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Short Communication

Cryptic speciation of hormogastrid earthworms revealed by mitochondrial and nuclear data

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ABSTRACT

Species delimitation of earthworms has been difficult to determine with certainty due to their structural simplicity. We sequenced fragments of COI, 16S, t-RNAs and 28S for 202 Hormogastridae individuals from the central Iberian Peninsula and three outgroup taxa.

A morphological constancy was found but a high genetic diversity suggests the presence of five cryptic allopatric species. Results showed a pattern of isolation by distance and a positive but weak correlation between some soil properties (coarse sand and total loam content) and genetic distances, which indicates that these populations may have been shaped genetically but not morphologically, by the environment.

1. Introduction

The existence of species difficult to distinguish using morphological characteristics was first discussed by Mayr (1948) and more recently, the increasing use of molecular data in taxonomy has led to an ever-increasing discovery of cryptic species, especially within invertebrate groups and specifically in earthworms (Chang et al., 2008; King et al., 2008).

The taxonomy of earthworms is very limited due to their structural simplicity and because the diagnostic characteristics can be variable and overlap among taxa (Pérez-Losada et al., 2009). DNA taxonomy and associated molecular tools could be the only way to reveal the true level of biodiversity (Proudlove and Wood, 2003).

Darwin described earthworms as the most important animal group in the history of the world (Darwin, 1881). Presently, there is a great deal of work being conducted on the applied ecology and biology of earthworms (Edwards, 2004) and without the appropriate species boundaries the results could be confounding.

The family Hormogastridae Michaelsen 1900 consists of four genera and 22 species of large and middle-sized earthworms from the western Mediterranean (Cobolli-Sbordoni et al., 1992). The genus *Hormogaster* Rosa 1887 contains the highest diversity with 18 species, 16 of which are found in the northeast of the Iberian Peninsula and only *Hormogaster elisae* Álvarez 1977 in the central area.

Novo et al. (2009) found high genetic divergences among populations of *H. elisae* in the central area of the Iberian Peninsula, which lead to the suggestion that different cryptic species might coexist within this area.

In the present work a more intensive sampling effort was conducted and more gene regions were studied. A single DNA region or an arbitrary level of divergence cannot define species boundaries by themselves. Therefore, nuclear genes should be used in addition to the mtDNA data (King et al., 2008, and references herein). We included both the mitochondrial markers proposed as most informative in delimiting species boundaries, namely COI (Hebert et al., 2003) and 16S ribosomal DNA (Blaxter, 2004), and the nuclear 28S ribosomal RNA gene. The latter region has been shown to provide an increased resolution at higher taxonomic levels in earthworms (Pérez-Losada et al., 2009), and would provide a robust confirmation of the proposed cryptic speciation in Novo et al. (2009).

We also investigated whether this high genetic diversity is influenced by soil factors or whether it is caused by historical events. Finally, we examined whether the variations found in the size of the specimens are consequences of different soil conditions (i.e., phenotypic plasticity) or whether they are related to genetic evolution.

2. Materials and methods

2.1. Sampling and morphological study

A total of 202 clitellate hormogastrid earthworms were collected in 16 locations from the central Iberian Peninsula (Table 1). We tried to locate more populations in the southern part of the sample area but the highest density was located in the north. Additionally

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Table 1
Hormogastrid sampling localities in the central Iberian Peninsula and the number of individuals sequenced for each gene fragment. The abbreviations used in the data analysis are shown (Abbr.).

Locality	Abbr.	NCOI	N16S–t-RNAs	N28S	Location[a]	Altitude (m)
Anchuelo	ANC	9	6	2	N40°28′50.2″ W3°14′33.5″	780
Boadilla del Monte	BOA	12	6	2	N40°25′50.2″ W3°55′30.9″	667
Cabrera	CAB	12	6	2	N40°51′25.9″ W3°37′18.2″	1029
Cubillo de Uceda	UCE	12	6	2	N40°49′38.7″ W3°25′19.5″	883
Fresno del Torote	FRE	2	2	2	N40°35′51.8″ W3°24′42.0″	660
Lozoyuela	LOZ	22	7	2	N40°56′51.9″ W3°37′16.2″	1036
Molar	MOL	12	11	2	N40°44′22.9″ W3°33′53.1″	753
Navas de Buitrago	NAV	12	6	2	N40°56′21.0″ W3°35′38.1″	994
Paracuellos del Jarama	JAR	12	6	2	N40°30′36.9″ W3°31′59.1″	674
Pardo	PAR	12	2	2	N40°31′11.0″ W3°47′42.7″	662
Redueña	RED	26	6	2	N40°48′46.7″ W3°36′06.2″	797
Sevilla la Nueva	SEV	12	6	2	N40°20′41.9″ W4°00′48.9″	644
Siguero	SIG	11	6	2	N41°11′06.1″ W3°37′07.4″	1073
Soto del Real	SOT	12	6	2	N40° 46′30.5″ W3°46′42.6″	985
Tres Cantos	TRE	12	6	2	N40° 36′46.9″ W3°40′41.1″	675
Venturada	VEN	12	6	2	N40° 48′07.7″ W3°37′19.6″	890

[a] The global location was calculated using a handheld global positioning system (GPS).

we collected: *Xana omodeoi* Diaz-Cosín et al. 1989, included as an outgroup, and *H. riojana* Qiu and Bouché, 1998 and *H. pretiosa* Michaelsen 1899 as *Hormogaster* references from distant locations of the Iberian Peninsula (Fig. S1, Supplementary data). The specimens were studied focusing on characteristics described by Qiu and Bouché (1998).

2.2. Environmental assessment

The most important factors affecting earthworm distribution such as soil texture, pH, total nitrogen and organic oxidable carbon were measured (see Hernández et al., 2007, and references herein for methods).

A Mantel test with 10,000 permutations was implemented in ARLEQUIN v. 3.1 (Excoffier et al., 2005) to correlate the genetic distances of the COI gene with the differences between localities for soil factors and earthworm size (in absolute values). A multiple regression analysis was performed to evaluate the relationship between the soil factors and the earthworm size (STATISTICA v. 6.1).

2.3. Molecular and data analyses

Genomic DNA was extracted using the DNeasy Tissue Kit (QIAGEN). Regions of COI, 16S rRNA, tRNA Leu, Ala, Ser and Leu and 28S rRNA genes were amplified (see primers in Table S1 and conditions in Table S2, Supplementary data). Sequencing was performed in the Scientific Park of Madrid (Spain). Sequences were then aligned with CLUSTALX (Thompson et al., 1997).

2.3.1. Population genetics analyses

Estimates of variability were computed with ARLEQUIN v. 3.1 (Excoffier et al., 2005). Mean genetic differentiation among and within populations was estimated using Kimura's (1980) 2-parameter model of sequence evolution. Pairwise Φ_{ST} were calculated and AMOVA was performed, generating 1000 permutations for statistical confidence. The correlation of genetic and geographical distances (Φ_{ST} for COI gene and straight line distance between sample sites for this study) was explored with a Mantel test with 10,000 permutations for significance estimate.

2.3.2. Haplotype networks

Haplotype networks were constructed with a 95% parsimony connection limit in TCS v. 1.21 (Clement et al., 2000). For COI, due to the high number of haplotypes found and the high divergences among them, the network was constructed excluding the more variable third codon positions.

2.3.3. Phylogenetic relationships

All phylogenetic analyses were performed for each gene partition (COI, 16S–tRNA, 28S) and for the concatenated sequence. Gblocks (Talavera and Castresana, 2007) was used to eliminate those positions that could not be unambiguously aligned.

Data partition congruence was assessed using the ILD test as implemented in WINCLADA v. 1.00.08 (Nixon, 2002). Heuristic searches were performed on 100 pseudoreplicates with five rounds of random addition of taxa holding two trees per iteration to a maximum of 10 trees.

JMODELTEST v. 0.1.1 (Posada, 2008) was used to select the evolutionary model that best explained the data.

Bayesian phylogeny estimation was performed in MRBAYES v. 3.1.2 (Ronquist and Huelsenbeck, 2003). In the concatenated matrix unlinked nucleotide substitution models selected by JMODELTEST were specified for each gene fragment.

Maximum likelihood (ML) analyses were performed with Phyml v. 2.4.4 (Guindon and Gascuel, 2003) including α and I values provided by JMODELTEST.

Maximum parsimony (MP) trees were inferred using TNT v. 1.1 (Goloboff et al., 2003). Heuristic searches with 1000 rounds of random addition of taxa holding 10 trees per round, up to a total of 10,000 trees, were performed. The best trees were then submitted to an extra round of TBR swapping.

Clade support under the ML and MP approaches was assessed using bootstrap with 1000 pseudoreplicates.

3. Results

3.1. Morphological features

All the individuals were consistent in a set of characteristics unique to *H. elisae* that strongly separate this species from the remaining species of *Hormogaster:* clitellum in (13)14(15)–26(27)28, tubercula pubertatis in 22(23)–25(26), two tubular spermathecae pairs in 9 and 10, with the pair in 10 much larger, and typhlosole with five lamellae within a minimum of 30 or 40 segments.

Significant differences were found when comparing the lengths of specimens (ANOVA, $F_{12.98} = 29.801$, $P < 0.001$). *Post hoc* comparisons demonstrated that three size groups could be established: (I)

SIG and PAR; (II) LOZ; (III) the remaining populations (Table S3, Supplementary data).

3.2. Relationships of genetic distances with size or soil properties

Soil characteristics are shown in Table S3, Supplementary data. Mantel tests indicated that there were significant correlations between COI genetic distances and differences in coarse sand ($r = 0.271$, $P = 0.024$) and total loam ($r = 0.273$, $P = 0.026$). Neither the genetic distances nor the soil properties were correlated with the size of the earthworms.

3.3. Genetic diversity, genetic divergence and population structure

Eighty and 51 exclusive to locality haplotypes were found for COI and 16S–tRNA fragments, respectively, and six haplotypes were found for 28S, which were shared in some occasions (see Section 3.4). The global values of haplotype and nucleotide diversity were $H = 0.98$ and $\pi = 0.128$ for COI, $H = 0.98$ and $\pi = 0.072$ for 16S–tRNA and $H = 0.63$ and $\pi = 0.005$ for 28S. For detailed information on genetic diversity see Table S4, Supplementary data.

Genetic divergence values for mitochondrial genes fragments are shown in Table S4, Supplementary data (within populations) and Table 2 (between populations). AMOVA results exhibited a population structure demonstrating that more than 90.49% of the genetic variation was due to differences among populations. The Φ_{ST} value was high (up to 0.90), indicating significant population genetic structure. All pairwise comparisons of Φ_{ST} among populations were significantly high, ranging from 0.376 to 0.999 for COI and from 0.329 to 1 for 16S–tRNA, indicating a lack of gene flow among the populations.

Mantel test for COI showed positive correlation between genetic and geographical distances among populations ($r = 0.372$, $P = 0.0028$, $r^2 = 0.138$).

3.4. Haplotype networks

The haplotype network for COI exhibited each population not connected to the remainder with the exception of JAR and VEN, with some haplotypes clustered together. Excluding the third codon positions the result was still represented as five different networks (not shown) with the following populations: (I) ANC; (II) BOA and SEV; (III) PAR; (IV) SIG and SOT; (V) the remaining populations. The same five groups although connected, can be distinguished for 28S gene network (Fig. 1).

According to the TCS programme, group (V) would contain the ancestral haplotype, which is present in the highest number of populations.

When reconstructing the network with 16S–tRNA (not shown), 10 different networks were recovered.

3.5. Phylogenetic relationships

The size of fragments after Gblocks was 648 bp for COI, 770 bp for 16S–tRNA, 739 bp for 28S and 2152 bp for the concatenated sequence.

All of the trees (Bayesian, ML, MP) generated with different partitions and the concatenated data set produced congruent topologies. Moreover, the ILD test did not find significant incongruence among them ($P > 0.05$). JMODELTEST indicated that the best models for the data were GTR + I + G for the 16S–tRNA and 28S partitions and Trn + I + G for COI. For the last, we also used GTR + I + G for the analyses because it was the second best model selected by AIC.

The Bayesian inference tree of the combined data set is shown in Fig. 2. The ML tree for that set had a log likelihood of −10,200.40212 and parsimony analysis yielded five most parsimonious trees ($L = 1510$, CI = 0.49, RI = 78). Their bootstrap values are shown in Fig. 2. The main phylogenetic relationships were well supported and exact in every tree, whereas some relationships at a lower level differed among analyses.

Morphospecies *H. elisae* constitutes a monophyletic group that is separated from the other *Hormogaster* species. Nine evolutionary units were identified: (I) MOL, TRE, RED, JAR, VEN; (II) LOZ, CAB, NAV; (III) FRE; (IV) UCE; (V) SOT, SIG; (VI) ANC; (VII) SEV; (VIII) BOA; (IX) PAR. The positions of (III) and (IV) differ among analyses but they always appear clustered with (I) and (II), overall representing a monophyletic group.

3.6. Differentiation between proposed species

We propose the existence of at least five different cryptic species within morphospecies *H. elisae* following Wiens and Penkrot (2002). Divergence values of mitochondrial genes were calculated for these species (Table S5, Supplementary data).

Table 2
Mean K2P genetic divergence (in percentage of changes) between pairs of hormogastrid populations in the central Iberian Peninsula obtained from COI (below the diagonal) and 16S–tRNA (above the diagonal) data. Reference species are included. See Table 1 for locality complete name.

	ANC	BOA	CAB	UCE	FRE	LOZ	MOL	NAV	JAR	PAR	RED	SEV	SIG	SOT	TRE	VEN	*H. r*	*H. p*	*X. o*
ANC	x	12.55	9.84	9.60	8.92	10.37	9.18	10.68	8.76	12.79	8.76	12.53	9.90	9.84	8.97	8.84	12.19	13.15	12.76
BOA	17.81	x	13.42	12.23	13.19	13.36	12.43	13.70	12.52	7.27	12.42	3.21	13.74	13.66	12.35	12.23	17.39	17.12	15.75
CAB	16.29	18.99	x	7.45	5.97	1.34	5.01	2.01	4.93	12.79	4.68	13.26	8.30	8.12	4.46	4.66	17.91	17.95	16.18
UCE	18.74	22.06	17.95	x	6.85	7.02	7.01	7.32	6.61	11.69	5.93	11.59	7.67	7.40	6.35	6.38	19.11	18.53	15.55
FRE	16.37	20.15	15.33	16.41	x	6.14	5.62	5.92	4.99	13.35	4.78	13.00	8.93	8.67	4.77	5.09	18.76	17.90	15.74
LOZ	17.22	18.88	5.00	17.67	14.95	x	4.97	2.10	4.62	12.72	4.37	13.23	8.23	8.05	4.53	4.50	17.83	18.09	16.18
MOL	16.93	19.99	12.38	16.39	12.65	13.11	x	4.96	1.98	12.06	1.48	12.54	7.66	7.56	1.08	1.68	18.25	18.13	15.62
NAV	16.96	19.30	4.00	17.15	14.18	4.73	11.19	x	4.89	13.33	4.52	13.67	8.07	7.85	4.51	4.86	18.21	18.24	16.61
JAR	15.23	18.96	10.81	14.35	12.15	11.53	6.08	11.46	x	11.84	0.98	12.62	7.62	7.46	1.15	0.44	18.31	17.84	15.56
PAR	18.31	10.99	20.27	19.94	19.86	19.57	18.97	19.31	19.89	x	11.80	7.13	12.95	12.63	12.05	11.73	18.65	18.03	18.14
RED	14.91	17.52	9.64	12.75	11.57	10.83	5.84	10.15	3.50	17.72	x	12.72	7.31	7.20	0.77	0.70	18.22	17.85	15.45
SEV	17.06	6.55	20.07	21.04	17.95	18.46	20.19	19.36	17.83	11.41	16.58	x	13.67	13.47	12.76	12.45	19.05	18.13	16.20
SIG	15.22	19.71	17.70	19.19	15.51	17.28	15.19	17.82	14.48	17.97	12.85	18.30	x	0.55	7.45	7.59	19.01	17.92	15.09
SOT	15.73	18.95	18.45	19.64	15.37	17.59	15.88	17.92	15.48	18.73	14.05	18.57	2.03	x	7.21	7.38	18.93	17.98	15.66
TRE	14.89	16.72	10.79	14.15	12.04	10.86	3.45	10.09	5.69	16.05	4.37	17.28	12.71	13.31	x	0.93	18.03	17.34	15.06
VEN	15.34	18.46	10.49	13.52	11.94	11.34	5.69	10.60	1.45	18.88	2.05	17.59	14.54	15.19	4.55	x	18.35	17.77	15.47
H. riojana	19.73	18.64	20.07	23.21	21.41	22.04	20.87	21.07	20.23	17.45	19.60	18.82	20.94	21.34	18.36	20.0	x	6.62	15.04
H. pretiosa	21.87	22.51	21.26	24.53	21.79	21.59	22.11	20.14	21.69	20.97	20.92	23.18	23.25	23.34	19.95	21.42	15.83	x	12.67
X. omodeoi	20.85	21.39	21.54	22.84	21.20	21.35	21.27	20.86	20.12	19.26	18.43	21.21	22.15	21.14	18.58	19.65	21.03	19.98	x

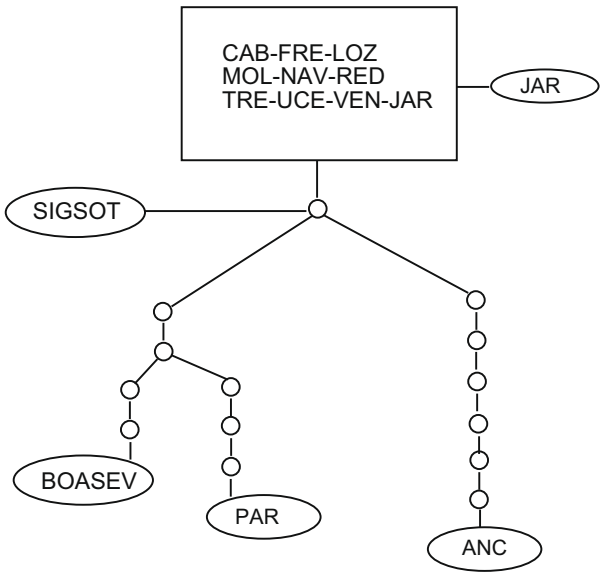

Fig. 1. TCS network for 28S gene fragment. Each haplotype is named with the abbreviation (see Table 1) of the localities where it was found. The size of the ovals and rectangle (ancestral haplotype according to TCS) depends on haplotype frequency. Empty circles represent intermediate haplotypes (unsampled). Each branch represents one mutational step and the branch length is meaningless.

An AMOVA (Table S6, Supplementary data) showed that most of the genetic variation was due to differences among species.

4. Discussion

4.1. Species-level diversification

The phylogenetic tree shows that *H. elisae* constitutes a monophyletic group separated from other *Hormogaster* species that are clustered together, in spite of being separated by a long geographical distance. Nevertheless, this homogeneous group presented very high divergent lineages. This differentiation is already captured at the population-level, as shown by the AMOVA, the high Φ_{ST} and divergence values, the exclusivity of mitochondrial haplotypes and the disconnected haplotype networks represented by TCS. All together, these results indicate a high degree of isolation and a lack of gene flow between populations.

Considering our data and following Wiens and Penkrot (2002) we propose the presence of five cryptic species within the complex of *H. elisae* (Fig. 2). The network constructed with the 28S gene precisely separated the five proposed species. This gene has been used in earthworms to elucidate phylogenetic relationships at higher taxonomic levels (Pérez-Losada et al., 2009) and hence its differentiation of the five groups is a robust confirmation of their specific value.

Moreover, the same five groups are shown to be disconnected networks for COI when excluding third positions and are distinguished as independent evolutionary lineages in every phylogenetic tree. The divergence values between proposed species were high and ranged from 9.49% to 18.31% for COI, and from 5.84% to 13.05% for 16S–tRNA, being similar to those found between different species (15.8% for COI and 6.62% for 16S between *H. riojana* and *H. pretiosa*) and making feasible the hypothesis of different cryptic species within *H. elisae*. Such high divergence values embrace the mean pairwise K2P species divergence of 11.3% (between congeneric species of various animals) and 15.7% (between annelid species) found in COI by Hebert et al. (2003) and similar values have been reported in other studies of clitellate annelids (see Novo et al., 2009 for examples). The AMOVA also exhibited significant structure when grouping the populations into these species. The cryptic species 1 showed a high intra-specific divergence (10.43% for COI and 4.27% for 16S), which should be investigated in the future. Indeed, UCE and FRE could be another example of cryptic speciation because they are independent evolutionary lineages but their position is uncertain as they showed differences among analyses. Besides, we captured only two individuals from FRE, which is not a sufficient sample size to support a conclusion. Finally, we included all these divergent populations within sp. 1 in order to be conservative because they shared the 28S haplotype. Fig. 1 shows that there is a population (JAR) with two 28S different haplotypes. This population is in the border of the sampled area for sp. 1 and could indicate the potential differentiation of that population in a future.

4.2. Cryptic speciation and morphological stasis

The morphological constancy found in *H. elisae* does not allow us to distinguish the five proposed species without the help of molecular techniques, which become particularly important in studies of non-visual invertebrates that live in cryptic environments (Proudlove and Wood, 2003).

These discordant patterns of morphological and molecular evolution (morphological stasis) can be common in soil environments where chemical signalling may play a more important role than morphology in sexual selection (Lee and Frost, 2002) and has been shown in taxa that occur in isolating environments, such as amphipods (Lefébure et al., 2006) and other earthworms (King et al., 2008). Typically, cryptic species have distinct, nonoverlapping geographical distributions (King et al., 2008, and references herein), as is the case in the five proposed species, perhaps because allopatric isolation limits gene flow between areas of suitable habitat. Stabilising selection may be imposed by the specialised environment and it minimises or eliminates the morphological change that can occur during speciation (Bickford et al., 2007). A pattern of allopatric speciation was also suggested for earthworms in Chang et al. (2008), whereas King et al. (2008) found highly divergent lineages even in sympatry.

Extreme subsurface conditions may also produce morphological constraints that could be responsible for a morphological convergence over large periods of time (Lefébure et al., 2006 and references herein). Indeed, the extreme conditions (low organic matter, cold winters and hot and dry summers) in the central area of Iberian Peninsula, which make the study sites unsuited for most species of earthworms, may have provoked this morphological stasis.

The difference in size between populations cannot be justified by a genetic differentiation. This is the opposite of the results recovered by Heethoff et al. (2004) who observed in *Octolasion tyrtaeum* two distinct size classes that represented two well-separated lineages for COII. In our case, these differences in size could then be attributed to environmental variations such as food availability (i.e., carbon content) and other soil properties. Nevertheless, we found no correlation for the measured soil factors, so a broader analysis may be necessary.

4.3. What causes these deep divergences

A pattern of isolation by distance was found and also a correlation was observed between genetic divergences and coarse sand and total loam. This could mean that the present-day scenario could be due to colonization in the past and a high fragmentation of populations, which have been shaped genetically, but not morphologically, by their environment. This is the first study that has demonstrated that environmental factors may have an influence on earthworms' genetic evolution. In contrast, Lentzsch and

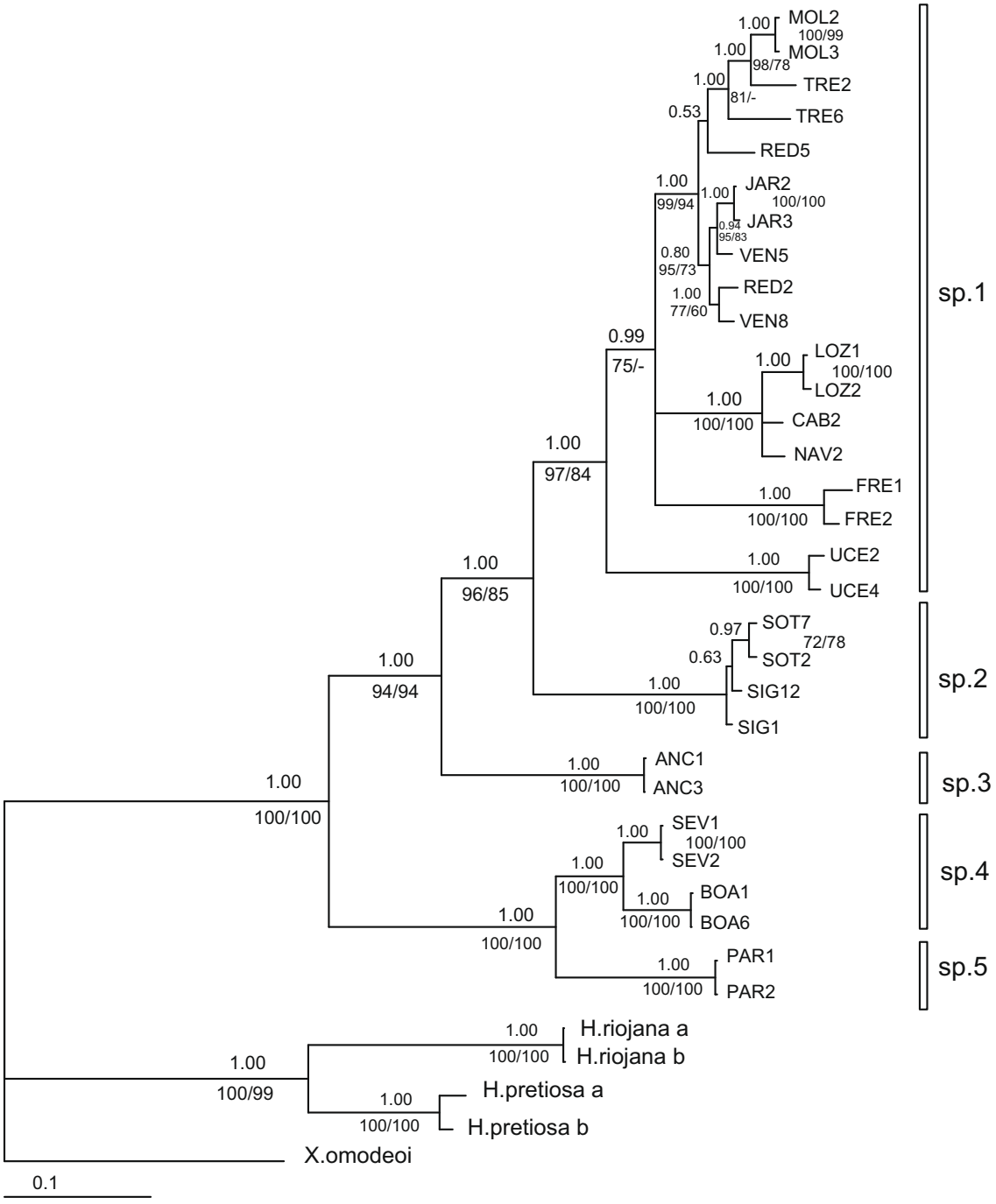

Fig. 2. Bayesian inference tree based on the analysis of the concatenated data set in morphospecies *H. elisae*. Posterior probability values are shown above branches when they are >0.5. Bootstrap values (1000 pseudoreplicates) of coincident associations given by ML and MP are shown below branches when they are >50% (ML/MP). Each haplotype is named with the abbreviation of its locality (see Table 1) followed by its number. The proposed species are indicated (see Section 4).

Golldack (2006) indicated that the distribution of *A. caliginosa* genotypes was not related to soil properties. Nevertheless, the relationship between earthworm distribution and soil factors is well known (Edwards, 2004) and specifically *H. elisae*'s abundance is proved to be positively correlated with total and coarse sands and negatively with clay, nitrogen, carbon and coarse loam contents (Hernández et al., 2007).

The high divergences found and the population structuring can be explained by the poor dispersion capacity of earthworms of around 2–4 m/year. This pattern of isolation by distance may be due to a spread of the earthworms via active or nonanthropogenic passive dispersal (e.g., by birds, mammals, wind or waterways) although there is limited evidence for the latter cause (Cameron et al., 2008 and references herein). This limited dispersion ability, together with the endogeic nature of *H. elisae* and the extreme environment facilitate the high isolation of populations and the absence of gene flow between them.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.04.010.

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