cross-breeding experiments. These types of experimentations have already found the evidence of reproductive isolation between *Eisenia fetida* and *Eisenia andrei* (Domínguez et al., 2005), and between cryptic lineages of the *Hormogaster elisae* (Marchán et al., 2017).

5. Conclusion

We showed that the *Pontoscolex corethrurus* complex consists of at least four cryptic species. Further, based on modern sampling from the type locality for *P. corethrurus*, we suggest that L1 represents *P. corethrurus* sensu stricto. Morphologically cryptic diversity in *P. corethrurus* could be problematic given its presumed wide distribution and common use in soil studies, as different species may have different impacts on and respond differently to the environment. It is possible that published studies in the past may have dealt with species that were not necessarily *P. corethrurus* sensu stricto (L1) but the risk is relatively low since L1 is the most widespread species in the complex. We echo the advice of Martinsson and Erséus (2017): it is important to define species boundaries as objectively as possible, especially for taxa used as biological models. One key to objectivity, especially with the continuous progress of our ability to delimit biological units, is to make results reproducible by preserving voucher specimens, and by publically releasing sequences (Wen et al., 2017).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.02.021.

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