

Article

# A Combined Molecular and Morphological Approach to Explore the Higher Phylogeny of Entimine Weevils (Coleoptera: Curculionidae), with Special Reference to South American Taxa

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**Abstract:** The Entiminae are broad-nosed weevils constituting the most diverse subfamily of Curculionidae, with over 50 tribes. We performed Bayesian and Maximum Parsimony combined phylogenetic analyses with the main objective of testing higher-level relationships and the naturalness of the major Neotropical and Southern South American (Patagonia and Andes) tribes, including some members from other regions. We compiled a data matrix of 67 terminal units with 63 Entiminae species, as well as four outgroup taxa from Cyclominae, by 3522 molecular (from nuclear 18S rDNA and 28S rDNA, and mitochondrial 16S rDNA and COI gene sequences) and 70 morphological characters. The resulting trees recover a clade Entiminae with a monophyletic Cylydrorhini and *Premnotrypes* branching off early. The tree resulting from parsimony analysis shows a clade of Leptopiini from the Australian region and another clade including taxa mainly distributed in the Palaearctic and Neotropical regions, but in the Bayesian tree the South American and Australian Leptopiini are grouped together. The mainly Palaearctic Entiminae (e.g., Brachyderini, Laparocerini, Otorhynchini, Peritelini, Polydrusini, Phyllobiini and Sciaphylini) form a subclade separated from Southern Hemisphere taxa. Among the latter, the well-supported Naupactini are the sister group of the South American Tanymecini, excluding *Platyaspistes*, herein transferred to Leptopiini (new placement). Another well-justified clade is Eustyliini–Geonemini, which also includes the enigmatic *Galapagonotus*, and the genus *Artipus*, thus corroborating its recent exclusion from Naupactini.

**Keywords:** Broad-nosed weevils; Entiminae; tribal relationships; combined evidence; ribosomal markers; structural alignment; mitochondrial COI; adults; larvae

## 1. Introduction

Entiminae constitute the most species-rich subfamily of Curculionoidea, with about 12,000 described species worldwide, classified into ca. 1370 genera and 54 tribes [1–3]. This subfamily includes mainly taxa that were grouped in the section Adelognatha of older Curculionidae classifications. With few exceptions, most entimine species have soil-dwelling larvae, many of them polyphagous. Monophyly of Entiminae is suggested by some synapomorphic characters in the adult (e.g., mandibles

with deciduous mandibular processes or their corresponding scar) [1] as well as the larva (e.g., cushion-like antennal sensorium) [4]. Molecular analyses consistently show the Entiminae as being part of a larger clade of broad-nosed weevils that also includes taxa classified in the Cyclominae and Hyperinae [5–10]. Such molecular studies, however, do not provide evidence supporting a clade Entiminae distinct from Cyclominae, except for a shared gene order rearrangement in the mitogenome [7,11]. The entimines and allied taxa have in common a relatively short, broad rostrum that is not used in oviposition site preparation; and their larvae are mostly ectophytic, feeding on roots in the soil or on aerial plant parts (some species with endophytic or semi-endophytic larvae, especially in Cyclominae) [3,12]. Among the structural features that would support the monophyly of “broad-nosed weevils” are the bilobed, largely sclerotized basal part of male sternite IX, the meso- and metatibiae apically either unarmed or mucronate (not uncinata) and the occurrence of iridescent scales with a particular nanostructure [3].

During recent decades, several specialists worldwide have done a considerable revisionary work on different tribes of Entiminae, providing useful morphological features to contribute to tribal diagnoses, to recognize genera and species, and to analyze their relationships. Most of the studies include taxa from a restricted biogeographic region, e.g., Entimini [13–15]; Eustylini and Geonemini [16–20]; Tanymecini [21–25]; Naupactini [26–29]; Sitonini [30]; Cyphicerini, Phyllobiini and Polydrusini [31,32]; Laparocerini [33–36]; Otiiorhynchini [37–40]; Tanyrhynchini [41,42]; Leptopiini (as Tropiphorini in recent studies) [43–46]; and Trachyphloeini [47]. However, the generic classification of the entimines into tribes, as in the last worldwide weevil catalogue [2], remains unsatisfactory, with many tribes probably not representing natural groups.

For historical reasons, weevil generic and suprageneric classifications remain essentially based on the Holarctic fauna. The relatively less studied entimines from other regions have been often classified in already well-known genera and tribes from the Holarctic region, but based on similar observed features which do not necessarily reflect unique common ancestry. A more detailed examination of the morphological features, however, suggests a deep divergence of the austral faunas relative to the Holarctic fauna, and a probable closer relationship between some Australian Entiminae and Cyclominae with those from southern Argentina and Chile [48–52].

In this contribution, we present results of a phylogenetic study focused on South American tribes of Entiminae. The study was based on nuclear and mitochondrial molecular markers combined with morphological data for a sample of species representing the Neotropical and Patagonian-Andean faunas as well as entimines from other regions in both southern and northern hemispheres, including some South American Cyclominae used as outgroups.

The main hypotheses to be tested are as follows: that the Neotropical and Patagonian-Andean members of the tribes Cylydrorhinini, Entimini, Eudiagogini, Eustylini, Geonemini, Leptopiini, Naupactini, Premnotrypini and Tanymecini form a clade separated from the mainly Palaearctic tribes (e.g., Brachyderini, Laparocerini, Otiiorhynchini, Peritelini, Polydrusini, Phyllobiini and Sciaphylini) and Oriental Entiminae (e.g., Celeuthetini); and that the South American species of Leptopiini are related to genera of this tribe occurring in the Australian region. In addition, we are interested to address the phylogenetic positions of *Platyaspistes* Schoenherr and *Galapagonotus* Anderson & Lanteri, two South American genera with unclear affinities but currently classified in Tanymecini and Eustylini, respectively.

## 2. Materials and Methods

### 2.1. Specimens and Taxon Sampling

Table 1 lists the specimens and taxa used in the phylogenetic analyses, corresponding to 67 terminal species of Entiminae and Cyclominae, including valid names, acronyms, geographic data and GenBank accession numbers of DNA sequences for the four markers used.

The tribal classification used in this paper is based primarily on the world generic catalogue by Alonso-Zarazaga and Lyal [2], but incorporating some changes made subsequent to this publication. *Artipus* Sahlberg was classified in Geonemini and not in Naupactini [28]. The names of the Palaearctic species, genera and subgenera, and their assignment to tribes were updated according to Alonso-Zarazaga et al. [53]. Since the concept of Tropiphorini is very controversial (e.g., [53,54]), for the moment, we prefer to classify the genera from the southern hemisphere in the tribe Leptopiini, as suggested by Pullen et al. [55].

The classification of geographic regions follows Morrone [56] except for the Andean region in which case we recognized four biogeographic units: High Andes, Central Chile, Patagonia and Subantarctic.

We selected 63 species of Entiminae in 42 genera representing 17 tribes, as well as four species of Cyclominae in three genera from two tribes used as outgroups. The taxon sampling includes the tribes of Entiminae best representing the Neotropical and Patagonian-Andean faunas (Cylydrorhinini, Entimini, Eudiagogini, Eustylini, Geonemini, Leptopiini, Naupactini, Premonotrypini and Tanymecini), as well as some tribes typical of the Palaearctic fauna (Brachyderini, Laparacerini, Otiorhynchini, Peritelini, Phyllobiini, Polydrusini, and Sciaphilini) and some representatives of the dominant element of the Australian fauna in tribe Leptopiini [55] and Celeuthetini, corresponding to the Australian and Oriental regions. In Southern Chile and Argentina three species of *Polydrusus* Germar are associated with *Nothofagus* spp. trees [57,58]. In our study, the *Polydrusus* specimens from this area were not preserved for DNA extraction, and consequently we only analyzed Polydrusini species from Europe.

Samples of adult specimens assayed for molecular analyses were collected in different areas of Argentina and Brazil, using a beating sheet or a sweep net, or were provided by colleagues (see Acknowledgements). The material was stored in 96–100% ethanol at  $-20\text{ }^{\circ}\text{C}$  freezer until DNA extraction. Voucher specimens were deposited at the entomological collection of the Museo de La Plata, Argentina.

**Table 1.** Studied taxa, geographical sources and GenBank accession numbers. Codes in boldface indicate the new sequences produced for this study. \* Tribes of Outgroups, in Cyclominae.

Tribe	Species	Acronyms	Locality	Accession Codes			
				18S	28S	16S	COI-5P
<b>*Aterpini</b>	<i>Aegorhinus silvicola</i> Kuschel	CYC_Aegorhinus_sil	Argentina, Neuquén, San Martín de los Andes	<b>MH746278</b>	<b>MH746328</b>	<b>MH746378</b>	<b>MH734198</b>
	<i>Aegorhinus vitulus</i> (Fabricius)	CYC_Aegorhinus_vit	Argentina, Tierra del Fuego, Ushuaia	<b>MH746279</b>	-	<b>MH746379</b>	<b>MH734199</b>
<b>*Listroderini</b>	<i>Listroderes victus</i> Germain	CYC_Listroderes_vic	Chile, Cartagena, El Tabo, coastal dunes	<b>MH746280</b>	<b>MH746329</b>	<b>MH746380</b>	<b>MH734200</b>
	<i>Rhigopsidius piercei</i> Heller	CYC_Rhigopsidius_pie	Argentina, Jujuy	<b>MH746281</b>	<b>MH746330</b>	<b>MH746381</b>	<b>MH734201</b>
<b>Cylydrorhinini</b>	<i>Caneorhinus biangulatus</i> (Champion)	CYL_Caneorhinus_bia	GenBank source (Arg. Falklands Is.)	-	-	EF213994	-
	<i>Cylydrorhinus caudiculatus</i> (Fairmaire)	CYL_Cylydrorhinus_cau	GenBank source (Arg. Falklands Is.)	-	-	EF214094	-
	<i>Cylydrorhinus chilensis</i> (Blanchard)	CYL_Cylydrorhinus_chi	Argentina, La Rioja, Dto. Vinchina	<b>MH746286</b>	<b>MH746335</b>	-	<b>MH734202</b>
	<i>Cylydrorhinus aff. patagonicus</i> (Kuschel)	CYL_Cylydrorhinus_pat	Argentina, Jujuy, Dto. Susques	<b>MH746287</b>	<b>MH746336</b>	<b>MH746385</b>	<b>MH734203</b>
<b>Premnotrypini</b>	<i>Premnotrypes latithorax</i> (Pierce)	PRE_Premnotrypes_lat	Perú, Cuzco	<b>MH746316</b>	<b>MH746365</b>	<b>MH746416</b>	<b>MH734204</b>
<b>Leptopiini</b>	<i>Catasarcus impresipennis</i> (Boisduval)	LEP_Catasarcus_imp	GenBank source (Australia)	-	KF016401	KF016266	-
	<i>Catasarcus</i> sp.	LEP_Catasarcus_sp	GenBank source (Australia)	FJ867744	FJ867671	AJ495489	FJ867847
	<i>Cecyrota</i> sp.	LEP_Cecyrota_sp	New Zealand, Waikuku Beach, Surf Club	<b>MH746322</b>	<b>MH746371</b>	-	<b>MH734205</b>
	<i>Irenimus</i> sp.	LEP_Irenimus_sp	New Zealand, Ahuriri Scenic Reserve, Port Hills, Christchurch	<b>MH746324</b>	<b>MH746373</b>	<b>MH746422</b>	<b>MH734206</b>
	<i>Strangaliodes deserticola</i> Kuschel	LEP_Strangaliodes_des	Chile, Atacama	<b>MH746325</b>	<b>MH746374</b>	-	<b>MH734207</b>
	<i>Strangaliodes</i> sp. (aff. <i>deserticola</i> )	LEP_Strangaliodes_sp	Chile, Pichilemos	-	<b>MH746375</b>	<b>MH746423</b>	<b>MH734208</b>
	<i>Strangaliodes niger</i> (Blanchard)	LEP_Strangaliodes_nig	Argentina, San Juan	<b>MH746326</b>	<b>MH746376</b>	<b>MH746424</b>	<b>MH734209</b>
	<i>Vossius nebulosus</i> (Gyllenhal)	LEP_Vossius_neb	Argentina, Misiones, on way to Salto Tabai, close to Jardín America	<b>MH746323</b>	<b>MH746372</b>	-	<b>MH734210</b>
<b>Entimini</b>	<i>Entimus</i> sp.	ENT_Entimus_sp	Brazil, Rio de Janeiro, Coastal Restingas	<b>MH746327</b>	<b>MH746377</b>	<b>MH746425</b>	<b>MH734211</b>
<b>Eudiagogini</b>	<i>Eudiagogus rosenscholdi</i> Fahraeus	EUD_Eudiagogus_ros	GenBank source (United States of America)	AF250081	-	-	-
	<i>Pororhynchus aff. albolateralis</i> Hustache	EUD_Pororhynchus_alb	Argentina, San Juan, near Telteca	<b>MH746288</b>	<b>MH746337</b>	<b>MH746387</b>	<b>MH734212</b>
<b>Eustyliini</b>	<i>Compsus</i> sp.	EUS_Compsus_sp	GenBank source (South America)	-	KT350862	-	KT350650
	<i>Diaprepes abbreviatus</i> (Linnaeus)	EUS_Diaprepes_abb	GenBank source (Central America)	AY157729	KT350865	CN475651	HQ891432
	<i>Galapagonotus cuneiformis</i> Waterhouse	Galapagonotus_cun	Ecuador, Islas Galápagos, San Cristóbal, El Junco	<b>MH746282</b>	<b>MH746331</b>	<b>MH746386</b>	-
	<i>Oxyderces bimaculatus</i> (Hustache)	EUS_Oxyderces_bim	Argentina, Corrientes, Empedrado	<b>MH746289</b>	<b>MH746338</b>	<b>MH746388</b>	<b>MH734213</b>
<b>Geonemini</b>	<i>Artipus</i> sp.	GEO_Artipus_sp.	GenBank source (Caribbean)	-	KT350859	KP253881	HQ891428
	<i>Epicaerus</i> sp.	GEO_Epicaerus_sp	United States of America	<b>MH746290</b>	<b>MH746339</b>	<b>MH746389</b>	<b>MH734214</b>
	<i>Lachnopus</i> sp.	GEO_Lachnopus_sp	GenBank source (Caribbean)	AF389052	KT350910	-	HQ891465

Table 1. Cont.

Tribe	Species	Acronyms	Locality	Accession Codes			
				18S	28S	16S	COI-5P
<b>Naupactini</b>	<i>Atrichonotus taeniatus</i> (Berg)	NAU_Atrichonotus_tae	Argentina, Mendoza, Guaymallén	MH746296	MH746345	MH746395	MH537919
	<i>Hoplopactus lateralis</i> Arrow	NAU_Hoplopactus_lat	Brazil, San Pablo	MH746297	MH746346	MH746396	MH537920
	<i>Litostylus</i> sp.	NAU_Litostylus_sp	GenBank source (Caribbean)	-	KT350916	-	HQ891470
	<i>Naupactus auricinctus</i> Boheman	NAU_Naupactus_aur	Brazil, San Pablo	MH746298	MH746347	MH746397	MH537921
	<i>Naupactus aff chloraspis</i> Erichson	NAU_Naupactus_chl	Brazil, Bahia	MH746299	MH746348	MH746398	MH734215
	<i>Naupactus aff faldermanni</i> Boheman	NAU_Naupactus_fald	Brazil, Mato Grosso	MH746300	MH746350	MH746399	-
	<i>Naupactus leucoloma</i> Boheman	NAU_Naupactus_leu	Argentina, Mendoza	MH746301	MH746349	MH746400	MH537922
	<i>Naupactus aff peruvianus</i> Hustache	NAU_Naupactus_per	Perú, Cuzco, Dto. Machu Pichu, Puerta Ciudad Antigua	MH746302	MH746351	MH746401	-
	<i>Naupactus sulphurifer</i> Pascoe	NAU_Naupactus_sul	Argentina, Mendoza, Reserva Divisadero Largo	MH746303	MH746352	MH746402	-
	<i>Naupactus tarsalis</i> Boheman	NAU_Naupactus_tar	Argentina, Formosa, Dto. Matacos, 20 Km Ing. Juárez	MH746304	MH746353	MH746403	-
	<i>Naupactus viridisquamosus</i> Boheman	NAU_Naupactus_vir	Brazil, Mato Grosso	MH746305	MH746354	MH746404	
	<i>Naupactus xanthographus</i> (Germar)	NAU_Naupactus_xan	Argentina, Mendoza [except 16S, Buenos Aires, La Plata]	FJ867775	FJ867695	MH746405	NC0180354
	<i>Pantomorus ruizi</i> (Brèthes)	NAU_Pantomorus_rui	Argentina, Chubut, Trelew INTA	MH746306	MH746355	MH746407	MH537925
	<i>Symmathetes setarius</i> (Boheman)	NAU_Symmathetes_set	Brazil, Mato Grosso	MH746308	MH746357	MH746408	MH537923
<i>Symmathetes setulosus</i> Hustache	NAU_Symmathetes_setu	Argentina, Catamarca, Las Esquinas, Dpto. Valle Viejo	MH746307	MH746356	MH746406	MH537924	
<b>Tanymecini</b>	<i>Airosimus jacobi</i> (Hustache)	TAN_Airosimus_jac	Argentina, Misiones, Uruguay-i	MH746318	MH746367	MH746418	MH734216
	<i>Hadromeropsis aff. pallida</i> Howden	TAN_Hadromeropsis_pal	Argentina, Entre Ríos, Cerrito	MH746319	MH746368	MH746419	MH734217
	<i>Pandeleiteius platensis</i> Brèthes	TAN_Pandeleiteius_pla	Argentina, Mendoza, Reserva Ñacuñán	MH746320	MH746369	MH746420	MH734218
	<i>Pandeleiteius</i> sp.	TAN_Pandeleiteius_sp	GenBank source (United States of America)	-	HQ891475	-	HQ891475
	<i>Platyaspistes argentinensis</i> Kuschel	TAN_Platyaspistes_arg	Argentina, San Juan, Dto. Sarmiento-Pedernal, Camino de los Berros	MH746321	MH746370	MH746421	MH734219
<b>Brachyderini</b>	<i>Brachyderes incanus</i> Linnaeus	BRA_Brachyderes_inc	GenBank source (Europe)	-	KX551463	AJ495503	KJ962877
	<i>Caulostrophus subsulcatus</i> (Boheman)	BRA_Caulostrophus_sub	Italy, Genova, Voltri, Fiorino	MH746283	MH746332	MH746382	MH734220
	<i>Strophosoma (Strophosoma) melanogrammum</i> (Forster)	BRA_Strophosoma_mel	Italy, Vercelli, Val Sesia, Monte Tovo	MH746284	MH746333	MH746383	MH734221

Table 1. Cont.

Tribe	Species	Acronyms	Locality	Accession Codes			
				18S	28S	16S	COI-5P
Laparocerini	<i>Laparocerus (Atlantis) noctivagans</i> Wollaston	LAP_Laparocerus_noc	Portugal, Madeira, Rabaçal	MH746293	MH746341	MH746391	MH734222
	<i>Laparocerus (Fernandezius) subcalvus</i> Wollaston	LAP_Laparocerus_sub	Spain, Islas Canarias, Isla El Hierro, NE San Andrés	MH746292	MH746344	MH746394	-
	<i>Laparocerus (Guanchotrox) canariensis</i> Boheman	LAP_Laparocerus_can	Spain, Islas Canarias, Isla de Tenerife, El Portillo	MH746294	MH746342	MH746392	MH734223
	<i>Laparocerus (Laparocerus) morio</i> Boheman	LAP_Laparocerus_mor	Portugal, Madeira, Encumeada,	MH746295	MH746343	MH746393	MH734224
Otiorynchini	<i>Dodecastichus mastix</i> Olivier	OTI_Dodecastichus_mas	Italy, Pescara, La Maielletta	MH746309	MH746358	MH746409	MH734225
	<i>Otiorynchus (Dorymerus) sulcatus</i> Fabricius	OTI_Otiorynchus_sul	GenBank source (Europe)	AF250084	KU041875	AJ495482/ JN163969	KJ962464
	<i>Otiorynchus (Metopiorrhynchus) teretirostris</i> Stierling	OTI_Otiorynchus_ter	Italy, Vercelli, Val Sesia, Monte Tovo	MH746310	MH746359	MH746410	MH734226
	<i>Otiorynchus (Provadilus) alpicola</i> Boheman	OTI_Otiorynchus_alp	Italy, Pescara, La Maielletta	MH746311	MH746360	MH746411	MH734227
Peritelini	<i>Drouetius azoricus parallelirostris</i> Machado	PER_Drouetius_azo	Portugal, Islas Azores, Isla Terceira, Angra, Monte Brazil	MH746291	MH746340	MH746390	MH734228
	<i>Simo hirticornis</i> Herbst	PER_Simo_hir	Italy, Lecco, Lombardia, Lagi di Annone	MH746312	MH746361	MH746412	MH734229
Phyllobiini	<i>Phyllobius (Parnemoicus) viridicollis</i> Fabricius	PHY_Phyllobius_vir	Italy, Verona, Monti Lessini, Monte Castelberto	MH746313	MH746362	MH746413	MH734230
Polydrusini	<i>Liophloeus (Liophloeus) tessulatus</i> O.F. Müller	POL_Liophloeus_tes	Italy, Como, Lombardia, Piano del Tivano	MH746314	MH746363	MH746414	MH734231
	<i>Polydrusus (Eurodrusus) cervinus</i> Linnaeus	POL_Polydrusus_cer	GenBank source (Europe)	-	HQ883568	AJ495494	HQ883653
	<i>Polydrusus (Eurodrusus) confluens</i> Stephens	POL_Polydrusus_con	Italy, Como, Lombardia, Piano del Tivano	MH746315	MH746364	MH746415	MH734232
Sciaphilini	<i>Eusomus ovulum</i> Germar	SCI_Eusomus_ovu	Italy, Milan, Zelo Buon Persico, Fiume Adda [except COI, GenBank source]	MH746317	MH746366	MH746417	MH734233
	<i>Sciaphilus asperatus</i> Bordsdorff	SCI_Sciaphilus_asp	GenBank source (Europe)	JN619105	-	AJ495502	KJ962310
Celeuthetini	<i>Celeuthetes</i> sp.	CEL_Celeuthetes_sp	Vanuatu, Efate, Tagabe, Agr. Research Str, Port Vila	MH746285	MH746334	MH746384	MH734234

- means "unavailable".

## 2.2. Molecular Data

### 2.2.1. DNA Isolation, PCR Amplification and Sequencing

The experiments of DNA extraction and PCR amplification of genetic material were performed at the molecular laboratories of IADIZA-CONICET (Mendoza, Argentina) and IMBIV-CONICET-UNC (Córdoba, Argentina).

Total genomic DNA was extracted from adult voucher specimens using an adapted “salting out” protocol [59] or, alternatively, the DNeasy Blood and Tissue Kit (QIAGEN, MD, USA.). Tissue was processed from 1–2 legs or part of the thorax. Extracted DNA was stored at  $-20^{\circ}\text{C}$ . Four molecular markers (two nuclear and two mitochondrial) were used in this study: 18S rDNA (entire), 28S rDNA (regions D2, D3), 16S rDNA (regions IV, V) and COI (“barcode” or 5’ region).

Amplification and sequencing of the targeted loci was performed using primers listed in Table 2. Polymerase chain reaction (PCR) was performed in a 50  $\mu\text{L}$  volume: 10 pmol for each primer, 0.8 mM dNTPs (Genbiotech SRL, Buenos Aires, Argentina),  $\text{MgCl}_2$  50 mM to a final concentration of 2–4 mM, 5  $\mu\text{L}$  10  $\times$  Buffer, and 1.25 units of Taq DNA Polymerase (Invitrogen SA, Buenos Aires, Argentina). The 18S (~2000 bp) was amplified in two parts with the primer pairs E and F1094, and R1138 and P, with PCR conditions as in McKenna et al. [6]: 3 min at  $95^{\circ}\text{C}$ ; then 30 cycles of 1 min at  $94^{\circ}\text{C}$ , 1 min at  $60^{\circ}\text{C}$ , 1 min at  $72^{\circ}\text{C}$ ; final extension of 5 min at  $72^{\circ}\text{C}$ ; kept at  $4^{\circ}\text{C}$ . In few instances, another combination of primers was used to obtain two smaller 18S fragments of ~500 bp each that contain the variable regions V4 and V7–V9 of 18S [60]: 18S a07 and 18S b25, and V7V9up and V7V9down, with PCR conditions: 3 min at  $95^{\circ}\text{C}$ ; then 10 cycles of 30 s at  $95^{\circ}\text{C}$ , 30 s at  $46^{\circ}\text{C}$ , 30 s at  $72^{\circ}\text{C}$ ; then 30 cycles of 30 s at  $95^{\circ}\text{C}$ , 40 s at  $48^{\circ}\text{C}$ , 40 s at  $72^{\circ}\text{C}$ ; final extension of 10 min at  $72^{\circ}\text{C}$ . The fragment used of 28S (~700 bp spanning regions D2 and D3) was amplified with primers S3660 and A335, with PCR reactions as in Brown et al. [61]: 2 min at  $94^{\circ}\text{C}$ ; 40 cycles of 15 s at  $94^{\circ}\text{C}$ , 30 s at  $54^{\circ}\text{C}$ , 75 s at  $72^{\circ}\text{C}$ ; final extension of 7 min at  $72^{\circ}\text{C}$ . The 16S (~600 bp spanning regions IV and V) was amplified with primers N13398 and J12887, with PCR conditions as in Hundsdoerfer et al. [5]: 4 min at  $94^{\circ}\text{C}$ ; 5 cycles of 45 s at  $93^{\circ}\text{C}$ , 90 s at  $40^{\circ}\text{C}$ , 90 s at  $72^{\circ}\text{C}$ ; then 26 cycles of 45 s  $93^{\circ}\text{C}$ , 90 s at  $50^{\circ}\text{C}$ , 90 s at  $72^{\circ}\text{C}$ ; final extension of 10 min at  $72^{\circ}\text{C}$ . The COI gene was amplified with the primer pair LCO and HCO that produce the standard barcode fragment of 658 bp, but sometimes with the primers LCO and A3014 that allow a longer fragment of 1000–1300 bp; using in both cases the PCR conditions as in McKenna et al. [6]: 3 min at  $94^{\circ}\text{C}$ ; then 5 cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $42^{\circ}\text{C}$ , 90 s at  $72^{\circ}\text{C}$ ; then 34 cycles of 1 min at  $94^{\circ}\text{C}$ , 30 s at  $45^{\circ}\text{C}$ , 90 s at  $72^{\circ}\text{C}$ ; final extension of 5 min at  $72^{\circ}\text{C}$ . The PCR products were purified and bi-directionally sequenced with the Sanger method, by means of the Sequencing Service of “Unidad de Genómica de INTA-Castelar” (Buenos Aires, Argentina) or by Macrogen Inc. (Seoul, South Korea). Electropherograms were edited and contig assembled using ProSeq v.2.91 [62] or Sequencher v.5 (GeneCodes Corp.). All sequences were deposited in GenBank under accession numbers provided in Table 1.

### 2.2.2. Alignment of Sequences

The sequences of the ribosomal markers (nuclear 18S and 28S and mitochondrial 16S) were aligned using information on secondary structure of the rRNA genes to identify homologous positions [63]. The alignment was constructed manually using as reference the structural model of arthropod rRNA [64,65]. The program PAUP [66] was used for editing the annotated alignment as text file and for exporting the data in other formats for analyses. Regions identified as being of ambiguous alignment were bracketed to be excluded from phylogenetic analyses.

**Table 2.** List of primers used for PCR amplification and sequencing of the markers used in the phylogenetic analyses of Entiminae.

Marker	Primer	Sequence (5' > 3')	Sense	Reference
18S	E	CTG GTT GAT CCT GCC ACG T	F	[67]
	R1138	CGC CTT CGA ACC TCT AAC	R	[68]
	F1094	GGA TCG TCG CAA GAC GGA CAG AAG	F	[68]
	P	TAA TGA TCC TTC CGC AGG TTC ACC T	R	[67]
	18S a07	ATT AAA GTT GTT GCG GTT	F	[60]
	18S b25	TCT TTG GCA AAT GCT TTC GC	R	[60]
	V7V9up	TCC GAT AAC GAA CGA GAC TC	F	[60]
V7V9down	GTT ACG ACT TTT ACT TCC TC	R	[60]	
28S	S3660	GAG AGT TMA ASA GTA CGT GAA AC	F	[69]
	A335	TCG GAR GGA ACC AGC TAC TA	R	[70]
16S	N13398	CGC CTG TTT AWC AAA AAC AT	F	[5]
	J12887	CTC CGG TYT GAA CTC AGA TCA AGT	R	[5]
COI	LCO	GGT CAA CAA ATC ATA AAG ATA TTG G	F	[71]
	HCO	TAA ACT TCA GGG TGA CCA AAA ATC A	R	[71]
	A3014	TCC AAT GCA CTA ATC TGC CAT ATT A	R	[72]

### 2.3. Morphological Characters

External and internal (dissected) adult structures were observed with a Nikon SMZ1000 stereomicroscope (Tokyo, Japan), Leica S6D and MZ16 stereomicroscopes (Wetzlar, Germany); line drawings were done with a camera lucida attached to the former scope. Digital photographs were taken with cameras attached to the S6D and MZ16 stereomicroscopes. For dissections, we used standard entomological techniques [73]. The terminology used for morphological characters follows Marvaldi et al. [3], and Lanteri and del Rio [28]. Some characters have been illustrated to facilitate recognition of different character states (Figures 1–4).

For each terminal, we scored 70 discrete morphological characters, of which 62 correspond to the morphology of adults and 8 to larvae. The list of morphological characters is given in Table 3 and the data matrix is shown in Table S1. When larval specimen or genitalia from adult could not be examined, character states were scored with “?” and treated as missing data. For some taxa (e.g., *Aegorhinus* Erichson, *Listroderes* Schoenherr, *Cylydrorhinus* Guérin-Méneville, and *Entimus* Germar), larval information was available from specimens belonging to different, but clearly co-generic species of those included in the analysis (see [4,74,75]).

**Table 3.** List of the 70 morphological characters, character states and codes.

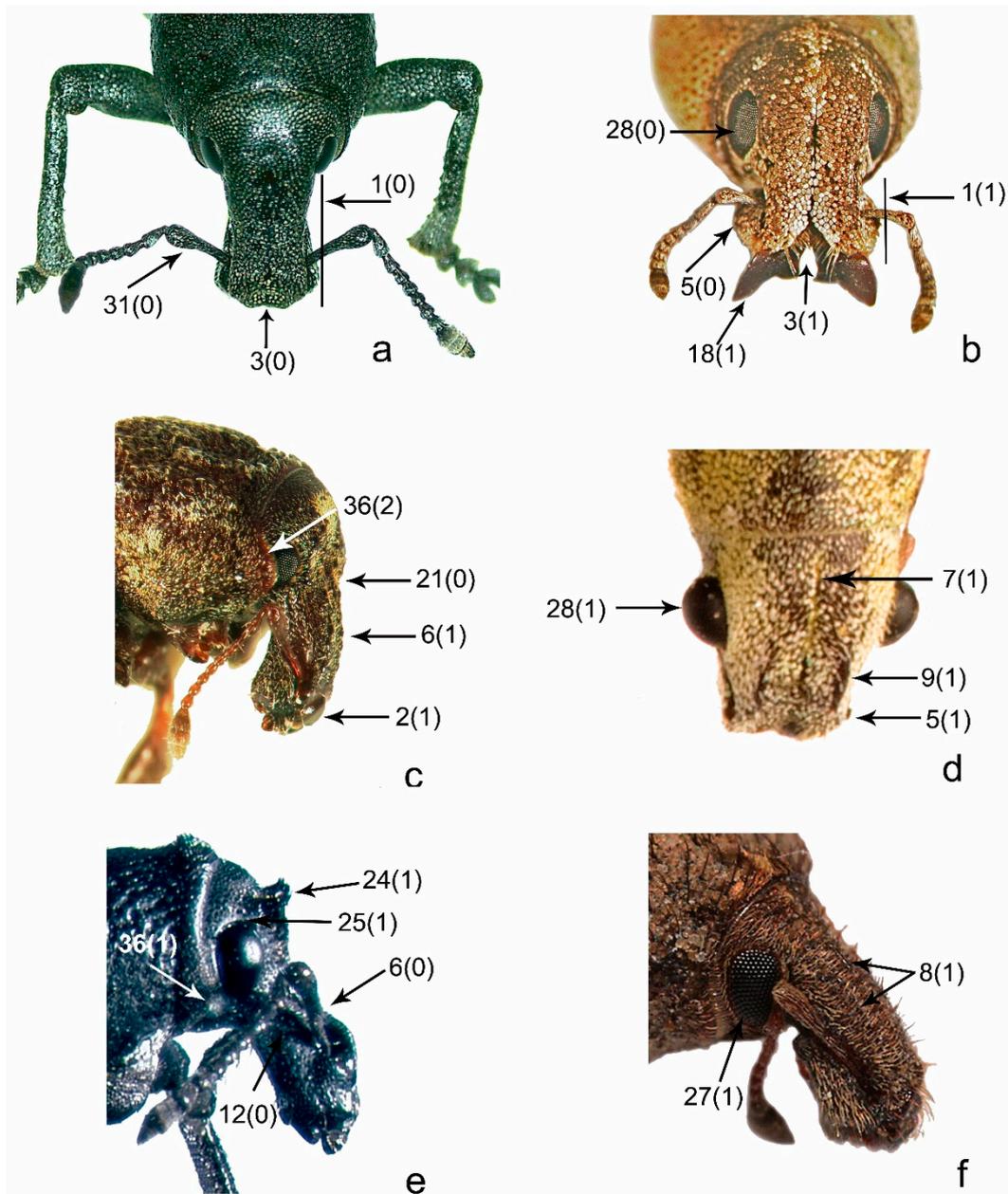
1	Rostrum, length: more than $1.25 \times$ as long as wide (minimum width, measured across apex without scrobes) (0) (Figure 1a); about as long as wide (slightly longer to slightly shorter) (1) (Figure 1b).
2	Rostrum, anterior portion of epistome: not raised and not projecting beyond anterior margin of rostrum (0); raised and projecting beyond anterior margin of rostrum (1) (Figure 1c).
3	Rostrum, outline of apex: slightly emarginated (0) (Figure 1a); V-shaped (1) (Figure 1b).
4	Rostrum, posterior part of epistome: denuded (0); covered with vestiture different from that of posterior part of rostrum (1) (Figure 5 in [28]).
5	Rostrum, expansion at apex (including pterigia): rostrum distinctly expanded at apex, ahead of antennal insertion (0) (Figure 1b); not to slightly expanded apex (sides subparallel or convergent towards apex) (1) (Figure 1d).
6	Rostrum, dorsal surface: impressed or flat (0) (Figure 1e); convex (1) (Figure 1c).
7	Rostrum, groove along midline: absent or indistinct (0); present (1) (Figure 1d).
8	Rostrum unicarinate or tricarinate (with central and lateral longitudinal carinae): not unicarinate or tricarinate (0); unicarinate or tricarinate (1) (Figure 1f).
9	Rostrum bicarinate (with pair of dorso-lateral carinae): not bicarinate (0); bicarinate (1) (Figure 1d).
10	Rostrum, scrobes in dorsal view: completely exposed, visible along their whole length (0) (Figure 3b); not completely exposed, only apical part partially visible (1) (Figure 3a).

Table 3. Cont.

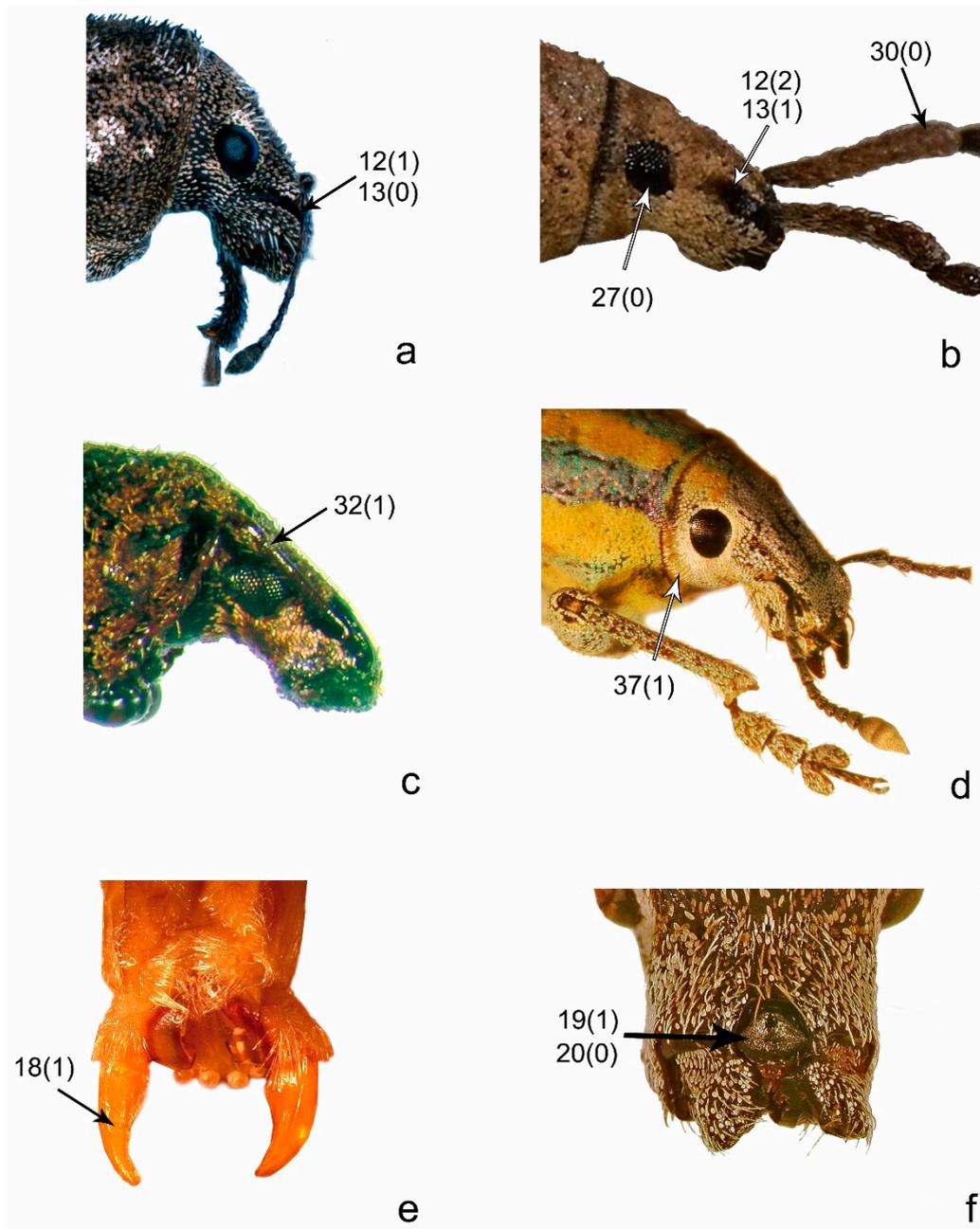
11	Rostrum, sulcus along pterigia in lateral view: absent to slight (0); deep (1).
12	Rostrum, curvature of scrobes in lateral view: strongly curved ventrad of head (0) (Figure 1e); curved downwards and passing below eyes (1) (Figure 2a); slightly curved and orientated towards eyes (2) (Figure 2b).
13	Rostrum, scrobes in lateral view: well-defined and slightly widened posteriad (0) (Figure 2a); vaguely defined, shallow and very widened posteriad (1) (Figure 2b).
14	Rostrum, anteocular impression: longitudinal, latero-dorsal (0) (Figure 1 in [14]); triangular, lateral (1) (Figure 5 in [14]); indistinct (2).
15	Rostrum, transversal sulcus between rostrum and head (ventral view): present (0); indistinct (1).
16	Rostrum, occipital sutures (ventral view): longitudinal, curved, usually connected with lower edge of eye (0) (Figure 4a); transversal relative to longitudinal axis of rostrum, not connected with lower edge of eye (1) (Figure 4b); reduced to a fovea (2) (Figure 4c).
17	Mouthparts, inner side on mandibles: with two or more teeth (0) (Figures 1 and 2 [76]); with a single tooth on distal end or lacking tooth (1) (Figures 7 and 8 in [18]).
18	Mouthparts, scar or lasting appendage on apical surface of mandibles: absent (0); present (1) (Figures 1b and 2e).
19	Mouthparts, prementum relative to maxillae: prementum incompletely concealing maxillae (phanerognathous condition) (0); prementum completely concealing maxillae (adelognathous condition) (1) (Figure 2f) (Figure 4d,e in [77]).
20	Mouthparts, long setae on prementum: absent (0) (Figure 2f); present (1) (Figures 9 and 10 in [18]).
21	Head, transversal furrow or impression between rostrum and forehead (dorsal view): present (0) (Figure 1c); absent (1).
22	Head, width of forehead relative to minimum width of rostrum (without pterigia): forehead wider than rostrum (0); forehead about same width to narrower than rostrum (1).
23	Head, orientation of setae behind forehead (vertex): setae anteriorly oriented (0); posteriorly oriented (1).
24	Head, superciliar arches: absent (0); present (1) (Figure 1e).
25	Head, circumocular sulcus: absent (0); present (1) (Figure 1e).
26	Head, large fovea on forehead: present (0); absent or very small (1).
27	Head, shape and position of eyes: rounded to slightly oval, oriented antero-posteriad (Figure 2b) (0); oval, oriented dorso-ventrad (1) (Figure 1f).
28	Head, convexity of eyes: flat (slightly protruding) (0) (Figure 1b); convex (protruding) (1) (Figure 1d); conical (strongly protruding) (2) (Figure 3a).
29	Eyes, position relative to anterior margin of pronotum: slightly separated from pronotum (by $1\times$ or less than $1\times$ the diameter of eyes) (0) (Figure 3c); largely separated from pronotum (by $1\times$ of more than $1\times$ the diameter of eyes) (1) (Figure 3b).
30	Antennae, shape and width of scape: clavate to spatulate, broad (0) (Figure 2b); clavate to capitate, slender (1) (Figure 3c).
31	Antennae, curvature of scape: straight (0) (Figure 1a); curved (1) (Figure 3b).
32	Antennae, scape in resting position: passing below eyes (0); passing across dorsal half of eyes or over eyes (1) (Figure 2c).
33	Antennae, length of scape: not reaching anterior margin of eyes (0); exceeding anterior margin of eye, but not exceeding anterior margin of pronotum (1); largely exceeding anterior margin of pronotum (2).
34	Antennae, relative length of funicle antennomeres 1 and 2: funicle antennomere 2 slightly shorter than 1 or both subequal (0); funicle antennomere 2 longer than 1 (1).
35	Antennae, length/ width ratio of funicle antennomeres 4–7: wider than long (0); about as long as wide (1); distinctly longer than wide (2).
36	Pronotum, postocular lobes: absent (0); present, not covering eyes (1) (Figure 1e); present, covering eyes (2) (Figure 1c).
37	Pronotum, long vibrissae on lateral margins: absent (0); present (1) (Figure 2d).
38	Pronotum, shape: subcylindrical to subconical, not impressed behind anterior margin (0); subhexagonal, impressed behind anterior margin (1) (Figure 3d).
39	Pronotum, basal margin: not constricted (0); strongly constricted (1) (Figure 3d in [28]).
40	Prosternum, channel to accommodate the rostrum in repose: absent (0); present (1).
41	Scutellum, shape: subtriangular (0); quadrate to rounded (1); suboval to subrectangular (longitudinal) (2) (Figure 3d); subcircular (3).
42	Elytra, striae 9 and 10: closer to each other on posterior 2/3 (0) (Figure 3f); equally separated along their extension (1) (Figure 3e); closer to each other on anterior 1/3 (2).
43	Elytra, outline of apex: entire (0); bifid (1) (Figure 3a,d in [28]).
44	Procoxae, position relative to anterior and posterior margins of prosternum: about equally close to both margins of prosternum (0); distinctly closer to anterior margin (1).
45	Femora, large tooth on ventral edge: absent (0); present on all femora (1) (Figure 3c).
46	Femora, width of profemora relative to width of metafemora: profemora about as wide as metafemora (0); $1.25\text{--}1.5\times$ as wide as metafemora (1); more than $1.5\times$ as wide as metafemora (2); narrower than metafemora (3) (Figures 2a and 3a,d,e in [28]).
47	Tibiae, mucro on protibiae: reduce to indistinct (0) (Figure 3b); moderate to large, not forming forceps with tuft of stiff setae (1) (Figure 3a); large, forming forceps with tuft of stiff setae (2).
48	Tibiae, line of denticles along inner edge: absent in all tibiae (0); present at least on protibiae (1) (Figure 3a).

Table 3. Cont.

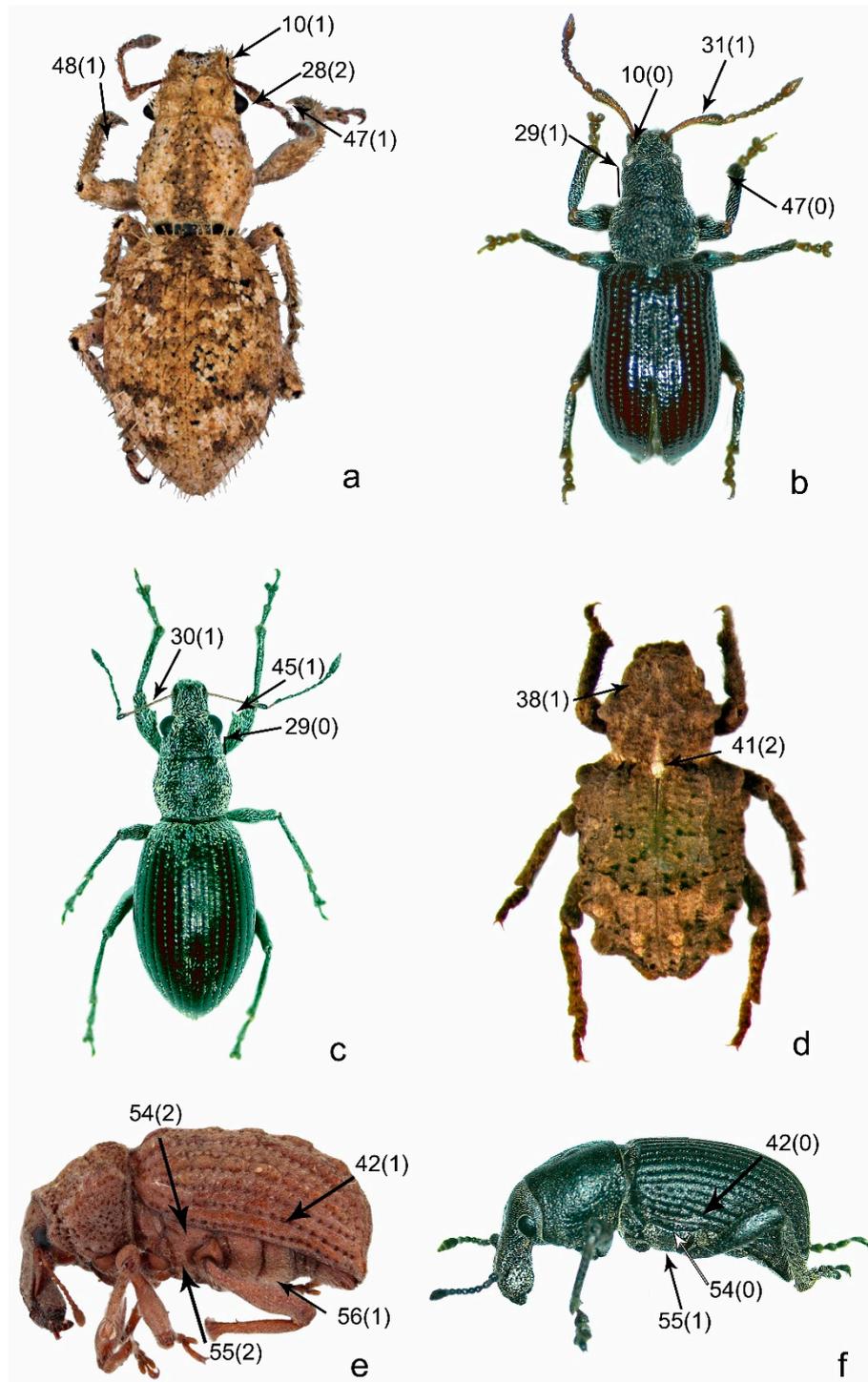
49	Tibiae, spurs at metatibial apex: present (0); absent (1).
50	Tibiae, true corbel at metatibial apex: absent (metatibial apex simple or corbel open); present (1) (Figures 5A, 5B–D in [3]).
51	Tibiae, false corbel (inner flange or corbel semi-enclosed): absent (0); present (1) (Figure 5E,F in [3]).
52	Tibiae, dorsal comb at metatibial apex (corbel ascending): absent (0); present (1) (Figures A–B and E–J in [78]).
53	Tarsal claws: free, widely separated (0) (Figure 4d); free, moderately separated (1) (Figure 4e); connate (2) (Figure 4f–g).
54	Metepisternum and metepisternal-metasternal suture: metepisternum wide, suture complete (0) (Figure 3f); metepisternum narrow, suture complete (1); metepisternum indistinct, suture incomplete, partially fused with metasternum (2) (Figure 3e).
55	Metasternum, length relative to metacoxae: about 2X as long as metacoxae (0); 1–2× as long as metacoxae (1) (Figure 3f); less than 1× as long as metacoxae (2) (Figure 3e).
56	Venter, convexity of ventrites: flat to slightly convex (0); ventrites 1 and 2 more convex than remaining ventrites (Figure 3e) (1); ventrites 1–4 very convex (2).
57	Venter, separation between metacoxae: equal to slightly narrower than width of each metacoxa (0); much wider than width of each metacoxa (1).
58	Venter, length of ventrite 2 relative to ventrites 3 + 4 (female): longer than 3 + 4 (0); about as long as to slightly shorter than 3 + 4 (1).
59	Female genitalia, shape of plate of sternite VIII: subtriangular (0); not subtriangular (1). (Figure 6a–g in [28])
60	Female genitalia, length of apodeme of sternite VIII: about as long as plate (0); distinctly longer than plate (1).
61	Female genitalia, distal gonocoxites and styli: gonocoxites membranous to slightly sclerotized, styli present (0); gonocoxites strongly sclerotized, styli absent (1) (Figure 7c,d in [28])
62	Female genitalia, pair of baculi along ovipositor: absent (0); present (1) (Figure 7b–e in [28]).
63	Larva, length and shape of antennal sensorium: longer than wide, subconical (0) (Figure 53 [75]; wider than long, cushion like (1) (Figure 2 in [74]).
64	Larva, shape of antennal sensorium in apical view: subcircular (0) (Figure 10 in [4]); elliptical (1) (Figure 13 in [4]).
65	Larva, number of ventral setae on maxillary mala: five (0) (Figure 56 in [75]); four (1) (Figures 7, 52, and 74 in [74]).
66	Larva, shape of posterior extension of premental sclerite of labium: acute at apex (0) (Figure 28 in [4]); truncate at apex (1) (Figures 33 and 34 in [4]).
67	Larva, accessory teeth on intermediate portion of mandibular cutting-edge: present (0) (Figure 20 in [4]); absent (1) (Figure 6 in [79]).
68	Larva, mandibular scrobe: sclerotized, similar to the rest of mandible (0) (Figure 55 in [75]); slightly sclerotized, paler than the rest of mandible (1) (Figure 6 in [79]).
69	Larva, labral rods: subparallel (0)(Figures 3–5 in [79]); divergent in their distal half (1) (Figures 1 and 2 in [79]); U-shaped (2) (Figures 50 and 60 in [74]).
70	Larva, number of setae on alar area: two (0) (Figure 9 in [74]); one (1) (Figure 7 in [79]).



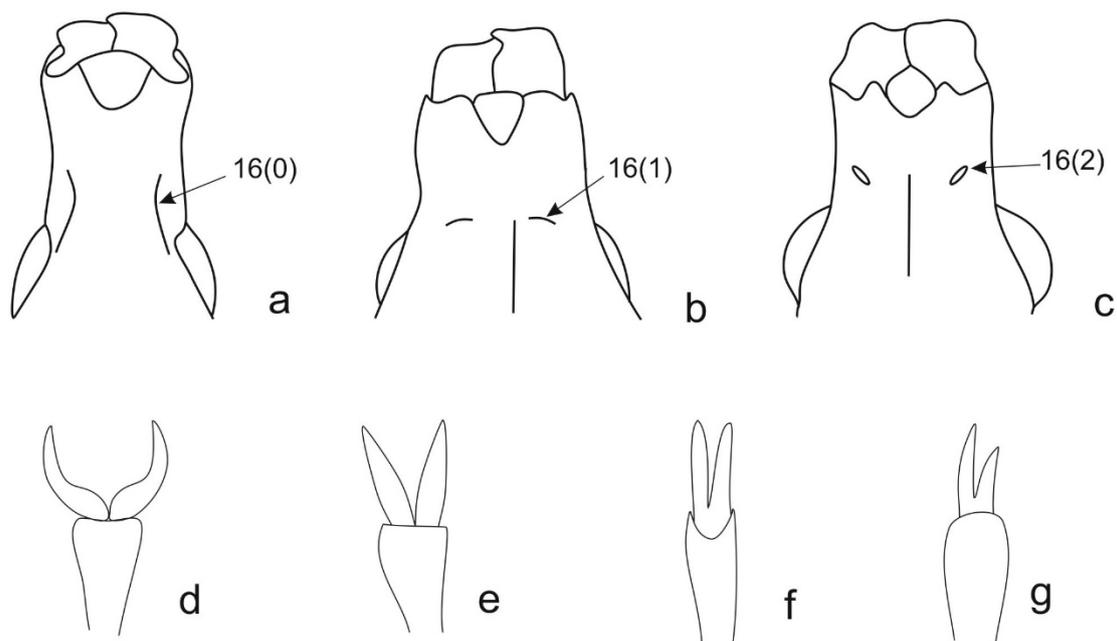
**Figure 1.** Morphology of adults, head and rostrum: (a) *Strangaliodes niger*, frontal view; (b) *Pororhynchus labeonis*, frontal view; (c) *Premnotrypes latithorax*, lateral view; (d) *Hoplopactus lateralis*, frontal view; (e) *Aegorhinus vitulus*, lateral view; and (f) *Listroderes costirostris*, lateral view. Arrows indicate characters and character states (between parentheses).



**Figure 2.** Morphology of adults, head and rostrum: (a) *Pantomorus postfasciatus*, lateral view; (b) *Simo hirticornis*, lateral view; (c) *Galapagonotus cuneiformis*, lateral view; (d) *Platyaspistes argentinensis*, lateral view; (e) *Entimus sastrei* (teneral adult), frontal view; and (f) *Naupactus xanthographus*, ventral view. Arrows indicate characters and character states (between parentheses).



**Figure 3.** Morphology of adults, habitus: (a) *Symmathetes setulosus*, dorsal view; (b) *Phyllobius viridicollis*, dorsal view; (c) *Eusomus ovulum*, dorsal view; (d) *Rhigopsidius piercei*, dorsal view; (e) *Premnotrypes latithorax*, lateral view; and (f) *Strangaliodes niger*, lateral view. Arrows indicate characters and character states (between parentheses).



**Figure 4.** Morphology of adults, occipital sutures and tarsal claws. Head and rostrum, ventral view, position of occipital sutures (character 16): (a) longitudinal (16.0); (b) transversal (16.1); (c) reduced to a fovea (16.2). Tarsal claws (character 53): (d) free, widely separated (53.0); (e) free, moderately separated (53.1); (f) connate, claws of equal length (53.2); and (g) connate, claws of different length (53.2).

#### 2.4. Phylogenetic Analyses

The molecular and combined data sets were analyzed using Bayesian Inference (BI) and Maximum Parsimony (MP) approaches.

The Bayesian analysis was performed using BEAST 2 v2.4.8 [80] on the Cipres Science Gateway (<http://www.phylo.org>) [81] with random starting trees without constraints. Each individual gene was treated as a separate partition in the analyses, resulting in a dataset comprising 3522 aligned nucleotide positions. The optimal substitution model for each partition was selected using the jModeltest software v.2.0 [82], on the basis of the corrected Akaike Information Criterion, as suggested by Burnham and Anderson [83]. We applied the following substitution models: TVM + I + G (16S, 28S), SYM + I + G (18S), TIM2 + I + G (COI) and Lewis MK for morphological data. We assumed a Yule speciation model and strict molecular clock. Clock and tree parameters were linked across partitions. All priors were left as the default values in BEAUti [80]. The analyses were run for a total of 30 million generations with sampling every 20,000 generations. The convergence of the runs was evaluated by accessing log files in TRACER v1.6 [84]. We generated a maximum clade credibility tree in TreeAnnotator v2.4.8 [80], using a burn-in of 10% (1500 trees) and visualized in FigTree v1.4.3 [85].

Parsimony analysis was conducted using TNT v1.5 [86], excluding third codon positions of COI and considering all other characters as un-weighted and non-additive. Gaps were treated as a fifth state (default in TNT). A heuristic search with TBR branch swapping was applied to a series of 500 random addition sequences, retaining 30 trees per replicate. A strict consensus tree was calculated when more than one most parsimonious tree (MPT) was obtained. Clade stability was evaluated by 1000 parsimony bootstrap replications [87]. For the MPTs we provided the basic parameters as total length (L), consistency index (CI) [88] and retention index (RI) [89]. The program WinClada [90] was used for character mapping (under unambiguous, fast and slow optimization options) and to prepare MP tree figures.

The outgroup *Aegorhinus silvicola* Kuschel (Cyclominae, Aterpini) was used to root the trees in both analyses. Posterior probability values (under BI) were considered as follows in the Results

and Discussion: 0.95–1, strong to maximal clade support; 0.70–0.94, low to moderate clade support; and <0.70, not supported.

### 3. Results

#### 3.1. Annotated Structural Alignment

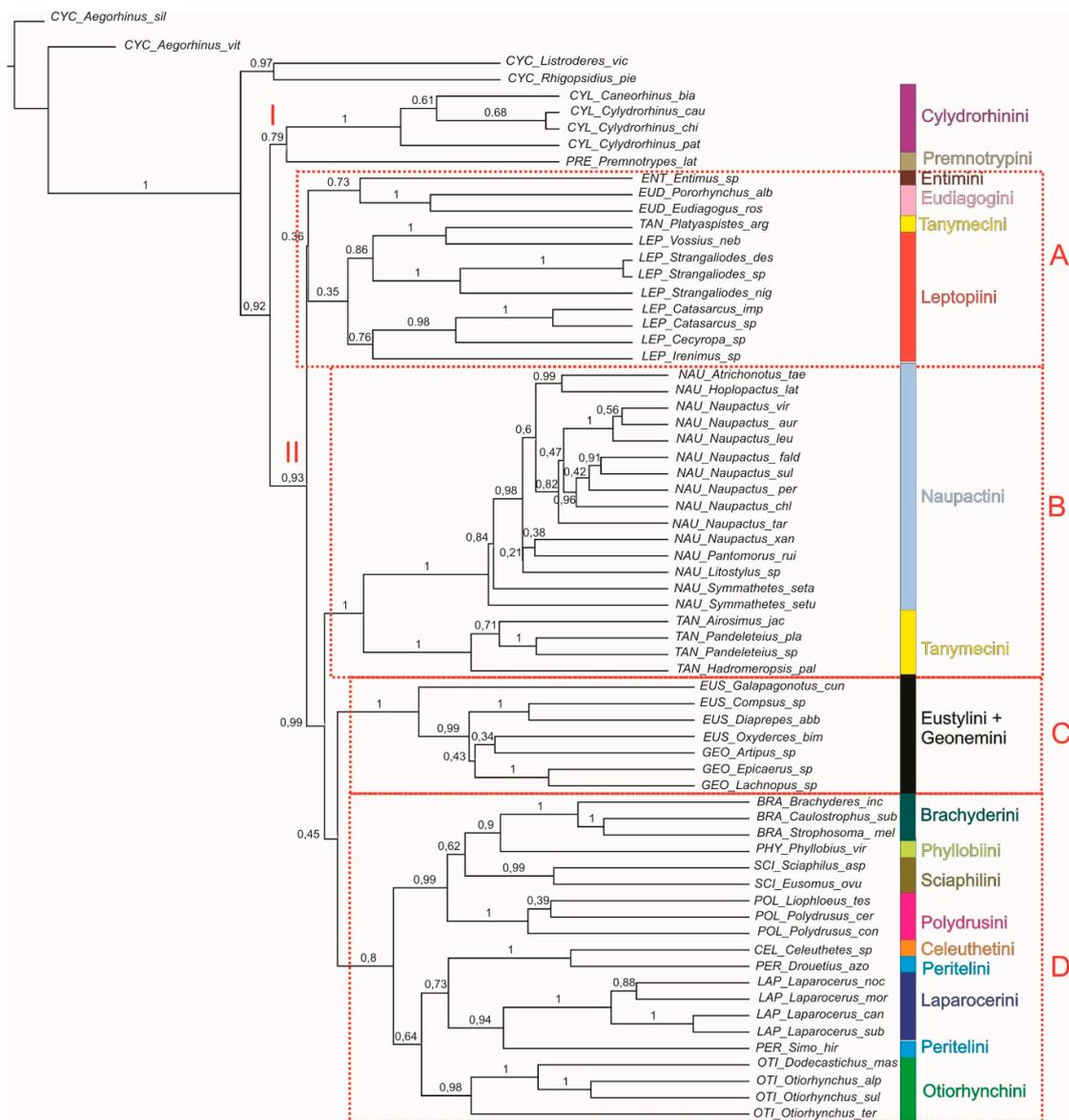
The annotated structural alignments of the entire 18S rRNA gene, the D2 and D3 expansion segments of the 28S rRNA gene and domains IV and V of the 16S rRNA gene performed for 63 entimine taxa and four cyclomine outgroups, are available online as Supplementary Materials (Table S2).

#### 3.2. Phylogenetic Trees

##### 3.2.1. Bayesian Analysis

The tree obtained from the Bayesian analysis is shown in Figure 5. Entiminae are monophyletic (PP 0.92), with the tribes Cylydorrhynini–Premnotrypini (Andes, Patagonia) (Clade I) retrieved as a sister group of the remaining members of this subfamily, which form a quite well-supported clade (Clade II). The latter contains four main groups (A–D) of which the first three include taxa from the Southern Hemisphere and the fourth from the Palaearctic and Oriental regions. Group A shows three subclades: the pair *Entimus*–*Eudiagogini*, the sampled Leptopiini from South America (*Strangaliodes* Schoenherr and *Vossius* Kuschel) with *Platyaspistes* (Tanymecini) as a sister group of *Vossius* (PP = 1), and the sampled Leptopiini from the Australian region (*Catasarcus* Schoenherr, *Cecyropa* Pascoe and *Irenimus* Pascoe); Group B includes the sister tribes Naupactini–Tanymecini; Group C includes the Eustylini–Geonemini tribes, even though they may not be reciprocally monophyletic; and Group D consists of entimines mainly from the Palaearctic and Oriental regions. Within Group D, there are three subgroups, one corresponding to Bachyderini, Phyllobiini, Sciaphilini and Polydrusini; another to Otiorhynchini; and a third to Laparocerini with *Simo* Dejean (Peritelini), sister of the strongly supported pair *Drouetius* Méquignon (Peritelini) and *Celeuthetes* Schoenherr.

The following entimine tribes are recovered as monophyletic, with either maximal or almost maximal support: Cylydorrhynini, Eudiagogini, Naupactini, Tanymecini (except for *Platyaspistes*), Brachyderini, Polydrusini, Sciaphilini, Otiorhynchini, and Laparocerini. Conversely, the relationships among tribes are moderate, weakly or not supported, except for the sister groups Tanymecini–Naupactini, Geonemini–Eustylini (not reciprocally monophyletic) and Phyllobiini–Brachyderini (among the Palaearctic taxa).

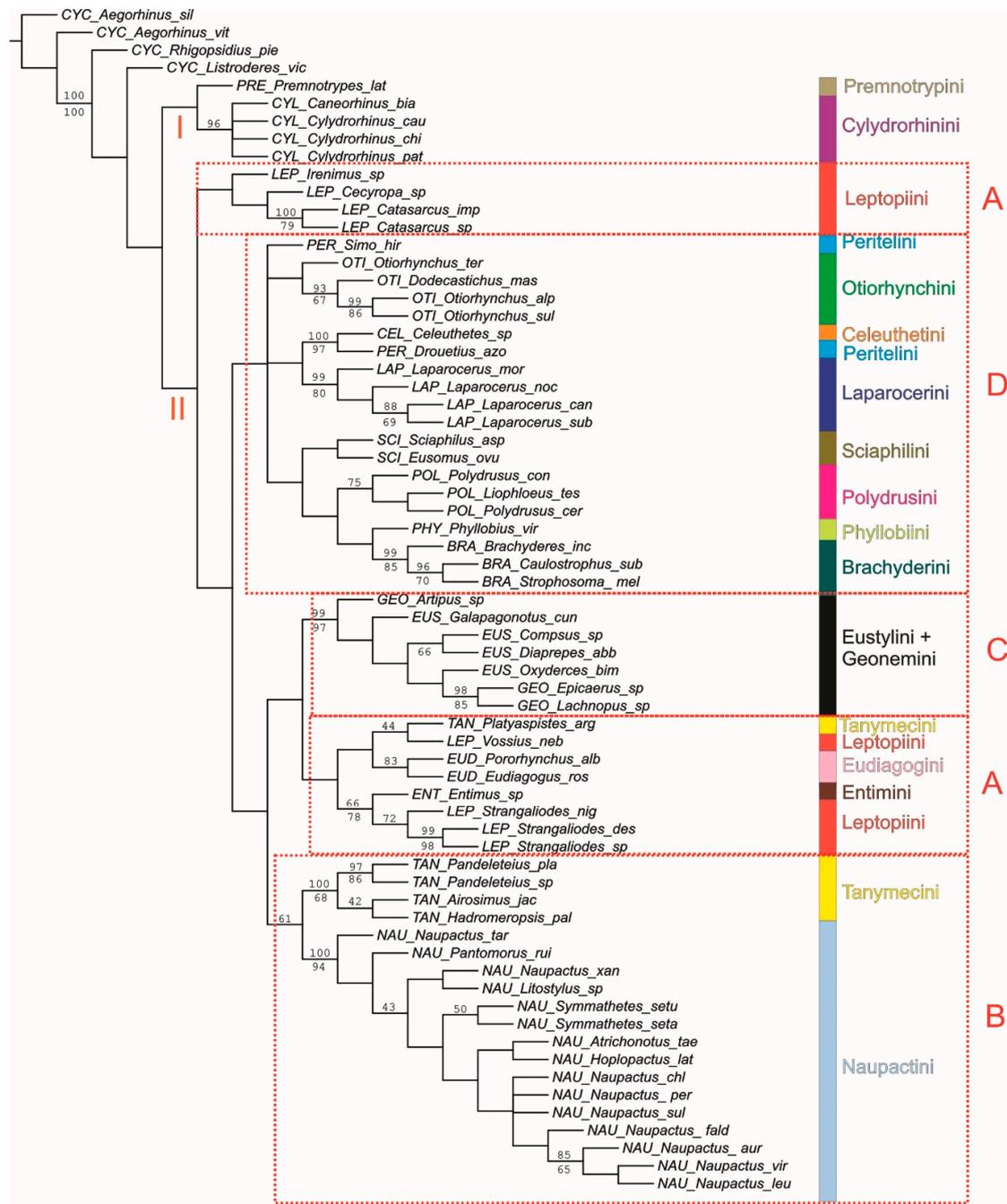


**Figure 5.** Phylogenetic tree of Entiminae resulting from partitioned Bayesian analysis (MCC) of DNA sequences from four markers and morphological data (see Section 2 Material and Methods). Posterior probability values indicated above branches. Clades discussed in the text are indicated in red (I, II, and A–D).

### 3.2.2. Combined Maximum-Parsimony Analysis and Synapomorphies

The Maximum Parsimony (MP) analysis yielded four most parsimonious trees ( $L = 3325$  steps;  $CI = 0.35$ ;  $RI = 0.53$ ), the strict consensus of which is shown in Figure 6. Bootstrap values over 40% are indicated above the branches of the tree. The main differences from the Bayesian (BI) tree are as follows: (1) The Leptopiini from the Australian region (*Catasarcus*, *Cecyropa* and *Irenimus*) are separated from those of South America (*Vossius* and *Strangaliodes*) (Group A of the BI tree is broken up). (2) The remaining entimines are grouped into two main clades, one including tribes mainly distributed in the Palearctic and Oriental regions, and the other including those from the Neotropics, Andes and Patagonia. (3) The latter clade includes Groups B and C and the Neotropical members of Group A of the BI, *Strangaliodes*–*Entimus*, the pair *Platyaspistes*–*Vossius*, and the Eudiagogini. (4) Within the Palearctic and Oriental Group D, the consensus tree shows that the basal relationships are not resolved, resulting in a polytomy of *Simo* (Peritelini), Otorhynchini, *Laparocerus* Schoenherr and *Celeuthetes*–*Droetius*,

and a clade of the remaining tribes, within which Polydrusini are closer to Brachyderini and Phyllobiini than Sciaphilini.



**Figure 6.** Strict consensus tree of four most parsimonious trees of Entiminae resulting from Maximum Parsimony analysis of DNA sequences from four markers and morphological data (see Material and Methods). Bootstrap values from the combined molecular and morphological data are indicated above branches and Bootstrap values from the separate molecular data are indicated below branches. Tribes are indicated in different colors. Clades recovered in the Bayesian tree are indicated in red.

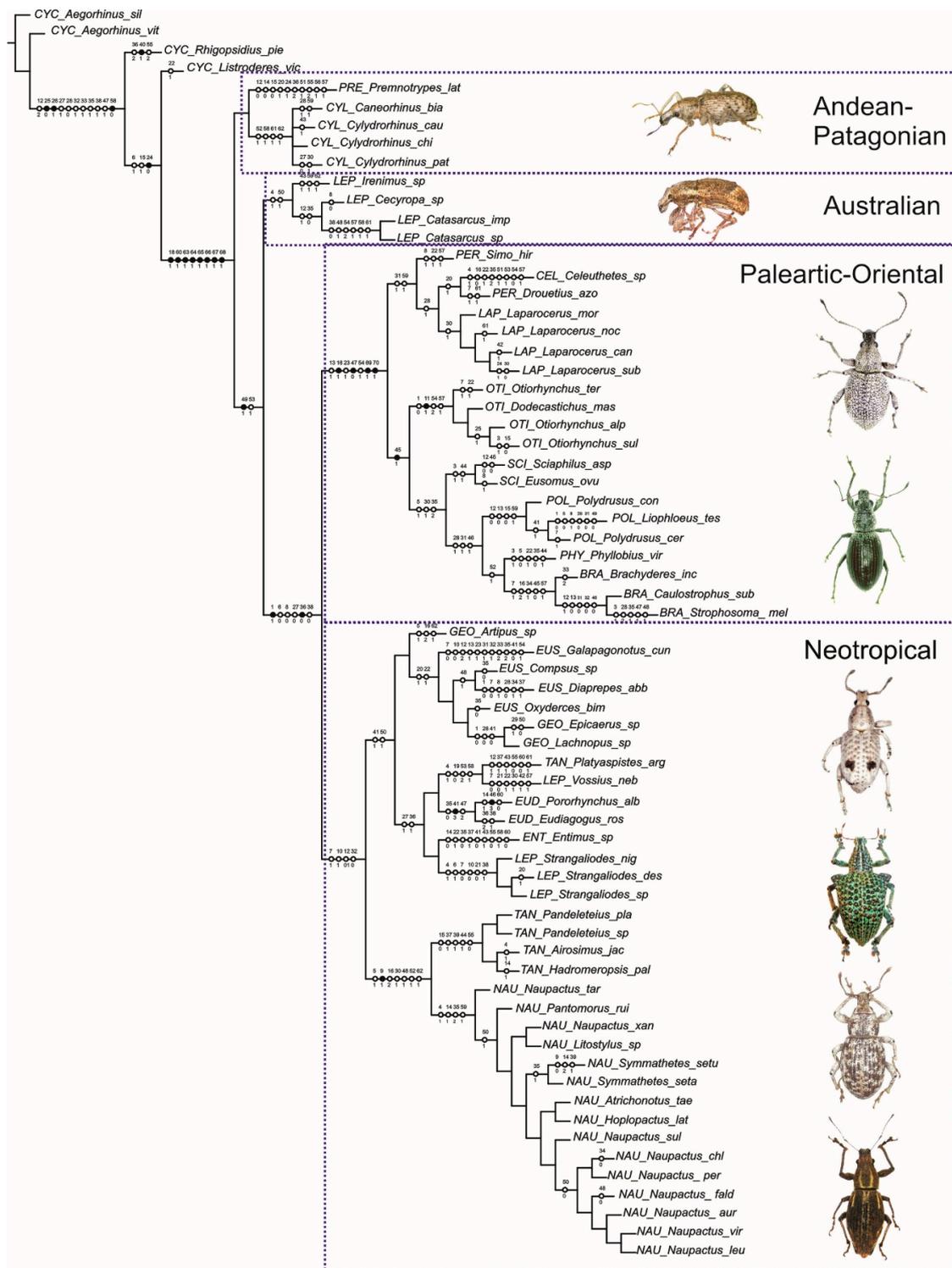
The selected MP tree (Figure 7) shows unambiguous optimization of morphological characters (see Figures S1 and S2 for character changes under fast and slow optimization options), with unique and homoplastic changes in black and white, respectively. It suggests that the monophyly of Entiminae

is mainly justified by the presence of a scar or lasting appendage in the mandibles (18.1) and several larval synapomorphies: antennal sensorium wider than long, cushion-like (63.1) and elliptical in apical view (64.1), maxillary mala with four setae (65.1), mandibles without accessory teeth on the intermediate portion of the cutting edge (67.1) and slightly sclerotized mandibular scrobes, which are paler than the rest of the mandible (68.1). The monophyly of entimines is also supported by two other adult mouthpart characters using the fast optimization (Figure S1): absence of two or more teeth on the inner margin of mandibles (17.1) and prementum completely concealing the maxillae (19.1); however, in Cylydrorhinini, these characters appear to have reversed to the primitive condition and the mandibular scar is sometimes markedly reduced.

Most Entiminae, except for Cylydrorhinini and Premnotrypini, share the synapomorphies of tibiae lacking spurs (49.1) and moderately separated tarsal claws (53.1), although this character shows further change. Some European groups such as Otorhynchini have tibial spurs but these are usually more slender and shorter than those of Cyclominae (Aterpini and Listroderini) and Cylydrorhinini. Other characters supporting the monophyly of this group under fast optimization (Figure S1) are: epistome not projecting beyond the anterior margin of the rostrum (2.0) and rostrum not separated from the forehead by a deep impression (21.1). However, rostrum and forehead are separated by a deep impression in several Leptopiini from Australia, mainly *Catasarcus*.

Except for the Australian Leptopiini, the remaining entimines form a clade supported by characters of the rostrum, which is usually less than  $1.25 \times$  as long as wide at the apex (1.1), has an impressed or flat dorsum (6.0) and is neither unicarinate nor tricarinate (8.0); eyes usually rounded (27.0); absence of postocular lobes (36.0); and a truncate-conical or subcylindrical pronotum (38.0), in contrast to the subhexagonal pronotum of most South American Listroderini, Cylydrorhinini, and Premnotrypini. This clade split into two major subclades, one including the Palaeartic and Oriental tribes and the other the mainly Neotropical tribes. The Palaeartic and Oriental subclade is justified by the following characters: slightly defined scrobes widening backwards (13.1), occipital sutures transversal relative to the longitudinal axis of the rostrum and usually not connected with eyes (16.1), setae behind eyes directed posteriad (23.1), reduced to indistinct mucro of protibiae (47.0), narrow metepisternum and complete metepisternal-metasternal suture (54.1), larvae with labral rods diverging in their distal half (69.1) and larvae with a single seta on the alar area (70.1). The Neotropical subclade is justified by a usually present rostral groove (7.1), scrobes partially visible in dorsal view (10.1) and strongly curved towards the ventral side of the head or passing below the eyes in lateral view (12.0–1), and a scape passing below the eyes (32.0).

The intertribal relationships within both Palaeartic and Oriental and Neotropical subclades are weakly supported by synapomorphies. Within the Palaeartic and Oriental subclade, the group that includes most of the tribes (i.e., all except Laparocerini, Peritelini and Celeuthetini) is mainly justified by the presence of teeth on the three pairs of femora (45.1). Within the Neotropical subclade, the relationship between Tanymecini and Naupactini is the best supported based on the following characters: rostrum with subparallel or convergent sides toward the apex (5.1), and usually bicarinate (9.1); occipital sutures reduced to a small fovea (16.2), a usually slender and capitate scape (30.1), inner margin of protibiae with a line of denticles (48.1), presence of dorsal comb at metatibial apex (52.1) and presence of baculi along the ovipositor (62.1). Most of these characters are also present in other tribes, e.g., Eustylini–Geonemini. Although baculi are also found along the ovipositor of Australian and South American Cylydrorhinini and Leptopiini, in these groups, they are more laterally placed and usually curved at the distal end.



**Figure 7.** Most parsimonious tree of Entiminae, selected from the four MPTs obtained after combined parsimony analysis. Morphological unambiguous character changes are indicated at branches, with numbers above and below corresponding to character and state, respectively; unique changes are in black and homoplastic changes in white. The most important clades are illustrated and highlighted with boxes and their main biogeographic areas are indicated.

### 3.2.3. Separate Molecular Analyses (Bayesian and Parsimony)

The monophyly of Entiminae was supported by the BI and MP trees derived from combined molecular and morphological data, but not by those obtained from molecular data only (Figures S3 and S4), because *Listroderes* and *Rhigopsidius* Heller were included in the ingroup. In addition, several suprageneric groups (e.g., tribes) were resolved as monophyletic independently of morphological evidence (e.g., Cylydrorhinini, Naupactini, Tanymecini (except for *Platyaspistes*), Eudiagogini, Brachyderini, Otiorhynchini (except for *Otiorhynchus* subgenus *Metopiorrhynchus*) and Laparocerini). Moreover, both the BI and MP molecular trees justify the relationships Geonemini–Euslylini (including *Artipus* and *Galapagonotus*) and *Celeuthetes*–*Drouetius*, while the relationship *Entimus*–*Strangaliodes* was supported by the BI molecular tree as well as the combined and molecular MP trees.

## 4. Discussion

### 4.1. Southern South American Entiminae and Cyclominae

The Cyclominae are widely distributed in the Southern Hemisphere [12,91,92], with some fossils being found in Antarctica [93], suggesting a Gondwanan ancestry [52]. The tribe Aterpini, used as outgroup in our analyses, is distributed in the Australian region (Australia, Tasmania, New Guinea, New Caledonia and New Zealand) and southern Argentina and Chile [94], along the southern Andes from 37° S to the Cape Horn, including the archipelago of southern Chile, Tierra del Fuego, South Georgia, and the Falkland Islands, an area mainly corresponding to the Subantarctic subregion [51,95,96].

Listroderini, Cylydrorhinini and Leptopiini are other tribes of Cyclominae and Entiminae with South American elements that would have close relatives in the Australian region [49,58,97–102]. However, they are not mainly associated to the Subantarctic and have diversified in northern and eastern areas, mainly Central Chile, Patagonia and the Andes. In comparison with Aterpini, these tribes are less clearly related to the Australian groups, probably because the latter areas have been subjected to more drastic geological and environmental changes after the separation of the southern continents [99]. It is worth mentioning that *Rhigopsidius* Heller and *Listroderes* Schoenherr are sister taxa in the Bayesian tree, consistent with the hypothesis of Oberprieler [91] that Rhythirrinini are confined to the African region and *Rhigopsidius* is assumed to belong to the tribe Listroderini. Morrone [52], who recovered the relationship of *Rhythirrinus* Schoenherr with *Rhigopsidius* based on a morphological cladistic analysis of Listroderini using other cyclomines as outgroups, considered that this inference required further confirmation. From a biogeographical point of view, it seems more plausible that *Rhigopsidius* is more closely related to other Andean genera of Listroderini than to any African Rhythirrinini. The morphology of *Rhigopsidius*, including that of the female genitalia [91,103], suggests that it is a Listroderini which has acquired new characters in response to a new environment. Indeed, the species of this genus inhabit the Andean region from southern Peru to Argentina at higher altitudes than do most other listroderines (mainly highland plateau of Puna), and their larvae bore into potato tubers [103], unlike the aerial and ectophytic larvae of most members of this tribe.

Several authors have classified Listroderini and Cylydrorhinini in the same subfamily Cylydrorhinae [104–107]. Their distribution ranges overlap but Cylydrorhinini mostly occur from 36° to 48° S, while Listroderini are mainly distributed from 48° S southward [48,52], including the Tristan da Cunha-Gough Islands [49]. Both tribes are phanero gnathous, with a subcylindrical rostrum which is frequently unicarinate or tricarinate and has a transversal impression separating it from the forehead. In addition, they show well-developed postocular lobes and oval, dorso-ventrally oriented eyes; a typical subhexagonal pronotum which is transversally impressed behind the anterior margin; tibiae with spurs and widely separated tarsal claws (Figure 4d). The latter two characters are most likely plesiomorphic and have not been found in other South American Entiminae. These tribes mainly differ in larval morphology [49,74], and the adults show the following differences: Listroderini lack mandibular scars and their ovipositor is reduced to a pair of distal gonocoxites, whereas Cylydrorhinini

have mandibular appendages or scar (though sometimes extremely reduced) and a long ovipositor with baculi.

The monophyly of Entiminae was recovered by our analyses based on molecular and morphological data (including Cylydrorhinini and excluding Listroderini), but not by the trees obtained from molecular data only. A more accurate analysis of the relationship between Cyclominae and Entiminae—with a particular focus on Listroderini and Cylydrorhinini—would be achieved by a modern taxonomic revision involving diverse, yet poorly studied, genera such as *Cylydrorhinus*. Moreover, such phylogenetic analysis should include increased taxon and character sampling of both groups and allied taxa.

The Clade I, Cylydrorhinini–Premnotrypini in trees from BI and MP analyses based on combined evidence, is rather weakly supported. Under slow optimization (Figure S2), this relationship is justified by having the anterior part of the epistome usually raised and projecting beyond the anterior margin of the rostrum (2.1). The Premnotrypini occur in the high Central Andes, from Colombia to northern Chile, within 2800–4500 masl. The adults show some particular features (e.g., presence of false corbels) and external similarities with *Rhigopsidius* (see Figure 3d) probably because adults and larvae share habits associated with development in potato tubers [108,109]. Interestingly, the geographic distributions of the South American groups that attack potatoes in the Andes (*Rhigopsidius* and *Premnotrypes* Pierce) are partially sympatric with those of their putative related taxa (Listroderini and Cylydrorhinini, respectively).

The Leptopiini (type genus *Leptopius* Oke endemic to Australia) sensu Kuschel [58,97] basically correspond to the entimines from the Southern Hemisphere, which are listed in Tropiphorini by Alonso-Zarazaga and Lyal [2]. They share some characters with Cylydrorhinini, particularly those of the rostrum and the hexagonal pronotum. However, they are adelognathous, as are most Entiminae, their tibiae lack spurs, their tarsal claws are less separated than in Cylydrorhinini, and their metatibial apex usually has true corbels (also referred to as close corbels). The leptopiines are particularly well-represented in extreme environments such as deserts, mountain areas and Subantarctic islands [58,97,101,102] although some also occur in temperate forests [110]. The external expansion of the protibiae in Leptopiini from deserts may represent an adaptation to this environment [45].

Kuschel [97] described several genera and species of leptopiines from arid and semiarid environments in Chile, Argentina and Peru [58,107]. More recently, new synonymies, new species and a new genus have been established [45,110,111]. Thompson [43] revised the Australian genus *Catasarcus* and Brown [46] studied some genera endemic to New Zealand, such as *Irenimus* and *Cecyropa*.

The South American Leptopiini, *Strangaliodes* and *Vossius* form a monophyletic group with those from the Australian region in the Bayesian tree (Figure 5), but not in the MP tree (Figures 6 and 7). The former result could be interpreted as a phylogenetic signal of an ancient southern connection, whereas the MP tree suggests that *Strangaliodes* is related to some typical Neotropical entimines. In fact, *Strangaliodes* is distributed in the Transition Zone sensu Morrone [56] between the Brazilian and Patagonian biotas, the latter being more related to the Southern Hemisphere fauna. The relationship *Strangaliodes*–*Entimus* is mostly justified by molecular evidence, recovered in both combined and molecular MP trees and in the BI molecular tree. According to previous phylogenetic analyses based on morphology, *Strangaliodes* would be closer to other southern South American leptopiines, e.g., *Geniocreminus* Kuschel and *Megalometis* Schoenherr, and less related to *Leptopius* and some genera from the Australian region [44]. Conversely, Entimini would be related to the Neotropical Lordopini [14]. Unfortunately, no samples of these groups were available in our study.

Although it is beyond the scope of our analyses, we consider that the wide concept of Tropiphorini (type genus *Tropiphorus* Schoenherr from the Palaearctic) as in Alonso-Zarazaga and Lyal [2] is not appropriate for the South American fauna, because the Palaearctic Tropiphorini exhibit a different combination of characters, such as mandibles without an obvious scar, absence of postocular lobes and connate tarsal claws. Therefore, we prefer to use the name Leptopiini as originally used by Kuschel [97] and more recently by Pullen et al. [55], at least until a more comprehensive study is carried out. According

to molecular analyses based on mitogenome data, the Palaearctic Tropiphorini would be related to Otiorhynchini [7,8]. Moreover, we disagree with the placement of *Strangaliodes* in the Palaearctic tribe Alophini (as a synonym of Tropiphorini) as proposed by Alonso-Zarazaga et al. [112], because of the synonymy of the monotypic genus *Ctenolobus* Debrochers based on a single specimen from Morocco, which was probably mislabeled.

#### 4.2. Neotropical Entiminae

Entimini, Eudiagogini, Naupactini and Eustylini have been based on Neotropical genera and are highly diversified in this region, particularly in tropical and subtropical forests, although some derived lineages have colonized other areas and a few genera or species occur in the Nearctic [28,113].

Entimini include 13 genera [14], most of which have been revised taxonomically [13,15,114,115]. They are composed of large species (about 20 mm long) with iridescent scaly vestiture and well-developed humeri and hind wings; their rostrum usually shows a median sulcus and the postocular lobes are always present. Females have a subtriangular sternite VIII, with a slightly longer than plate apodeme—as in most entimines—and a short ovipositor (about one-third the length of the abdomen) bearing styli and sclerotized proximal and distal gonocoxites [15]. The phylogenetic position of Entimini is doubtful because our analyses included a single species of *Entimus* and lacked representatives of other allied taxa, such as Lordopini [14].

Eudiagogini are probably close to Entimini (in the BI, Figure 5), as suggested by shared larval features [74], but their adults are differentiated by having several apomorphic characters: a very short rostrum with broad pterigia; very pronounced postocular lobes covering more than half of the eyes; laterally expanded mandibular lasting appendages in some species (see Figure 1b); protibiae bearing a hook-like micro, which forms a forceps with a tuft of stiff setae; metafemora being usually wider than the profemora; and metatibial apex showing a very broad, squamose corbel. The type genus *Eudiagogus* Schoenherr was taxonomically revised by Warner [116] and studied by O'Brien and Kovarik [117].

The results of both combined analyses indicated that Naupactini are monophyletic and closely related to Tanymecini, but this relationship was not recovered in the separate molecular analyses. Most species in our sampling belong to the *Pantomorus*–*Naupactus* complex [29], but other genera outside this complex (e.g., *Cyrtomon* Schoenherr, *Stenocyphus* Marshall, *Hadropus* Schoenherr, *Ericydeus* Pascoe, *Briarius* Fischer de Waldheim) are more similar to some Eustylini (e.g., *Compsus* Schoenherr, *Exodercus* Schoenherr) [28], probably by convergence.

The Eustylini *sensu* Franz [18] are an exclusively Neotropical assemblage with 20 genera and approximately 325 species, *Compsus* being the most diverse genus (104 species). This tribe is the subject of ongoing studies by Franz and colleagues [20,118,119], who suggested that Geonemini are paraphyletic relative to Eustylini [18]. According to our analyses, Eustylini become monophyletic when the Neotropical Geonemini are included. Moreover, we corroborated that *Artipus* belongs to the clade Eustylini–Geonemini, and is not a Naupactini (see [28]), while *Galapagonotus* Anderson & Lanteri and probably also *Coconotus* Anderson & Lanteri (not included) belong to Eustylini [120].

On the other hand, *Geonemus* Schoenherr as well as other genera currently assigned to Geonemini occur in the Palaearctic region (e.g., *Barynotus*) and we are doubtful about their close relationship with Neotropical Geonemini. This uncertainty is supported by results based on mitogenome data [8]. We think that Geonemini are probably valid for some genera outside the Neotropics, but this issue must be addressed by specialists.

Something similar occurs with Tanymecini, whose type genus *Tanymecus* Germar is from the Holarctic region. The Neotropical tanymecines have been extensively studied by Howden [21–25], particularly *Pandeleiteius* Schoenherr, *Hadromeropsis* Pierce and *Airosimus* Howden, which were included in our analyses and constitute a well-supported monophyletic group. Conversely, *Platyaspistes* Schoenherr, currently classified in the Tanymecini subtribe Piazomiina (type genus *Piazomiina* Schoenherr from Africa and Asia) has never been found to be related to the remaining Tanymecini but rather to *Vossius* (Leptopiini). The larval characters of *Platyaspistes* studied by Marvaldi [74] concur

with those of the leptopiine larvae (e.g., two alar setae) but not with those of the larvae of Piazomiina, as in *Piazomias* and *Leptomias* Faust, and other Tanymecini as in *Pachnaeus* Schoenherr, described by van Emden [121].

*Platyaspistes* includes five Chilean species distributed from the Atacama desert to Valparaiso (with one present in Argentina), and differs from the sampled Tanymecini and other Neotropical Entiminae in its tarsal claws, which are connate –as in most of the Holarctic entimines studied herein– but unequal in length. Moreover, the distal coxites of the ovipositor are strongly sclerotized and bifurcated and females show a particular type of oviposition, as observed in *Platyaspistes glaucus* Farhaeus [122] and in *P. argentinensis* Kuschel [123]. The eggs are laid between, and glued to, two adjacent surfaces of a host plant leaf which was previously folded by the female, in the same way as does *Cyphometopus marmoratus* (Blanchard) (currently classified in Leptopiini). The larvae emerge through a hole made by gnawing the meristem and complete their development in the soil as do other entimines [122].

Although there was not complete agreement between the results of the Bayesian and the Parsimony analyses regarding the Leptopiini, we propose to transfer *Platyaspistes* from Tanymecini to Leptopiini.

#### 4.3. Palaeartic, and Australian and Oriental Entiminae

Both analyses recovered the entimine tribes mainly distributed in the Palaeartic region as a monophyletic group. In our study, every tribe represented by more than one species/genus is monophyletic, although no definite conclusions can be drawn due to insufficient taxon sampling. In general terms, we conclude that Brachyderini, Phyllobiini, Polydrusini and Sciaphylini might be closer to each other than to Otiorhynchini, Laparocerini and Peritelini.

The phylogenetic position of *Drouetius* is controversial. It was formerly treated as a subgenus of *Laparocerus* [35] and later as a separate genus [33] and was assigned to Peritelini [33,53]. *Laparocerus* includes about 240 flightless species and subspecies endemic to the Atlantic islands of Macaronesia (Madeira, Selvagens, and Canary Islands), except for one species from northern Africa, Morocco [36], whereas *Drouetius* is endemic to the Azores Islands (northern Macaronesia), thus more distant geographically.

In a Bayesian analysis based on 16SrRNA, *Drouetius* was closer to *Peritelus* Germar than to *Laparocerus* [33] and probably for this reason it was transferred to Peritelini. In our analyses, *Drouetius* is the most closely related to *Celeuthetes* sp. and the pair *Drouetius*–*Celeuthetes* is within the same group as *Laparocerus* (Figures 6 and 7) or as *Laparocerus*–*Simo* (Peritelini) (Figure 5). The *Celeuthetini* are distributed in the Oriental or Indo-Australian region, mainly diversified across islands of the Pacific (New Guinea, Molucas, Sulawesi and Lesser Sunda Islands) [124]. According to a recent phylogenetic analysis, this tribe is most likely related to other Indo-Australian entimines, e.g., Pachyrhynchini [125] and, therefore, we believe that the close relationship *Drouetius*–*Celeuthetes* may not be recovered in further studies expanding the taxon sampling.

Our results support the hypothesis that the genus *Drouetius* is independent from *Laparocerus*, but reject the hypothesis that it belongs to Peritelini. Indeed, the BI tree (Figure 5) shows that the only Peritelini included in our analysis (*Simo*) is close to *Laparocerus* and not to *Drouetius*, and in the MP trees *Simo* is in an unstable position (Figure 6). A more complete taxon sampling is necessary to elucidate the correct tribal placement of *Drouetius*.

#### 4.4. General Remarks

Our study provides evidence for the monophyly of several Neotropical tribes, however, the relationships among the large clades are in general weakly supported by both analyses, preventing us from drawing firm conclusions. Notwithstanding this, results suggest that the Palaeartic entimines evolved independently from the typical Neotropical ones, e.g., Entimini, Eudiagogini, Eustylini, Naupactini, Tanymecini and other tribes not included in our analysis, such as Lordopini and Anypotactini. Future research should be aimed at expanding the geographic representation of

tribes/genera with disjoint distributions in both hemispheres or in different continents, as is the case highlighted by Kuschel [48] for the mainly Holarctic Polydrusini/*Polydrusus* occurring in southern South America. This information would be helpful to discriminate between old clades widely distributed in the past and artificial taxa defined on the basis of convergences.

The striking similarity between some derived lineages of Palaearctic and Neotropical entimines, attributed to the subfamily Brachyderinae in older classifications, e.g., *Strophosoma* Billberg (Brachyderini) and some species currently assigned to *Symmalthetes* Schoenherr or *Pantomorus* Schoenherr (Naupactini) is most likely due to convergent evolution.

As already suggested by Kuschel [48,50,94], some South American entimines (Leptopiini and Cylydrorhinini) as well as some cyclomines (Listroderini and Aterpini) are probably closely related to those of the Australian region. These groups are expected to be ancient and to occupy a relatively basal position with respect to the typical Neotropical tribes (e.g., Entimini, Eudiagogini, Eustylini and Naupactini).

Further investigation with more complete taxon and character samplings is needed to clarify all the tribal relationships within the Entiminae–Cyclominae clade. To achieve a natural tribal classification of the Entiminae, we propose considering not only morphological and molecular evidence, but also important biological features and information on the historical biogeography of the areas they inhabit.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1424-2818/10/3/95/s1>: Table S1: Morphological data matrix. Table S2: Annotated structure alignment of the ribosomal markers. Figure S1: Selected Most Parsimonious tree with morphological character changes under fast optimization. Figure S2: Selected Most Parsimonious tree with morphological character changes under slow optimization. Figure S3: Bayesian tree based on molecular data only. Figure S4: Strict consensus tree of 395 MPITs from parsimony analysis of molecular data only.

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