



## Research

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# The discovery of Iberobaeniidae (Coleoptera: Elateroidea): a new family of beetles from Spain, with immatures detected by environmental DNA sequencing

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The ongoing exploration of biodiversity and the implementation of new molecular tools continue to unveil hitherto unknown lineages. Here, we report the discovery of three species of neotenic beetles for which we propose the new family Iberobaeniidae. Complete mitochondrial genomes and rRNA genes recovered Iberobaeniidae as a deep branch in Elateroidea, as sister to Lycidae (net-winged beetles). Two species of the new genus *Iberobaenia*, *Iberobaenia minuta* sp. nov. and *Iberobaenia lencinai* sp. nov. were found in the adult stage. In a separate incidence, a related sequence was identified in bulk samples of soil invertebrates subjected to shotgun sequencing and mitogenome assembly, which was traced to a larval voucher specimen of a third species of *Iberobaenia*. *Iberobaenia* shows characters shared with other elateroid neotenic lineages, including soft-bodiedness, the hypognathous head, reduced mouthparts with reduced labial palpomeres, and extremely small-bodied males without strengthening structures due to miniaturization. Molecular dating shows that Iberobaeniidae represents an ancient relict lineage originating in the Lower Jurassic, which possibly indicates a long history of neoteny, usually considered to be evolutionarily short-lived. The apparent endemism of Iberobaeniidae in the Mediterranean region highlights the importance of this biodiversity hotspot and the need for further species exploration even in the well-studied European continent.

## 1. Introduction

In the beetles (Coleoptera), the most diverse order of insects, several thousand new species are described every year. Yet new high-level taxa are rarely discovered and most of the main lineages had already been described by the end of the nineteenth century. Altogether, 36 of the 179 extant families were proposed since 1950. Most of them have been elevated to the status of family as their divergent phylogenetic position became clear, whereas only a few were based on newly discovered specimens (see the electronic supplementary material). Not surprisingly, only one of the newly described families (Crowsoniellidae Iablokoff-Khnzorian, 1983) was collected in Europe, which can be considered the best-explored region worldwide. Here, three decades after the last discovery of a beetle family in Europe, we report a unique new lineage within the superfamily Elateroidea from Spain, for which we propose the taxonomic rank of family.

Despite the proverbial evolutionary success of the beetles [1], evident from enormous species richness and great morphological and ecological diversification, some lineages of Coleoptera are very species-poor. This includes some groups that are fairly common in the fossil record, such as the Archostemata, which today consists of approximately 40 species grouped in four highly

divergent families. Other ancient, but species-poor and rare lineages are exemplified by the families Aspidytidae, Meruidae, Crowsoniellidae, Jurodidae, Decliniidae, Lymexyliidae, Rhinorhipidae and others, all represented by one or a few species only, which mostly occur in unique habitats or exhibit localized distributions [2]. Similarly, the Elateroidea (click beetles, fireflies, soldier beetles and allies) includes several deep rooted families such as Omethidae including Telegeusinae, Omalisidae, Plastoceridae, Brachypsectridae and Rhagophthalmidae, which are species-poor (less than 100 known species worldwide, each) and generally rare in ecosystems (e.g. [3]). These groups, with few exceptions, exhibit females that are neotenic (retaining juvenile traits) to various degrees, and are either completely larviform, show a metamorphosed head but with the rest of the body larviform, or retain larval traits only in the abdomen. Soft-bodiedness and loosely connected abdominal sclerites resembling larval morphology possibly represent an initial stage of ontogenetic modifications [4]. Owing to the morphological similarities, families with these characteristics were traditionally grouped into a separate superfamily of soft-bodied lineages, the Cantharoidea ([5]; figure 1*b*) or as a monophylum within Elateroidea [7]. However, molecular data have shown the polyphyly of soft-bodied lineages, indicating the parallel evolution of soft-bodiedness and neoteny ([8], figure 1*b–c*).

Recently, when searching for neotenic for DNA analyses in the Iberian Peninsula, we encountered a series of elateroid specimens that could not be assigned to any known family. Independently, metagenomic sequencing of assemblages of beetles from soil biodiversity surveys in southern Spain recovered several partial and complete mitochondrial genome sequences that formed deep independent lineages in Elateroidea [9]. These sequences, obtained from mixtures of adult and larval specimens, indicated the existence of evolutionarily divergent taxa in the soil samples, which, however, could not be identified any further based on morphological characters. DNA sequences enabled us to link the adult males with the independently obtained metagenomic samples. Extensive phylogenetic analyses combined with the thorough study of adult and larval morphology confirmed the presence of a new family of Elateroidea. These findings confirm the Mediterranean basin as a reservoir of ancient lineages and a hotspot of diversity [10,11].

## 2. Material and methods

A total of 11 adult male specimens from several localities in the Murcia and Jaén provinces of southern Spain were studied. DNA from one specimen was extracted and deposited in the collection of Palacky University, Olomouc (UPOL) and the larvae and dry mounted specimens were deposited in the collections of the Natural History Museum, London (BMNH) and the collections of J. L. Lencina (JLC), A. Allen (AAC) and M. Baena (MBC). The full addresses of collections are given in authors' affiliations.

### (a) Molecular phylogenetics

#### (i) Four-marker dataset

Existing sequences for the four most widely used mtDNA (*cox1* and *rnl*) and nuclear (*SSU* and *LSU* rRNA) genes were compiled for the taxonomically most extensive dataset of Elateroidea to date (557 taxa, approx. 5300 bp), representing the main elateroid lineages and outgroup taxa [8,12]. Sequence data for two representatives of the newly discovered taxon were added to this matrix,

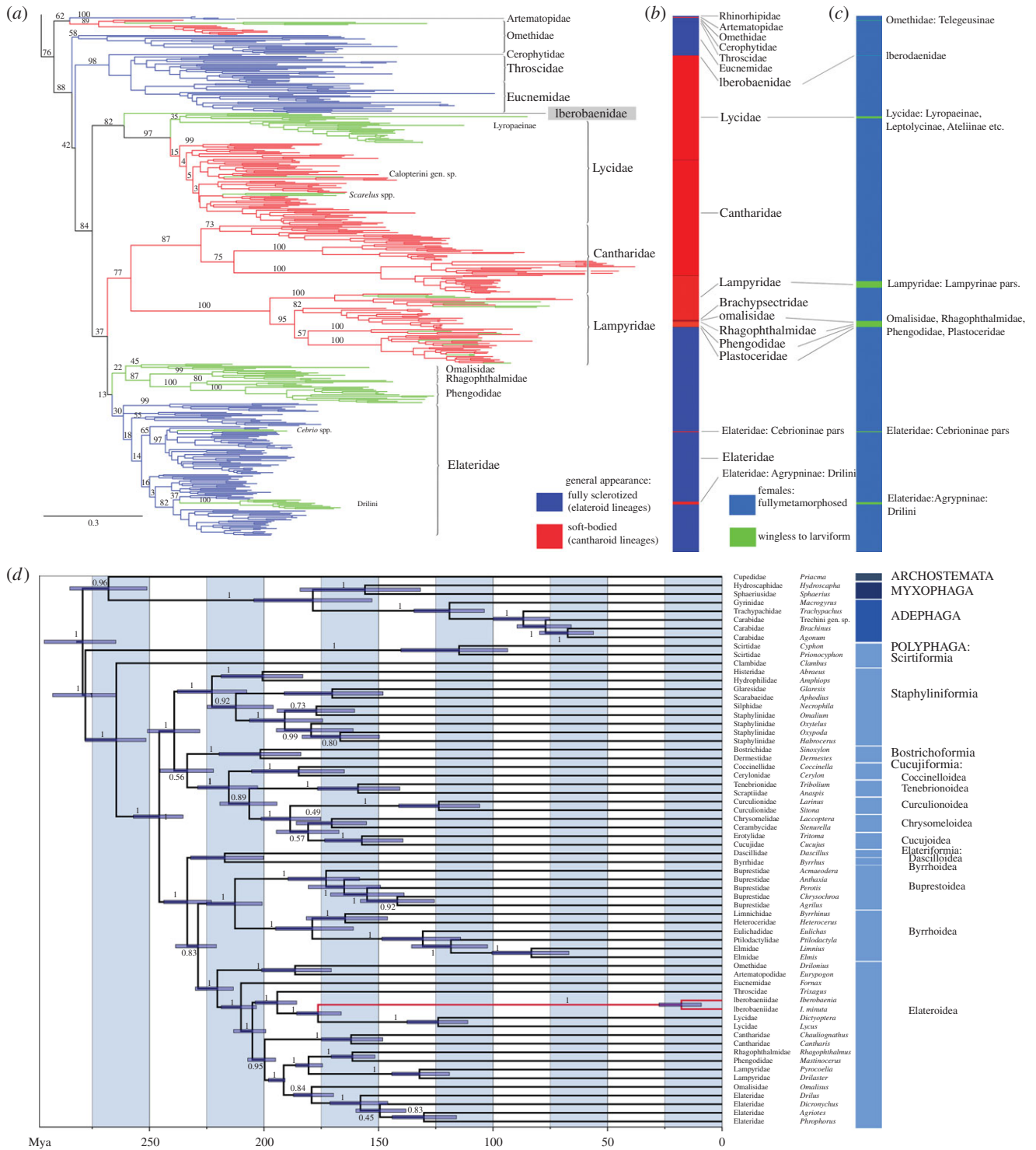
generating two versions of the four-marker dataset: (i) *v1* a chimerical terminal was assembled from the sequences of *cox1*, *SSU* and *LSU* for an adult specimen, sequenced using procedures reported by Bocakova *et al.* [13] (GenBank accession nos. KT339296–98; electronic supplementary material, table S1), and *rnl* sequence for the larval specimen BMNH1042541 of Andújar *et al.* [14] obtained from a full mitogenome sequence (see Mitogenome dataset paragraph); and (ii) *v2* two terminals were added including an adult (fragments as listed above) and the larva (*cox1* and *rnl* sequences of [14]). Sequences were edited using SEQUENCHER 4.9 (Gene Codes Corp., Ann Arbor, MI, USA). The length invariable *cox1* sequences were aligned using TRANSALIGN [15] and the rRNA fragments using default parameters of MAFFT 7.2 [16] and BLASTALIGN 1.2 [17].

Maximum-likelihood (ML) phylogenetic analyses using RAxML 7.3.1 [18,19] were conducted on the CIPRES web server to analyse individual gene alignments for *SSU*, *LSU* and *cox1* and the four-marker concatenated dataset (*SSU*, *LSU*, *rnl* and *cox1*), the latter partitioned by genes and by codon positions. All analyses were duplicated, with the ribosomal genes aligned using either MAFFT (Q-INS-I algorithm [16]) or BLASTALIGN. All ML tree searches were performed using 100 replicates for the best tree under a GTR+G+I model proposed by jMODELTEST2 [20], with bootstrap values calculated using the rapid algorithm [21] with 1000 bootstrap iterations under the GTRCAT model.

#### (ii) Mitogenome dataset

The full mitogenome sequence of the larval specimen BMNH1042541 was re-sequenced (accession number KT825140; [14] for details) to complement the partial mitogenome available from Andújar *et al.* [9]. Additional mitochondrial genomes were retrieved from GenBank (electronic supplementary material, table S2), and the 13 protein coding genes (PCGs) were extracted using GENEIOUS and individually aligned with TRANSALIGN. *SSU* and *LSU* rRNA sequences were retrieved from GenBank for the same species or, in a few cases, for the same genus (electronic supplementary material, table S3). The rRNA fragments were aligned using MAFFT (Q-INS-I algorithm) and concatenated with the PCGs. The final mitogenome dataset (mtDNA, *SSU*, and *LSU* genes; 64 taxa) additionally included the sequences obtained from the adult specimen. This dataset was used for phylogenetic inference using ML, as described above. Analyses were repeated applying: (i) a 3-partition scheme (*SSU+LSU+mtDNA*); (ii) an 8-partition scheme (six partitions by plus/minus strand and 1st, 2nd and 3rd codon position+*SSU+LSU*); (iii) a 15-partition scheme (partitions by genes); and (iv) a 38-partition scheme (partitions by gene and by codon position). Bayesian analyses on the concatenated matrix were performed using BEAST v. 1.81 [22] to estimate a phylogenetic tree and ages of diversification simultaneously. The best ML tree was used as a starting tree. Analyses were repeated using the four partition schemes as before, applying a GTR+G+I substitution model as above and an uncorrelated log-normal clock to each partition [23]. Polyphaga were designated as sister to the remaining clades [24–26]. A Yule speciation prior was applied and analyses were run for 150 million generations sampling one tree every 5000 generations. Consensus trees were estimated with TREEANNOTATOR [22] discarding the required fraction as a burn-in after checking the ESS of the tree likelihood and ensuring that values had reached a plateau in TRACER v. 1.6 (<http://beast.bio.ed.ac.uk/Tracer>).

Fossils relevant to the origin of Elateriformia and Scarabaeiformia [27,28] were used as calibration priors. The earliest fossils of Elateriformia date back to the Hettangian and Sinemurian deposits (190.8–201 Ma; Elateridae, *Elaterophanes* [29]) and the fossil of Scarabaeiformia to the Jurassic Formation of Switzerland (196.5–201.6 Ma; *Aphodiites* [30]). We applied a lognormal distribution as recommended for fossil calibration [31] with a minimum age hard bound at 190.8 Ma and with a 95% range of 190.8–228.5 Ma (offset: 190.8; log(mean): 0.01; log(s.d.): 2.2) as a prior for the node



**Figure 1.** (a) Phylogenetic hypothesis of Elateroidea inferred from the four-gene BLASTALIGN dataset using maximum-likelihood optimality criterion. *Iberobaenia* represented by a four-gene chimera. (b) Relative species diversity of soft-bodied and completely sclerotized elateroid lineages. (c) Phylogenetic position and relative species diversity of neotenic and completely metamorphosing lineages. The numbers of species are taken from Bocak *et al.* [2,6]. (d) Maximum clade credibility ultrametric tree resulting from the Bayesian analysis of 13 mtDNA genes, *LSU* and *SSU* rRNA in the program BEAST (8-partitions applied). (Online version in colour.)

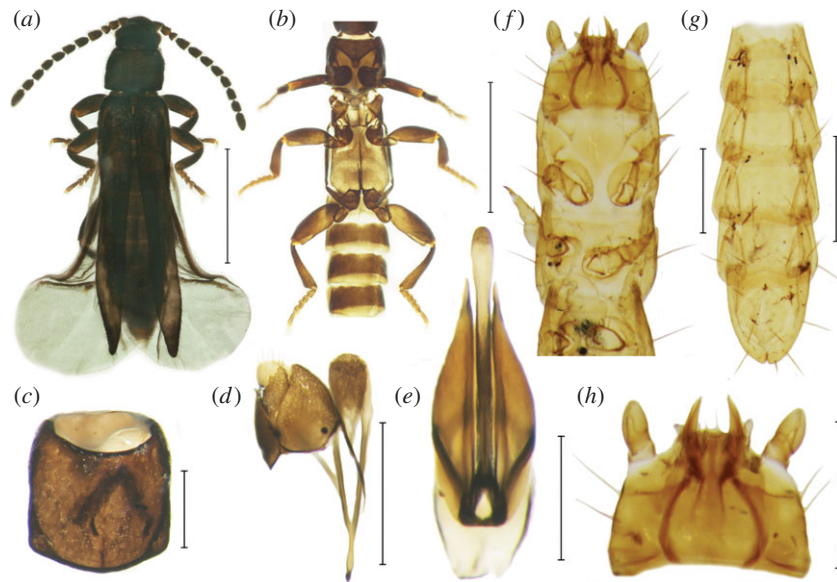
representing the split of Elateridae from other Elateroidea and a log-normal distribution with a minimum age hard bound at 196 Ma and a 95% range of 196–233 Ma (offset: 196; log(mean): 0.01; log(s.d.): 2.2) for the split between Scarabaeoidea and Staphylinioidea.

### 3. Results

#### (a) Molecular phylogenetic relationships of *Iberobaenia*

The well-resolved tree produced by the analyses of the four-marker dataset showed the clade of Omalidae,

Phengodidae, Rhagophthalmidae and Elateridae, deeply rooted Lycidae in an independent position and the newly discovered individuals were placed as the sister group of Lycidae with 62–85% bootstrap support (figure 1a, electronic supplementary material, figure S3). The relationships inferred from single-gene matrices varied, but the new lineage was never placed within any clade representing the existing families. When Lycidae were excluded, the new taxon was recovered as the sister to elaterid subfamilies Thylacosterninae + Lissominae, which have been difficult to place in molecular analyses (although placed in Elateridae based on morphology) and



**Figure 2.** Morphology of *Iberobaenia minuta* sp. nov. (*a–e*, adult) and *Iberobaenia* sp. (*f–h*, larva). (*a*) General appearance, (*b*) thorax ventrally, (*c*) pronotum, (*d*) terminal abdominal segments, (*e*) male genitalia, (*f*) thorax and head ventrally, (*g*) abdomen ventrally, (*h*) head ventrally. Scale bars: 1.0 mm (*a,b*), 0.5 mm (*g*), 0.25 mm (*c–f,h*). (Online version in colour.)

have a tendency to group with Lycidae using rRNA markers (e.g. [25]). The ML analyses of the mitogenome dataset recovered *Iberobaenia* in a clade containing besides Lycidae also *Mastinocerus* (Phengodidae) and Throscidae (electronic supplementary material, figure S4).

The mitogenome sequence (GenBank accession number KT339298) had a perfect match (100% similarity) with the *cox1* of the sequence from the same larval specimen (voucher BMNH 1042541). A second larval specimen (BMNH 1042563) differed by 1 of the 651 bp of the *cox1* gene (GenBank accession numbers AB123456–7). Both specimens were 92% similar to the adult specimen from Jaén, indicating they are different species, but closely related. The mitogenome showed a gene order unique among the Coleoptera, as gene rearrangement affected several PCGs, tRNAs and the control region [14]. This rearrangement explained the failure of the PCR amplifying the *rrnL–nad1* region using primers 16Sa and ND1A. Combined with 62 other mitogenomes of Elateriformia (14 697 positions), the analyses of two rRNA genes and 13 PCGs resulted in the well-supported sister relationship of *Iberobaenia* and Lycidae for the Bayesian analyses in BEAST (posterior probability of 1 with any data partitioning scheme applied; figure 1*d*). The calibration analyses with BEAST dated the split between these two lineages to the Jurassic at 170.7–176.6 Ma depending on the partitioning scheme (146.5–194.8, maximum 95% highest posterior density (HPD)). The ML analyses of the mitogenome dataset recovered the clade (Throscidae (Lycidae(*Mastinocerus*, *Iberobaenia*))) (electronic supplementary material, figure S4*a*). As the ML analyses of the mitogenome dataset merged *Mastinocerus* and *Iberobaenia* (electronic supplementary material, figure S4*a*), we constrained such topology in an additional dating analysis with eight partitions and parameters as above. The similarly deep split of *Iberobaenia* and its sister groups was inferred at 171.0 Ma (149.7–190.3, 95% HPD).

## (b) Morphology and taxonomy

### *Iberobaenia* gen. nov.

Type species: *Iberobaenia minuta* sp. nov.

**Diagnosis.** *Iberobaenia* are small-bodied beetles (1.9–2.5 mm) and similar to *Thilmanus* Gemminger, 1869 and *Paradrillus* Kiesenwetter, 1865 (Omalisidae) in general appearance (figure 2*a*), but have a hypognathous head and two-segmented labial palpi (electronic supplementary material, figure S1*c*). The prosternum is slightly transverse (figure 1*b*), with apical processes directed upwards, the pronotum and elytra do not bear any ridges or costae and the pronotum has rounded lateral margins (figure 1*c*). Legs are short, robust and non-compressed (figure 1*b*). The phallus is trilobate with slender pointed apexes of parameres; its phallobase is plate-like and v-shaped (figure 1*e*). All similar small-bodied neotenic beetles in Lycidae have developed apparent pronotal lateral edges, costae or at least papillae on the elytra, have a very short prosternum with two diverging processes at the apex, and compressed legs [32]. The full description is available in the electronic supplementary material.

The larva of *Iberobaenia* has two-segmented antennae with a finger-like process at the apex of the terminal antennomere similar to net-winged beetles (figure 1*h*; [33]). The larval mandibles of *Iberobaenia* are in opposite position, short, robust and with a sucking groove (figure 1*f,h*). Conversely, the larvae of Lycidae have slender, divergent mandibles consisting of two blades. The terminal abdominal segment of *Iberobaenia* is rounded with a deep notch (figure 1*g*); all net-winged beetle larvae have fixed urogomphi with variable length.

**Etymology.** The name *Iberobaenia* is derived from 'Iberia' referring to geographical origin of the taxon and in honour of M. Baena, the collector of the type species. Gender: feminine.

### *Iberobaenia minuta* sp. nov.

**Type material.** Holotype, male. SPAIN. Sierra de la Pandera, Valdepeñas de Jaén, 9.vi.2012, M. Baena leg. (UPOL). Paratypes, 2 males, same data (UPOL, MBC).

**Diagnosis.** *Iberobaenia minuta* is very similar to *I. lencinai* in size and general appearance, however, they differ in the more transverse prosternum in *I. lencinai* sp. nov. and in the male genitalia (electronic supplementary material, figure S1*a,b*).

**Description.** Male adult as in the diagnosis of the genus *Iberobaenia* and description of *I. minuta* in the electronic supplementary material.

**Etymology.** The specific epithet refers to the very small body of the male.

*Iberobaenia lencinai* sp. nov.

**Material studied.** Holotype, male. SPAIN, Molina de Segura, El Rellano, P. E. "Vicente Blanes", 15.iv–1.vi.2009, J. L. Lencina leg. (JLC). Paratypes: see the electronic supplementary material.

**Diagnosis.** See the differential diagnosis under *I. minuta* sp. nov. and description in the electronic supplementary material.

**Etymology.** The species name is a patronym in honour of the collector J. L. Lencina.

*Iberobaenia* sp.

**Material studied.** Larva, SPAIN, Camino Viejo a la Ermita, Sierra de Cabra, 37.481117N, 4.388536 W, 970 m, 6.xii.2012, grassland, C. Andújar & P. Arribas leg.; larva, SPAIN, Ermita Nta. Sra. de la Sierra, Sierra de Cabra, 37.490527N, 4.381292 W, 1145 m, 6.xii.2012, C. Andújar & P. Arribas leg. (BMNH).

### (c) Iberobaeniidae fam. nov.

The phylogenetic relationships, ancient divergence and morphology justified the establishment of a new taxon at the rank of family and we thus define monogeneric Iberobaeniidae fam. nov. The diagnosis of the family is the same as that of the genus *Iberobaenia* gen. nov.

## 4. Discussion

### (a) Relationships and morphological traits of Iberobaeniidae

The trees inferred from the four-marker dataset consistently supported *Iberobaenia* as a sister group to the Lycidae (figure 1a, electronic supplementary material, figure S3). The same relationships were recovered by the BEAST analysis of the mitogenome data (figure 1d) and the morphology of adult males and larvae supports this hypothesis. The only analyses inconsistent with this placement were obtained after removing the Lycidae, which grouped Iberobaeniidae with the elaterid subfamily Lissominae in the four-marker dataset, and in the ML analysis of the rRNA/mtDNA with (Lycidae(*Mastinocerus*, *Iberobaenia*)) clade (electronic supplementary material, figure S4). However, these spurious relationships are a problem associated with the other sequences, not with *Iberobaenia*. Lissominae has a tendency to group with Lycidae [25], and close relatives of *Mastinocerus* (e.g. *Rhagophthalmus*) were never found with Lycidae in all previous analyses [8,13,25]. No morphological character of Lissominae or *Mastinocerus* supports a close relationship to Lycidae and *Iberobaenia*. Iberobaeniidae share the trilobate phallus and mouthparts adapted for sucking liquids with other Elateroidea [3,33]. Adult *Iberobaenia* differs from Lycidae by the filiform male antennae with a long antennomere 2, the absence of the sharp lateral edge and strengthening structures in the pronotum, the elytra without costae or tubercles, and the uncompressible legs (figure 1a,b). Yet, some characters are shared between both families, such as the v-shaped phallobase that is similar to those in some Lyropaeini [34] and the short prosternum with two apical processes (although these are directed upwards; figure 1b). Females are absent in our sample and we suppose that they remain larviform or at least incompletely metamorphosed when sexually mature, with a short lifespan

and cryptic lifestyle. Lycidae contain several lineages with completely larviform females and small-bodied males, although in many cases (e.g. *Alyculus* Kazantsev, 1999, *Antennolycus* Bocakova & Bocak, 1999) the supposedly neotenic females have never been encountered, similarly to the Iberobaeniidae. The larvae of *Iberobaenia* differ from Lycidae in the uncompressible body, mandibles in opposing positions and able to bite (unlike Lycidae exhibiting mandibles with closely positioned bases and divergent tips that are not able to bite), fully sclerotized cranium and absent urogomphi (figure 1f–h). Both families share two-segmented larval antennae with a unique dorsal slender peg of the apical antennomere (figure 2h). The two-blade mandibles remain a synapomorphy of Lycidae, to the exclusion of Iberobaeniidae. All diagnostic morphological characters of Iberobaeniidae are summarized in the electronic supplementary material.

*Iberobaenia* males resemble various neotenic small-bodied elateroid genera (e.g. *Antennolycus*, *Paradrilus*, *Thilmanus*), including taxa with vestigial or absent elytra (*Alyculus*, *Cautires apterus* [2,6]). Additional morphological traits shared among neotenic elateroid lineages are found in the mouthparts, including a rounded, small mouth opening (electronic supplementary material, figure S1c), vestigial mandibles, reduced maxillae and labium and a lowered number of palpomeres, as these have been found in *Iberobaenia*. Our phylogenetic analyses confirm that shared morphological traits encountered in neotenic elateroids developed independently multiple times (figure 1c). Convergences in these traits have to be seen in the context of miniaturization of males, i.e. sexually linked body size differentiation [34,35] and hypothesized to be a result of the K- and r-strategy of the respective sexes [4]. Miniaturization is linked to the absence of the pronotal carinae and elytral costae as strengthening structures. Similarly, the patterns of four or nine longitudinal elytral costae are widespread in Elateroidea (e.g. Omalisidae, Lycidae, Omethidae) and the substantial reduction of costae is characteristic for neotenic forms with an extremely small body [32,36].

### (b) Evolutionary biology and distribution

The split between Iberobaeniidae and Lycidae was dated with elateriform and scarabaeiform fossils [27–29], using a conservative approach by applying minimum ages to the stem clades of these taxa. The time-calibrated phylogeny placed the radiation of most families of Elateroidea to the Jurassic and the origin of Iberobaeniidae at 170.7–176.6 Ma depending on partitioning scheme (figure 1d). The inferred dates agree with the supposed early origins of beetle families proposed by Hunt *et al.* [37] and Misof *et al.* [24], but the age of Elateroidea (approx. 217 Ma) is slightly older than those inferred by McKenna *et al.* [25]. Their dating of Elateroidea was based on click beetles in the Karatau deposits (152 Ma) when Elateridae were already a diversified lineage (greater than 100 species; [38]), and in agreement with our date for the origin of Elateridae. Despite these differences, either dating approach infers Iberobaeniidae as a very ancient lineage. This supports the taxonomic rank of family.

The deep separation of Iberobaeniidae and the shallow separation of the two species of *Iberobaenia* near the tip make it impossible to date the origin of neotenic development along the terminal branch with any degree of precision. However, neotenic lineages in Lycidae and other early branches of Elateroidea (Omalisidae, Phengodidae, Rhagophthalmidae)

are placed deeply in the tree and have undergone diversification, albeit at a low rate [4]. Thus, the Iberobaeniidae may represent another example of an ancient neotenic lineage, despite being species-poor. Equally, various neotenic lineages of Lycidae and Omalidae exhibit high endemism and relict occurrence [4,36], as found in Iberobaeniidae. Neotenic females are apterous and short-lived during the adult stage, and thus due to their limited dispersal, population persistence is predicted to require geological and climatic stability [35,39]. Additionally, the discovery of a new beetle family in Europe, only the second since the nineteenth century, emphasizes the need for the protection of natural habitats and, in combination with the high level of threat from human activities, justifies the designation of the western Mediterranean as a biodiversity hotspot [11]. Further morphological and palaeontological studies are needed for understanding of the evolutionary history of neotenic lineages.

Finally, novel molecular tools provide new approaches for recognizing these divergent lineages. Most neotenic lineages of Lycidae, Omalidae and also Iberobaeniidae have been collected either by sweeping the lowest strata of the vegetation or by sifting ([6,32]; M. Baena and M. Geiser 2012, personal communication). This presumably targets the males during short mating flights, while we detected the larvae in the deep soil that presumably identifies their main ecological affinities.

Assemblages of soil arthropods composed of different life stages and usually minute individuals constitute a huge challenge for taxonomists. Therefore, both knowledge of deep soil species diversity as well as life history of many known taxa remains incomplete. Shotgun sequencing of bulk specimen samples, followed by assembly of mitochondrial genomes and phylogenetic analysis against a set of reference sequences [9,40], now provides a new approach to search for these undiscovered lineages, for a more complete knowledge of the major branches of the tree of life and for the identification of regions of exceptional high phylogenetic diversity.

**Data accessibility.** The DNA sequences reported in this article can be accessed in GenBank under accessions KT339296–98 and KT825140.

**Authors' contributions.** L.B. and A.P.V. participated in the study design, R.K. and C.A.F. carried out the molecular laboratory work and data analyses, all authors contributed to the draft of the manuscript and discussed results. All authors gave final approval for publication.

**Competing interests.** We have no competing interests.

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