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Understanding the biogeography of a group of earthworms in the Mediterranean basin—The phylogenetic puzzle of Hormogastridae (Clitellata: Oligochaeta)

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ABSTRACT

Traditional earthworm taxonomy is hindered due to their anatomical simplicity and the plasticity of the characteristics often used for diagnosing species. Making phylogenetic inferences based on these characters is more than difficult. In this study we use molecular tools to unravel the phylogeny of the clitellate family Hormogastridae. The family includes species of large to mid-sized earthworms distributed almost exclusively in the western Mediterranean region where they play an important ecological role. We analyzed individuals from 46 locations spanning the Iberian Peninsula to Corsica and Sardinia, representing the four described genera in the family and 20 species. Molecular markers include mitochondrial regions of the cytochrome *c* oxidase subunit I gene (COI), 16S rRNA and tRNAs for Leu, Ala, and Ser, two nuclear ribosomal genes (nearly complete 18S rRNA and a fragment of 28S rRNA) and two nuclear protein-encoding genes (histones H3 and H4). Analyses of the data using different approaches corroborates monophyly of Hormogastridae, but the genus *Hormogaster* is paraphyletic and *Hormogaster pretiosa* appears polyphyletic, stressing the need for taxonomic revisionary work in the family. The genus *Vignysa* could represent an early offshoot in the family, although the relationships with other genera are uncertain. The genus *Hemigastrodrilus* is related to the *Hormogaster elisae* complex and both are found in the Atlantic drainage of the Iberian Peninsula and France. From a biogeographic perspective Corsica and Sardinia include members of two separate hormogastrid lineages. The species located in Corsica and Northern Sardinia are related to *Vignysa*, whereas *Hormogaster pretiosa pretiosa*, from Southern Sardinia, is closely related to the *Hormogaster* species from the NE Iberian Peninsula. A molecular dating of the tree using the separation of the Sardinian microplate as a calibration point (at 33 MY) and assuming a model of vicariance indicates that the diversification of Hormogastridae may be ancient, ranging from 97 to 67 Ma.

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1. Introduction

The Mediterranean basin is a hotspot of outstanding biodiversity and should have priority for conservation policies, and therefore should benefit from biogeographic and evolutionary studies (Blondel and Aronson, 1999; Myers et al., 2000). Numerous studies have centered on the biogeography of circum-Mediterranean groups (e.g., Carranza and Arnold, 2003; Santos et al., 2007; Murienne and Giribet, 2009; Ribera et al., 2010), but only a few have focused on earthworms (Annelida, Clitellata, Oligochaeta) (Novo et al., 2009, 2010a), a group of terrestrial invertebrates with a great biogeographical potential due to narrow ecological requirements and their supposed old age. Bouché (1972) suggested that hormogastrid earthworms were already present in the Iberian plate in the Late Cretaceous, occupying areas that would coincide mainly with their present distribution. The edge of the Iberian Plate underwent drastic changes that affected the Betic–Rifean range, Balearic Islands, Kabylies, Corsica, Sardinia and Calabria (see Magri et al., 2007). It is in all these areas, once forming a primitive Iberian plate, where hormogastrids should be sought for in order to understand their evolutionary and biogeographic relationships. To date, hormogastrids have been discovered in all these regions, with the exception of the Betic and Rif ranges.

Hormogastridae currently includes 22 species in four genera (plus eight subspecies or varieties) of large to mid-sized (approximately 5–90 cm in length) earthworms distributed almost exclusively in the western Mediterranean (Díaz Cosín et al., 1989; Cobolli-Sbordoni et al., 1992) where they play an important ecological role aerating the soil and recycling nutrients (Bouché,

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1972). Traditionally, earthworm systematics has been based principally on morpho-anatomical features (Bouché, 1972; Sims and Gerard, 1999). Nevertheless, their taxonomy, and specifically that of hormogastrids has remained unstable and their phylogenetic relationships are uncertain because of the low number of characters available for study (Pop et al., 2003; Álvarez, 1977). The shape and number of spermathecae have been used as taxonomic characters, but they can vary with developmental stage. Other characters used in species diagnoses, such as the extension of the clitellum or the developmental degree of tubercula pubertatis, also show ontogenetic and reproductive differences. Although morphology still has an important role for species recognition in hormogastrids (Omodeo, 1956; Bouché, 1972; Qiu and Bouché, 1998), the degree of homoplasy in many characters is high, probably reflecting high levels of phenotypic plasticity.

In two recent studies, Novo et al. (2009, 2010a) detected high genetic diversity among populations of the *Hormogaster elisae* species complex in the central Iberian Peninsula, as well as between this species complex and other hormogastrid species. These studies questioned the status of *H. elisae* as a valid species and discussed its affinities within the family. In order to re-evaluate the status of *H. elisae*, and to test species and genus delimitations in Hormogastridae, we expanded both taxon sampling and the number of molecular markers and inferred the phylogenetic history of the family by using data from nine molecular markers. We also used geological events occurring during the evolution of the Mediterranean basin as a calibration point to provide an estimate of the age of diversification of the family.

2. Materials and methods

2.1. Sampling and morphological study

Mature individuals, representing 20 of the 22 described species of Hormogastridae, were collected from 30 localities in the Iberian Peninsula, France (including Corsica) and Sardinia, including the type locality of each known species. Additional data from the study of Novo et al. (2010a) in Central Spain were included, making for a total of 46 localities (Tables S1 and S2, Fig. 1). We included an immature specimen collected near the shore of the Ter river (approximately 9 km from Girona, Spain) that was analyzed for allozymes in Cobolli-Sbordoni et al. (1992), and identified as *Hormogaster pretiosa* cf. *hispanica* (HPA). Amplification of specimens from Elba, Sardinia, and France, also provided by P. Omodeo, was unsuccessful.

The type locality of *Hormogaster redii redii* (Ghilarza [GHI], Sardinia, Italy) (Rosa, 1887), the type species of the family, was sampled for this study. In addition an individual of this species from Alghero (ALG, Sardinia, Italy) was kindly provided by Magualida Ricoy. A new species, whose diagnosis will be presented (M. Novo, work in progress), was collected at Sant Joan de les Abadesses (SAN, Girona, Spain). The type locality of *Hormogaster multilamella* (Etxauri, Navarra, Spain) was visited several times but no specimens were obtained. Amplification of the target regions from a formalin-fixed specimen kindly provided by *El Museo de Zoología de la Universidad de Navarra* was also unsuccessful. Specimens collected in Talarn (TAL, Lleida, Spain) do not match the morphological description of *Hormogaster lleidana*, and we refer to them as *Hormogaster* sp. These two species, *H. multilamella* and *H. lleidana*, are thus the only described hormogastrids missing from this study. However, the identity of these two species is questionable, as Qiu and Bouché (1998) based their morphological descriptions on a single adult specimen for *H. multilamella* and one adult and one juvenile for *H. lleidana*, which could explain the difficulties in collecting the specimens in the first case and the mismatch morphology in the second.

All individuals were washed in distilled water and preserved in ca. 96% EtOH at −20 °C for subsequent molecular work. A portion of the integument (ca. 25 mg) was carefully cleaned under a stereomicroscope to remove soil particles and associated fauna. Subsequently, integument samples were hydrated and preserved at −80 °C until DNA extraction.

2.2. DNA extraction, gene amplification and sequencing

Total genomic DNA was extracted from a tissue sample from the integument using the DNeasy Tissue Kit (QIAGEN) eluting twice with 70 μL of buffer. Molecular markers included mitochondrial regions of the cytochrome *c* oxidase subunit I gene (COI), 16S rRNA gene and tRNA Leu, Ala, and Ser, two nuclear ribosomal genes (complete 18S rRNA and a fragment of 28S rRNA) and two nuclear protein-encoding genes (histone H3 and histone H4) (see Table 1 for primer information). We also tried amplifying the Internal Transcribed Spacer gene (ITS) in two fragments with the primers: 9F: 5′–GTA GGT GAA CCT GCG GAA GG–3′ (Carranza, 1997) and 5.8Srev: 5′–GCG TTC AAA GAC TCG ATG ATT C–3′ for ITS1 and 5.8Sfor: 5′–GAA TCA TCG AGT CTT TGA ACG C–3′ and 28Srev: 5′–GTT AGT TTC TTT TCC TCC GCT T–3′ for ITS2, but only ITS1 was consistently amplified, and only in two of the populations studied (CER: *Hormogaster sp.* and GRA: *Hormogaster eserana*). Only two nucleotide changes were detected among them.

Polymerase chain reactions (PCR) (50 μL) included 0.6 μL of template DNA, 1 μM of each primer, 200 μM of dNTPs (Invitrogen), 1X PCR buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems,) and 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems). DMSO (1 μL) was included for the 28S rRNA reaction. PCR reactions were carried out using a GeneAmp Multicycler Ep gradient (Eppendorf), and involved an initial denaturation step (5 min at 95 °C) followed by 35 or 40 cycles including denaturation at 95 °C for 30 s, annealing (ranging from 49 °C to 60 °C) for 30 s or 1 min, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. PCR products were resolved by 1% agarose gel electrophoresis, visualized by ethidium bromide fluorescence and purified using MultiScreen PCR Plates and a Vacuum Manifold (Millipore). The purified products were sequenced directly with the same primer pairs used for amplification. Each sequence reaction contained a total volume of 10 μL including 3 μL of the PCR product, 0.32 μM of one of the PCR primer pairs, 0.25 μL of ABI BigDye 5X sequencing buffer and 0.5 μL of ABI BigDye Terminator (Applied Biosystems). The sequencing reactions involved an initial denaturation step (5 min at 95 °C), and 30 cycles (95 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min). The BigDye-labeled PCR products were cleaned using Performa DTR Plates (Edge Biosystems,). The sequence reaction products were then analyzed using an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems).

Chromatograms were visualized in Sequencher v.4.7 (Gene Codes Corporation) to obtain the assembled sequences. These were compared against the GenBank database with the BLAST algorithm (Altschul et al., 1997). All new sequences have been deposited in GenBank (Tables S1 and S2).

2.3. Data analysis

2.3.1. Phylogenetic relationships

Sequences of the polychaete *Arenicola marina* and other clitellates were included as outgroups. These sequences were kindly provided by the Protostome AToL project (PI G. Giribet), downloaded from GenBank (see Table S3), or generated de novo (histone H4 for *Aporrectodea trapezoides*).

Data analyses were based on two approaches, a traditional two-step approach (multiple sequence alignment + phylogenetic analysis) and a single-step approach with direct optimization (Wheeler,

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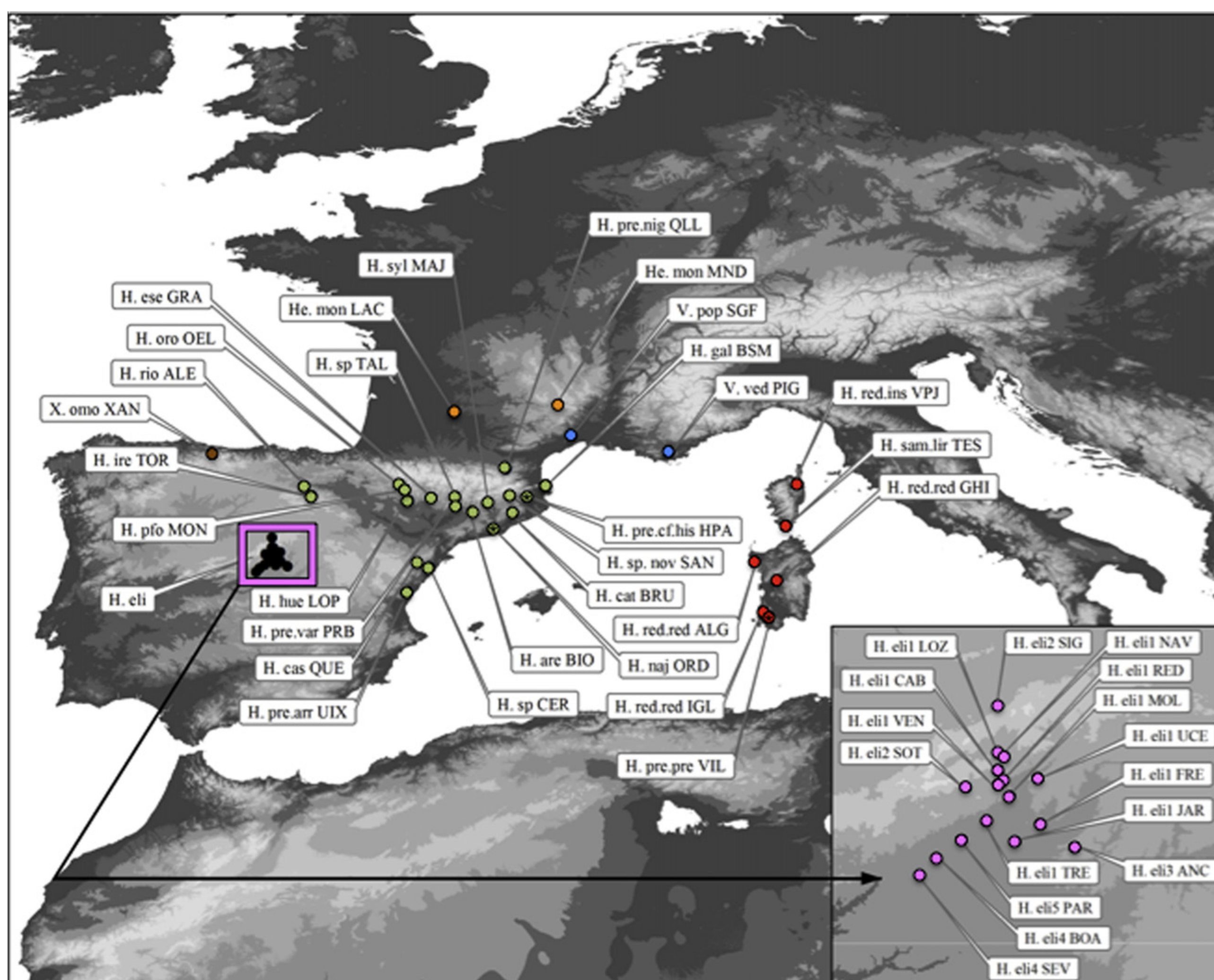

Fig. 1. Sampling localities. The abbreviation of the species found in each locality is indicated (see in Table S1 the complete name of species and localities). Colors match those used in the phylogenetic trees. ORD, HPA y VIL are shown with an asterisk since they form the clade used for tree calibration (see phylogenetic trees). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Sequences of primers used to amplify and sequence gene fragments in this study.

Amplified region (size in bp.)	Primer name	Primer sequence (5′–3′)	Reference
COI (648)	LCO1490 HCO2198	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
16S-tRNAs (759–793)	16S-tRNA-Leu-Ala-Ser-Leu-LumbF2 Ho_16sRa:	CGACTGTTTAACAAAAACATTGC GCACTATTCTGCCAYCTTGT	Pérez-Losada et al. (2009) Novo et al. (2010a)
18S (1701)	1F 4R 3F 18Sbi 18Sa2.0 9R	TACCTGGTTGATCCTGCCAGTAG GAATTACCGCGGCTGCTGG GTTCGATTCCGGAGAGGGA GAGTCTCGTTCGTTATCGGA ATGGTTGCAAAGCTGAAAC GATCCTTCCGCAGGTTCACCTAC	Giribet et al. (1996), Whiting et al. (1997)
28S (738–739)	28S-F1 28S-R1	GAGTACGTGAAACCGTCTAG CGTTTCGTCCCCAAGGCCTC	Pérez-Losada et al. (2009)
H3 (328)	H3a F H3a R	ATGGCTCGTACCAAGCAGACVGC ATATCCTTRGGCATRATRGTGAC	Colgan et al. (1998)
H4 (183)	H4F-2S H4F-2er	TSCGIGAYAACATYCAGGGIATCAC CKYTTIAGIGCRTAIACCACRTCCAT	Pineau et al. (2005)

1996). For direct optimization we used parsimony as the optimality criterion, as implemented in the program POY v.4.1.2 (Varón et al., 2010). Tree searches were conducted by a combination of random addition sequences with subtree pruning and regrafting (SPR) and tree bisection and reconnection (TBR) branch swapping followed by multiple rounds of tree fusing (Goloboff, 1999,

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2002). The protein-encoding genes were "pre-aligned" as their alignments were trivial due to lack of length variation. We undertook a sensitivity analysis of 10 parameter sets varying the contributions of indels and base transformations (Wheeler, 1995) and used an index of character congruence for selecting the optimal parameter set. For the sensitivity analyses data were analyzed in combination and for each independent partition. Nodal support was estimated by bootstrap resampling (Felsenstein, 1985) with 100 pseudoreplicates.

For the two-step approach sequences of each individual gene were aligned in CLUSTALX v. 2.0.12 (Thompson et al., 1997) with default settings and concatenated with PHYUTILITY (Smith and Dunn, 2008). jModelTest v. 0.1.1 (Posada, 2008) was used to select the best-fit evolutionary model using the Akaike information criterion (AIC; Akaike, 1973). The phylogenetic hypothesis was then estimated with 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 and the nucleotide substitution estimates were allowed to vary independently between each partition. Parameters in MRBAYES were set to two million generations and 4000 trees were sampled for every 500th generation, using the default random tree option to initiate the analysis. The analysis was performed twice and all sample points prior to the plateau phase were discarded as burn-in. The remaining trees were combined to find the maximum *a posteriori* probability estimate of phylogeny. Because we detected a saturation pattern in third codon positions of COI and H3 using DAMBE (Xia and Xie, 2001), a second phylogenetic tree was also constructed without these positions. We also explored the results after an alignment with MAFFT v.6 (Katoh et al., 2005; online server: http://mafft.cbrc.jp/alignment/server/). Finally, trees were inferred with mitochondrial and nuclear fragments separate using the same settings as indicated for the general analysis, to test for possible conflicting signal among these two sets of genes.

A maximum likelihood (ML) analysis was performed with PhyML v. 2.4.4 (Guindon and Gascuel, 2003) using simultaneous NNI to estimate tree topology. The best-fit evolutionary model selected by jModelTest for the concatenated sequences was specified and the program optimized α and I values. Clade support was evaluated by 1000 replicates of non-parametric bootstrapping (Felsenstein, 1985).

2.3.2. Estimating divergence times

All the phylogenetic trees were calibrated by using the separation between *H. pretiosa pretiosa* from Sardinia (VIL) and the continental species clustering in the same clade, *H. pretiosa* cf. *hispanica* collected in the shore of the river Ter (HPA) and *Hormogaster najaformis* (ORD), assuming a divergence time for these lineages going back to the separation of the Corso-Sardinian microplate from continental Europe. This is justified due to the low dispersal ability of earthworms in general (Ligthart and Peek, 1997; Hale et al., 2005) and of hormogastrids in particular (Novo et al., 2010b; Omodeo and Rota, 2008). This low vagility is best explained by the endogeic nature of hormogastrids. As a calibration point we use the most current estimates for the separation of the occidental Mediterranean microplates (33 Ma; Schettino and Turco, 2006). It is of course well understood that this minimum age only applies if there has been no transoceanic dispersal of hormogastrid species, an assumption that we accept given the biology of the animals of choice.

To our knowledge, there are no similar substitution rate estimates in earthworms that could be used for comparison to our data. Buckley et al. (2011) constructed an ultrametric tree with BEAST for New Zealand megascolecids, including 16S rRNA and 28S rRNA but their age estimation was relative due to the lack of a calibration point. Also, Chang et al. (2008) offered a rough estimate for the COI rate of a megascolecid species complex, but this is not comparable to our estimate of the combined mitochondrial genes.

Clockness of the data (constant substitution rate) was tested by means of a likelihood-ratio test in jModelTest. The Bayesian tree and the evolutionary model parameters employed were introduced in PaupUP v. 4.0b10 (Calendini and Martin, 2005) and its likelihood values were estimated with and without the assumption of a molecular clock. The comparison of these values in jModelTest was significant, indicating the lack of a molecular clock (the substitution rates vary along the branches of the tree). Therefore r8s v. 1.71 (Sanderson, 1997, 2006) was used for estimating divergence times. Initially the truncated Newton (TN) algorithm was used to do a cross-validation with eight smoothing factors. Divergence times were then estimated using the penalized likelihood (PL) method and the optimal smoothing value and fixing the age of the separation between continental "*H. pretiosa*" (see below) and the Sardinian population (where this species was originally described by Michaelsen, 1899) at 33 Ma. The analyses were then repeated with both the Bayesian and maximum likelihood trees, including and excluding outgroups. Another analysis was conducted with the implied alignment (Wheeler, 2003; Giribet, 2005) from POY under the optimal parameter set (221). The implied alignment was then introduced in PAUP* 4.0b3 (Swofford, 2002) to estimate branch lengths and finally the divergence times were calculated with r8s.

We finally obtained an ultrametric tree by means of Bayesian estimations as implemented in BEAST v.1.6.1. (Drummond and Rambaut, 2007), including the same evolutionary models as in the phylogenetic analyses for each gene partition. The well-supported node of all the earthworms was constrained to be monophyletic and the analysis was performed under an uncorrelated lognormal relaxed clock and a Yule diversification model. The age of the calibration clade was set to a normal distribution with mean of 33 MY and a 95% confidence interval. BEAST was run for 50 millions of generations, saving trees at every 5000th generation. The "burnin" was set to 2000 in TreAnnotator v.1.6.1. after visualizing the results with Tracer v.1.5. (Drummond and Rambaut, 2007).

3. Results

3.1. Morphological features

The morphological characters from the individuals collected in Cervera del Maestre (CER, Castelló, Spain), match those described by Zicsi (1970) for *H. pretiosa* from the same locality. These characters, however, do not match the original description by Michaelsen (1899) and emended by Omodeo (1956). Therefore we use the name *Hormogaster* sp. for these specimens for the reasons outlined below. Figs. 2 and 3 illustrate important morphological features of the species included in this study.

3.2. Gene diversity and phylogenetic relationships

Values of genetic variability for the Hormogastridae data set are shown in Table 2. The mitochondrial COI and 16S-tRNA were the most variable regions, showing a mean percentage of uncorrected pairwise divergence between 14.5% and 17.4%. The nuclear genes were less variable, showing a mean percentage of uncorrected pairwise divergence of around 3% for histones (being H3 more variable than H4) and around 2% for 28S rRNA. The least variable gene was 18S rRNA, showing a mean percentage of uncorrected pairwise divergence of only 0.23%. Within the

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Fig. 2. Morphological features of the different genera of hormogastrids. *Xana omodeoi* (A–C), specimens (A) spermathecae from one side (B) and typhosole (C). *Vignysa* (D–G). D: Spermathecae from one side of *V. popi*: E: Anterior part of a *V. popi* specimen collected in Saint Gely du Fesc (France); F: Blood vesicles of *V. popi*; G: Pair of spermathecae of *V. vedovinii*. *Hormogaster* (H and I). Anterior part of a *Hormogaster redii insularis* specimen (H: Volpajola, Corsica, France) and a *Hormogaster samnitica lirapora* specimen (I: Col de la Testa, Corsica, France). *Hemigastrodrilus monicae* (J–M). J: Femenine pores (pointed by the arrows), located in the ventral area; K: Specimens of *He. monicae* collected in Cardayre (France); L: Morren gland; M: Spermathecae from one side.

Hormogastridae data set indels were only found in the 16S-tRNA and 28S rRNA genes.

In the parsimony analysis with direct optimization the combination of parameter sets that minimized the incongruence among partitions was "221" (Table 3). By applying this optimal combination only one tree of 10,933 weighted steps was found (Fig. 4).

The multiple sequence alignment of the concatenated sequence data used for the two-step phylogenetic analyses was 4547 bp in length. Likelihood ratio tests performed with jModelTest indicated that the best-fit models of sequence evolution are GTR + I + G for the concatenated data set, as well as for all individual partitions, with the exception of histone H4, which adjusts to a HKY + I model.

The 50% majority-rule consensus tree generated by MRBAYES for this alignment is shown in Fig. 5 (BI). The topology was identical whether the third codon positions for COI and histone H3 were excluded or not, but posterior probability values varied slightly (not shown). The maximum likelihood tree for all data for the aligned data set (*ln*L = –37,467.335496) is also shown in Fig. 5 (ML). The analyses after alignment of sequences with MAFFT showed similar results, with the previously well-supported clades being the same.

All trees share common features such as the monophyletic nature of Hormogastridae (support is low in the direct optimization analysis), the paraphyly of *Hormogaster*, which includes the genera *Xana* and *Hemigastrodrilus* in all trees and *Vignysa* in some of the analyses, or the polyphyly of *H. pretiosa*. Many of the identified clades are also shared among analyses. The *H. elisae* species complex constitutes a lineage generally related to *Hemigastrodrilus* (MP and ML), but no other *Hormogaster* species appear in this clade. The *Hormogaster* species from the NE Iberian Peninsula and S France form another stable clade, with two exceptions, *H. najaformis* (ORD) and the *H. pretiosa* cf. *hispanica* specimen from the Ter river, both forming a clade with the population of *H. pretiosa pretiosa* from Sardinia (VIL) and with *Xana omodeoi*. A clade formed by *H. redii* and *Hormogaster samnitica lirapora* from Corsica and Sardinia sometimes appears related to *Vignysa* (BI and ML analyses). However, this latter genus appears to have diverged earlier from the rest of the family in the direct optimization analysis (Fig. 4).

The trees built with mitochondrial and nuclear genes separately (Fig. S1) are generally congruent with the trees obtained from the combined analysis—our preferred hypothesis—, but there are differences worth highlighting. When mitochondrial genes were analyzed alone, lumbricids nest within homogastrids, forming a clade with *Vignysa*. In the tree based on nuclear sequence data alone, *Xana* and *Vignysa* form a clade with high support values, a result not obtained in other analyses. These genes also support a subdivision of the clade that contains most of the species from the NE Iberian Peninsula and S France.

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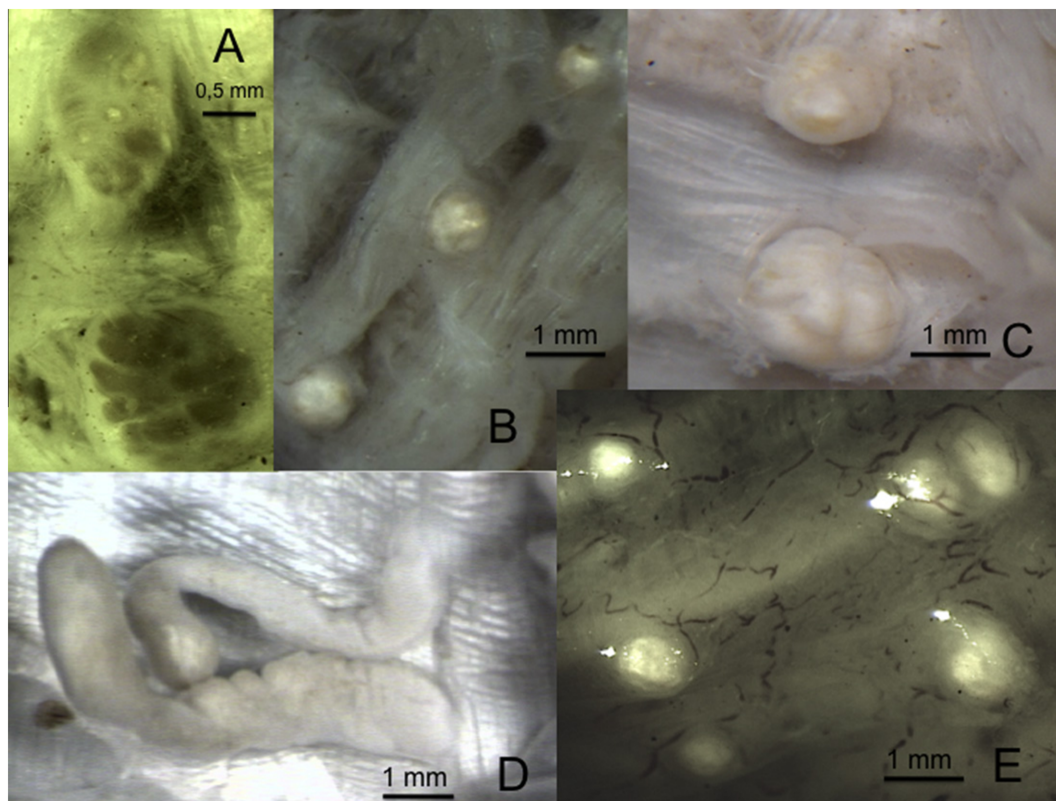

Fig. 3. Spermathecae of different described species of *Hormogaster*. A: *H. pretiosa nigra*, collected in Quillan (France); B: *H. redii insularis*, collected in Volpajola (Corsica, France); C: *H. riojana*, collected in Alesanco (Spain); D: *H. elisae*, collected in Siguero (Spain); E: *H. samnitica lirapora*, collected in Col de la Testa (Corsica, France).

Table 2
Diversity values of the gene fragments used for the analyses within Hormogastridae. *N*: Number of individuals analyzed (in the case of the histones, two alleles per individual are analyzed, due to the presence of ambiguities). *NHAP*: Number of identified haplotypes; *H*: Haplotypic diversity; π: Nucleotidic diversity; *S*: Number of polymorphic sites; Parsimony IS: Number of Parsimony Informative Sites; *Ts*: Number of Transitions; *Tv*: Number of Transversions; *Dp*: Mean number of pairwise differences (*N*: Total number; %: percentage). Standard deviation of the estimates shown in parentheses.

	COI	16S-tRNA	H3	H4	28S	18S
N	45	46	46 (92)	44 (88)	45	46
NHAP	45	46	44	24	29	19
H	1 (0.0047)	1 (0.00145)	0.98 (0.0032)	0.91 (0.0188)	0.93 (0.03)	0.90 (0.03)
π	0.174 (0.085)	0.144(0.070)	0.036 (0.018)	0.027 (0.014)	0.021 (0.011)	0.002 (0.001)
Sites	648	806	328	183	739	1701
Gaps	0	77	0	0	3	0
Mutations	498	532	86	34	89	22
S	262	366	64	26	73	21
Parsimony IS	250	225	63	26	49	21
Ts	350	334	53	21	50	19
Tv	148	198	33	13	39	3
Dp (*N*)	113.04 (49.44)	116.45 (50.89)	11.87 (5.42)	4.88 (2.40)	15.58 (7.09)	3.87 (1.98)
Dp (%)	17.44 (7.63)	14.45 (6.31)	3.62 (1.65)	2.67 (1.31)	2.11 (0.96)	0.23 (0.12)

3.3. Divergence time estimates

When the separation of the Western Mediterranean microplates was used as a calibration point, both the maximum likelihood and the Bayesian analyses (Fig. 5) show concordant dates and the inclusion or exclusion of the outgroups had no effect on the dates. The calibration of the tree from direct optimization (Fig. 4) has some differences due to the topological disagreement mostly of *Vignysa*, but the main dates coincide. Fig. 6 illustrates the results of the tree generated with BEAST, that showed a later diversification date than alternative methods. Hormogastrids seem to have started diversifying at 97–67 Ma (this range shows the values of the different methods), and the clade formed by the *Hormogaster* species from the NE Iberian Peninsula and S France is dated at 56–46 Ma. Perhaps most interesting is the old age of the *H. elisae* species complex, dated at 42–37 Ma. The recovered substitution rates were similar among the analyses. As an example, the values from the analyses with r8s and the Bayesian tree were 0.28% (sd. 0.16%) substitutions per site per million years for the concatenated data set, 0.52% (sd. 0.20%) for the mitochondrial genes and 0.017% (sd. 1.822 e-10) for the nuclear genes.

4. Discussion

All phylogenetic analyses of the combined data show the monophyly of the family Hormogastridae, which is well supported in the probabilistic two-step analyses, as well as in the direct optimization approach, albeit with low support values in the latter.

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Table 3
Tree lengths for the different partitions analyzed and congruence values (ILD) for the combined analysis of the six loci with different parameter set combinations (left column). The first numeral used in the parameter set corresponds to the ratio between indel/transversion and the following two numbers correspond to the ratio between transversion/transition; for example, 111 corresponds to equal weights and 121 corresponds to a indel/tranversion ratio of 1 and to a transversion/transition ratio of 2:1, thus, the indels and tranversions have a cost of 2, and transitions have a cost of 1. (For a list of the specific step matrices that this involves see Giribet et al., 2002). 3221 indicates an indel opening of 3, and transversion = transition = 2, and an indel extension of 1. The optimal ILD value is highlighted in italics.

	COI	16S-tRNA	H3	H4	28S	18S	All	ILD
111	2927	2210	324	57	651	637	6980	0.02493
121	4059	3332	479	81	1009	964	10150	0.02227
141	6273	5492	779	127	1679	1592	16,332	0.02388
211	2927	2457	324	57	737	732	7415	0.02441
221	4059	3771	478	81	1154	1152	10,933	*0.02177*
241	6273	6353	779	127	1970	1970	17,872	0.02238
411	2927	2811	324	57	839	905	8066	0.02517
421	4059	4457	479	81	1358	1495	12,197	0.02197
441	6273	7700	779	127	2383	2654	20,396	0.02353
3221	2927	2701	324	57	810	797	7798	0.02334

The only significant discrepancy among analyses seems to be due to possible conflict between the nuclear and mitochondrial genes, as the latter group lumbricids with *Vignysa*. In a similar case by Briones et al. (2009) it was concluded that 16S rRNA and COI have limited discriminatory power above the genus level. Nevertheless, and in contrast to our combined approach, Jamieson (1988) suggested non-monophyly of Hormogastridae based on morphological characters, suggesting that *Vignysa* was related to lumbricids whereas *Hemigastrodrilus* and *Hormogaster* were more closely related to microchaetids and glossoscolecids. Future analyses including representatives of these additional earthworm families should be able to fully test Jamieson's phylogenetic scheme.

The geological scenario found at the moment when Hormogastridae may have started its diversification (ca. 97–67 Ma) suffices for explaining their current distribution without the need to invoke oceanic dispersal. This date range agrees with the late Cretaceous to the beginning of the Tertiary postulated by Bouché (1972) for the origin of the Lumbricoidea families, and suggests that the current distribution of the different genera was probably achieved during the early Cenozoic. Omodeo and Rota (2008) however, use 180 million years for the split of two megadrile genera (see Omodeo, 2000). This was based on estimates of the divergence time between congeneric species of both sides of the Atlantic (Omodeo, 2000), but it is not applicable for the present study because substitution rates in earthworms are highly variable even among members of the same family (Novo, 2010). Of course, our

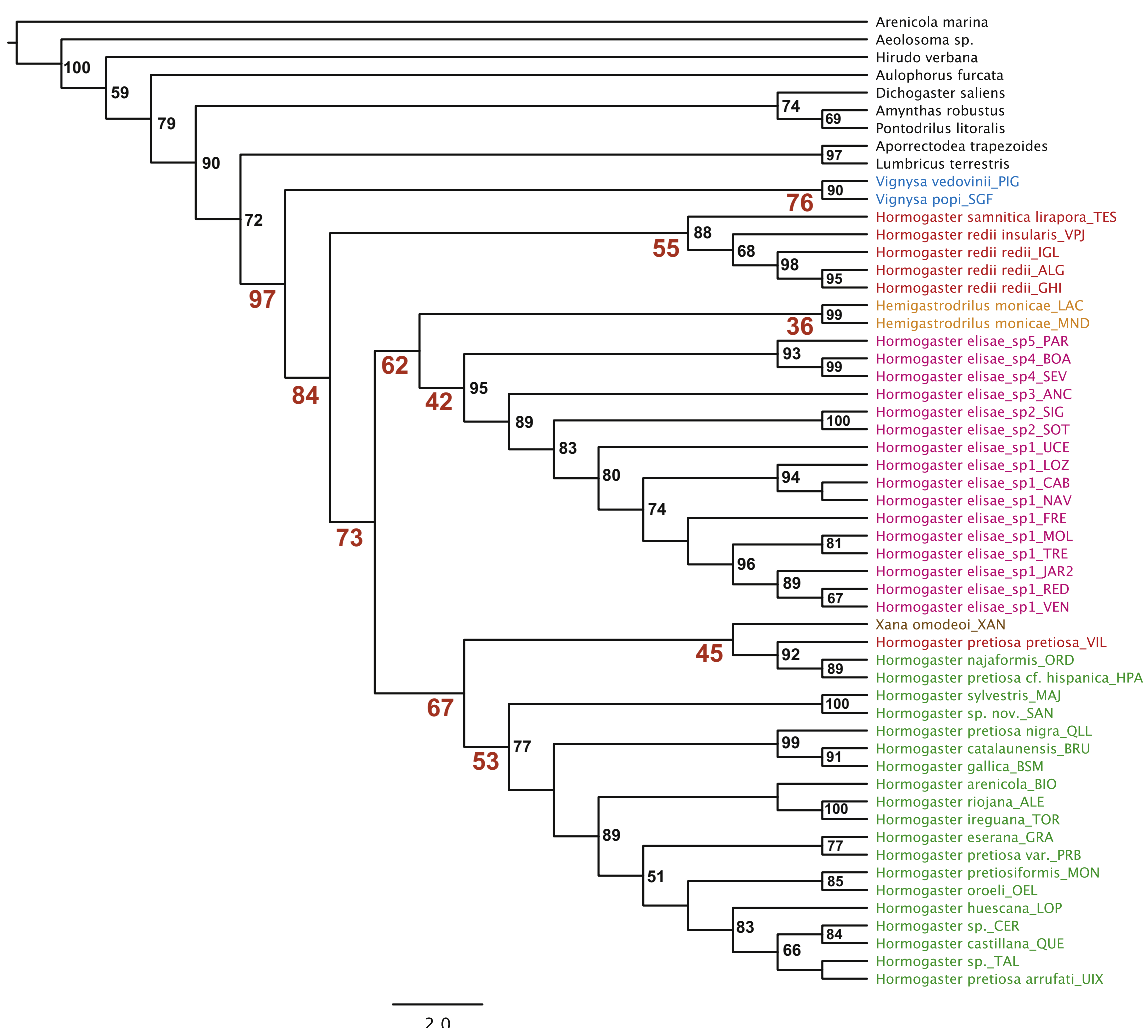

Fig. 4. Most parsimonious tree for parameter set 221 of 10,933 weighted steps built with POY and based on the six gene fragments amplified for hormogastrids. Bootstrap values (100 pseudoreplicates) are shown on the nodes. Below the nodes of the main clades, the dates generated by r8s are shown in red (MY). The colors for the terminals match those used in the map from Fig. 1: *Hemigastrodrilus* in orange, *Vignysa* in blue, *Xana* in brown, *Hormogaster* from Corsica and Sardinia in red, *Hormogaster* from the NE of the Iberian Peninsula and S of France in green, *Hormogaster elisae* species complex in pink, outgroups in black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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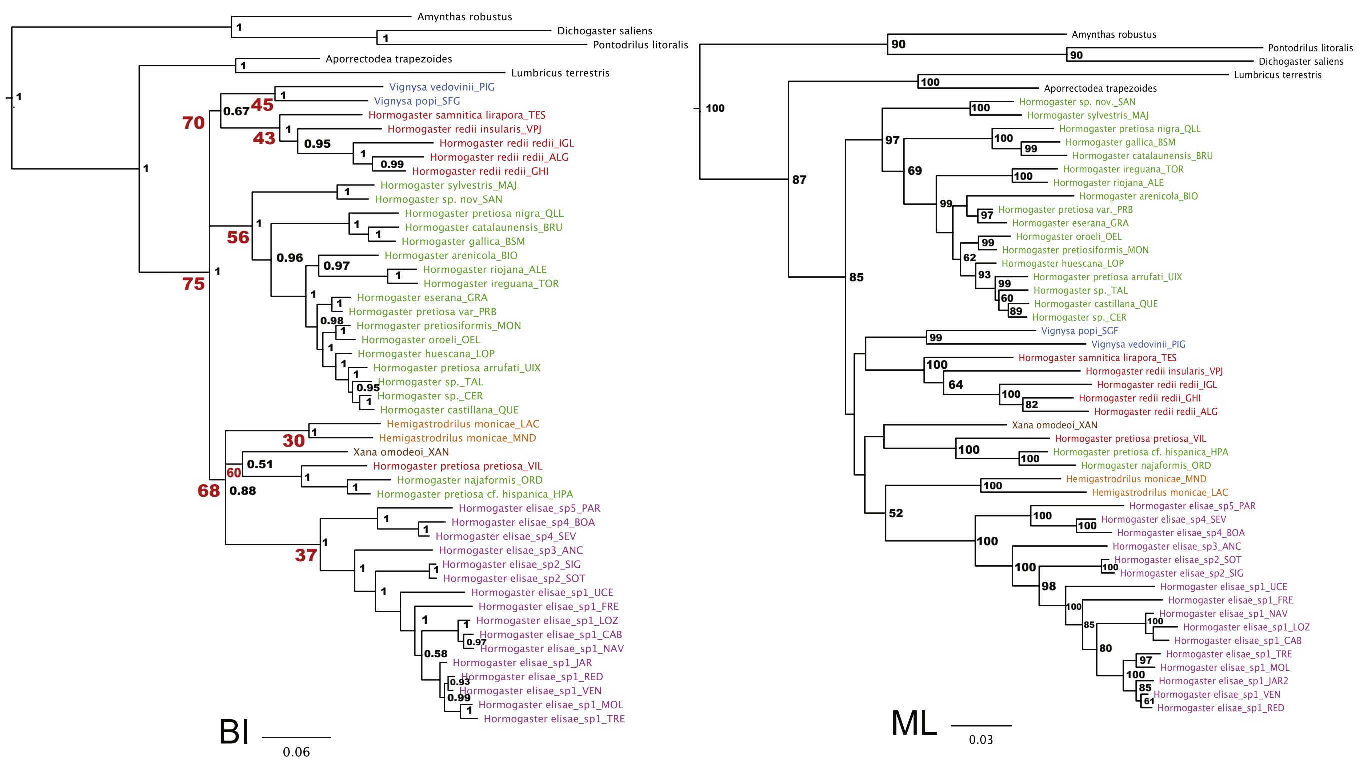

Fig. 5. Left: Bayesian phylogenetic hypothesis (BI) based on the six gene fragments amplified for hormogastrids. Posterior probability values are shown on the nodes. Below the nodes of the main clades, the dates generated by R8S are shown in red (MY). Right: Maximum likelihood tree (ML) (log likelihood: −37,467.335496), based on the six gene fragments amplified for hormogastrids. Bootstrap values (1000 pseudoreplicates) are shown on the nodes. Colors as in Figs. 1 and 4. Some outgroups were deleted from the trees. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

estimates are contingent to our assumption that the cladogenetic event leading to *H. pretiosa pretiosa* from Sardinia (VIL) and the continental specimens of *Hormogaster* was due to a vicariant event during the separation of the Corso-Sardinian microplate from continental Europe. If this were the case, the cladogenetic event could still be older than the geologic event used for the calibration, and therefore our estimate for the divergence of the family would appear younger than it was.

During the late Cretaceous the Iberian Peninsula was divided into an Eastern and a Western area separated by water (Andeweg, 2002), perhaps explaining the current absence of hormogastrids in the westernmost area of the Iberian Peninsula. The Betic range was then occupied by a water mass that closed when this range emerged (approximately 11 MY ago; Andeweg, 2002), explaining also their absence in the SE Iberian Peninsula, acting the flooded area first and the mountain range later, both, as geographic barriers to dispersal. The current distribution of *Xana* remains unforeseen, appearing isolated in Asturias, in the North of the Iberian Peninsula. The hypothesis of an origin of *Xana* from *Vignysa*, due to their shared presence of two anterior gizzards (instead of three, as in the remaining hormogastrids), is rejected by our combined analyses, but the nuclear genes, support a relationship among the two genera. A second hypothesis of dispersal of *Xana* through the land bridge that appeared in the North of the Iberian Peninsula between the western and eastern areas, approximately 36 MY ago seems more plausible (Andeweg, 2002), but the surrounding areas (Cantabria and the Basque Country), have never yielded hormogastrid specimens (Briones, 1991). Whether this distribution is ancient or the result of recent extinction due to competition with other species, remains a mystery, but interestingly other ancient Iberian lineages of terrestrial invertebrates, as mite harvestmen, are also present in Asturias but missing from many other adjacent areas, paralleling the distribution of *Xana* and *Vignysa* (Murienne and Giribet, 2009).

According to the present analyses, the genus *Hormogaster* is paraphyletic, as it includes the members of several other hormogastrid genera. A denser sampling of *Vignysa* and *Hemigastrodrilus* (there are no additional known populations of *Xana*) is necessary to further test these relationships. Moreover there are probably several undescribed hormogastrid species in regions of suitable habitat that remain unexplored. In the present work the sampling focused on the type localities of all the known species but there are ample areas in the Iberian Peninsula where earthworms have not been studied. In addition, even though we expected to find *Hormogaster gallica* in Sant Joan de les Abadesses (Girona), a new species was found instead (M. Novo work in progress). Likewise, in Talarn (Lleida) the specimens collected do not match the description of *H. lleidana*, reported from this locality by Qiu and Bouché (1998). These findings indicate that a greater sampling effort may be required to have a better representation of the diversity of this earthworm family.

The central area of the Iberian Peninsula has already been surveyed extensively (Novo et al., 2010a) and a unique speciation event is responsible for explaining the diversity of Hormogastridae that has persisted in this region. *H. elisae* is the only morphospecies found, but it includes several cryptic genetic lineages (Novo et al., 2009, 2010a), contrary to what happens in other regions of the Iberian Peninsula, where several hormogastrid morphospecies can be identified in an area of similar size. In the current study the *H. elisae* species complex constitutes an independent evolutionary lineage from other *Hormogaster*, closely related to *Hemigastrodrilus*, but erecting a new genus for the species complex is unwarranted until the taxonomy of the family can be critically revised (authors' work in progress).

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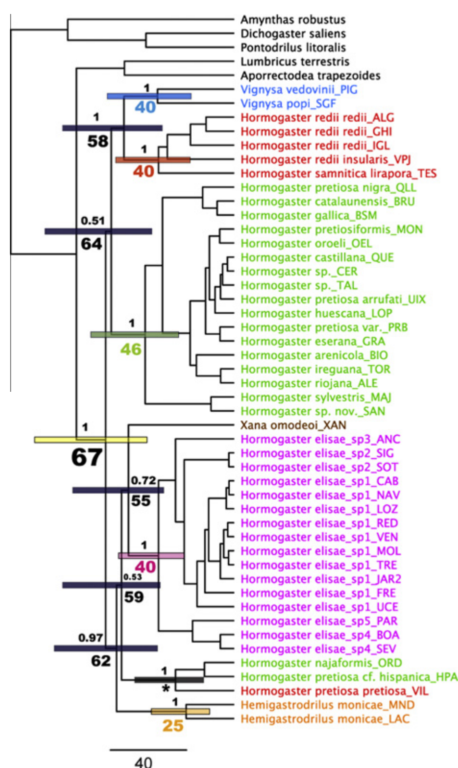

Fig. 6. Ultrametric tree obtained with BEAST using the combined data. The estimated dates (MY, below the bars) and their 95% confidence bars are shown for the main clades in the same colors as in previous figures. Posterior probability values for these clades are shown above bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

It can also be concluded that the hormogastrid species present in Corsica and Sardinia have received two different genetic influences. One includes *H. redii* and *H. samnitica lirapora*, related to *Vignysa* in some analyses, but of unresolved position (appears basal in the direct optimization analysis, unresolved in the Bayesian analysis and derived in the maximum likelihood analysis). It is plausible that in this first lineage cryptic species exist, as the genetic divergences found between populations of *H. redii* are very high (authors' work in progress). The second genetic lineage only occurs in the SW area of Sardinia, where *H. pretiosa pretiosa* was originally described. This species is closely related to some *Hormogaster* species from the NE Iberian Peninsula, again, paralleling the distribution of other organisms, such as the cyphophthalmid genus *Parasiro*, found in the NE Iberian Peninsula and S France, Corsica and Sardinia (Juberthie, 1958; de Bivort and Giribet, 2004; Murienne and Giribet, 2009). The SW area of Sardinia has been proposed as an independent biogeographic region, based on studies of plant endemicity, unique vegetation and geolitological, geomorphological, paleogeographical and bioclimatic characteristics (Bacchetta et al., 2007). Moreover, it seems that this corner was separated from the main part of the island by water, which was subsequently filled with alluvial deposits in the Campidano plain (Omodeo and Rota, 2008). The presence of two genetic influences in the Corso-Sardinian system is therefore easily explained, as the Northern area of the islands has always been closer to Southern France whereas the Southern area was closer to the NE Iberian Peninsula (Andeweg, 2002). Following Omodeo and Rota (2008), the native fauna of the Corso-Sardinian system originated from the terranes that currently form Catalonia and to a lesser extent from those that gave origin to Provence. Hence, most species found in Sardinia probably originated in the Pyrenees, whereas most species from Corsica originated in the Provence or from territories West of the Alps. Nevertheless, the Italian Peninsula should be explored in order to assess the possible influences of its earthworm fauna to the fauna in the islands, as it seems that in the case of *Parasiro* closely related populations are found both in the islands and the Italian Peninsula (de Bivort and Giribet, 2004), while the species from the NE Iberian Peninsula seem to be more distantly related.

H. pretiosa, as understood nowadays, is polyphyletic, suggesting the need of revisionary taxonomy in the group (authors' work in progress). Cobolli-Sbordoni et al. (1992) found high genetic distances between "*H. pretiosa*" from the Iberian Peninsula and those of the islands, but not all the described subspecies were included in that analysis, and others were described subsequently to that study. But the data were not sufficient to falsify its monophyly and the authors indicated that any proposal for a revision of the nomenclature of *H. pretiosa* could be only justified after the examination of more material from Catalonia. Our data are clear in that respect and indicate that "*H. pretiosa*" constitutes multiple species in unrelated lineages. The type specimen of *H. pretiosa pretiosa* is from Sardinia and many of the NE Iberian specimens formerly identified as *H. pretiosa* may belong to a different species, as they show deep genetic divergences as well as important differences in the number and position of spermathecae. Omodeo and Rota (2008) indicated that Cobolli-Sbordoni et al. (1992) had already proposed the name *Hormogaster hispanica* for the populations from Catalonia, but this is inaccurate, as these authors simply stated that the high divergence values found between populations they assigned to *H. pretiosa* suggested the subdivision of this morphospecies into three sibling species, but they did not assign species status to these three lineages—which include *H. pretiosa* cf. *hispanica*. Later, Rota (1993) described a new species for the third lineage, *H. gallica*, but never assigned species status to *H. pretiosa hispanica*.

The phylogenetic hypothesis generated under direct optimization is perhaps the most credible from a biogeographical point of view, in spite of the low bootstrap support values. This hypothesis shows *Vignysa* as the sister clade to the remaining species of the family. Following this tree, two clades differentiate afterwards, one present in Corsica and the Northern area of Sardinia and another including the Atlantic lineage inclusive of *Hemigastrodrilus* and *H. elisae* and the lineage present in the NE Iberian Peninsula and S France. The latter clade includes two groups, one with *H. najaformis* (ORD), *H. pretiosa* cf. *hispanica* (HPA), *H. pretiosa pretiosa* from Sardinia (VIL) and *Xana*, and another with the remainder of the *Hormogaster* species.

Xana is again challenging morphological reasoning, as its position in the trees implies multiple origins of the third gizzard or a regression to the two-gizzard state in one occasion. *Xana* inhabits soils that are very different from those of other hormogastrids (Novo, 2010). The higher content of clay in these soils could explain the regression of this character to a two-gizzard state, as *Xana* has no need for such an exhaustive grinding of the soil as required in a sandier soil. Therefore it is plausible that selective pressure has contributed to eliminating the third gizzard in this genus. Nonetheless, as discussed above, *Xana* and *Vignysa* do form a clade in the analyses using only conserved nuclear genes, and this correlates with the apomorphic presence of two gizzards in both genera. In addition to the number of gizzards, other morphological characters also show homoplasy; the shape of the spermathecae, tubular in *H. elisae* and in *Vignysa popi*, multiple in *H. najaformis* and *H. samnitica lirapora*, or multilocular in some *Hormogaster* species of the NE Iberian clade and *Vignysa vedovinii*.

The results of this work have important implications for the taxonomy of the family, evidencing that it should be revised and that generic designations must be tested phylogenetically. In addi-

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tion, species definitions are also deficient in some cases, as in *H. elisae* and *H. pretiosa*, calling perhaps for combined diagnosis of species using molecules in addition to morphology (see for example Edgecombe and Giribet, 2008; Cook et al., 2010). There are still lots of mysteries around hormogastrid systematics and the information at hand is only sufficient to delimit the backbone of this phylogenetic and biogeographic puzzle. Additional sampling effort and exploration of new geographic regions in the Western Mediterranean basin are necessary in order to fill in the missing pieces.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.05.018.

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