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Phylogeny and Evolution of the Genus *Abax*: A Genetic and Biosystematic Approach (Carabidae, Pterostichini)

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ABSTRACT

In this article, we carry out a study of the genus *Abax* (Carabidae, Pterostichini) based on mitochondrial and nuclear markers to test hypotheses about its evolutionary history in an integrative taxonomical framework. The subgenera *Pterostichoabax* (four species) and *Abacopercus* (monotypic) are likely natural taxa supported by all data. *Pterostichoabax* was nested within the species of the nominal subgenus *Abax*, which is rendered polyphyletic. The subgenus *Abax* is subdivided into a few clades that include two or more taxa and correspond to monophyletic lineages. The subdivisions within *Abax parallelepipedus* and *Abax pyrenaeus* are related to the geographical origin and may correspond to current subspecies. The results support hypotheses about a close relationship within the species of the POBE group (*A. pilleri*, *A. oblongus*, *A. baenningeri*, and *A. exaratus*); between *A. carinatus*, *A. pyrenaeus*, and *A. parallelus*; the basal position of *A. schueppeli*; and the monophyly of *Pterostichoabax*. In contrast, hypotheses about a close relationship between the species pairs *schueppeli-carinatus*, *parallelepipedus-fiorii*, and *ovalis-parallelus* were not supported by molecular results. The evolutionary history of *Abax* has been traced back to the Middle Tortonian (10.3 MYA). Successive lineage splits during the second half of the Miocene gave rise to the main lineages, with rapid intraspecific radiation during the Pleistocene. Lineage diversification included moderate ecological, behavioral, and molecular changes accompanied by morphological conservatism and diversified geographical patterns. Our results constitute a framework for future studies on the evolutionary radiation of the genus *Abax* and other carabids during the Miocene in the western Palearctic and contribute to developing a natural classification of the genus.

1 | Introduction

The genus *Abax* Bonelli, 1810, is a group of ground beetles (Carabidae) of the tribe Pterostichini. It is distributed across temperate Europe and is rather homogeneous on a morphological basis, as all species look very similar: the dorsum is shiny black, the body is robust, the appendages are often entirely black, and the body size is ~15–18 mm (Figure 1). Ecologically, there are somewhat more diverse forest-dwelling species inhabiting the Carpathian Mountains, the Alps, and the Pyrenees; five species have colonized lowland temperate forests from Great Britain to central and oriental Europe, the Balkans, and Turkey, reaching western Iran, and several taxa have colonized alpine meadows [1, 2]. The Alps between Switzerland, Italy, Slovenia, and Austria are the present geographical core of the genus; there are 15 species

FIGURE 1 | Habitus of *Abax parallelepipedus parallelepipedus* (Piller and Mitterpacher, 1783; male), from Weinheim (Odenwald, Germany). Bar equals 2.82 mm.

of *Abax* in Italy [3], most of which are in the Alps, with 18 species in total in this region.

Many of these species live in sympatry with others, which often makes it difficult to identify them with certainty. Indeed, there is no updated key for the identification of all the species of *Abax*. Concerning the imagoes, a key for the whole group has been drafted (Brandmayr, unpublished), using similar characters to those listed by Magrini and Degiovanni [3] for Italian species. Knowledge of the larval morphology of most taxa is nearly complete [4, 5]. Recent contributions to the taxonomy of the genus that are not mentioned in the list of the last Palearctic Catalogue [1] are from the works of Zanella [6, 7] who resolved the status of several subspecies in addition to synonymic problems. Similarly, *Abax sexualis* Fairmaire, 1859 became a synonym for *A. pyrenaeus pyrenaeus* (Dejean, 1829) after the male holotype was examined [8]. After these changes, the genus is presently divided into the monotypic subgenus *Abacopercus* Ganglbauer, 1891; the subgenus *Abax* Bonelli, 1810, with 13 species; and the subgenus *Pterostichoabax* Schauberger, 1927, with four species.

The evolutionary history of *Abax* was outlined [2] according to available data on morphology, geographical distribution, ecological preferences, and ethology. These authors postulated that primitive *Abax* were forest dwellers that gave rise to the oldest lineages, *A. schueppeli* Palliardi, 1825 and *A. pyrenaeus*; that most taxa are the result of Pliocene-Pleistocene disjunctions; and that ecological shifts within the *A. parallelepipedus* (Piller et Mitterpacher, 1783) lineage originated in the subgenus *Pterostichoabax* and the *A. oblongus* (Dejean, 1821) group. Nevertheless, these hypotheses have seldom been explored using molecular data [9, 10] although many sequences of *Abax* are available in the GenBank and BOLD databases. The objective of our research was to get molecular data for most *Abax* species based on a wide sampling during the last decades, and combine this knowledge with information from morphology, ecology, behavior, and biogeography to test these hypotheses in an integrative way and develop an improved taxonomic classification of the genus. To this aim, we have sequenced most species and compared the phylogenetic inferences with those resulting from nonmolecular characters. Our results show that the genus *Abax* is a young monophyletic group of insects with a notable radiation during the Miocene and the Quaternary, that includes well-characterized natural groups together with other uncertain phylogenetic and taxonomic relationships.

2 | Materials and Methods

2.1 | Sample Collection

We collected most of the *Abax* samples analyzed over the last 20 years in different countries (Table 1). Specimens were collected by hand, by live traps baited with beer and apple, or by means of pitfall traps containing propylene glycol as a preserving agent, and then immediately killed in 96% ethanol. The taxon names follow Bousquet [1], except for the recent nomenclatural changes proposed by Zanella [6, 7]. The voucher specimens are deposited in the scientific collection of the Department of Zoology and Physical Anthropology, University of Murcia (Spain).

2.2 | DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from the third right leg of specimens using a glass fiber plate DNA extraction protocol [11].

The COI region of the DNA was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [12]. Amplification of the 28S region was performed using the primers LS58F (D1) (5′-GGGAGGAAAAGAAACTAAC-3′) and LS998R (D3) (5′-GCATAGTTCACCATCTTTC-3′) [13]. These genomic markers are commonly used for phylogenetic studies, which allows for comparisons with previous results and other sequences in public databases. Other markers were tried but their amplification was not successful. Amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, US), using the Phire Animal Tissue Direct PCR kit (Thermo Scientific, US). The total reaction volume was 12.5 μL, containing 2 μL of sample DNA for each reaction. The PCRs consisted of initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, and a final extension of 10 min at 72°C for COI. For 28S, the PCRs consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 60 s and 72°C for 1 min, and a final extension of 10 min at 72°C. After amplification, 2 μL of each PCR product was loaded into a 2.5% agarose gel stained with RedSafeTM (iNtRON Biotechnology, Seongnam, South Korea) for visualization under a compact system for gel documentation (Analytik Jena, Germany).

Sequencing was carried out either by Macrogen Spain or Secugen S.L. (Madrid, Spain).

2.3 | Sequence Analyses

The number of obtained sequences was 96 for COI and 98 for 28S. The sequences we imported into Geneious (www.geneious.com), and the low-quality ends were trimmed. The sequence alignment for each marker was performed in the same program using MAFFT [14]. The total lengths of the alignments were 658 bp for COI and 1028 bp for 28S. We included *Abax* sequences from the BOLD (Supporting Information Table S1) and GenBank (Supporting Information Table S2) databases and sequences of

TABLE 1 | List of species and number of sequenced individuals (COI and 28S) of the genus *Abax*.

Species	Locality	COI	28S
Subgenus *Abacopercus* Ganglbauer, 1891			
A. schueppeli schueppeli Palliardi, 1825	Sibiu RO	1	1
Subgenus *Abax* Bonelli, 1810			
A. baenningeri Schauberger, 1927	Grigna Mt. IT	3	1
	Foppolo IT	—	1
A. carinatus sulcatus A. Fiori, 1899	Basovizza IT	3	1
	Kozina SL	4	1
	Blagoevgrad BU	2	2
	Dolno Dzyan BU	1	1
	Rila BU	—	1
A. contractus (Heer, 1841) (formerly *continuus* Ganglbauer, 1891)	Chiavedano IT	4	4
	Oropa Mt IT	3	3
A. exaratus (Dejean, 1828)	Val Sessera IT	5	6
A. fiorii Jakobson, 1907 (formerly *angustatus* Fiori, 1896)	Cainallo Pass IT	2	2
A. oblongus (Dejean, 1821)	Grigna Mt. IT	9	8
	Bocchetta IT	—	1
A. ovalis (Duftschmid, 1812)	Basovizza IT	1	2
	Kozina SL	1	2
	Rila BU	1	1
	Predela1 BU	—	1
	Predela2 BU	—	1
A. parallelepipedus parallelepipedus (Piller et Mitterpacher, 1783)	P. Nac. Ordesa SP	1	1
	Pineta SP	2	2
	Añisclo SP	1	2
	Peñacerrada SP	3	2
	Labastide FR	3	3
A. parallelepipedus parallelepipedus (formerly *audouini*) (Dufour, 1851)	Setcases SP	1	1
A. parallelepipedus parallelepipedus (formerly *germanus* Schauberger, 1927)	Freiburg GE	1	1
A. parallelepipedus parallelepipedus (formerly *subpunctatus* Dejean, 1828)	Kozina SL	2	2
A. parallelepipedus sp	Sibiu RO	2	3
A. parallelepipedus curtulus Fairmaire, 1856	Fagnano IT	1	1
	Sella Leonessa IT	1	1
A. parallelepipedus inferior Seidlitz, 1887	Tornedo IT	2	1
	Cimolais IT	3	1
	Sequals IT	2	1
	Prescudin IT	1	—
	Muzzana IT	2	4
	Val Camonica IT	—	1
	Cainallo Pass IT	—	1
A. parallelepipedus lombardus A. Fiori, 1896	Clusone IT	2	1
	Grigna Mt. IT	3	1
A. parallelus parallelus (Duftschmid, 1812)	Sibiu RO	1	1
	Isola Morosini IT	3	1
	Freiburg GE	1	1
	Oblik CZ	2	4
A. pilleri Csiki, 1906	Jezersko SL	1	1
	Tornedo IT	1	1
	Cimolais IT	1	1
A. pyrenaeus pyrenaeus (Dejean, 1828)	Setcases SP	2	3
	Oriental Pyrenees SP	1	1
A. pyrenaeus jeannei Ortuño et Arribas, 1992	Port Comte SP	2	3
	Moli Fornols SP	1	1
	Sierra Serrat SP	1	—
Subgenus *Pterostichoabax* Schauberger, 1927			
A. beckenhauptii schatzmayri J. Müller, 1926	Prescudin IT	1	2
A. ecchelii venetianus J. Müller, 1926	Piancavallo N. Park IT	1	1
A. springeri J. Müller, 1925	Cimolais IT	3	2
	Prescudin IT	—	1
A. teriolensis Schauberger, 1921	Mt. Obante IT	3	4

Abbreviations: BU, Bulgaria; CZ, Czech Republic; FR, France; GE, Germany; IT, Italy; RO, Romania; SL, Slovenia; SP, Spain.

other Pterostichini from GenBank as outgroups: *Nirmala indica* (JX535619), *Pterostichus niger* (JX535631) and *Trigonotoma morvani* (JX535620). The sequences were uploaded to GenBank. The accession numbers are PV068201 and PV125164-PV125258 for COI sequences, and PV056229–PV056326 for 28S sequences.

We built a concatenated matrix in which we included one 28S sequence and one COI sequence from each taxon and population, selecting the most complete sequences. Maximum likelihood phylogenetic analyses were carried out in IQTREE 2.2 [15] for the COI, 28S, and the concatenated matrices, searching for the most appropriate partition scheme [16] and model [17], and including ultrafast bootstrapping with 1000 replicates [18]. Bayesian inference analyses were carried out in MrBayes 3.2.7 [19], which was run for 5 million generations. A tree was sampled every 5000 generations, and the first 25% of trees were discarded as burn-in. The most appropriate nucleotide substitution model for each matrix was the GTR+I+G model, which was calculated in jMODELTEST 2.1.10 [20].

The concatenated matrix was analyzed with BEAST 1.10.4 [21] using the rates calculated for the COI fragment in *Carabus* [22]: normal distribution with a mean of 0.0145 substitutions per site per million years and a standard deviation of 0.0001. We tried different combinations of clock models (strict clock, lognormal relaxed clock and exponential relaxed clock) and tree models (constant coalescent and birth-death) and compared the outputs in TRACER 1.6 [23] to choose the most appropriate model combination using Bayes factors. The best combination was a constant coalescent tree model with a lognormal relaxed clock model, but it produced an aberrant tree, probably due to a model-data misfit or being stuck in a local maximum, so we chose the second-ranked model combination, the birth-death coalescent tree model with a lognormal relaxed clock. The consensus tree was created in TreeAnnotator (distributed with BEAST) with a 10% burn-in.

The concatenated matrix was partitioned for each analysis, with one partition corresponding to the 28S fragment and three partitions for the three different codon positions of the COI marker. The COI matrix was also partitioned for the individual analyses.

3 | Results

3.1 | Molecular Data: Major Clades

While the topology of the individual matrices (COI and 28S, Supporting Information Figures 1 and 2) was more variable, the topology obtained in the analyses using the concatenated matrix (Figures 2–4) was completely identical in both IQTREE and BEAST analyses (Figures 3, 4) and equivalent to that obtained in MrBayes (Figure 2) if we see past the unresolved basal node, indicating the robustness and reliability of our results. Most species are shown as monophyletic lineages clearly separated from each other, and only a few interspecific nodes show a lower support. According to the calibrated analysis (Figure 4), the genus *Abax* originated around 10 MYA.

In our trees (Figures 2–4), the following clades were supported in all analyses (from bottom to top): (i) the monotypic subgenus *Abacopercus* made up of *A. schueppeli*; (ii) a clade composed of *A. carinatus* and *A. pyrenaeus*; (iii) the species *A. parallelus*; (iv) the subgenus *Pterostichoabax*, composed of the species *A. beckenhauptii*, *A. ecchelii*, *A. springeri*, and *A. teriolensis*; (v) a clade composed of *A. contractus* and a subclade formed by *A. fiorii*, and the POBE species complex (*A. pilleri*, *A. oblongus*, *A. baenningeri*, and *A. exaratus*); and (vi) a large clade that includes *A. parallelepipedus* and *A. ovalis*.

The genus *Abacopercus* is shown in a basal position in most analyses (Figures 3, 4), although MrBayes does not fully resolve this relationship (Figure 2). This lineage would have diverged from the other *Abax* around 9.97 MYA.

In the main *Abax* lineage, two major subclades diverged ~9.14 MYA (Figures 3, 4): *A. parallelus*, *A. carinatus*, and *A. pyrenaeus* on one hand; and all the other species on the other. Within the first major subclade, *A. carinatus* and *A. pyrenaeus* are always shown as sister species, diverging 6.74 MYA. The species *A. parallelus* is closely related to them, having diverged from their ancestor 8.36 MYA.

The subgenus *Pterostichoabax* makes up a monophyletic clade, diverging 7.4 MYA from the closest lineage, and it is nested within the taxa belonging to the subgenus *Abax*. The relationships between its member species are not clearly resolved in any analysis.

The species *A. contractus* and *A. fiorii* are closely related in all the analyses to a group of very recently diverging (Figure 4) species that we call the POBE complex (*A. pilleri*, *A. oblongus*, *A. baenningeri*, and *A. exaratus*). The relationships between the POBE species are not fully resolved.

Finally, *A. ovalis* is related to *A. parallelepipedus* (formerly *A. ater*), which is a species with a broad geographical distribution that explains why many individuals from different countries have been included in our study. A notable diversity of haplotypes was found, with their relationships seemingly related to the geographical origins of the individuals.

4 | Discussion

4.1 | Integration of Molecular Characteristics Into *Abax* Systematics

The aim of this article is to provide a robust framework for future studies of the genus *Abax* and contrast past hypotheses about the evolution of this genus with newly obtained mitochondrial and nuclear molecular data, analyzed through different methodologies, and including a temporal framework.

Molecular data clearly support that the genus *Abax* is a monophyletic taxon well differentiated from *Percus* Bonelli, 1810, and related genera, as noted by previous works [9]. This conclusion also applies to the subgenera *Pterostichoabax* and *Abacopercus*. However, the clade of *Pterostichoabax* is consistently interspersed within the species belonging to the subgenus *Abax*, which indicates that this latter subgenus is a polyphyletic taxon that should be redefined.

Adults of the four species of *Pterostichoabax* share features such as 5th tarsomeres with spines on the ventral side, a 7th interval of elytra not keeled at the base, and the absence of humeral teeth on the elytra. Similarly, these species share the same parental care behavior [24] and are geographically and ecologically close, that is strictly tied to the alpine and subalpine grass mats habitat [2]. The four species included in this subgenus are found in the Southeastern Alps in Slovenia, Italy, and Austria. These data suggest that the subgenus is a natural taxon. A more detailed analysis, including more samples and markers, may corroborate the hypothesized close relationship between the species

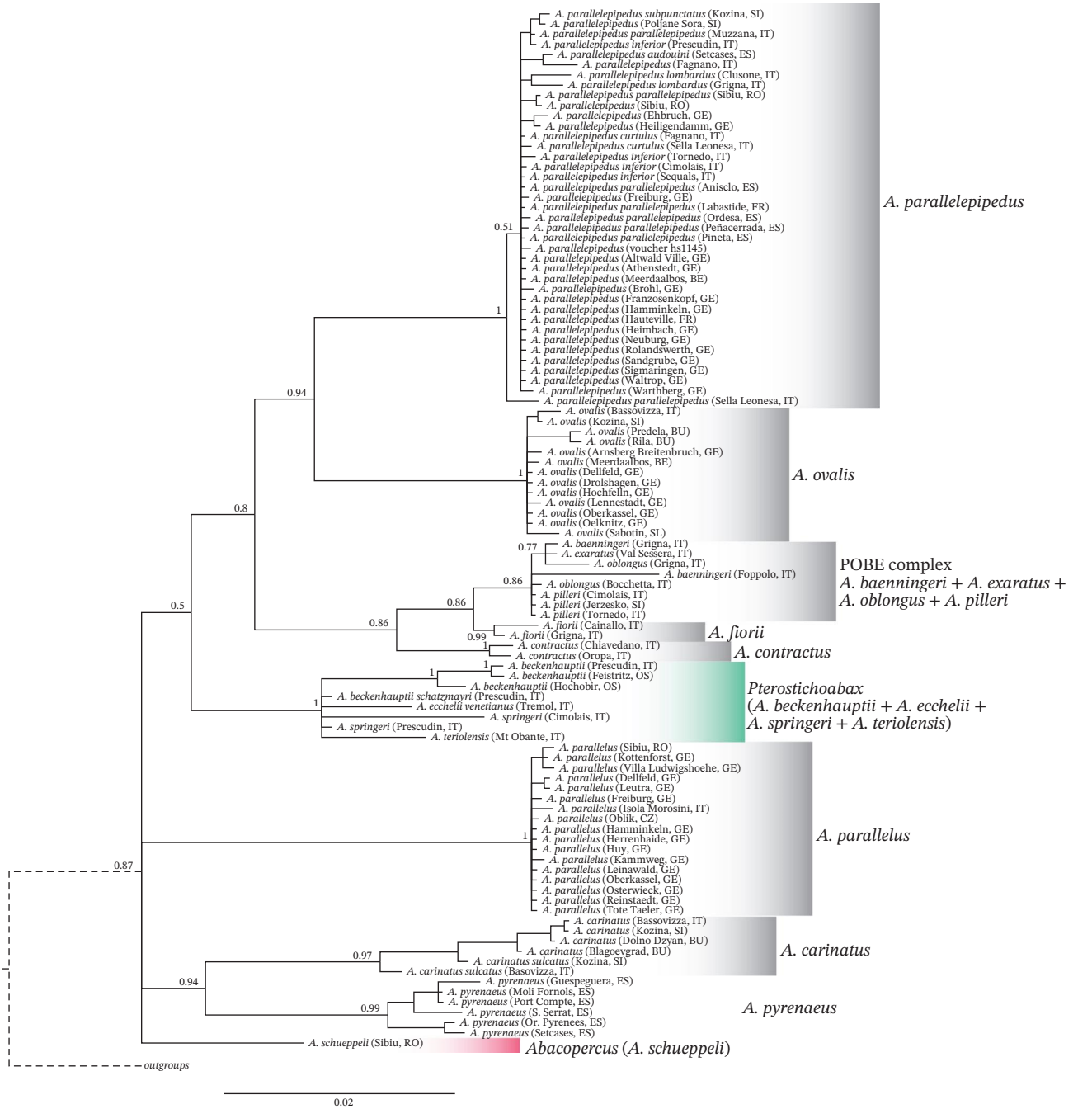

FIGURE 2 | Bayesian inference phylogeny of the genus *Abax* based on the concatenated matrix obtained in MrBayes. The numbers on the nodes indicate posterior probability values.

pairs *springeri–beckenhauptii* and *ecchelii–teriolensis*, which are not fully resolved in our analyses. One possible cause of this lack of resolution is that these taxa may have experienced introgression events along their evolutionary history, creating a conflict between the mitochondrial and nuclear markers used in this study. This can also explain the incongruencies observed between the trees obtained from the individual matrices (Supporting Information Figures 1 and 2) and from the concatenated matrix (Figures 2–4).

The integration of molecular results with other characters is congruent in the case of *Abax schueppeli*, as it shows a basal position relative to all the other *Abax* in most of the molecular analyses, and morphologically is the largest species, lacks an elytron basis, the 7th stria is notably carinate and shows punctuated striae (monotypic subgenus *Abacopercus*). It is the easternmost species of *Abax* and is somewhat isolated in the Carpathian Mountains. The hypothesized inclusion of *A. carinatus* within the subgenus *Abacopercus* because this species also shows a marked 7th carinate stria [3] lacks molecular support according to our results.

A close relationship between species of the POBE clade (*Abax pilleri*, *A. oblongus*, *A. baenningeri*, and *A. exaratus*) was already hypothesized [2, 24] based on their ethology, ecology, biogeography, and morphology. These species, together with *A. arerae* Schauberger, 1927 and perhaps *A. benellii* Magrini et Degiovanni, 2013, possibly make up a monophyletic group corresponding to the proposed

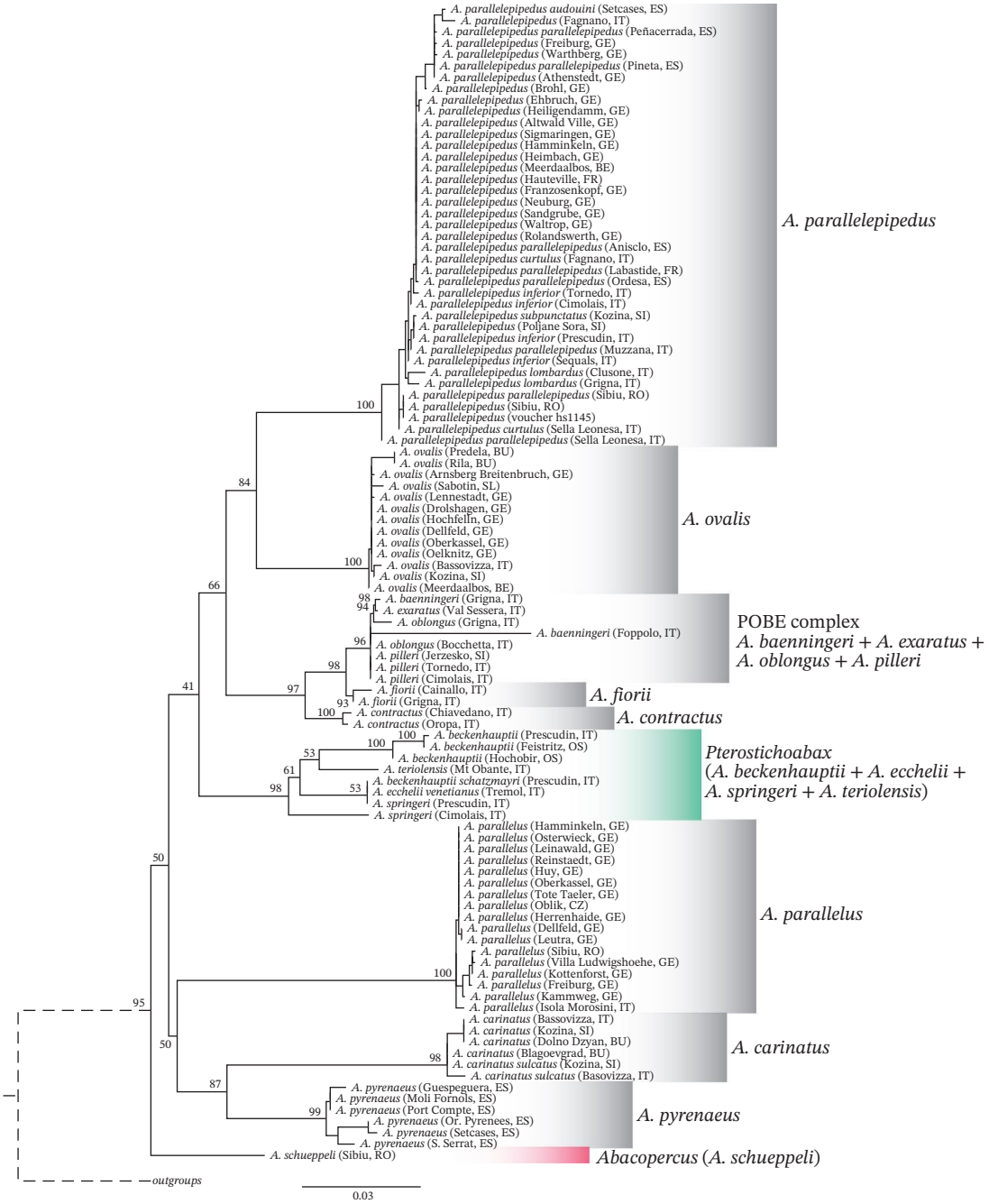

FIGURE 3 | Maximum likelihood phylogeny of the genus *Abax* based on the concatenated matrix obtained in IQTREE. The numbers on the nodes indicate bootstrap support values.

exaratus group [3], characterized by a moderate body size (less than 17.5 mm), a moderately keeled 7th stria, a minute humeral tooth of elytron, and a smooth frontal furrow. All these species are found on the southern side of the Alps, from Piemonte to the Julian and Slovenian Alps, and given the recent age of their clade they may still be undergoing a speciation process. Within this group, *A. benellii* is differentiated by the peculiar apex of the aedeagus [3]. Ecologically, some of these species (e.g., *A. oblongus* and the related *A. arerae*) have secondarily colonized alpine grasslands over the timberline, with larvae active in soil crevices [2]. Therefore, integration of different character sets suggests that the POBE group also makes up a natural lineage.

The species *A. fiorii* and *A. contractus* consistently showed up near the POBE clade in most analyses. It has been hypothesized that *A. fiorii* should be considered a subspecies or a closely related entity of *A. parallelepipedus* on a morphological basis [25, 26] but our molecular results showed a clear separation between these taxa. In *A. contractus* the 5th tarsomeres lack spines on the ventral side, which would place it near *A. ovalis*, *A. parallelus*, and *A. pyrenaeus* in a morphological key. However, this character is homoplastic, as it may be present or lacking in taxa as *A. carinatus sulcatus* [3]. On the other hand, geographically, *A. contractus* is related to the POBE clade and to *A. fiorii*, in agreement with our molecular results.

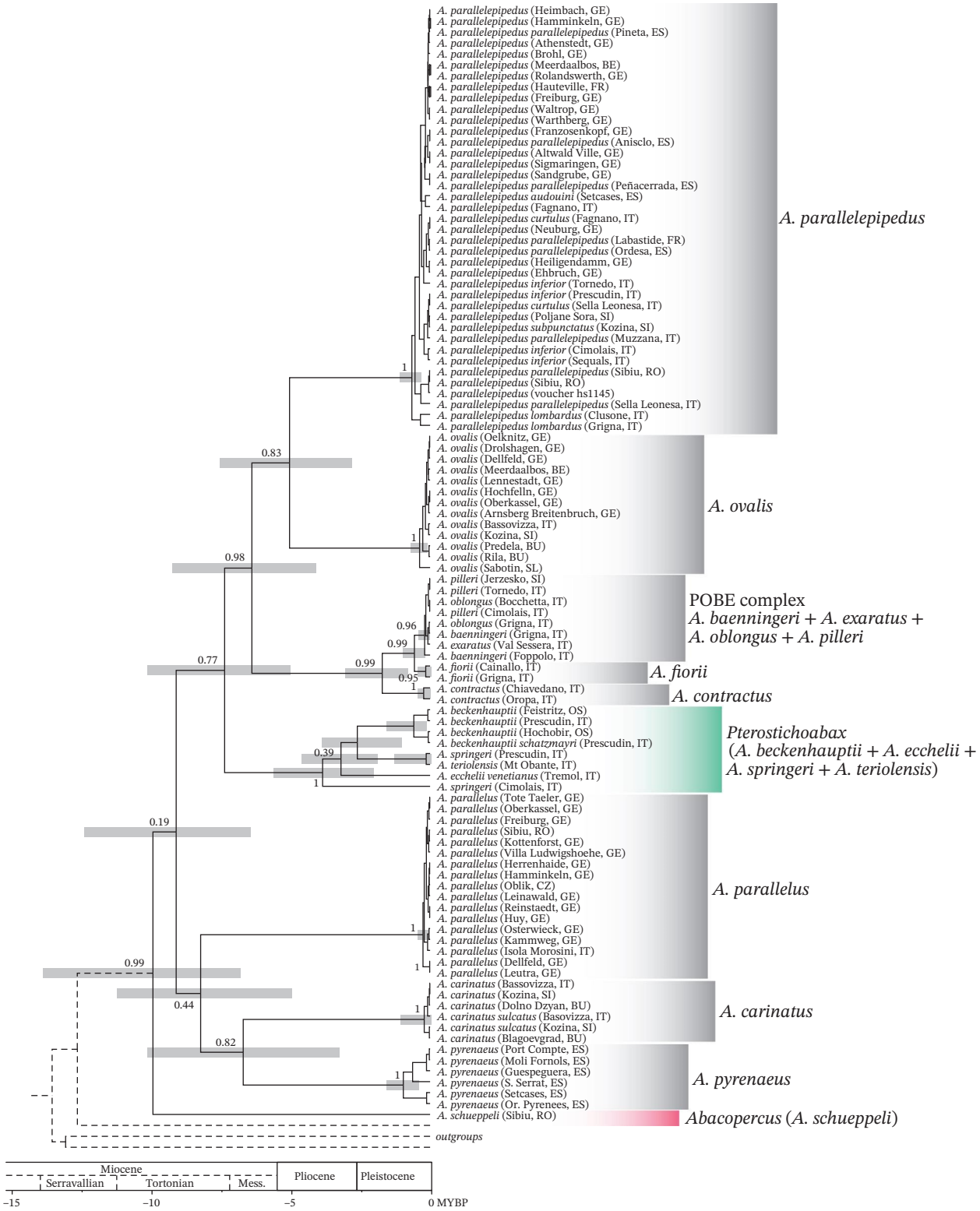

FIGURE 4 | Chronogram of the genus *Abax* based on the concatenated matrix obtained in BEAST. The numbers on the nodes indicate posterior probability values. Bars in the nodes represent 95% confidence intervals. A time scale is shown at the bottom for reference.

Abax carinatus and *A. pyrenaeus* were hypothesized to be closely related taxa according to male genitalia and other morphological characteristics; both species are mostly forest dwellers. These characteristics are consistent with the relatedness of both species in our molecular results. Both species share the ancestral state of larvae with only four terminal joints of urogomphi in aged stages (Figure 5) [5, 24] and show an overlapping distribution in plain areas of Europe. The hypothesized close relationship between *A. parallelus* and *A. ovalis* [2] is not supported by our molecular data.

Abax ovalis and *A. parallelepipedus* are closely related in all the molecular analyses, but this relationship is not supported by nonmolecular characters. The external morphology of adults of

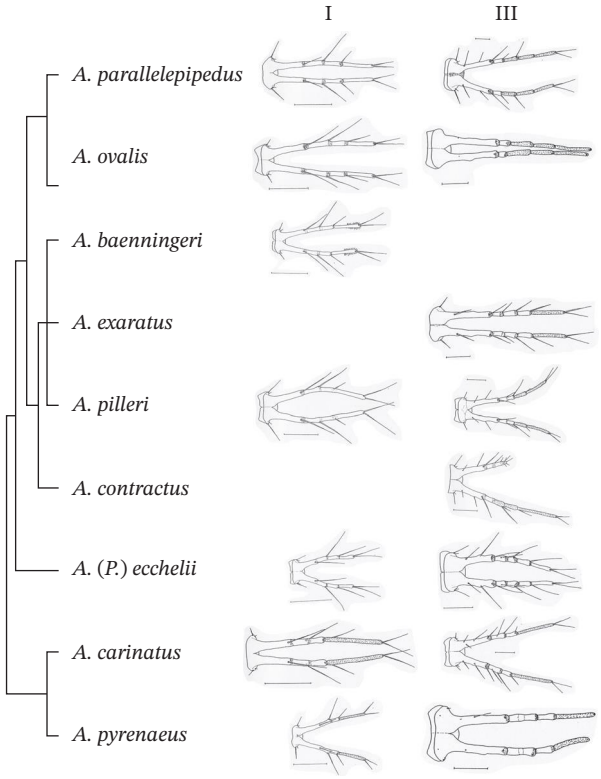

FIGURE 5 | Urogomphi of *Abax* larvae according to the thesis by Marano [5]. The topology of the tree follows the ultrametric BEAST tree (Figure 4), including only the species for which there is morphological data available. Capital Roman numbers on top indicate first or third stages of the larvae. Bars equal 1 mm.

the two species is quite different, and the same differences are found regarding the aedeagus shape, the evaginated aedeagus membranous sac, and the larval characteristics, which are more primitive in *A. ovalis* [2]. The behavior of parental care is more advanced in *A. ovalis* (egg watchers) than in *A. parallelepipedus* (mud cells) [24]. If the separation of the lineages of both species indeed took place before the onset of the Pliocene (Figure 4), then evolutionary changes occurred at a rapid pace to give rise to the current differences between the two species.

The most striking of our molecular results is that the nominal subgenus *Abax*, as currently considered, is not a monophyletic lineage, as the subgenus *Pterostichoabax* is included in every case within it, in contradiction with morphological grounds based on potential homoplastic characteristics, such as the spiny 5th tarsomeres, the umbilicate series of elytron sides, the humeral tooth, body size, pronotum shape, and so on. Furthermore, on ecological, ethological, and geographical grounds, this subgenus is diverse and lacks any pattern denoting monophyly. Therefore, further studies are needed to determine the phylogeny of this subgenus, and determine if it should be divided into at least the two main lineages into which it is split: (a) *A. parallelus*, *A. carinatus*, and *A. pyrenaeus* on one hand, and (b) the rest of the species on the other.

4.2 | Intraspecific Genetic Diversity

We find intraspecific genetic diversity in the sequences of most *Abax* taxa, especially in species with wide geographical distributions such as *A. parallelepipedus* and *A. parallelus*. In the last Palearctic Catalogue, there are nine recognized subspecies of *A. parallelepipedus* [1]. According to a recent revision [6], *A. parallelepipedus subpunctatus* and *A. parallelepipedus audouini* are synonyms of *A. parallelepipedus parallelepipedus*. On the other hand, the name *A. contractus*, once regarded as a subspecies of *A. parallelepipedus*, was given to the species *A. continuus*, invalidating this name based on priority, after the reexamination of the type specimen [7]. Both conclusions are supported by our molecular data, as *A. parellepipedus* subspecies do not form separate groups within this species, and *A. contractus* is related to *A. parallelepipedus* but makes up its own clade (Figures 2–4). The sequences of individuals initially identified with the names of the proposed synonyms (*subpunctatus*, *audouini*) were related to those of individuals of *A. parallelepipedus inferior* (Supporting Information Figures S1, S2), which suggests that they should indeed be identified as belonging to this last subspecies.

The species of the POBE group presented slight genetic diversity without a clear geographical pattern, as interspecific differences overlapped with intraspecific differences. As noted above, the four species of the group are closely related according to different sets of traits and probably make up a monophyletic group that corresponds to a species complex that is still undergoing speciation, probably after population isolation during the last glacial period.

Populations of *A. contractus* inhabiting the Chiavedano and Oropa Mountains do not make up separate clades and even share the same 28S haplotypes (Supporting Information Figures S1, S2). These findings, combined with the results of the concatenated tree (Figures 2–4), suggest that they are populations that recently originated or that still have some degree of contact.

Abax parallelus is a widespread species inhabiting Central Europe and the Balkans. Its specimens showed moderate variation in their sequences, and only individuals from the isolated population of Isola Morosini (NE Italy) appeared to be somewhat distinct (Figure 3, Supporting Information Figure S2).

Abax carinatus showed little genetic diversity although individuals with distant geographical origins were sequenced (Figures 2–4). In contrast, *A. pyrenaeus* presented two distinct clades (Figures 2, 4), possibly corresponding to the two subspecies *A. pyrenaeus pyrenaeus* and *A. pyrenaeus jeannei*. The first subspecies is found in the easternmost part of the Eastern Pyrenees (Figure 6). On the west, *A. pyrenaeus jeannei* is found, while *A. pyrenaeus pyrenaeus* is distributed in the easternmost localities; with the population of the Sierra del Serrat, laying between both subspecies and forming a somewhat distinct lineage. The populations of *A. pyrenaeus* are scattered across the central and easternmost Pyrenean peaks [27], which might explain our finding of notable genetic diversity associated with geographical isolation.

4.3 | Evolution of Larval Morphology and Parental Clade in *Abax*

To test hypotheses about the evolutionary history of *Abax*, it is necessary to consider not only the morphology of adults but also that of larvae, and the parental care provided by adults. Larval morphology shows notable differences in the shape and pubescence of the urogomphi (Figure 5) [5]. *A. carinatus* and *A. parallelus* exhibit three-jointed urogomphi at all instars, and the last joint is

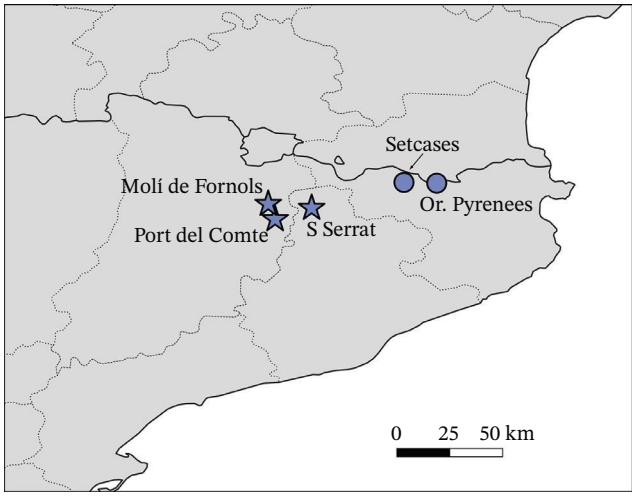

FIGURE 6 | Distribution map of the subspecies of *Abax pyrenaeus*. Circles show localities corresponding to sequenced individuals of *A. pyrenaeus pyrenaeus*, stars show localities of *A. pyrenaeus jeannei*. Map source: Natural Earth.

very long and hairy in the first instar. *Abax pyrenaeus* has a distinct 3rd-instar morphology. Based on the basal position of these species in the phylogeny, it is hypothesized that this state is primitive for the genus. The aged instars of most species include four-jointed urogomphi (except for *A. baenningeri*), which are hairy according to the last two or three articles (except for *A. exaratus*). This urogomphi shape is considered a derived state in agreement with the hypothesis formulated above. In the subgenus *Pterostichoabax*, urogomphi pubescence was perhaps lost in aged instars, and the articles became shorter and more "*Pterostichus*-like", probably an adaptation to life on alpine mats and screes.

It seems that the urogomphi shape reflects both habitat features and phylogenetic affinities; thus, its phylogenetic meaning still needs to be disentangled. In this context, the study of the larvae of *A. schueppeli* and a careful reexamination of the previously fully neglected subspecific differences in larval morphology are crucial.

Concerning parental care, the most primitive stage corresponds to *A. carinatus*, whereas the advanced "mud cell" behavior stage (Brutvorsorge Ib) was attained in *A. pyrenaeus*, the *A. parallelepipedus* lineage, the *A. exaratus* group, and the subgenus *Pterostichoabax* [24]. A different stage (IIa) was developed in *A. parallelus* and *A. ovalis*, consisting of ovipositor modifications to simply lay eggs on the nest surface, with additional parental care aimed at protecting the clutch (egg watcher). These parental behaviors have also evolved independently within *Abax* lineages, as suggested by our molecular data, at least with respect to behavior at stage IIa. The phylogenetic signal of these characteristics is likely obscured by functional (convergent) adaptations to environmental factors [2].

4.4 | Evolutionary History of *Abax*

According to our results, the ancestors of *Abax* possibly existed in the Middle Tortonian period (~10.3 MYA, Figure 4), an estimation that differs from that indicated by previous works [9], who estimated that they were already present in the Burdigalian period 16.6 MYA. After the middle Tortonian, the primitive *Abax* stock split into a main lineage (subgenera *Abax* and *Pterostichoabax*), and the *A. schueppeli* lineage (Figures 2–4). Immediately after, this second lineage split into two large sublineages, one corresponding to the ancestors of *A. parallelus*, *A. carinatus*, and *A. pyrenaeus*, and the second corresponding to the remaining *Abax* taxa.

At the end of the Tortonian, a lineage separated from the ancestors of *A. parallelus*, and gave origin to the lineages leading to *A. carinatus* and *A. pyrenaeus* during the Middle Messinian. The separation of these last two species (one in Italy and the Balkans, and the other in northern Iberian Peninsula) might have been influenced by the climatic and ecological changes driven by the drastic desiccation of the Mediterranean Sea during this period.

The other main clade split into three main stocks. The first corresponds to the ancestors of the subgenus *Pterostichoabax*, the second corresponds to the ancestors of the POBE clade + *A. fiorii* + *A. contractus*, and the last corresponds to the ancestors of *A. ovalis* + *A. parallelepipedus*. This last clade separated at the beginning of Zanclean, 5.1 MYA, and this separation may also have been driven by the Messinian crisis or its aftereffects, although both species now overlap, having expanded their distribution ranges since then.

Intraspecific diversification within most lineages has likely been recent, during the last half of the Pleistocene, according to the short length of terminal branches (Figure 4). Only some species of *Pterostichoabax* show deeper lineages that reach the Pliocene.

4.5 | Summary of the Evolution of *Abax*

The present knowledge suggests the following hypotheses about the evolutionary history of *Abax*:

1. The most different species on morphological grounds is *A. schueppeli*, which shows autapomorphy based on elytron characters, together with a large body size, a prominent 7th keeled stria and strong punctures on the striae. It seems to represent the closest extant taxon to the ancestors in morphology. *Abax carinatus* shows a carinate 7th stria (not as strongly developed as that in *A. schueppeli*) and striae punctured with variable intensity. However, on molecular grounds, it shared a common ancestor with *A. pyrenaeus* and is somewhat more distant from *A. parallelus* and *A. schueppeli*. The parental care of *A. parallelus* (which shows a reduced ovipositor) is highly differentiated, being of the egg-watcher type. In contrast, *A. carinatus* is the only species that oviposits directly in the soil (Brandmayr, unpublished), a behavior that likely evolved in *A. pyrenaeus* to lay eggs in mud cells (Brandmayr, unpublished). The oviposition behavior of *A. schueppeli* is still unknown, but the female terminalia are well developed and without signs of reduction. To date, there are no autapomorphies joining all these taxa. Therefore, the systematics of these species still require further research. The hypothesis of a strict affinity between *Abax parallelus* and *A. ovalis* based on larval morphology [24] was correctly questioned [4] as it is not supported by our molecular data.
2. The lineage represented by the subgenus *Pterostichoabax* lacks any morphological synapomorphy. The absence of spiny 5th tarsomeres or a peculiar shape of the 7th stria is found in other lineages; in the four species of the subgenus,

the urogomphi of the aged larvae are glabrous (Figure 5), a state also found in other *Abax* taxa. However, in addition to molecular data, other characteristics agree with the postulated monophyly of the subgenus, namely parental care, the geographical distribution, and habitat preferences.

3. The POBE clade (*Abax pilleri*, *A. oblongus*, *A. baenningeri*, and *A. exaratus*) is morphologically and geographically uniform and may represent a lineage originating on the southern slopes of the Central Alps that has remained in this area. If it is assumed that spines of the 5th tarsomeres were lost in *A. contractus*, this latter species could be added to this group, a hypothesis also supported by a combination of morphological characteristics, although none of them are autapomorphies. *Abax fiorii* is close to the POBE clade on a molecular basis, and it shares a similar morphology. This species lives in sympatry with *A. baenningeri*, which, in turn, has contact zones along the Adige and the Ticino River valleys with the allopatric taxa *A. pilleri* and *A. exaratus*. These species show moderate variation in the urogomphi of larvae, which is somewhat distinct in *A. exaratus* (Figure 5).

4. The sister relationship between *A. parallelepipedus* and *A. ovalis* is difficult to explain when external morphology, aedeagus details, larval structure (the 3rd instar stage), and ecological preferences are considered. It is postulated that a common ancestor to both species existed before the end of the Miocene, and each lineage evolved independently, developing different adaptations to their respective environments.

In conclusion, the genus *Abax* is a lineage of the tribe Pterostichini that possibly originated after the middle Miocene, when other lineages of the subtribe Molopina had already formed. The ancestors of *Abax* were likely inhabitants of temperate forests of Europe and radiated into several lineages that colonized both areas above the timberline line to occupy alpine grasslands and alluvial plains of Europe, denoting a notable ecological potential to adapt to the dynamic events affecting Europe during the Miocene. However, the distribution of *Abax* is strongly limited to the Atlantic bioclimatic zone of the Iberian Peninsula, indicating the inability of the genus to colonize Mediterranean areas, where species of the related genus *Percus* occupy their niche. If climatic change favors more dry and hot conditions in the next few decades, it is expected that some species of *Abax* will be displaced to northern latitudes or may even become extinct.

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Ethics Statement

There is nothing to declare regarding beetle samples.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study will be openly available in DIGITUM (Universidad de Murcia) at http://hdl.handle.net/10201/150447, after the publication. The DNA sequences are available in GenBank, with accession numbers PV068201, PV125164–PV125258, and PV056229–PV056326.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. (*Supporting Information*)

Table S1. Codes of the sequences of the genus *Abax* retrieved from the Bold database for the phylogenetic analyses. Table S2. Codes of the sequences of the genus *Abax* retrieved from the GenBank database for the phylogenetic analyses. Supporting Figure 1: Bayesian Inference phylogeny of the genus *Abax* based on the COI matrix obtained in MrBayes. The numbers on the nodes indicate posterior probability values. Supporting Figure 2: Maximum Likelihood phylogeny of the genus *Abax* based on the 28S matrix obtained in IQTREE. The numbers on the nodes indicate bootstrap support values.