



## Contribution to the knowledge of the genus *Boucheona* (Oligochaeta, Hormogastridae) in France with a newly described species and a redescribed species

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### Abstract

Hormogastrid earthworms are found in the diversity hotspot of the Franco-Iberian domain, together with the better-known family Lumbricidae. Integrative systematics (the combination of morphological, molecular and ecological data) have increased our knowledge of the diversity and evolutionary history of these earthworms, highlighting unresolved taxonomic conflicts. One example of a species group in need of integrative taxonomic revision is the genus *Boucheona* in France. In this work, we analyzed their diversity using previously published data together with additional data obtained from recently sampled localities. Molecular data including DNA barcodes and additional markers enabled us to reconstruct Bayesian and time-calibrated phylogenies to discuss the evolutionary relationships among the different taxa, and to propose hypotheses regarding their biogeographical history. Based on our results, four species of *Boucheona* are present in Southern France, including two new taxa. Morphological distinctness and molecular phylogenetics results supported the status of four populations as the newly described *Boucheona corbierensis* sp. nov., as well as the status of “*Hormogaster pretiosa* var. *nigra*” as an independent species, redescribed as *Boucheona tenebrae* sp. nov. These results provide a new perspective of the importance of the genus *Boucheona* in southern France, as the possible evolutionary origin of a clade of giant anecic earthworms with unknown (but probably remarkable) impact on ecosystem functioning across their range.

**Key words:** Earthworms, alpha taxonomy, integrative systematics, biogeography, soil diversity

### Introduction

Earthworms (Crassieclitellata, Annelida) are one of the most abundant components of soil fauna in temperate regions, representing up to 60% of the soil fauna biomass (Turbé *et al.* 2010). In addition, they exert a strong influence in the soil habitat and all related trophic networks and biogeochemical cycles (Lavelle *et al.* 2006, 2016). Paradoxically, compared to other invertebrate groups such as mollusks or arthropods, the relative focus on this taxon has been relatively weak resulting in a taxonomic deficit estimated to be above 50% (Decaëns *et al.* 2013). While this is particularly the case in the tropics (e.g. Decaëns *et al.* 2016), the situation also applies in temperate regions such as Western Europe, where several renowned taxonomists have made concerted efforts to describe this elusive fauna (Michaelsen 1900, 1925; Pop 1947; Bouché 1972; Qiu & Bouché 1998a). The Franco-Iberian domain (Csuzdi *et al.* 2011), which includes the Iberian Peninsula, the Balearic Islands, Andorra, mainland France, Corsica and Sardinia, is largely recognized as a hotspot of earthworm diversity. It harbours several endemic genera of Lumbricidae

Rafinesque-Schmalz 1815 and is considered the centre of origin and diversification of the family (Marchán *et al.* 2021). It is also the home of a less diverse, yet relevant earthworm family: the Hormogastridae Michaelsen, 1900. Members of this family were relatively unknown until the 1970s, but the advent of molecular phylogenetics at the start of the 21st century enabled more information to be obtained about their evolution and phylogenetic relationships. One of the most interesting findings was the contrasting patterns of morpho-molecular diversification in this family. While some species groups developed a high level of morphological diversity associated with low genetic interspecific divergence, other species groups suffered almost complete morphological stasis coupled with strong genetic differentiation, a phenomenon usually known as cryptic speciation (Bickford *et al.* 2007; Novo *et al.* 2012).

Another interesting finding regarding the macroevolutionary pattern in hormogastrid earthworms is the correlation between body size and one of their evolutionary novelties: the multilamellar typhlosole (Marchán *et al.* 2016a). Composed of several longitudinal lamellae (from 3 to 23), this structure provides a relatively large intestinal surface area, which may compensate for a lower surface area/volume ratio (Haldane 1926) as well as improving nutrient absorption in poorly organic soils. For this mainly endogeic group, increasing body weight (from the smallest - 0.5 gr - to the heaviest - 100g) appears to have been the main evolutionary pressure for the development of highly complex typhlosoles, allowing them to occupy a unique niche by evolving large to giant geophagous life forms. Interestingly, some species display adaptations consistent with an ecic (vertical burrowing, litter feeding) lifestyle, although the cumbersome multilamellar typhlosole is expected to hinder strong longitudinal contractility (which is important for anecic earthworms).

Most species of the Hormogastridae are endemic to the Iberian Peninsula (22 species, 5 genera), with a small fraction known from insular and continental Italy (5). In France, the known diversity is relatively low (7 species, 4 genera), which contrasts with the large number of endemic Lumbricidae found in this country, and most French species are widely distributed and locally abundant in the Mediterranean ecosystems of Occitanie and Provence (Bouché, 1972).

Among these French taxa, the species formerly known as *Hormogaster praetiosa* (Michaelsen, 1899) is emblematic of a conflicting taxonomical history. Bouché (1972) assigned several populations of Pyrénées-Orientales and Aude (plus an isolated population from Bouches-du-Rhône) to *Hormogaster praetiosa*, originally described from Sardinia but also cited from Spain. He also proposed the variety *Hormogaster praetiosa* var. *nigra* Bouché, 1972 for an almost black, giant population from Quillan, a name currently unavailable according to the ICZN due to being an infrasubspecific name published after 1961. More recently, Rota (1993) examined new material from the populations of Banyuls-sur-Mer (Pyrénées-Orientales) and La Fare-les-Oliviers (Bouches-du-Rhône) and described a new species, *Hormogaster gallica* Rota 1993, but made no statement about the other populations of “French *Hormogaster praetiosa*”. Novo *et al.* (2011) applied molecular phylogenetic techniques to all the hormogastrid species described at the time (including several new species described by Qiu & Bouché 1998b), and found that the name *Hormogaster praetiosa* had been assigned to at least three independent lineages of earthworms (Sardinian, French and Spanish) while it should be restricted to the populations in Sardinia (from where the species was described). *Hormogaster praetiosa* var. *nigra* and *Hormogaster gallica* showed a close phylogenetic relationship together with *Hormogaster catalaunensis* Qiu & Bouché, 1998 and other morphologically similar species from Catalonia. Based on an integrative approach, Marchán *et al.* (2018a, 2018b) elevated this clade of hormogastrids to the genus *Boucheona*. Additional populations of the former “French *Hormogaster praetiosa*” were included in the analyses, suggesting they belonged to an undescribed species more closely related to “*Boucheona nigra*” than to *Boucheona gallica*. The lack of specimens from the isolated population of La Fare-les-Oliviers precluded any further taxonomic actions.

In this work, specimens from the missing population previously assigned to *Boucheona gallica*, as well as new specimens of the undescribed *Boucheona* species and of “*Boucheona nigra*”, are studied using an integrative approach including standard morpho-anatomical scanning electron imaging of genital chaetae and molecular phylogenetics, in order to clarify the taxonomic status of these important elements of the French soil fauna.

## Materials and methods

### Specimens and sampling

For the present study, 21 specimens of eight French populations of *Boucheona* (Hormogastridae) were studied. Four of the populations (PCORB, RIV, BSM, and QUILL) were previously sampled (Novo *et al.* 2011; Marchán *et*

al. 2016a, 2018b) and the specimens were deposited in the collection held by the Faculty of Biology, Universidad Complutense of Madrid (UCMLT, Spain). The other four populations were sampled in spring and autumn of 2021. The locations and number of individuals are shown in Table 1.

**Table 1.** Species, code, locality information, number of specimens studied (n), and GenBank accession numbers of the populations of *Boucheona* under study.

Species	Code	Locality	n	COI	16S	28S
<i>Boucheona corbierensis</i> sp nov.	PCORB	Portel des Corbières, France 43.038697, 2.947892	3	KT246385	KT246408	KT246433
<i>Boucheona corbierensis</i> sp nov.	RIV	Rivesaltes, France 42.757328, 2.886342	4	KT246386	KT246409	KT246434
<i>Boucheona corbierensis</i> sp nov.	#536	Bizanet, Quillanet, France 43.146, 2.894	1	DFM-0968	-	-
<i>Boucheona corbierensis</i> sp nov.	#1072	Durban-Corbières, France 42.993, 2.792	3	DFM-0972 - DFM-0974	-	-
<i>Boucheona rosae</i>	#1083	Prunet-et-Belpuig, France 42.552, 2.614	3	DFM-0969 - DFM-0971	-	-
<i>Boucheona gallica</i>	#1040	La Fare-les-Oliviers, France 43.550929, 5.229772	5	To be provided	To be provided	To be provided
<i>Boucheona gallica</i>	BSM	Banyuls-sur-Mer, France 42.468894, 3.152286	1	HQ621974	HQ621867	HQ621945
" <i>Boucheona nigra</i> "	QUILL	Quillan, France 42.880222, 2.170000	1	HQ621988	HQ621882	HQ621959

Earthworms were collected by soil digging and were hand-sorted, rinsed with water and fixed in 96% ethanol. Sampling and handling of specimens followed ethical considerations and conformed to Directive 2010/63/EU. Species were identified using the external and internal morphological characters used by Rota (1993) and Qiu & Bouché (1998b).

### Morphological data collection

Morphological characters included those features traditionally used for hormogastrids and other earthworms, with emphasis on most of the characters used in Marchán *et al.* (2018): thickened septa, average length of clitellum, position of spermathecal pores, type and repetition of spermathecae, backward displacement of dorsal insertion of septum 9/10, average number of segment, average weight, type of pigmentation, presence/absence and degree of development of cephalic keels, position of *tubercula pubertatis*, typhlosole lamellae, lateral expansions of clitellum, number of gizzards and first nephridia with caecae. Scanning electron microscopy images of the genital chaetae of five French *Boucheona* populations were obtained following Marchán *et al.* (2016b).

### DNA isolation and sequencing

One to three individuals from each newly sampled locality were selected for DNA barcoding. Small ventral integument tissue samples were assembled in 96-well plates and shipped for processing at the Centre for Biodiversity Genomics at the University of Guelph (Canada, Ontario). After total genomic DNA extraction using a CTAB-based approach, the standard DNA barcode for animals (Hebert *et al.* 2003) – a 658bp fragment of mitochondrial marker cytochrome c oxidase subunit 1 (COI) – was amplified using a primer cocktail C\_LepFolF-C\_LepFolR (Hernandez-Triana *et al.* 2014). Sequencing reactions were carried out with the same primer cocktail and products were subjected to a PureSeq-MP (Aline Biosciences, Woburn, USA) cleanup before Sanger sequencing in an ABI 3730XL DNA sequencer. Consensus sequences from automatically assembled contigs (subsequently reviewed and manually edited when needed) were uploaded to BOLD (www.boldsystems.org; Ratnasingham & Hebert 2007) along with trace files, specimen data and images.

Three representative members of population #1040 were chosen for further analysis. Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) from ventral integument samples of approximately 5 x 5 mm. Regions of the genes for the nuclear 28S rRNA (28S) and mitochondrial 16S rRNA (16S), tRNAs Ala, Leu and Ser (*tRNAs*) were amplified using the polymerase chain reaction (PCR) using the following primers: 16S-

tRNA (16SF2: CGACTGTTTAAACAAAACATTGC, 16SR2: GTTAAACCTGTGGCACTATTC); 28S (RD33F: GAAGAGAGAGTTCA AGAGTACG, RD5b: CCACAGCGCCAGTTCTGCTTAC). PCR condition were as follows: 94°C – 3', (94°C – 30'', 45(COI)-50(16S, 28S)°C – 45'', 72°C – 1') 40 cycles, 72°C – 5', 4°C – ∞. PCR products were purified and sequenced by the C.A.C.T.I Genomics service (University of Vigo). DNA sequences were uploaded to GenBank. Accession numbers are shown in Table 1 together with accessions of the other 4 French populations of *Boucheona*.

Additional sequences reported by Novo *et al.* (2011) and Marchán *et al.* (2016, 2018b), including representatives of all the species of the genus *Boucheona*, were downloaded from GenBank and used as a reference dataset.

### Phylogenetic analyses and time-calibrated phylogenetic inference

Sequences were aligned using MAFFT v.7 (Kato & Standley 2013), with default settings, and concatenated using BioEdit (Hall 1999), resulting in a matrix of 2,011 bp. The best fitting evolutionary model for each partition was selected using jModelTest v. 2.1.3 (Darriba *et al.* 2012) by applying the Akaike information criterion (AIC; Akaike 1973) and the Bayesian information criterion (BIC; Schwarz 1978). *tRNAs* were analyzed as a separate partition from *16S*. GTR + I + G was selected as best-fitting evolutionary model for *COI* and *28S* and HKY+I+G was selected for *16S*, *tRNAs*.

Bayesian Inference of the phylogeny was estimated using MrBayes v.3.2.6 (Ronquist *et al.* 2012) as implemented in CIPRES Science Gateway V. 3.3 (Miller *et al.* 2010). The analysis was performed with default parameters, and each of the two independent runs was set to 50 million generations sampling every 5,000th generation (10,000 trees). Twenty-percent of the trees were discarded as burn-in, with remaining trees combined and summarized on a 50% majority-rule consensus tree.

An ultrametric tree was generated using BEAST v. 1.10 (Suchard *et al.* 2018). Each partition was trimmed using GBlocks (Castresana 2000) under the less stringent parameters, with the best-fitting evolutionary model (shown above) as the evolutionary model for each. An external calibration of 40-73 mya was implemented as a uniform prior for the root of *Boucheona*, obtained from a BEAST analysis that included species of all genera of Hormogastridae, *Criodrilus lacuum* and *Diporodrilus pilosus* as outgroups (detailed in Suppl. Material 1).

A Yule diversification model and an uncorrelated lognormal relaxed clock were specified. Two parallel runs were performed, each including 50 million generations, sampling every 5,000th generation. Tree and log files were combined in Logcombiner v.1.10 (Suchard *et al.* 2018) by resampling at a lower frequency (10,000) and the results were visualized in Tracer v. 1.7.1 (Rambaut *et al.* 2018). The final tree was generated using TreeAnnotator v.1.10 (Suchard *et al.* 2018) with a burn-in of 2000 generations.

Uncorrected average pairwise genetic distances based on the *COI* and *16S* markers were obtained in MEGA 11.

### Collection abbreviations

UCMLT Faculty of Biology, Universidad Complutense of Madrid (Spain)

MNHN Muséum national d'Histoire naturelle of Paris (France)

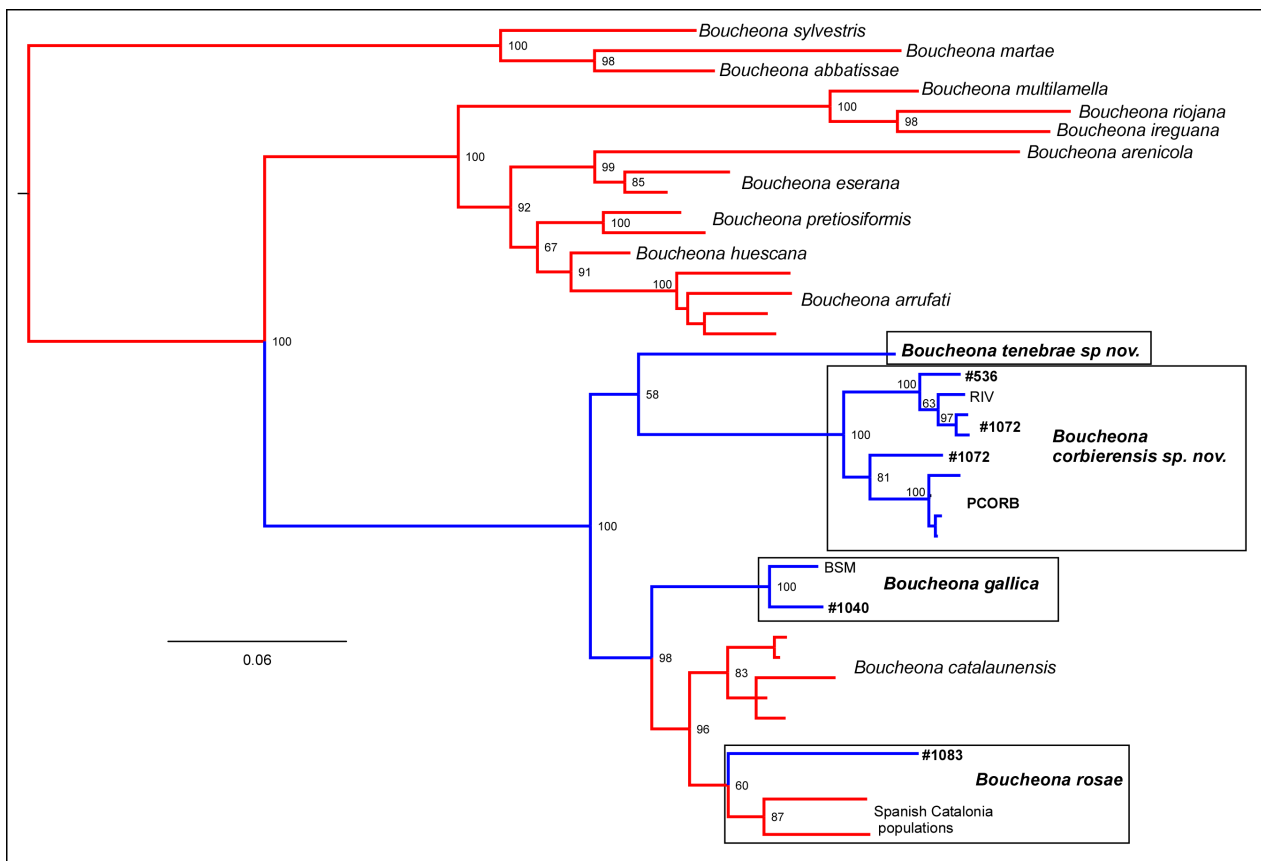
ECOSOLS Eco&Sols laboratory (Montpellier, France)

## Results

### Phylogenetic analyses

Bayesian Inference of the phylogenetic tree (Figure 1) recovered three main clades within *Boucheona*: the Northern Catalonian *Boucheona sylvestris* (Qiu & Bouché, 1998), *Boucheona martae* Marchán *et al.*, 2018 and *Boucheona abbatisae* (Novo & Diaz Cosin, 2012), a clade comprising eight species from Navarra- La Rioja- Huesca- Eastern Catalonia and the Valencian Community, and a clade comprising the Northeastern Catalonian *B. catalaunensis* and *Boucheona rosae* Marchán *et al.*, 2018 and French *B. gallica*, "*B. nigra*" and the populations PCORB, RIV, #1040, #536, #1072 and #1083.

The individuals from #1040 formed a clade with *B. gallica* from BSM (type locality), with short branch lengths consistent with different populations of the same species. The individuals from #1083 formed a clade with *B. rosae* from Spanish Catalonia, but with low support. The individuals from PCORB, RIV, #536 and #1072 formed a well delimited and independent clade, which appeared more closely related to "*B. nigra*" than to *B. gallica*, but the relationship was weakly supported. This clade is further described under the name of *Boucheona corbierensis* sp. nov..



**FIGURE 1.** Bayesian inference of the phylogenetic relationships between the known species of *Boucheona* based on the concatenated sequence of the molecular markers *COI-16S-28S*. The populations of focus in this work are shown in bold. Posterior probability values are shown above branches. Red branches indicate Spanish populations and blue branches indicate French populations.

The ultrametric tree (Figure 2) estimated the ages of divergence between the French-Catalan species of *Boucheona* at around 22.15-11.58 mya. The populations of *B. corbierensis* sp. nov. were estimated to have diverged at 6.9 mya (3.1-12.2 95% highest posterior density interval), while the populations of *B. gallica* (BSM and #1040) would have diverged at 3.7 mya (1.5-7.4 95% HPD).

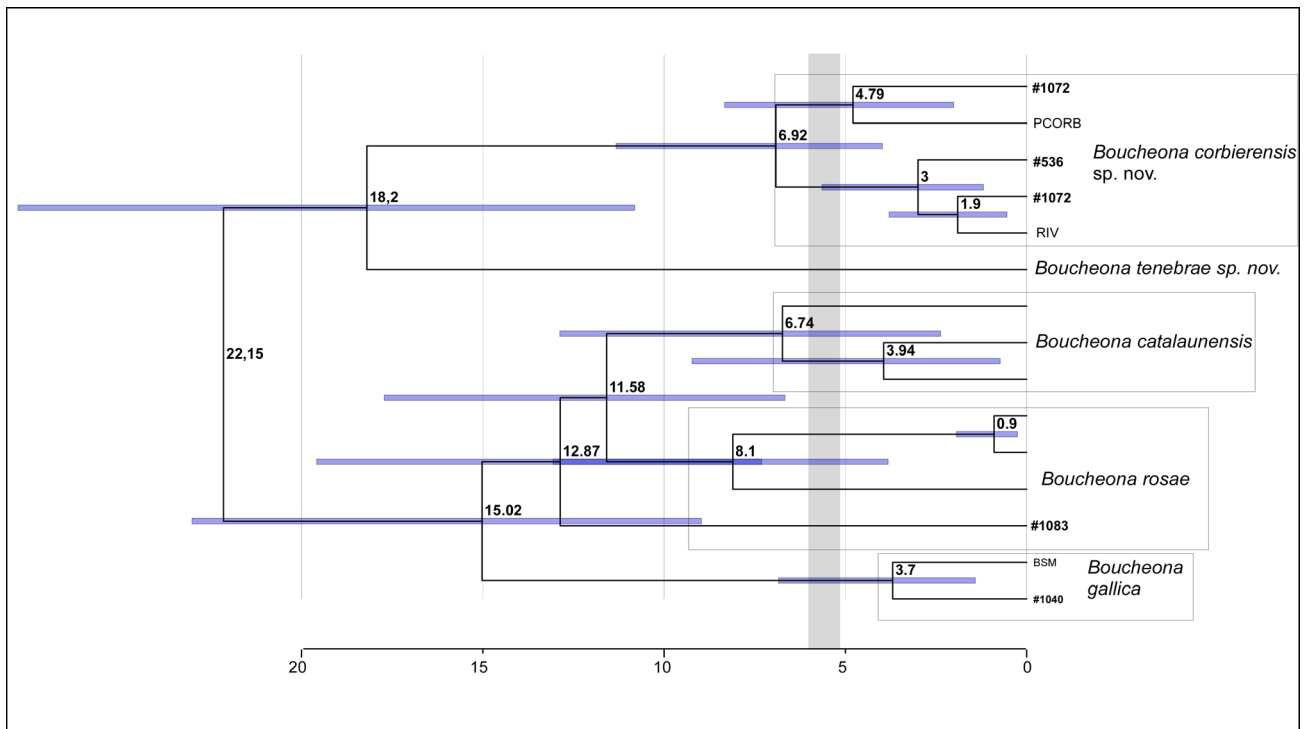
COI uncorrected average pairwise genetic distances within *B. corbierensis* sp. nov. were small (4%), i.e. smaller than the genetic distance between the populations of *B. rosae* (7%). Meanwhile, the COI genetic distances between *B. corbierensis* sp. nov. and the closely related species “*B. nigra*”, *B. rosae*, *B. catalaunensis* and *B. gallica* (including both BSM and #1040) ranged between 14% and 14.7%, i.e. larger than distances between most species of *Boucheona* (9.9%-13.4%). The same was found for the 16S, as the genetic distance between the populations of *B. corbierensis* sp. nov. and the aforementioned species ranged between 5.3 and 6.7%, while the interspecific distance for the other taxa ranged between 2.5 and 5.5%.

### Morphological characters

The studied individuals from population #1040 were consistent with the description of *B. gallica* provided by Rota (1993). The studied individuals from the population #1083 were consistent with the description of *B. rosae* provided by Marchán *et al.* (2018).

Genital chaetae of populations PCORB, RIV, BSM and #1040 were very similar: lanceolate with strong dorso-ventral differentiation and ornamented tip (Fig. 3). The general shape of the tip of the chaetae was that of a broad dorso-ventrally flattened rhombus. A long portion of the distal part of the tip has a smooth surface, while the rest is ornamented by the usual hairy grooves. A moderate, longitudinal dorso-ventral depression can be observed in some of the chaetae. Chaetae of the QUILL population were damaged and their shape could not be described.





**FIGURE 2.** Detail of the ultrametric tree based on the molecular markers *COI-16S-28S* focused on the Catalan-French *Boucheona* clade. Mean heights (ages) are shown besides nodes, and 95% highest posterior density intervals are shown as blue bars. The grey bar indicates the approximate interval corresponding to the Messinian Salinity Crisis.

## Discussion

### Systematic implications

Morphological distinctness and molecular phylogenetics results support the status of the populations PCORB, RIV, #536 and #1072 as an undescribed *Boucheona* species, as well as the status of “*Boucheona nigra*” as an independent species which must be redescribed due to its unavailable (invalid) name.

### Phylum Annelida Lamarck, 1802

### Subphylum Clitellata Michaelsen, 1919

### Class Oligochaeta Grube, 1850

### Superorder Megadrili Benham, 1890

### Order Haplotaxida Michaelsen, 1900

### Family Hormogastridae Michaelsen, 1900

### Genus *Boucheona* Marchán, Fernández, Díaz Cosín & Novo, 2018

#### *Boucheona corbierensis* Marchán & Decaëns sp. nov.

urn:lsid:zoobank.org:act:B39B20F1-1882-4A89-88C5-00FCABA96CC4

#### *Hormogaster praetiosa* Michaelsen, 1899 in Bouché (1972) (partial)

## Etymology

The species name is derived from the natural region of Corbières where the type specimens were collected.

## Material examined

### Holotype

FRANCE • Adult; Occitanie, Aude, Portel-des-Corbières 19/03/2021; leg. D. F. Marchán, T. Decaëns, Y. Capowiez; Locality PCORB: 43.039109 2.949103; cleared meadow between pine forest and orchard; BOLD Sample ID: DFM-0030; deposited in MNHN.

### Paratypes

FRANCE • 2 specimens, one adult and one subadult; same data as holotype: BOLD Sample ID: DFM-0029 and DFM-0031; deposited in MNHN. 4 specimens, adults; Occitanie, Pyrénées Orientales, Rivesaltes 15/04/2012; leg. D. F. Marchán, R. Fernández; Locality RIV: 42.757328 2.886342; garrigue; voucher: UCMLT00395, UCMLT00396, UCMLT00397, UCMLT00398; deposited in UCMLT. 1 specimen, adult; Occitanie, Aude, Bizanet, Quillanet 18/11/2021; leg. D. F. Marchán; Locality #536: 43.146, 2.894; abandoned culture; BOLD Sample ID: DFM-0968; deposited in ECOSOLS. 3 specimens, adults; Occitanie, Aude, Durban-Corbières 18/11/2021; leg. D. F. Marchán; Locality #1072: 42.993, 2.792; grassland; BOLD Sample ID: DFM-0972, DFM-0973, DFM-0974; deposited in ECOSOLS.

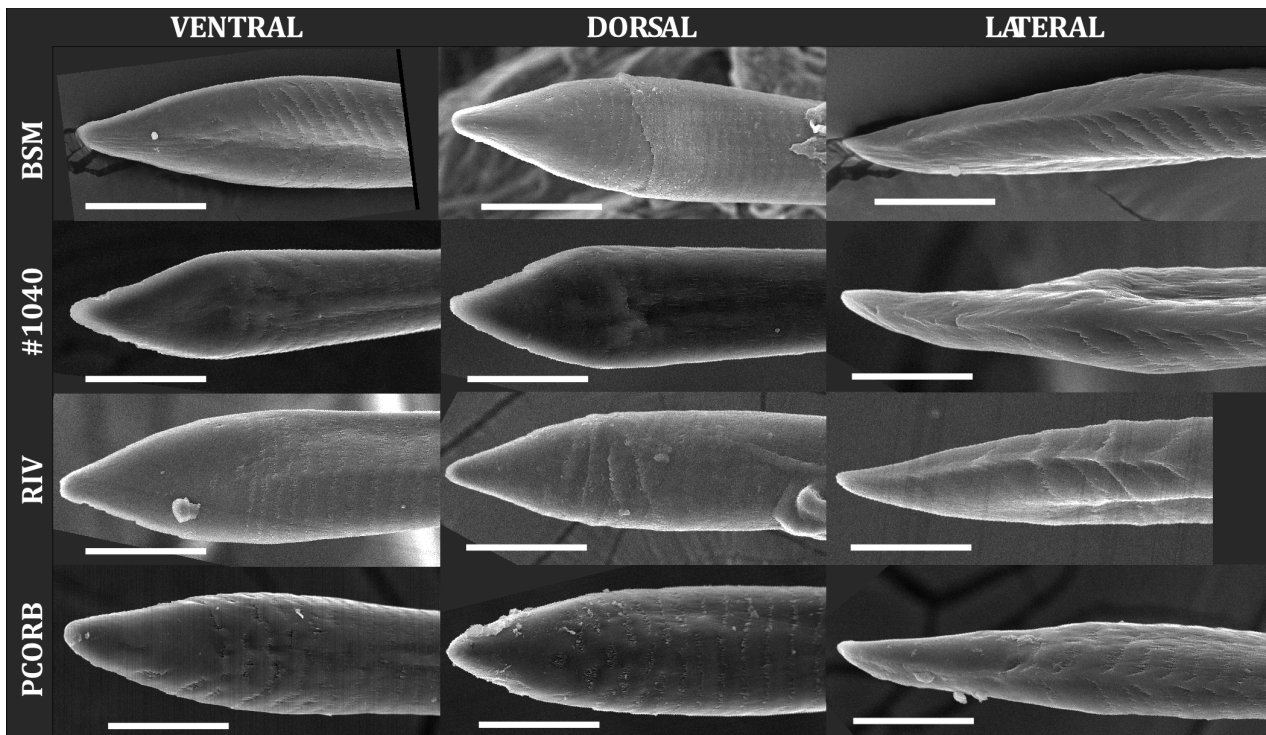


FIGURE 3. Scanning electron micrographs of genital chaetae of four populations of French *Boucheona*. Scale bar: 10  $\mu$ m.

## Diagnosis

Pigmentation brownish-grey to dark brown, average length (fixed) of 21.8 cm (17.5–24 cm), average weight of 15.1 gr (13.5–16.7 gr), average number of segments of 437 (376–501). Clitellum extending over segments (XIII)XIV–XXIX(XXX), *tubercula pubertatis* as a continuous line on segments XXII–XXIX, multichamber disc-shaped spermathecae in intersegments 9/10 and 10/11, typhlosole composed of a maximum of 15 lamellae, first nephridial caecae in XI.

## Morphological description

External morphology. All quantitative characters were measured in mature, whole individuals. Average length (fixed): 21.8 cm (17.5–24 cm). Average weight: 15.1 gr (13.5–16.7 gr). Average number of segments: 437 (376–501). Pigmentation: Brownish grey to dark brown on living specimens, with pigmentation neatly interrupted at the level

of chaetae b (Figure 4a). Clitellum may be darker brown or lighter grey in colour, depending on the stage of development. Dorsally grey, ventrally beige on fixed specimens. Prostomium prolatic, strongly developed and with a small invagination delimiting a small prostomium-like lobe (Fig 4b); longitudinal striation on segments I, II and III. Strongly developed cephalic keels on segments I to XXIII (Fig. 4b, 4c). Closely paired chaetae; interchaetal ratio at segment XXXX, aa: 78, ab: 1, bc: 9, cd: 1, dd: 71. Nephridial pores in a row between chaetae b and c (very close to b). Spermathecal pores at intersegments 9/10 and 10/11 at the level of cd. Male pores open over chaetae ab at intersegment 15/16, surrounded by heart-shaped porophores. Female pores in segment XIV at the same level as male pores. Clitellum ring shaped- (only when wholly developed) extending over segments (XIII)XIV-XXIX(XXX) (Figure 4c). *Tubercula pubertatis* as a continuous line on segments XXII-XXIX. Papillae of chaetae ab in variable positions between segments XI, XII, XIII, XIV, XVII-XXII.

Internal anatomy. Funnel-shaped, strongly thickened septa in 7/8 to 11/12. Clearly developed hearts in 6–11. Three shiny, strongly muscular gizzards in VI, VII and VIII. Morren's glands and intestinal gizzard not apparent. First section of the intestine slightly dilated. Gut content frequently includes vegetal fibers. Typhlosole begins around segments XX with 13 lamellae, increasing to 15 lamellae in some individuals. Anterior nephridial bladders U-shaped with very close branches and no apparent caecum until segment XI. Bladders gradually flatten towards the end of the body, taking on the usual elongated shape.

Fraying testes and iridescent seminal funnels in segments X and XI. Two pairs of voluminous, grainy seminal vesicles in XI and XII. Ovaries and female funnels in XIII, ovisacs in XIV. Two pairs of multichamber disc-shaped spermathecae in intersegments 9/10 and 10/11, the posterior pair larger. Genital chaetae lanceolate with strong dorso-ventral differentiation and ornamented tip (Fig. 3a,b). The general shape of the tip of the chaetae is that of a broad dorso-ventrally flattened rhombus. A long portion of the distal part of the tip has a smooth surface, while the rest is ornamented by the usual hairy grooves. A moderate, longitudinal dorso-ventral depression can be observed in some of the chaetae. Average diameter: 34 (widest part)-24  $\mu\text{m}$ . Average length: 1150  $\mu\text{m}$ .

## Remarks

*Boucheona corbierensis* sp. nov. resembles *B. catalaunensis*, *B. gallica*, *Boucheona tenebrae* sp. nov. and *B. rosae* in the position of the clitellum (XIV-XXIX, XXX), type and position of spermathecae (multichamber disc-shaped spermathecae in intersegments 9/10 and 10/11) and pigmentation (dorsal, dark brown-grey). They differ from all of these close relatives in the position of the *tubercula pubertatis* (XXII-XXIX), from *Boucheona tenebrae* sp. nov. in its smaller size and lighter pigmentation, and from *B. catalaunensis* and *B. gallica* in the position of the first nephridial caecae (XI vs XII) and in the maximum number of typhlosole lamellae (15 vs 13).

COI uncorrected average pairwise distances and topology of multilocus molecular phylogenetic trees support the status of *B. corbierensis* sp. nov. as independent from other morphologically similar species.

### Distribution and ecology

*B. corbierensis* sp. nov. is known from two localities of Aude and Pyrénées Orientales and is probably present in another 3 localities (from Bouché (1972) putatively assigned by geographic proximity) in an area of 1,200 km<sup>2</sup> (Figure 5). It inhabits sclerophyllous woods and shrublands, sometimes occupying edges of neighbouring orchards.

Laboratory experiments (Capowiez in prep.) have shown that *B. corbierensis* sp. nov. behaves like an anecic earthworm, which is consistent with its pigmentation, morphology and gut content (vegetal fibers).

## *Boucheona tenebrae* Marchán & Novo sp. nov.

urn:lsid:zoobank.org:pub:FA29B304-DD7C-4472-BDE2-618B80557709

## *Hormogaster praetiosa* var. *nigra* Bouché, 1970

## Etymology

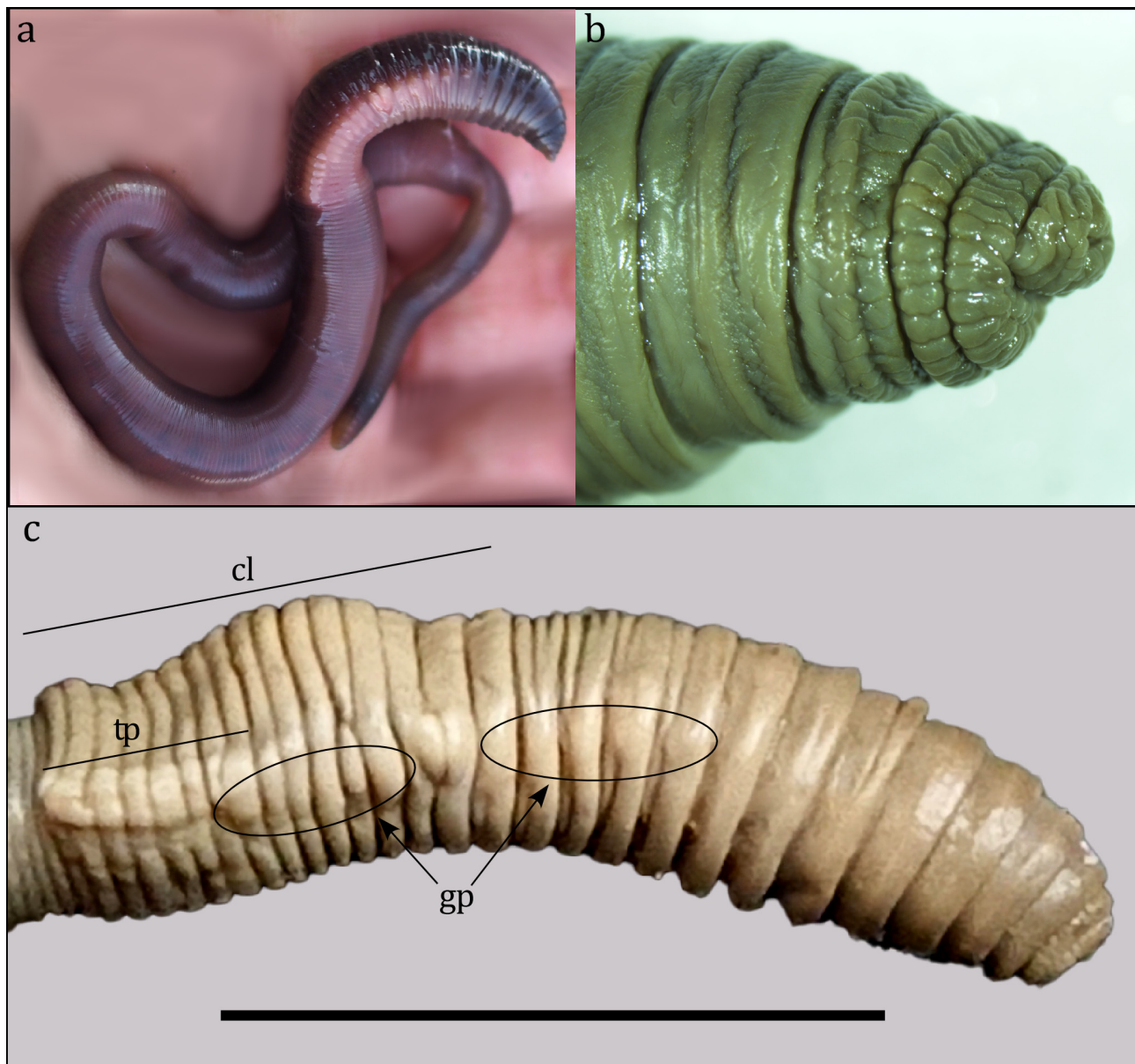
This species name refers to its very dark, almost black pigmentation.

## Material examined

## Holotype



FRANCE • Adult; Occitanie, Aude, Quillan 19/04/2008; leg. M. Novo, R. Fernández; Locality 1 (QUILL): 42.880222 2.170000; shrubland on a pronounced slope; voucher UCMLT00393; deposited in the UCMLT.



**FIGURE 4.** External morphology of *Boucheona corbierensis* sp. nov. a) Live specimen. b) Detail of the cephalic region, fixed specimen. c) Cephalic and clitellar region of a fixed specimen. cl: clitellum; tp: *tubercula pubertatis*; gp: genital papillae. Scale bar: 3 centimeters.

#### **Paratypes**

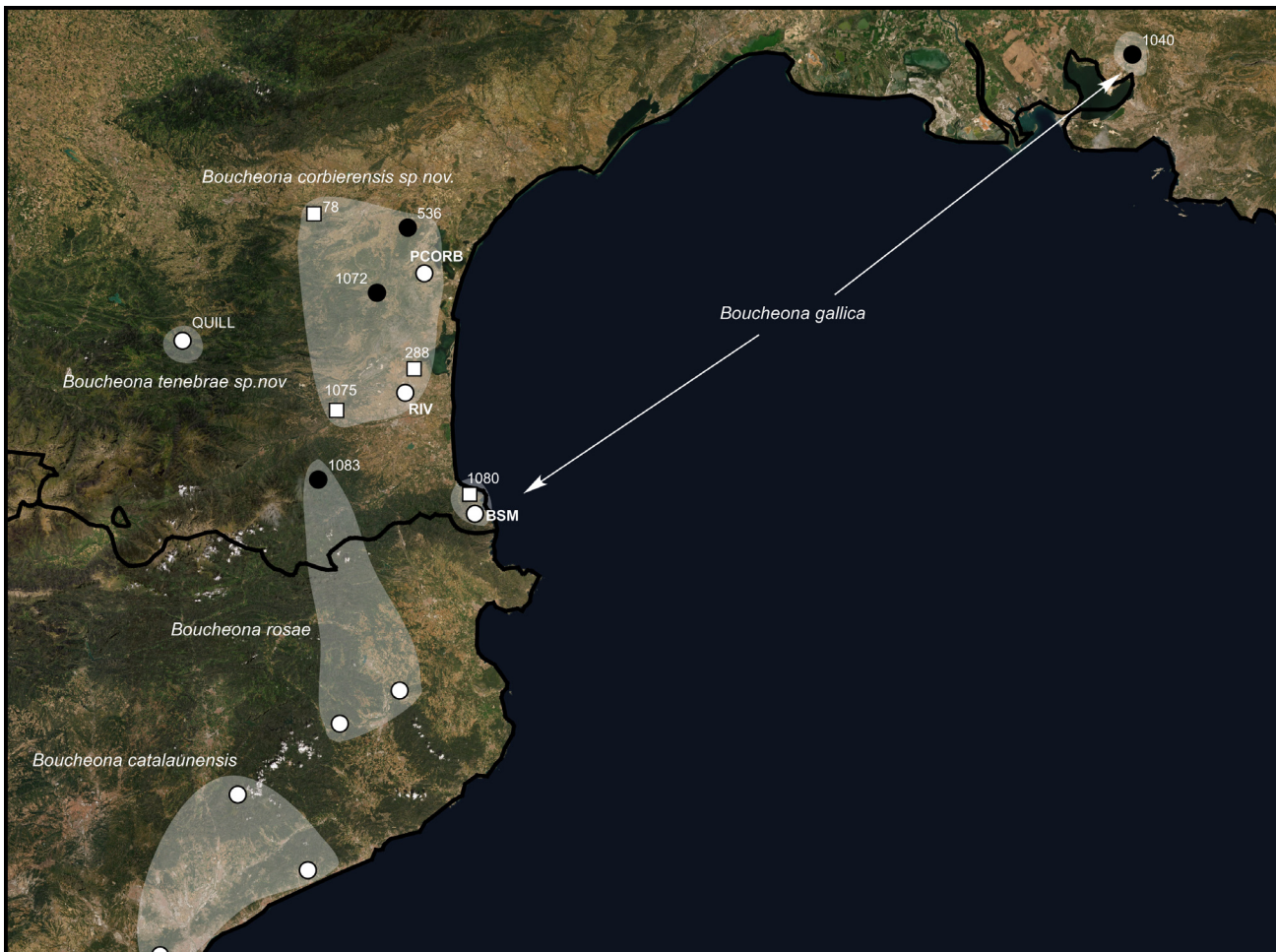
FRANCE • 1 specimen, adult; 16/04/2012; leg. D. F. Marchán, R. Fernández, same data as holotype; voucher UCMLT00394; deposited in UCMLT.

#### **Diagnosis**

Pigmentation almost black with purple iridescence, length reaching 50 cm, weight reaching 27.36 gr, number of segments reaching 600. Clitellum extending over segments XIV-XXX, *tubercula pubertatis* as a continuous line which narrows towards the end on segments XXII-XXVII(XXVIII), first thickened septum in 6/7, multichamber disc-shaped spermathecae in intersegments 9/10 and 10/11, typhlosole composed of a maximum of 13 lamellae, first nephridial caecae in XI.

## Morphological description

External morphology. Recently collected specimens (used for molecular information) are incomplete and damaged, thus some characters refer to the description of *Hormogaster praetiosa* var. *nigra* (Bouché, 1970)



**FIGURE 5.** Known localities of *Boucheona corbierensis* sp. nov., *Boucheona tenebrae* sp. nov. and their closest relatives. White dots indicate previously sampled and analyzed populations. Black dots indicate populations sampled and analyzed during this work. White squares indicate localities from Bouché (1972), which were putatively assigned to the different species due to geographic proximity.

Length: 50 cm according to Bouché 1970 (living specimens?). Weight (holotype): 27.36 gr (20-25 according to Bouché 1970). Number of segments (holotype): 580 (more than 600 according to Bouché 1970). Pigmentation: Almost black with purple iridescence on living specimens, with pigmentation neatly interrupted at the level of chaetae b. Dorsally dark grey, ventrally beige on fixed specimens. Prostomium prolobic, strongly developed and with a small invagination delimiting a small prostomium-like lobe; longitudinal striation on segments I, II and III. Strongly developed cephalic keels on segments I to XIII. Closely paired chaetae; interchaetal ratio at segment 40, aa: 50, ab: 1, bc: 8, cd: 1, dd: 50. Nephridial pores in a row between chaetae b and c (very close to b). Spermathecal pores on intersegments 9/10 and 10/11 at the level of cd. Male pores open over chaetae ab at the intersegment 15/16, surrounded by heart-shaped porophores. Female pores in segment XIV at the same level as male pores. Clitellum ring shaped-shaped (only when wholly developed) extending over segments XIV-XXX. *Tubercula pubertatis* on XXII-XXVII(XXVIII) as a continuous line which narrows towards the end. Papillae of chaetae ab in variable positions between segments XIII, XIV, XVII-XXIII, XXVIII, XXIX.

Internal anatomy. Funnel-shaped, strongly thickened septa in 7/8 to 11/12, moderately thickened in 6/7. Clearly developed hearts in VI-XI. Three shiny, strongly muscular gizzards in VI, VII and VIII. Morren's glands and intestinal gizzard not apparent. First section of the intestine slightly dilated. Typhlosole begins around segments XX with 9 lamellae, which swiftly increase to a maximum 13 lamellae and the decrease to 11 lamellae. Anterior nephridial bladders U-shaped with very close branches and no apparent caecum until segment XI. Bladders gradually flatten towards the end of the body, taking the usual elongated shape.

Fraying testes and iridescent seminal funnels in X and XI. Two pairs of voluminous, grainy seminal vesicles in XI and XII. Ovaries and female funnels in XIII, ovisacs in XIV. Two pairs of multichamber disc-shaped spermathecae in intersegments 9/10 and 10/11, the posterior pair bigger.

### Remarks

*Boucheona tenebrae* sp. nov. resembles *B. catalaunensis*, *B. gallica*, *B. corbierensis* sp. nov. and *B. rosae* in the position of the clitellum (XIV-XXIX,XXX) and type and position of spermathecae (multichamber disc-shaped spermathecae in intersegments 9/10 and 10/11). It differs from these close relatives in its larger size (length, weight, number of segments), its darker pigmentation (almost black vs brown-dark brown) and the position of the first thickened septum (6/7 vs 7/8). In addition, it differs from *B. corbierensis* sp. nov. in the position of the *tubercula pubertatis* (XXII-XXVII(XXVIII) vs XXII-XXIX) and maximum number of typhlosole lamellae (13 vs 15).

COI uncorrected average pairwise distances and topology of multilocus molecular phylogenetic trees support the status of *B. tenebrae* sp. nov. as independent from other morphologically similar species.

Distribution and ecology

*B. tenebrae* sp. nov. is known from a single locality of Aude (Figure 5). It inhabits shrublands.

### Biogeographic implications

The newly described species *B. corbierensis* sp. nov. and *B. tenebrae* sp. nov., together with *B. gallica*, appear as the earliest branching species of a clade of Franco-Catalan hormogastrids containing *B. gallica*, *B. catalaunensis* and *B. rosae*. This suggests that the ancestral range of the clade was located in the actual position of the regions of Aude and Pyrénées Orientales in Southern France, and that species further diversified and expanded towards Catalonia. The paleotopography of the Eastern Pyrenées during the Cenozoic remains unresolved, but most authors agree that after collision-related uplift ceased at around 30-28 mya, an elevated, low relief peneplain existed until rapid uplift resumed at 12-10 mya (Gunnel *et al.* 2009; Suc & Fauquette 2012). This paleogeographic scenario is compatible with the estimated divergence times, with an isolation of the French-Catalan lineage from the rest of the *Boucheona* species at around 44 mya, followed by diversification and range expansion towards the south between 22-13 mya and allopatric speciation of French and Spanish species afterwards. In order to test the correlation between paleogeographic events and earthworm diversification in this region, performing a comparative study with other earthworm taxa such as *Scherotheca* Bouché, 1972 or *Proselodrilus* Bouché, 1972 may prove helpful.

*B. corbierensis* sp. nov. has a wide distribution range for an hormogastrid, demonstrating its ecological versatility and success, being well established in sclerophyllous Mediterranean habitats. *Boucheona tenebrae* sp. nov. has very different distribution, with a single known location in the foothills of the Pyrénées at altitudes of around 400 m and under more humid conditions. The more specialized traits of *Boucheona tenebrae* sp. nov. (giant size, very dark pigmentation) may have limited the dispersal of this species compared to *B. corbierensis* sp. nov. which presents more generalist traits.

Interestingly, both known populations of *B. gallica* show a striking disjunction (separated by over 200 km of sea), which is unusual in Hormogastridae. As the estimated divergence time for those populations overlapped significantly with the Messinian Salinity crisis, it is possible that this species took advantage of the large area in the Gulf of Lyons exposed by the lowered sea level to expand towards the east of France. The later marine ingression would have restored the coastline, eliminating the intermediate populations between Banyuls-sur-Mer and La Fareles-Oliviers. Interestingly, this distribution is shared with the genus *Cataladrilus* Qiu & Bouché 1998 (Marchán *et al.* 2020). Alternative explanations, such as human mediated transport, seem unlikely as the estimated age of divergence precedes the Anthropocene by more than one million years (at least). It is also possible that *Boucheona gallica* is widely distributed in Mediterranean France but intermediate populations have not been sampled yet; they could have been isolated or even destroyed by the intense human activity in the area.

Both *B. gallica* and *Boucheona tenebrae* sp. nov. appear to be prime targets for evaluation of conservation status. The known geographic and occurrence ranges of these species are remarkably small, in the first case with two extremely isolated populations that are distant from each other and surrounded by highly anthropized areas, and in the second case limited to a single population (but in a better-preserved habitat). Those species are potentially vulnerable to habitat disturbance due to their probably K life strategies (large body size, slow development, late sexual maturity, low reproductive rate) and specialized lifestyle (deep burrowing anecics). *B. corbierensis* sp. nov. appears to be more frequent across its range, but more information is needed about its genetic diversity across the putative range and about the connectivity between populations and habitat preferences before it can be classified as of Least Concern.



These large-to-giant anecic earthworms are expected to have a strong influence on ecosystem functioning in the soils they inhabit, in a similar way to the strikingly convergent *Scherotheca* (their relatively more well-known lumbricid counterpart). Interestingly, both taxa display remarkable non-overlapping ranges, with *Boucheona* dominating in Spanish Catalonia and *Scherotheca* being more frequent and diverse in Southern France (sometimes in a checkerboard pattern). Whether this biogeographic pattern responds to preferences for non-overlapping environments or to biotic interactions such as interspecific competition remains to be tested.

## Conclusions

Integrative systematics of the French populations of *Boucheona* enabled identification of four species including two that turn out to be new to science, which highlights the importance of Southeastern France for the evolution of this genus. *B. gallica* and *Boucheona tenebrae* sp nov. showed extremely narrow or fragmented ranges, while *B. corbierensis* sp nov. and *Boucheona rosae* (also present in Spain) appeared to be more widely distributed. Our study represents a significant contribution to the knowledge of the diversity and biogeography of the genus, which should be helpful for further studies into their biology and ecological preferences, as well as for more applied aspects such as their potential role as ecosystem service providers and evaluation of their conservation status.

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## Supplementary Material 1

### External calibration

The dataset used in Marchán et al (2018), including representatives of most known Hormogastridae species, as well as sequences from *Criodrilus lacuum* and *Diporodrilus pilosus* (published in Domínguez et al. 2015; Pérez Losada et al. 2015) were used to generate an ultrametric tree with BEAST v. 1.10 (Suchard et al. 2018). Each partition was trimmed with GBlocks (Castresana 2000) under the less stringent parameters, with the best-fitting evolutionary model (shown above) as the evolutionary model for each. The following calibration points were implemented as uniform priors: 87–170 mya for the Criodrilidae + Lumbricidae + Hormogastridae clade; 82–147 mya for the Lumbricidae+Hormogastridae clade. These age intervals correspond to the 95% HPD (highest posterior density) intervals obtained by Marchán et al. (2017).

A Yule diversification model and an uncorrelated lognormal relaxed clock were specified. Three parallel runs were performed, each including 50 million generations, sampling every 5,000th generation. Tree and log files were combined in Logcombiner v.1.10 (Suchard et al. 2018) by resampling at a lower frequency (15,000) and the results were visualized in Tracer v. 1.7.1 (Rambaut et al. 2018). The final tree was generated by TreeAnnotator v.1.10 (Suchard et al. 2018) with a burn-in of 2000 generations.