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Mitochondrial phylogenomics reveals the sister relationship between the endogean Mediterranean raymondionymine weevils and the remaining 51,000+ Curculionidae (Coleoptera)

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Abstract

The tribe Raymondionymini has long been neglected in phylogenetic studies. The tribe is characterized by uncertain monophyly, fluctuating taxonomic status, and a composition prone to instability. All raymondionymine weevils are wingless and have eyes either completely absent or, rarely, consisting of a single ommatidium. With body lengths predominantly below three millimeters, they inhabit deep soil environments and are infrequently collected. The core of this tribe comprises nine genera distributed in Europe and around the Mediterranean region and encompassing 76 species, while six additional genera include 17 species distributed in USA (California), Mexico, Ecuador, Venezuela, Russian Far East, and Madagascar. Here, we present eight new mitogenomes, complemented by one publicly available, encompassing all but two Mediterranean genera of raymondionymine weevils. We used publicly available Curculionoidea mitogenomes to compile an all-inclusive dataset with 391 terminals and a reduced dataset with 61 terminals representing main families of Curculionoidea and subfamilies within Curculionidae. Our maximum likelihood and Bayesian phylogenetic analyses, employing both DNA and amino acids datasets under alternative partition schemes, consistently produced congruent phylogenies. Our results show that the Mediterranean raymondionymines form a strongly supported clade, and their easternmost and morphologically distinct genus *Ubychia* is sister to the rest of them. Most notably, our results consistently recover a sister relationship between the clade of Mediterranean raymondionymine weevils and a clade encompassing all remaining Curculionidae. Consequently, we propose a revision of weevil taxonomy: (i) Our target group is removed from the non-monophyletic subfamily Brachycerinae; (ii) this clade is resurrected to its former subfamily level within Curculionidae, as the subfamily Raymondionyminae **stat. rev**; (iii) the nine Mediterranean genera *Alaocephala*, *Alaocyba*, *Coiffaitiella*, *Derosasius*, *Ferreria*, *Raymondiellus*, *Raymondionymus*, *Tarattostichus*, and *Ubychia* compose Raymondionyminae **stat. rev**; (iv) and non-Mediterranean genera *Alaocybites*, *Bordoniola*, *Gilbertiola*, *Homosomus*, *Neoubychia*, and *Schizomicrus* are considered as "incertae sedis" pending further phylogenetic corroboration. We hypothesize that the remaining Brachycerinae and the non-Mediterranean representatives within Raymondionyminae constitute a series of species-poor early-diverging lineages representing currently unrecognized subfamilies of Curculionidae.

Key words

Shot-gun sequencing, mitochondrial metagenomics, Brachycerinae, Raymondionymini, Raymondionyminae, endogean, deep soil

1. Introduction

The limits of the superfamily Curculionoidea have not been disputed given the easily observable possession of the adult rostrum that defines the clade. Similarly, the monophyly of the so called "true" weevils, classified as the family Curculionidae, is well supported based on both molecular and morphological data (Oberprieler et al. 2007; Shin et al. 2018; Haran et al. 2023; Li et al. 2023). The internal classification within the hyperdiverse Curculionidae, consisting of at least 51,000 extant species (Oberprieler et al. 2007), is more complex despite the outstanding phylogenetic efforts based on both morphological (Davis 2017; Kuschel 1995; Marvaldi et al. 2002; Thompson 1992) and molecular characters (Gillett et al. 2014; Gunter et al. 2016; Haran et al. 2013; McKenna et al. 2009; Mugu et al. 2018; Shin et al. 2018). The current subfamily classification and the phylogenetic consensus are best summarized by Shin et al. (2018). These authors confidently resolved Curculionidae as the sister clade to Brentidae. Within Curculionidae they found two well-supported clades accounting for over 95% of true weevil species diversity: (clade 1) Dryophthorinae plus Platypodinae and (clade 2) Bagoinae sister to "higher weevils". The "higher weevils" are distributed in two species-rich clades: the CEGH clade (Cyclominae, Entiminae, Gonipterini, Hyperinae) and the CCCMS clade (Conoderinae, Cossoninae, Curculioninae, Molytinae, Scolytinae). The non-monophyletic rest of the family, represented in Shin et al. (2018) by a grade including seven genera in three lineages, was grouped into the subfamily Brachycerinae, including raymondionymine weevils as a tribe in agreement to Oberprieler (2014) and opposed to their status as a family proposed by Alonso-Zarazaga and Lyal (1999).

Given the phylogenetic uncertainties in the early evolution of Curculionidae, subfamily Brachycerinae has been defined as "the evolutionary twilight zone of true weevils" (Grebennikov and Anderson 2021). As currently delimited (Oberprieler 2014), Brachycerinae includes about 1,350 species (about 2.7% of the documented diversity of true weevils) classified in seven tribes: Brachycerini, Cryptolaryngini, Erirhinini (including the genus *Ocladius* Schönherr), Himasthlophallini, Tanysphyrini, Myrtonymini, and our target group Raymondionymini. Most of Brachycerinae are fully eyed and often volant, however, Raymondionymini as well as Myrtonymini and some species of two other tribes (Erirhinini and Himasthlophallini) contain eyeless or nearly eyeless and wingless soil-inhabiting species. Raymondionymini groups a series of tiny species (smaller than three millimetres in body length), almost exclusively eyeless (some *Alaocybites* Gilbert have a single ommatidium) and wingless, inhabiting the deep soil with a spotty local distribution. Difficulties associated with the study of deep soil fauna, that typically can only be collected through soil-washing, likely explain why Raymondionymini was represented within the aforementioned analyses by a single Californian terminal, either *Schizomicrus* Casey (McKenna et al. 2009; Shin et al. 2018; Li et al. 2023) or *Gilbertiola* Osella (Davis 2017).

These sampling difficulties in obtaining deep soil beetles, which are often known only by the typical series or from only the type localities widely scattered across the Globe (Morrone and Hlaváč 2017), has also limited the extend of our current study. Our target is the lineage putatively formed by the European/Mediterranean representatives of the tribe Raymondionymini. Of the 93 species and 15 genera currently included within the tribe Raymondionymini, 76 species and 9 genera have an endemic distribution in Europe and around the Mediterranean region (Morrone and Hlaváč 2017) (Fig. 1): *Alaocephala* Ganglbauer (1 sp.), *Alaocyba* Perris (10 spp.), *Coiffaitiella* Osella (6 spp.), *Derosasius* Ganglbauer (1 sp.), *Ferreria* Alonso-Zarazaga and Lyal (2 spp.), *Raymondiellus* Ganglbauer (15 spp.), *Raymondionymus* Wollaston (28 spp.), *Tarattostichus* Ganglbauer (2 spp.), and *Ubychia*

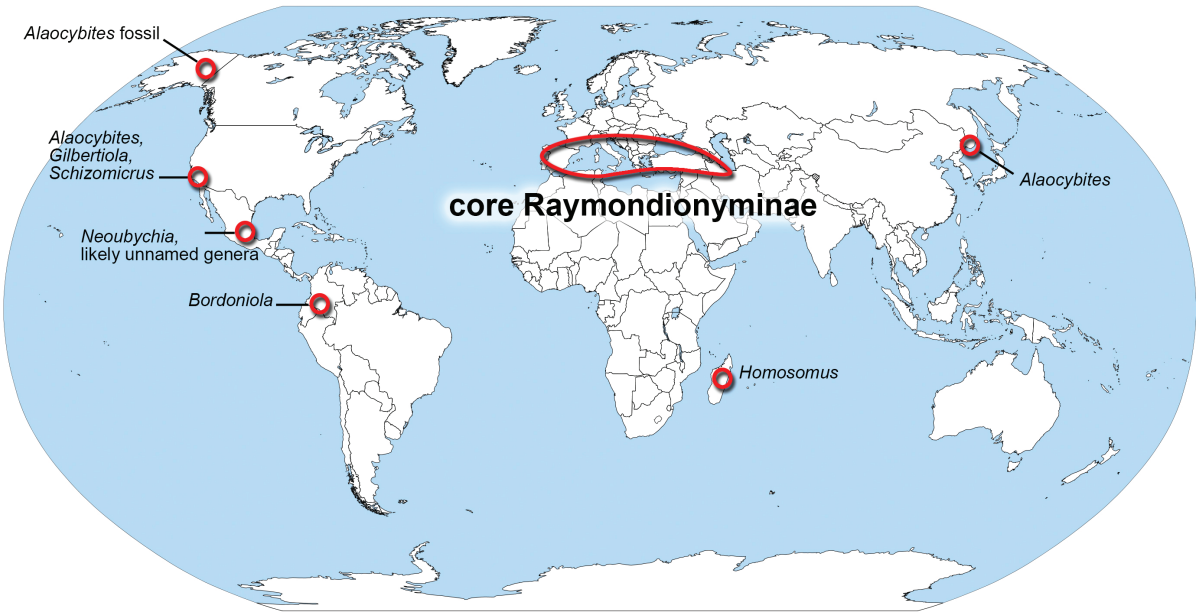

Figure 1. Composition and geographic distribution of Raymondionymini redefined as Raymondinyminae stat. rev.

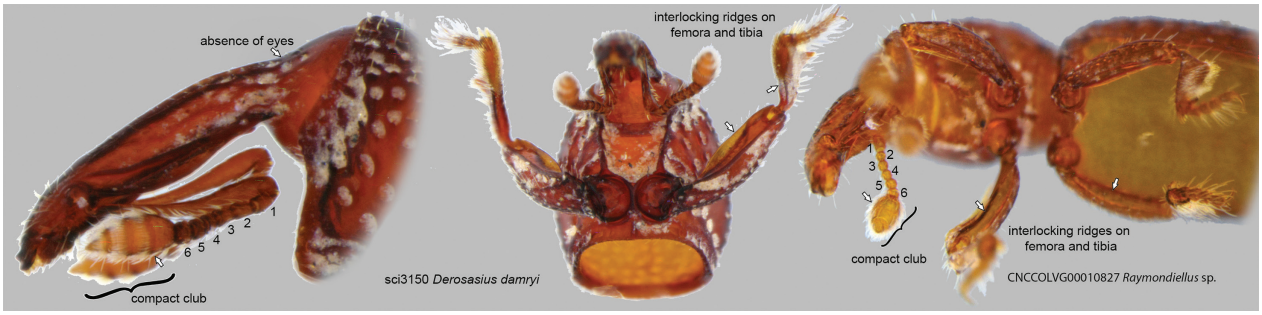

Figure 2. Morphological synapomorphies of Raymondionyminae.

Rost (11 spp.). *Ubychia* is the only genus which is going far out of the Mediterranean region reaching the Caucasus and Iran. All these beetles examined in sufficient detail share at least four potential morphological synapomorphies (Fig. 2): (i) the antennal funicle with five or six antennomeres, (ii) all tibiae and femora with interlocking ridges and groves on the ventral surface, (iii) all legs with tarsi with four, subequal tarsomeres (fig 1G in Grebennikov 2010) and (iv) orthocerous type of male genitalia. Although never a subject of a focused phylogenetic analysis, the Mediterranean raymondionymine weevils were thought to be monophyletic (Grebennikov 2010; Grebennikov and Anderson 2021).

Three phylogenetic studies tangentially addressed the monophyly and/or sister group relationship of raymondionymine weevils. All of them, however, were limited in their design and, therefore, remained inconclusive in their findings. Grebennikov (2010) in a morphology-based phylogenetic analysis suggested that the genus *Alaocybites*, distributed in California and Russian Far East, is an unlikely member of the tribe. Grebennikov and Anderson (2021), in a three-marker DNA analysis, detected Mediterranean *Alaocyba* and *Raymondiellus* as a strongly supported clade lacking, however, a well-supported sister group. The only included American representative of the tribe, tentatively assigned to *Bordoniola* Osella although likely representing an unnamed genus, was only distantly related to the European clade. Finally, Andújar et al. (2019) generated the mitogenome of *Coiffaitiella* Osella, the only presently known mitogenome for the group, and recovered it as a sister to the rest of the true weevils in a dataset of 39 soil-dwelling beetles, however with a highly incomplete representation of Curculionidae lacking among others any additional Brachycerinae.

Our study was triggered by the availability of difficult-to-obtain DNA-grade specimens of European raymondionymine weevils, our technical expertise in assembling mitochondrial genomes and phylogenetics, and the availability of a mitogenome dataset for weevils that was demonstrated highly informative (Andújar et al. 2019; Gillett et al. 2014; Haran et al. 2013). Although lacking any non-European representative, our dataset has enabled us to address for the first time two relevant phylogenetic questions: (i) is the Mediterranean core of the brachycerine tribe Raymondionymini monophyletic and, if "yes", (ii) are they forming a clade robustly placed as sister to all remaining true weevils (Curculionidae) as tentatively found by Andújar et al. (2019)? Despite the remaining uncertainties about the phylogenetic placement of other non-Mediterranean Raymondionymini, corroboration of these two hypotheses and the phylogenetic trees we provide represent a step towards the understanding of the early evolution and diversification within the hyperdiverse Curculionidae.

2. Material and methods

A total of 16 specimens representing six of the nine described genera of European Raymondionymini are here firstly DNA extracted and barcoded (Table 1). Taxonomic identification and barcode sequences were used to confirm the species status, and for one representative of each of the eight sampled species the mitogenome has been sequenced, de novo assembled, and annotated (Table 1; Fig 3). The Raymondionymini mitogenome dataset is completed with a representative from an additional genus previously sequenced by the authors (Andújar et al. 2019). We additionally generated the mitogenome of *Notaris scirpi* (Fabricius) (Brachycerini) (Table 1, Fig. 3).

2.1. DNA extraction and barcoding

DNA extraction was conducted from whole specimens (excepting the large-bodied *N. scirpi* for which a leg was used) using non-destructive procedures and Omega Mag-Bind® Blood and Tissue DNA Kit (Omega Bio-tek) in the KingFisher robotic system (Thermo Fisher Scientific inc.). PCR amplification was done for the 5' end COI gene (standard barcode region for Metazoa; Hebert et al. 2003) using degenerate Folmer barcode primers (FoldF: 'TCNACNAAYCAYAARRAYATYGG; FoldR: 'TANACYTCNGGRTGNCCRAARAAYCA') (Folmer et al. 1994; Yu et al. 2012). PCR conditions were: 10 min at 95°C in 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 48°C, and 3 min at 72°C; 10 min at 72°C, and holding at 10°C. PCR products were cleaned using exonuclease and rapid alkaline phosphatase, and were Sanger-sequenced with ABI technology in Macrogen, Spain. This procedure was applied to 16 Raymondionymini specimens and *Notaris scirpi* (Table1). We used Geneious Prime 2023 to vi-

Table 1. Specimens of Curculionidae: Brachycerinae (including those of the tribe Raymondionymini herein re-classified as the subfamily Raymondionyminae) used in our DNA analyses. An asterisk (*) indicates sequences retrieved from GenBank. Two and three asterisks (** and ***) indicate two mitogenomes, each obtained twice from independent libraries, corresponding to specimens CNCCOLVG000010827 and CNCCOLVG000010826 in Grebennikov and Anderson (2021), respectively. Coordinates are indicated in Decimal Degrees.

Taxa	Voucher code	Barcode GB accession	Mitogenome GB accession	Country	Latitude	Longitude	Tribe
Ubychia sp1.	sci3153	PP949483	PP889716	Croatia	45.356	14.766	Raymondionymini
Ubychia sp2.	sci3141	PP949471	PP889720	Georgia	41.6514	41.764	Raymondionymini
Ubychia sp2.	sci3142	PP949472		Georgia	41.6514	41.764	Raymondionymini
Raymondiellus sp.	sci3592**	PP949484	PP889721	Italy	39.26	8.65	Raymondionymini
Raymondiellus sp.	sci3148	PP949478		Italy	39.234	8.599	Raymondionymini
Raymondiellus sp.	sci3149	PP949479		Italy	39.234	8.599	Raymondionymini
Derosasius damryi	sci3150	PP949480	PP889719	Italy	40.534	9.584	Raymondionymini
Coifaitiella sp.	BMNH 1041911	n.a.	MK692586*	Spain	36.772637	–5.423984	Raymondionymini
Ferreria marqueti	sci3621	PP949486	PP889715	Spain: Canary Islands	28.497137	–16.345822	Raymondionymini
Ferreria marqueti	sci3143	PP949473		England	52.034	–2.423	Raymondionymini
Ferreria marqueti	sci3144	PP949474		England	52.034	–2.423	Raymondionymini
Ferreria marqueti	sci3145	PP949475		England	52.034	–2.423	Raymondionymini
Alaocyba sp.	sci3595***	PP949485	PP889722	Italy	39.26	8.65	Raymondionymini
Alaocyba sp.	sci3146	PP949476		Italy	39.234	8.599	Raymondionymini
Alaocyba sp.	sci3147	PP949477		Italy	39.234	8.599	Raymondionymini
Raymondionymus laneyriei	sci3151	PP949481	PP889718	France	43.191	6.371	Raymondionymini
Raymondionymus lavagnei	sci3152	PP949482	PP889717	France	43.954	3.647	Raymondionymini
Notaris scirpi	CNCCOLVG00008489	n.a.	PP889723	Poland	51.54	17.86	Brachycerini
Brachicerus muricatus	BMNH 696973	n.a.	JN163970*	France	n.a.	n.a.	Brachycerini
Lissorhopthus oryzophilus	n.a.	n.a.	MW732716*	China: Ningxia	n.a.	n.a.	Erirhinini
Echinocnemus sp.	CG210	n.a.	MH404139*	Australia	n.a.	n.a.	Erirhinini
Ocladius sp.	CG288	n.a.	MH404142*	RSA	n.a.	n.a.	Erirhinini

sualize and edit chromatograms and to generate an HKY distance matrix and UPGMA tree to explore similarity between specimens. The agreement between the obtained barcode sequences and the morphological identification of specimens corroborated the presence of 8 species of Raymondionymini belonging to 6 genera.

2.2. Mitogenome sequencing and assembly

One representative per species was selected for mitogenome sequencing and assembly following the mitochondrial metagenomics approach (Andújar et al. 2015; Crampton-Platt et al. 2015; Dettai et al. 2012), where complete mitochondrial genomes are assembled from shotgun sequencing of specimen DNA mixtures. The dsDNA concentration of raw DNA extracted from each specimen was measured using a Qubit 2.0 Fluorometer (Life Technologies Corp., Carlsbad, CA), and five TruSeq nano DNA libraries were constructed respectively from five equimolar DNA pools, each of these including one or several target specimens of this study plus a number of other Coleoptera from distant lineages (Table 1). Sequencing was performed with the Illumina MiSeq platform (Illumina Inc., San Diego, CA) (2 × 300 bp; 700–900 bp insert size), aiming a coverage of 1% of the MiSeq run per specimen. Illumina output was processed with Trimmomatic 0.30 (Bolger et al. 2014) for Illumina adapter removal. Reads were subsequently filtered using Blast 2.2.27 (Altschul et al. 1990) against a reference database including 2344 mitochondrial genomes longer than 5,000 bp retrieved from NCBI nucleotide database (accessed 5th November 2020). Retrieved mitochondrial reads were then assembled using RAY 2.3.1 (Boisvert et al. 2012) (–K 61; –minimum-seed-length 100 –minimum-contig-length 1000), SPADES 3.14 (Prjibelski et al. 2020) (–k 21,33,55,77,99,127), and IDBA 1.1.3 (Peng et al. 2012) (–maxk 300 to –mink 50). The resulting contigs from the three assemblers were re-assembled in Geneious using the de novo assembly function and showed wide overlap, minimizing potential problems associated with the formation of chimeric mitogenome sequences. Obtained mitogenomes were annotated using gene predictions with MITOS (Bernt et al. 2013) with additional manual editing performed in Geneious. All mitogenomes were structured following the putatively ancestral gene order for the Coleoptera. Finally, mitogenomes were unambiguously assigned a taxonomic identity by comparison against the COI barcode sequences obtained from the same specimens with PCR-Sanger sequencing (see above). For two of these specimens (Table 1), mitoge-

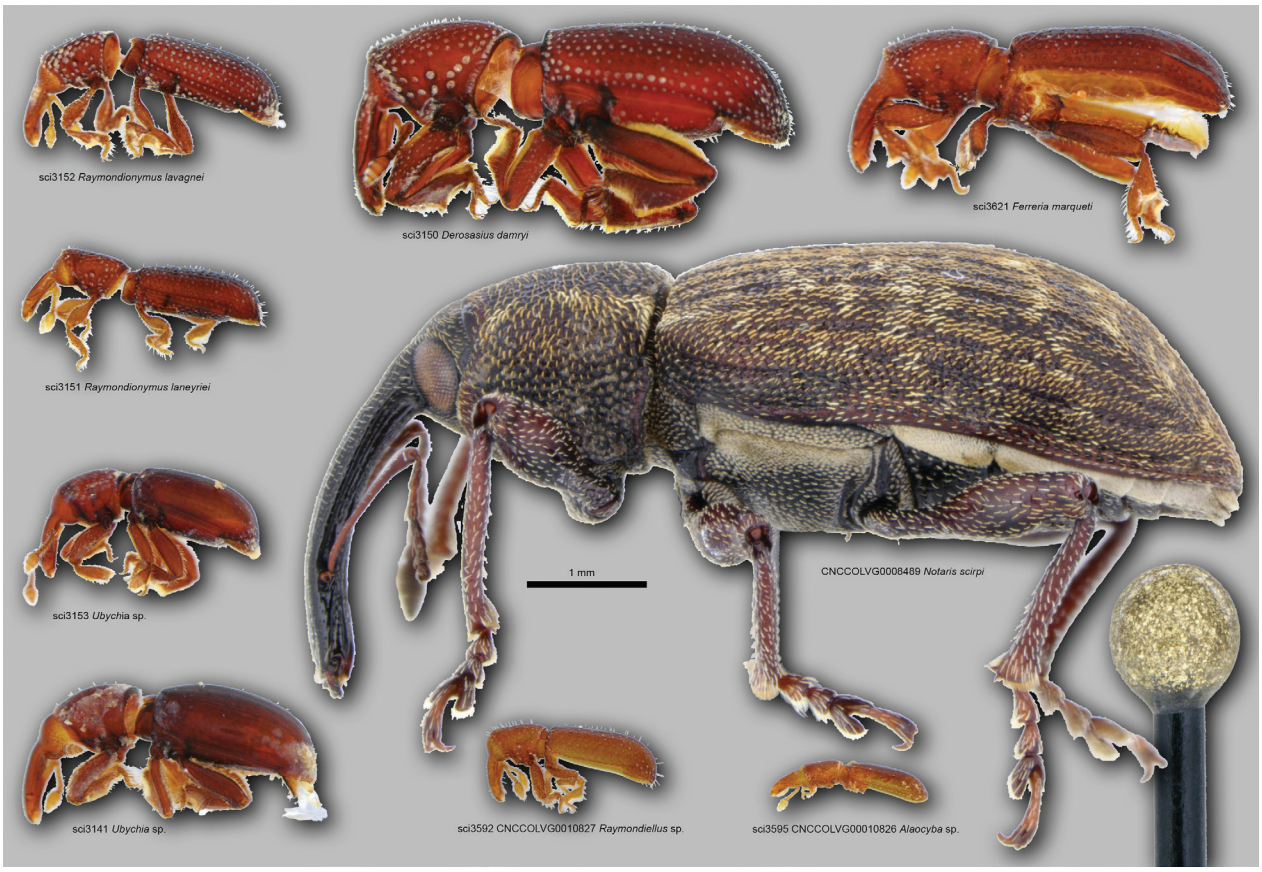

Figure 3. Habitus of specimens used to sequence nine new mitogenomes. *Notaris* represents Brachycerinae; the remaining eight specimens represent Raymondionyminae. Head of entomological #3 pin is added for scale to emphasize small body size of Raymondionyminae.

nome sequencing was performed twice in two independent libraries, allowing to corroborate reliability of obtained sequences.

2.3. Generating a mitogenomic guide weevil tree

A first and preliminary analysis was designed to construct a guide tree including our nine newly generated mitogenomes plus available Curculionoidea mitogenomes within the NCBI nucleotide database. This guide tree was to serve two purposes. Firstly, we wanted to preliminarily replicate the basal weevil branching events reported in earlier studies and summarized in Shin et al. (2018). Secondly, the obtained tree will guide the selection of the near and distant outgroups for a subsequent and statistically more exhaustive analysis using a lesser number of terminals. For these purposes, all 423 mitogenomes longer than 5000 bp classified as Curculionoidea available from GenBank on 14th July 2021 were downloaded. Of these, 59 mitogenomes classified as unspecified Curculionoidea were excluded. From the remaining 382 mitogenomes, 94 lacked gene annotations and where de novo annotated in MITOS as indicated above. The final dataset included 391 gene annotated mitogenomes. Single gene datasets for each of the 13 protein coding genes (PCGs) and the two ribosomal genes were extracted using Geneious and individually trimmed and aligned using the FFT-G-INS-i algorithm of MAFFT (Katoh et al. 2002). Individual gene alignments were concatenated, yielding (i) a dataset of 15 genes and 13,491 bp (Preliminary Dataset 1; PD1); (ii) a dataset with exclusively the 13 PCGs and a length of 10,842 bp (PD2); and (iii) a dataset with amino acids sequences obtained from the 13 PCGs (invertebrate mitochondrial code) with a length of 3,856 AAs (PD3). These three datasets were used for Bayesian inference with PhyloBayes (Lartillot and Philippe 2004) running 2 chains under a GTR-CAT model for a minimum of 5,000, 6,000 and 3,500 generations respectively for PD1, PD2, and PD3. A consensus tree was obtained for each dataset combining trees from both chains after discarding the first 2000 generations as a burn-in fraction. In this and all subsequent analyses we used FigTree (Rambaut 2012) to visualize the obtained topologies.

2.4. Thorough phylogenetic analyses with a reduced dataset

We designed our restricted phylogenetic analysis based on congruence between trees obtained for the preliminary dataset (391 terminals) and the well-resolved weevil topology of Shin et al. (2018). The Raymondionymini ingroup finally included eight newly generated mitogenomes plus that of *Coiffaitiella* (MK692586, Andújar et

al. 2019) (Table 1). The nearest outgroup was formed by four mitogenomes of Brachycerinae available from GenBank, plus the newly generated mitogenome of *Notaris scirpi*. The distant true weevil outgroup consisted of the representatives of the following clades/subfamilies (Shin et al. 2018): Dryophthorinae (5 mitogenomes), Platypodinae (3), Bagoinae (1) and "higher" weevils consisting of the CEGH clade (9) and CCCMS clade (19). To adequately place Curculionidae within the Curculionoidea phylogenetic framework, we added representatives of Brentidae (3 mitogenomes; the supposed sister group of Curculionidae), Attelabidae (3), Anthribidae (3), and Nemonychidae (1). We rooted all topologies on the clade formed by Anthribidae plus Nemonychidae consistently with the current hypotheses on the first split within weevils (Oberprieler et al. 2007). In total, 61 mitogenomes were analysed, nine of them newly generated. The 13 protein coding genes (PCGs) and the two ribosomal were individually extracted, aligned, and trimmed as before in Geneious, with the exception of ribosomal genes that were aligned using the online version of MAFFT with the Q-INS-i algorithm (Katoh and Toh 2008).

Individual gene alignments from the reduced dataset were concatenated, yielding (i) a dataset of 15 genes and 12,603 bp (Reduced Dataset 1; RD1); (ii) a dataset with exclusively the 13 PCGs and a length of 10,191 bp (RD2); and (iii) a dataset with amino acids sequences obtained from the 13 PCGs (invertebrate mitochondrial code) with a length of 3,396 AAs (RD3). These three datasets were used for maximum likelihood (ML) and Bayesian phylogenetic analyses. ML trees were obtained using RAxML v.8 (Stamatakis 2014) and IQTree (Nguyen et al. 2015), in both cases using gene partitions (DNA and AA datasets RD1, RD2 and RD3) and gene and codon partitions (DNA datasets RD1 and RD2). RAxML analyses were conducted on the CIPRES Science Gateway (Miller et al. 2010), applying an independent GTRGAMMA (DNA datasets) or PROTCATGTR model (AA dataset) to each data partition. The best scoring ML tree was selected among 1,000 searches on the original alignment with different randomized parsimony starting trees. Support values were obtained with 1,000 bootstrap replicates (Felsenstein 1985). IQTree analyses were run on the IQ-TREE web server at http://iqtree.cibiv.univie.ac.at (Trifinopoulos et al. 2016) using the best fitting substitution model for each gene partition as estimated with ModelFinder (Kalyaanamoorthy et al. 2017). Nodal support was obtained by 1,000 ultrafast bootstrap (UFBoot) replicates (Minh et al. 2013). For each dataset and partitions scheme, IQTree analyses were repeated twice. PhyloBayes (Lartillot and Philippe 2004, Lartillot et al. 2013) analyses were done on the CIPRES Science Gateway, running 2 independent chains under a GTR-CAT model. For each dataset (RD1, RD2 and RD3) analyses were duplicated, allowing to run on CIPRES for 48 and 72 hours respectively (and using between 64 and 96 cores). A consensus tree was obtained for each dataset combining trees from both chains after discarding the first 500 generations as a burn-in fraction. All together we conducted 21 phylogenetic analyses on the reduced datasets, as summarised in Table 2.

Table 2. Results of 21 phylogenetic analyses of 61 Curculionoidea mitogenomes focussing on the monophyly and phylogenetic position of Raymondionyminae. Columns two to seven define various analytical parameters (DNA or proteins, software used, number of genes, partition scheme, number of replicates, and the representative replicate shown in Table S2). Column taxonomic abbreviations: BRE: Brentidae; CUR: Curculionidae; Dry: Dryophthorinae; Pla: Platypodinae; Ray: Raymondionyminae; Bag: *Bagous*, Bra: *Brachycerus*; Ech: *Echinocnemus*; Lis: *Lissorhoptrus*; CEGH: the CEGH clade (Cyclominae, Entiminae, Gonipterini, Hyperinae); CCCSM: the CCCMS clade (Conoderinae, Cossoninae, Curculioninae, Molytinae, Scolytinae). Cell values and colour represent statistical support for respective branches: >94 (dark grey), 90-94 (gray), 70-89 (light gray), <70 (white).

DNA/ proteins	Software	Genes	Partitions	Max-diff * (Phylobayes)	Mean-diff * (Phylobayes)	Total reps	Rep n	BRE	CUR	BRE + CUR	Ray	Cur–Ray	Cur- (Ray, *Lis*, *Ech*)	Dry	Pla	Dry + Pla	(Pla + *Bra*) + Dry	CEGH	CCCMS	CEGH + CCCMS	(CEGH + CCCMS) + *Bag*
proteins	IqTree	13	Bygene			2	1	100	100	100	100	100	94	93	100	none	91	100	100	72	100
proteins	IqTree	13	Bygene			2	2	100	100	100	100	100	94	90	100	none	90	100	100	71	100
proteins	Phylo-bayes	13	N.a.	0.228	0.00929	2	1	100	100	97	100	96	72	59	100	none	72	100	100	50	95
proteins	Phylo-bayes	13	N.a.	0.212	0.01116	2	2	100	100	100	100	93	72	63	100	94	none	100	100	74	100
proteins	RAxML	13	Bygene			1 (1000 searches)	1	100	100	100	100	90	68	60	100	93	none	100	100	74	99
DNA	IqTree	13	Bygene			2	1	100	100	100	100	99	96	93	100	none	95	100	100	92	97
DNA	IqTree	13	Bygene			2	2	100	100	100	100	99	97	96	100	none	97	100	100	93	98

DNA/ proteins	Software	Genes	Partitions	Max-diff * (Phylobayes)	Mean-diff * (Phylobayes)	Total reps	Rep n	BRE	CUR	BRE + CUR	Ray	Cur–Ray	Cur-(Ray, *Lis*, *Ech*)	Dry	Pla	Dry + Pla	(Pla + *Bra*) + Dry	CEGH	CCCMS	CEGH + CCCMS	(CEGH + CCCMS) + *Bag*
DNA	IqTree	13	Bygeneand bycodon			2	1	**100**	**100**	**100**	**100**	**100**	**95**	79	**100**	none	89	**100**	**100**	**90**	**97**
DNA	IqTree	13	Bygeneand bycodon			2	2	**100**	**100**	**100**	**100**	**100**	**96**	84	**100**	none	89	**100**	**100**	88	**97**
DNA	RAxML	13	Bygene			1 (1000 searches)	1	**100**	**100**	**100**	**100**	85	73	67	**100**	none	60	**100**	**100**	72	67
DNA	RAxML	13	Bygene			1 (1000 searches)	1	**100**	**100**	**100**	**100**	**94**	none	52	**100**	none	none	**100**	**100**	69	54
DNA	Phylobayes	13	N.a.	0.290	0.01496	2	1	**100**	**100**	**100**	**100**	**91**	69	58	**100**	**93**	none	**100**	**100**	72	**99**
DNA	Phylobayes	13	N.a.	0.312	0.01718	2	2	**100**	**100**	**100**	**100**	**90**	70	57	**100**	**94**	none	**100**	**100**	72	**99**
DNA	IqTree	15	Bygene			5	1	**100**	**100**	**100**	**100**	**100**	**95**	**99**	**100**	none	83	**100**	**100**	88	**97**
DNA	IqTree	15	Bygene			2	2	**100**	**100**	**100**	**100**	**100**	**96**	**100**	**100**	none	82	**100**	**100**	89	**95**
DNA	IqTree	15	Bygeneand bycodon			2	1	**100**	**100**	**100**	**100**	**100**	**96**	**99**	**100**	none	65	**100**	**100**	80	98
DNA	IqTree	15	Bygeneand bycodon			2	2	**100**	**100**	**100**	**100**	**100**	**96**	**99**	**100**	none	64	**100**	**100**	85	**98**
DNA	RAxML	15	Bygene			1 (1000 searches)	1	**100**	**100**	**100**	**100**	88	61	88	**100**	none	39	**100**	**100**	70	56
DNA	RAxML	15	Bygene			1 (1000 searches)	1	**100**	**100**	**100**	**100**	**92**	none	86	**100**	none	none	**100**	**100**	72	44
DNA	Phylobayes	15	N.a.	0.368	0.01648	2	1	**100**	**100**	**100**	**100**	**96**	none	none	**100**	**99**	none	**100**	**100**	**99**	**99**
DNA	Phylobayes	15	N.a.	0.0998	0.00484	2	2	**100**	**100**	**100**	**100**	**95**	none	none	**100**	**99**	none	**100**	**100**	**99**	**99**

* Largest (maxdiff) and mean (meandiff) discrepancy observed across all bipartitions after burn-in in Phylobayes analyses: maxdiff < 0.1: good run; maxdiff < 0.3: acceptable; 0.3 < maxdiff < 1: the sample is not yet sufficiently large)

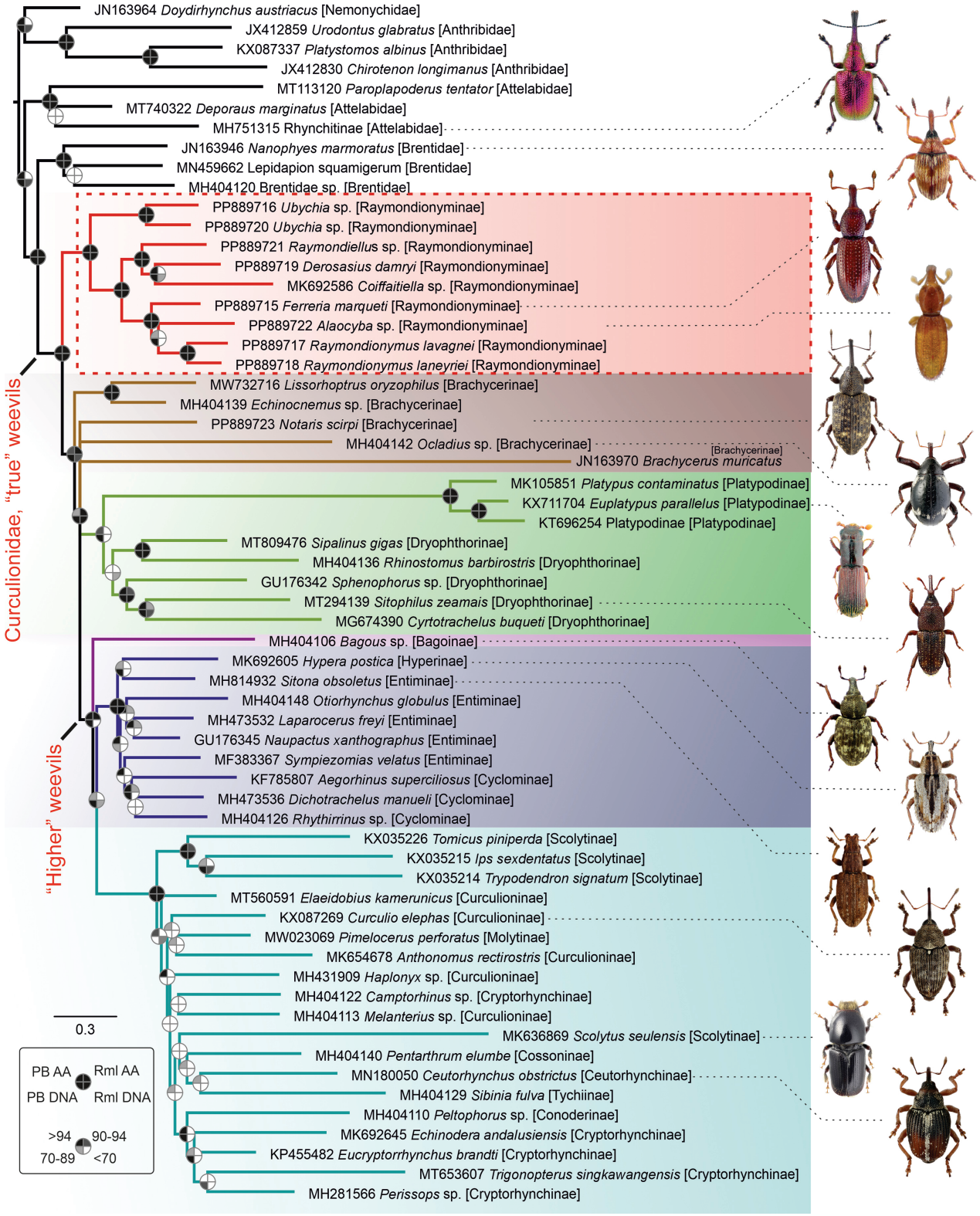

Figure 4. Maximum likelihood phylogenetic tree obtained with PhyloBayes AA dataset including nine Raymondionyminae (branches in red) and 51 other weevils. Circles indicate support values. Habitus images of congeneric (not necessarily conspecific) specimens were taken by us (*Alaocyba* sp.), by Udo Schmidt (*Ferreria marqueti* (Aubé)), by Ilya Zabaluev (*Sitophilus zeamais* (Motschulsky) and *Bagous meregallii* Caldara et O'Brien), and by Kirill Makarov (*Rhynchites bacchus* (Linnaeus), *Nanophyes marmoratus* (Goeze), *Notaris scirpi* (Fabricius), *Ocladius salicorniae* (Olivier), *Platypus* sp., *Sitona obsoletus* (Gmelin), *Curculio aino* Kono, *Scolytus ratzeburgi* Janson, and *Ceutorhynchinae sinicus* Voss); not to scale; used with permission.

3. Results

Reassembly within Geneious of contigs generated with IDBA, SPADES, and RAY showed wide overlap and a perfect match with barcode cox1 sequences generated using Sanger sequencing, allowing to unambiguously identify newly generated mitogenomes. The two pairs of specimens each representing the genera *Alaocyba* and *Raymondiellus* that were included in two independent libraries (Table 1) yielded identical mitogenomes (Fig. S1).

Mitogenomic guide weevil phylogenetic trees obtained with the preliminary DNA and AA datasets (Supplementary Material 1) were highly congruent among themselves and with the basal weevil dichotomies found by Shin et al. (2018) and Li et al. (2023). Curculionidae was found monophyletic (pp = 1) and sister to Brentidae forming a well supported clade (pp = 1). Within Curculionidae, the brachycerine tribe Raymondionymini was recovered as a strongly supported clade (pp = 1) sister to a clade grouping the rest of Curculionidae (pp = 0.98 with PD1; pp = 0.92 with PD2; pp = 0.88 with PD3) (Figs S2–S4). Internal distribution of the clade formed by non-raymondionymine true weevils was highly consistent with Shin et al. (2018). Brachycerinae (without Raymondionymini) appears as a grade including three early splitting lineages (*Notaris* Germar, *Ocladius* Schoenherr, and *Brachycerus* Olivier) and the highly supported (pp = 1) clade of *Lissorhoptrus* LeConte and *Echinocnemus* Schoenherr. The remaining taxa form two main clades. One clade groups Dryophthorinae plus Platypodinae, consistently with previous DNA (Mugu et al. 2018) and morphological (Davis 2017) analyses .The second clade groups Bagoinae sister to "higher weevils", the latter composed of two highly supported clades (pp = 1) corresponding to those named by Gunter et al. (2014) and subsequently consistently recovered (e.g., Shin et al. 2018) as the CEGH clade (Cyclominae, Entiminae, Gonipterini, Hyperinae) and the CCCMS clade (Conoderinae, Cossoninae, Curculioninae, Molytinae, Scolytinae).

The reduced dataset included 61 terminals selected to represent all main lineages described above. Completeness score for the alignment as estimated in AliStat (Wong et al. 2020) was 0.93, 0.97, 0.97 for RD1, RD2 and RD3 datasets respectively (additional summary statistics on datasets are shown in Table S1). The 21 analyses performed with different datasets (DNA vs AA), under different partitions schemes, and using maximum likelihood and Bayesian inference resulted in topologies remarkably similar among themselves (Figs 4, S5–S25), to that from the 391 mitogenomes analysis, and to that of Shin et al. (2018) and Li et al. (2023). In all 21 analyses, highly supported monophyletic true weevils (Curculionidae) were resolved as a sister clade to Brentidae (Fig. 4) with high support (Table 2). All nine mitogenomes of raymondionymine weevils from the Mediterranean region were consistently grouped into a strongly supported clade sister to the rest of true weevils (Fig. 4). The internal relationships within this lineage were well-resolved (Fig 4). The basal dichotomy was defined by two sister species of the genus *Ubychia* and the rest of raymondionymines. The non-*Ubychia* rest of the lineage was formed by two strongly supported clades: the genera *Coiffaitiella*, *Derosasius*, and *Raymondiellus* sisters to the genera *Alaocyba*, *Ferreria*, and *Raymondionymus*. Both species of the latter genus included in the analysis formed a strongly supported clade.

All 21 restricted analyses recovered the non-raymondionymine rest of the family Curculionidae as a clade. In 13 of these analyses this clade was strongly supported (Bootstrap ≥ 95; pp ≥ 0.95; Table 2), six analyses showed support between 90 and 94, with only two analyses having a support between 85 and 89. This clade always contained the following five species-rich clades at the rank of subfamily or higher (Table 2): (1.) the CCCMC clade, (2.) the CEGH clade, (3.) these two together, (4.) these two together sisters to *Bagous*, and (5.) the subfamily Platypodinae. The subfamily Dryophthorinae was mainly monophyletic, but two analyses recovered it paraphyletic with respect to Platypodinae. Dryophthorinae and Platypodinae formed a clade in six analyses; in 13 other analyses, the brachycerine genus *Brachycerus* was nested in this clade as a sister to Platypodinae. All other five representatives of Brachycerinae never formed a clade, and the only consistent well supported relationship among them was the clustering of *Lissorhoptrus* and *Echinocnemus*. This lineage was recovered as a sister clade to the rest of non-raymondionymine Curculionidae in all but four analyses (as shown in Fig 4; Table 2). In all analyses, the bark beetle genus *Scolytus* Geoffroy was not most closely related to the clade of three other scolytine representatives (Fig. 4); the latter always formed the sister to the rest of non-sclolytine members of the CCCMS clade.

Considering these results, we implement the following taxonomic acts within the family Curcculionidae: (i) the tribe Raymondionymini is removed from the non-monophyletic subfamily Brachycerinae and resurrected to its former subfamily level as Raymondionyminae **stat. rev.**; (ii) the Mediterranean genera *Alaocephala*, *Alaocyba*, *Coiffaitiella*, *Derosasius*, *Ferreria*, *Raymondiellus*, *Raymondionymus*, *Tarattostichus*, and *Ubychia* are retained within Raymondionyminae **stat. rev.**; and (iii) all non-Mediterranean genera previously placed within Raymondionymini (genera *Alaocybites*, *Bordoniola*, *Gilbertiola*, *Homosomus* Richard, *Neoubychia* Gilbert and Howden, and *Schizomicrus*) are considered as "incertae sedis" within the subfamily Raymondionyminae **stat. rev.** pending further phylogenetic corroboration.

4. Discussion

4.1. Consistency with the earlier weevil phylogenies

Our results are remarkably consistent with the gradually emerging phylogenetic framework of weevil (see Introduction). Our topologies display the composition and ar-

rangement of the main weevil clades (Figs 4, S2–S25), as well as their statistical support (Table 2), nearly identical to those obtained for the analyses of hundreds of protein coding nuclear genes by Shin et al. (2018) and (Li et al. 2023). These consistent results include the monophyly of Curculionidae and its sister relationship with Brentidae, Brachycerinae as a grade of early splitting lineages within Curculionidae, the sister relationships of Dryophthorinae and Platypodinae, and a well supported group including Bagoinae plus the well supported clades CEGH and CCCMS as found by Shin et al. (2018). Also consistent between both studies is the lower support for the internal relationships within CEGH and CCCMS clades and a topology suggesting the polyphyletic nature of several large subfamilies within Curculionidae, such as Molytinae and Curculioninae. The herein recovered non-monophyly of Scolytinae, even though highly unlikely given morphological and biological data (summarized in Johnson et al 2017), has also been detected by Shin et al. (2018) and Mugu et al. (2018). These two groups of scolytines correspond to the basal split within this subfamily, between Scolytini and the rest (Pistone et al. 2018) and currently we consider this result as an artefact of our analysis.

Four of the five non-raymondionymine Brachycerinae genera here studied (i.e., *Ocladius*, *Brachycerus*, *Lissorhoptrus* and *Echinocnemus*) were among the seven Brachycerinae analyzed by Shin et al. (2018) and (Li et al. 2023). *Ocladius* and *Brachycerus* are consistently found as early splitting lineages in the case of Shin et al. (2018). Each of these lineages include an additional genus here not sampled (*Ocladius* + *Schizomicrus* and *Brachycerus* + *Synthocus*), whereas in the case of Li et al. (2023) *Schizomicrus* appears as an additional early splitting lineage. The additional Brachycerinae early splitting lineage consistently found corresponds to the supported clustering of *Lissorhoptrus* and *Echinocnemus*, with disagreement regarding their position. In 17 of our 21 analyses we found *Lissorhoptrus* + *Echinocnemus* as a sister clade to the rest of non-raymondionymine Curculionidae, whereas results of Shin et al. (2018) supported the sister relationship of a clade formed by *Lissorhoptrus* + *Echinocnemus* + *Tanysphyrus* with the clade grouping Platypodinae + Dryophthorinae. Further analyses with additional Brachycerinae representatives will be required to improve our knowledge on the early evolution of the polyphyletic Brachycerinae, where special attention should be placed to avoid long branch attractions effects among multiple early splitting lineages. In this sense, it will be especially relevant incorporating additional molecular markers from the nuclear genome to our dataset, in order to discard phylogenetic wrong conclusions derived from the use of single marker dataset and the potential misleading effect of compositional heterogeneity and long branch attractions (Sheffield et al. 2009; Song et al. 2010). Despite of these potential limitations, complete mitochondrial genomes have been shown to be highly informative and robust for relatively deep phylogenetic nodes as those targeted in the current study (e.g., Cameron et al. 2007; Liu et al. 2018; Talavera and Vila 2011; Timmermans et al. 2015, and including Curculionidae Gillett et al. 2014), with long branch attraction problems minimised and high consistency with nuclear markers reported when protein sequences are used and/or the site heterogeneous mixture model (CAT; Lartillot and Philippe 2004; Lartillot et al. 2013) are applied, as performed in this study (Liu et al. 2018; Timmermans et al. 2015).

Within the lineage formed by the Mediterranean raymondionymines, we have found that the genus *Ubychia* forms the sister clade to the rest of the subfamily in all our analyses. This result is consistent with (i) *Ubychia* being the easternmost representative of the subfamily and the only genus inhabiting the Caucasus and Elburs mountains (Hlaváč and Nakládal 2018), and (ii) with its notably distinct morphology, by having elytra smooth, rather than striate and deeply punctured (Fig. 3).

4.2. The monophyly and phylogenetic position of raymondionymine weevils

Mediterranean raymondionymine weevils, here represented by seven of the nine known genera, form a strongly supported lineage sister to the remaining Curculionidae, in agreement with the phylogenetic position reported by Andújar et al. (2019) for the genus *Coiffaitiella*. This statistically significant basal dichotomy within the family Curculionidae, together with the documented polyphyletic nature of the subfamily Brachycerinae, points to the need of a reclassification from their current taxonomic rank as a tribe within the subfamily Brachycerinae. Two alternatives are allowable: to consider raymondionymines either as a subfamily of the family Curculionidae, or elevate them to the status of a full beetle family as previously suggested by Alonso-Zarazaga and Lyal (1999). We advocate for the former option based on both morphological and physiological traits. Curculionidae is definable by at least five morphological synapomorphies (Oberprieler et al. 2007), all five are observable in raymondinymines: (i) adult geniculate antennae, (ii) compact adult antennal club, (iii) 3–4 dorsal folds in the larval abdominal segments, (iv) a prothoracic position of the thoracic spiracle in larvae, and (v) the frontal sutures of the larval head blocked by a frontoepicranial bracon. Despite the limited knowledge of raymondinymine larvae, these three characters are distinguished on the larva of *Raymondionymus perrisi* (Grenier), the only member of the subfamily with documented immature stages (Remillet 1968a). Although limited, available knowledge on feeding habits also support the inclusion of Raymondionyminae within Curculionidae. As presently defined, true weevils (Curculionidae) is a monstrous clade of some 51,000 species sister to the family Brentidae containing about 4,000 species (Oberprieler et al. 2007). Unlike other weevils feeding predominantly on fungi and conifers, the Brentidae plus Curculionidae clade is cladistically defined by an evolutionary novel colonizing of angiosperms and their use as the food source (Oberprieler et al. 2007). Among 55,000+ species of this clade, only a few feed on non-angiosperms. All such cases are

likely subsequent evolutionary novelties. The only record of Raymondionyminae feeding habits (Remillet 1968b) indicates that at least *Raymondionymus perrisi* feeds on roots of angiosperm trees and shrubs, which is consistent with the herein proposed placement of this species inside Curculionidae.

The absence within our analyses of representatives from the six non-Mediterranean genera (19 species) prevents any conclusion about the global monophyly and the limits of the group. The relationship between the non-Mediterranean genera and the Mediterranean lineage has been previously questioned (Grebennikov 2010; Grebennikov and Anderson 2021), as the non-Mediterranean members were added to the group mostly based on the easily converging and often misleading similarity of small, eyeless, wingless, and depigmented deep soil dwellers. In this sense, using a morphological dataset, Grebennikov (2010) suggested that the genus *Alaocybites*, distributed in California and Russian Far East, is an unlikely member of the tribe. Similarly, Grebennikov and Anderson (2021) using a three DNA-marker dataset found no support for the relationship between an American unnamed genus standing close to *Bordoniola* and a well supported clade formed by the Mediterranean genera *Alaocyba* and *Raymondiellus*. European representatives were not included in the study by Shin et al. (2018), where the only raymondionymine weevil analysed corresponded to the genus *Schizomicrus* from California, which is not represented in our study. Still, the sister relationship between *Schizomicrus* and *Ocladius* in Shin et al. (2018), and the lack of such relationship between the European raymondionymines and *Ocladius* in our study, may be interpreted as an indirect evidence for the non-monophyly of the here resurrected subfamily Raymondionyminae.

Consequently, the phylogenetic position of all non-Mediterranean genera previously placed within Raymondionymini, including *Alaocybites* (two species endemic to California and two species endemic to the Russian Far East), *Bordoniola* (seven species in Ecuador and Venezuela), *Gilbertiola* (two species in California), *Homosomus* (three species in Madagascar), *Neoubychia* (monotypic in Mexico), and *Schizomicrus* (monotypic in California), can not be established according to current phylogenetic or morphological evidence and are left as incertae sedis within Raymondionyminae **stat. rev.**, requiring further phylogenetic corroboration. Given the results of the present study, we hypothesize that additional currently undetected species-poor early offshoots of the true weevil radiation might await taxonomic recognition as subfamilies of Curculionidae. Most likely these lineages are hidden in what we consider the evolutionary twilight zone of true weevils. The latter is formed by the remaining members of the herein circumscribed subfamily Brachycerinae and the six non-Mediterranean genera of the subfamily Raymondionyminae. Bringing these intuitively classified organisms under a phylogenetic spotlight will significantly improve our knowledge on the early evolution and allow to fine-tune the systematics of the charismatic and megadiverse clade of true weevils.

5. Conclusions

Our mitogenomic phylogenetic analyses using maximum likelihood and Bayesian inferences recovered congruent topologies, which show that the Mediterranean raymondionymines form a strongly supported clade with a sister relationship with a clade encompassing all remaining Curculionidae. Remarkably, our findings align closely with a recent phylogenetic reconstruction by Shin et al. (2018), based on 522 protein-coding nuclear genes, although this study did not include Mediterranean raymondionymine weevils. Consequently, we propose a revision of weevil taxonomy by removing our target group from the non-monophyletic subfamily Brachycerinae and re-classifying this clade as the true weevil subfamily Raymondionyminae. The inclusion of non-Mediterranean raymondionymine genera within the subfamiliy Raymondionyminae is a practical decision pending further phylogenetic corroboration. Non-Mediterranean raymondionymine genera together with the remaining Brachycerinae are hypothesized to form a series of species-poor early-diverging lineages representing currently unrecognized subfamilies of Curculionidae.

6. Declarations

Author contributions. Carmelo Andújar: Methodology, Formal analysis, Investigation, Resources, Writing – Original Draft, Writing – Review and Editing. **Peter Hlaváč**: Investigation, Resources, Writing – Review and Editing. **Vasily Grebennikov**: Conceptualization, Methodology, Investigation, Resources, Writing – Original Draft, Writing – Review and Editing, Project administration.

Conflict of interest. The authors declare that there is no conflict of interest.

Data availability statement. The molecular data newly generated for this study is available in GenBank. Accession numbers PP889715–PP889723 for mitogenomes and PP949471–PP949486 for cox1 barcode sequences.

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Supplementary Material 1

Figures S1–S25

Authors: Andújar C, Hlaváč P, Grebennikov VV (2024)
Data type: .pdf
Explanation notes: Figure S1. ML tree obtained in Geneious using FastTree for the 16 newly generated barcode sequences. — **Figure S2.** Consensus tree obtained from PhyloBayes for the Preliminary Dataset 1 (DNA; 15 genes; 391 terminals). — **Figure S3.** Consensus tree obtained from PhyloBayes for the Preliminary Dataset 1 (DNA; 13 genes; 391 terminals). — **Figure S4.** Consensus tree obtained from PhyloBayes for the Preliminary Dataset 1 (AA; 13 genes; 391 terminals). — **Figure S5.** Tree estimated with IqTree with the Reduced Dataset 3 (AA, 13 genes, 61 terminals). Replicate 1. — **Figure S6.** Tree estimated with IqTree with the Reduced Dataset 3 (AA, 13 genes, 61 terminals). Replicate 2. — **Figure S7.** Tree estimated with RAxML with the Reduced Dataset 3 (AA, 13 genes, 61 terminals). — **Figure S8.** Consensus tree obtained from PhyloBayes with the Reduced Dataset 3 (AA, 13 genes, 61 terminals). Replicate 1. — **Figure S9.** Consensus tree obtained from PhyloBayes with the Reduced Dataset 3 (AA, 13 genes, 61 terminals). Replicate 2. — **Figure S10.** Tree estimated with IqTree with the Reduced Dataset 2 (DNA, 13 genes, 61 terminals) partitioning by gene. Replicate 1. — **Figure S11.** Tree estimated with IqTree with the Reduced Dataset 2 (DNA, 13 genes, 61 terminals) partitioning by gene. Replicate 2. — **Figure S12.** Tree estimated with IqTree with the Reduced Dataset 2 (DNA, 13 genes, 61 terminals) partitioning by gene and by codon. Replicate 1. — **Figure S13.** Tree estimated with IqTree with the Reduced Dataset 2 (DNA, 13 genes, 61 terminals) partitioning by gene and by codon. Replicate 2. — **Figure S14.** Tree estimated with RAxML with the Reduced Dataset 2 (DNA, 13 genes, 61 terminals) partitioning by gene. — **Figure S15.** Tree estimated with RAxML with the Reduced Dataset 2 (DNA, 13 genes, 61 terminals) partitioning by gene and by codon. — **Figure S16.** Consensus tree obtained from PhyloBayes with the Reduced Dataset 2 (DNA, 13 genes, 61 terminals). Replicate 1. — **Figure S17.** Consensus tree obtained from PhyloBayes with the Reduced Dataset 2 (DNA, 13 genes, 61 terminals). Replicate 2. — **Figure S18.** Tree estimated with IqTree with the Reduced Dataset 1 (DNA, 15 genes, 61 terminals) partitioning by gene. Replicate 1. — **Figure S19.** Tree estimated with IqTree with the Reduced Dataset 1 (DNA, 15 genes, 61 terminals) partitioning by gene. Replicate 2. — **Figure S20.** Tree estimated with IqTree with the Reduced Dataset 1 (DNA, 15 genes, 61 terminals) partitioning by gene and by codon. Replicate 1. — **Figure S21.** Tree estimated with IqTree with the Reduced Dataset 1 (DNA, 15 genes, 61 terminals) partitioning by gene and by codon. Replicate 2. — **Figure S22.** Tree estimated with RAxML with the Reduced Dataset 1 (DNA, 15 genes, 61 terminals) partitioning by gene. Fig. S23. Tree estimated with RAxML with the Reduced Dataset 1 (DNA, 15 genes, 61 terminals) partitioning by gene and by codon. — **Figure S24.** Consensus tree obtained from PhyloBayes with the Reduced Dataset 1 (DNA, 15 genes, 61 terminals). Replicate 1. — **Figure S25.** Consensus tree obtained from PhyloBayes with the Reduced Dataset 1 (DNA, 15 genes, 61 terminals). Replicate 2.

Link: https://doi.org/10.3897/vz.74.e112684.suppl1

Supplementary Material 2

Table S1

Authors: Andújar C, Hlaváč P, Grebennikov VV (2024)
Data type: pdf
Explanation notes: Summary statistics for the alignments used estimated with AliStat (Wong et al. 2020) and MEGA (Tamura et al. 2013).

Link: https://doi.org/10.3897/vz.74.e112684.suppl2