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Molecular and Morphological Phylogenetic Analysis of *Naupactus* Dejean (Curculionidae: Entiminae) and Allied Genera: The Dilemma of Classification

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Abstract: *Naupactus* (Curculionidae: Entiminae) is the most speciose weevil genus of the tribe Naupactini. The main objective of this work is to recognize species groups within *Naupactus* and to analyze the relationships between this and other Neotropical genera. For this purpose, we compiled a combined data matrix of 60 terminal units corresponding to 40 species for which we recorded 812 molecular and morphological characters (763 and 49 respectively), which were analyzed by Maximum Parsimony and Bayesian analyses. The single tree obtained from each analysis was rooted with *Cyrtomon inhalatus*. The species of *Naupactus* were recovered as different monophyletic groups, some of them closer to other genera of Naupactini (*Lanterius*, *Teratopactus*, *Pantomorus* and *Parapantomorus*) than to species of the same genus. We conclude that *Naupactus* is non-monophyletic, even though most species can be recognized based on a particular combination of morphological characters, which are probably symplesiomorphic. To be consistent with the cladistic principles, some genera diversified in marginal areas of the *Pantomorus*-*Naupactus* complex should be synonymized with *Naupactus*; however, these nomenclatural changes may not ensure a generic definition based on synapomorphies. We prefer to be conservative about the current classification until more evidence is available. The only nomenclatural amendments proposed herein are the transference of *Naupactus inermis* Hustache to *Lanterius* and of *N. setarius* to *Symmalthetes*.

Keywords: Neotropical region; broad-nosed weevils; Naupactini; *Pantomorus*-*Naupactus* complex; phylogeny; COI; combined evidence

1. Introduction

Naupactini is one of the most diverse tribes of broad-nosed weevils in the Neotropical Region [1,2]. It includes about 500 described species, several of them with agricultural importance, that probably represent half of its real diversity. This tribe may not be monophyletic in its present definition because it includes some genera from Africa, New Guinea, North America and an extinct genus from the Baltic Amber [3], which probably do not belong to this tribe. However, all the Neotropical genera and a few ranging also in the Nearctic region (e.g., *Phacepholis* Horn, *Ericydeus* Pascoe) [4,5] are likely to have a more recent common ancestor, as suggested by previous phylogenetic analyses based on morphological and combined data [2].

The generic definition of *Naupactus* has varied according to different authors [6–11]. For example, Morrone [12] includes in *Naupactus* most of the South American species traditionally placed in *Pantomorus* Schoenherr [13]. The actual number of species is uncertain due to the lack of a comprehensive revision. According to the last checklists of American weevils [12–14], there are more than 200 nominal species of *Naupactus*. However, it is possible that some of them should be synonymized because they are morphotypes, geographic races or males and females of the same species, others should be transferred to other genera of Naupactini, while many new species still remain to be described.

A preliminary phylogeny of 54 genera of Naupactini based on 69 terminal units (type species or species representative of these genera) and a set of 100 morphological characters suggests that there are three main clades (I, II and III), the latter being the most diversified in species and genera and divided into three major subclades (A, B and C) [2]. The analysis revealed that *Naupactus* belonged to clade III and the fact that the three species analyzed (types of genera considered synonyms of *Naupactus*) were distributed in subclades B and C would indicate that the genus is not monophyletic [2]. Likewise, phylogenetic analyses of a small set of *Naupactus* from South America [15] and from Central and North America [16] showed that the genus is not monophyletic, with species being placed close to the root of the tree.

In this study, we increased the sample size by adding more species of *Naupactus* and other genera of the *Pantomorus*-*Naupactus* complex [2,4,15–18] to recognize well-supported groups in an attempt to gain further insight into a natural generic classification of the naupactines. The recognition of natural groups (either genera or species groups) is essential to understand the evolution of certain characters, e.g., oviposition habits and parthenogenetic reproduction [15,19] and for testing hypotheses about historical biogeography [1,20].

The specific objectives of this contribution are as follows:

1. To recognize different groups of *Naupactus* and to analyze the relationships among them and with other genera of the *Pantomorus*-*Naupactus* complex: *Aramigus* Horn, *Atrichonotus* Buchanan, *Eurymetopus* Schoenherr, *Floresianus* Hustache, *Galapaganus* Lanteri, *Hoplopactus* Chevrolat, *Lanterius* Alonso-Zarazaga and Lyal, *Pantomorus* Schoenherr, *Parapantomorus* Emden, *Phacepholis* Horn, *Symmathetes* Schoenherr and *Teratopactus* Heller.
2. To test the monophyly of *N. leucoloma* Boheman species group [10] and *N. xanthographus* (Germar) species group [21].

2. Materials and Methods

2.1. Taxon Sampling and Morphological Characters

Samples of adult specimens were obtained from Argentina, Brazil, Ecuador, Uruguay, Mexico and Central America (Dominica Island). The new specimens included in this paper were collected from different areas of Argentina and Brazil; they were captured on wild and cultivated plants using a beating sheet or a sweeping net. The material was stored at $-80\text{ }^{\circ}\text{C}$ or in 100% ethanol at $4\text{ }^{\circ}\text{C}$ for molecular analysis. One leg of each specimen was removed for DNA sequencing. Most voucher specimens were deposited at the entomological collection of the Museo de La Plata, Argentina.

Externally visible and dissected structures were observed with a Nikon SMZ1000 stereomicroscope (Japan). For dissections we used standard entomological techniques [18]. The terminology used for morphological characters follows Marvaldi et al. [22], and Lanteri and del Río [2].

We selected 58 specimens corresponding to 40 species and 12 genera of Naupactini recovered in Clade III, subclades B and C *sensu* Lanteri and del Río [2], plus species representative of two genera placed outside this clade, *Cyrtomon* Schoenherr and *Litostylus* Faust, which were used as outgroups. The taxon sampling includes almost all genera of the *Pantomorus*-*Naupactus* complex (except *Alceis* Billberg) and species of the main groups of *Naupactus*. For the phylogenetic analyses we consider only

terminals for which COI sequences were available. Unfortunately, we were not able to get molecular sequences for the type species of *Naupactus*, *N. rivulosus* (Olivier) from South America.

The complete valid names, acronyms, geographic data and accession numbers for the terminal units are given in Table 1. Some species are represented by more than one specimen because they show different morphotypes and/or haplotypes.

Table 1. List of the 60 terminal taxa included in the phylogenetic analysis of the tribe Naupactini, valid names, acronyms used in the analysis, geographic data and genbank accession numbers and source for the terminal units. * The acronyms of *Lanterius inermis* and *Symmalthetes setarius* correspond to the species names previous to the nomenclatural actions taken in this work.

Species	Acronyms	Location	Accession Numbers	Source
<i>Cyrtomon inhalatus</i> (Germar)	<i>Cyrtomon_inh</i>	AR, Entre Ríos, Victoria	MH537926	
<i>Litostylus</i> sp.	<i>Litostylus_sp</i>	Dominica Island, Warner, Caribbean	HQ891471.1	
<i>Aramigus tessellatus</i> Say, morph. <i>tessellatus</i>	<i>Aramigus_tes_tes</i>	AR, Buenos Aires, Otamendi	MH537929	
<i>Aramigus tessellatus</i> Say, morph. <i>pallidus</i>	<i>Aramigus_tes_pal</i>	AR, Buenos Aires, Punta Lara	MH537928	
<i>Aramigus tessellatus</i> Say, morph. <i>santafecinus</i>	<i>Aramigus_tes_san</i>	AR, Buenos Aires, La Plata	MH537927	
<i>Aramigus conirostris</i> (Hustache)	<i>Aramigus_con1</i>	UR, San José, Libertad	MH537930	
	<i>Aramigus_con2</i>	AR, Entre Ríos, Paraná	U25295	[23]
<i>Atrichonotus taeniatus</i> (Berg), morph. <i>taeniatus</i>	<i>Atrichonotus_tae_tae</i>	AR, Mendoza, Guaymallén	MH537919	[23]
<i>Atrichonotus taeniatus</i> (Berg), morph. <i>pictipennis</i>	<i>Atrichonotus_tae_pic</i>	AR, Buenos Aires, Arrecifes	MH537931	
<i>Eurymetopus birabeni</i> Kuschel	<i>Eurymetopus_bir</i>	AR, Buenos Aires	AY790877	[23]
<i>Eurymetopus fallax</i> Boheman	<i>Eurymetopus_fal</i>	AR, Buenos Aires	AY790878	[23]
<i>Floresianus sordidus</i> Hustache	<i>Floresianus_sor1</i>	AR, Misiones	MH537932	
	<i>Floresianus_sor2</i>	BR, RG do Sul, Santa María	MH537933	
<i>Galapaganus galapagoensis</i> (Linell)	<i>Galapaganus_gal</i>	EC, Galápagos, San Cristobal	AF015914	[23]
<i>Hoplopactus lateralis</i> Arrow	<i>Hoplopactus_lat</i>	BR, São Paulo	MH537920	[23]
* <i>Lanterius inermis</i> (Hustache)	<i>N_inermis1</i>	AR, Misiones, Urugua-í	MH537908	IBOL MLPCU0411
	<i>N_inermis2</i>	AR, Misiones, Urugua-í	MH537909	IBOL MLPCU0412
	<i>N_inermis3</i>	AR, Misiones, PP Moconá	MH537910	IBOL MLPCU0407
<i>Lanterius micaceus</i> (Hustache), morph. <i>micaceus</i>	<i>Lanterius_mic_mic1</i>	AR, Misiones, Urugua-í	MH537911	IBOL MLPCU0420
	<i>Lanterius_mic_mic2</i>	AR, Misiones, Urugua-í	MH537912	IBOL MLPCU0418
<i>Lanterius micaceus</i> (Hustache), morph. <i>villosipennis</i>	<i>Lanterius_mic_vil</i>	AR, Misiones, Urugua-í	MH537913	IBOL MLPCU0427
<i>Naupactus auricinctus</i> Boheman	<i>N_auricinctus</i>	BR, São Paulo	MH537921	[23]
<i>Naupactus cervinus</i> Boheman	<i>N_cervinus1</i>	AR, Misiones, Cerro Azul	JX440490.1	[23]
	<i>N_cervinus2</i>	BR, Misiones, Oberá	GQ406843.1	[23]
	<i>N_cervinus3</i>	AR, Córdoba, Río Cuarto	GQ406828.1	[23]
<i>Naupactus cinereidorsum</i> Hustache	<i>N_cinereidorsum</i>	AR, Córdoba	AY770388	[23]
<i>Naupactus condecoratus</i> Boheman	<i>N_condecoratus</i>	AR, Misiones, PP Moconá	MH537914	IBOL MLPCU00406
<i>Naupactus cyphoides</i> (Heller)	<i>N_cyphoides</i>	AR, Misiones, San Ignacio	MH537942	
<i>Naupactus dissimilis</i> Hustache	<i>N_dissimilis</i>	AR, Misiones, Yacutinga	MH537940	
<i>Naupactus dissimulator</i> Boheman	<i>N_dissimulator1</i>	AR, Misiones, PP Las Araucarias	MH537915	IBOL MLPCU0041
	<i>N_dissimulator2</i>	AR, Buenos Aires, Punta Lara	JX440494	[23]
<i>Naupactus leucoloma</i> Boheman	<i>N_leucoloma1</i>	AR, Mendoza	MH537922	[23]
	<i>N_leucoloma2</i>	AR, Entre Ríos, Victoria	MH537934	
<i>Naupactus minor</i> (Buchanan)	<i>N_minor1</i>	AR, Entre Ríos	AY790881	[23]
	<i>N_minor2</i>	AR, Buenos Aires	EU264960	[23]
<i>Naupactus navicularis</i> Boheman	<i>N_navicularis</i>	BR, São Paulo	AY790882	[23]

Table 1. Cont.

Species	Acronyms	Location	Accession Numbers	Source
<i>Naupactus peregrinus</i> (Boheman)	<i>N_peregrinus</i>	AR, Entre Ríos, Concordia	MH537935	
<i>Naupactus purpureoviolaceus</i> Hustache	<i>N_purpureoviolaceus</i>	AR, Entre Ríos, Concordia	MH537936	
<i>Naupactus stupidus</i> Boheman	<i>N_stupidus</i>	ME, Oaxaca, Salina Cruz	GU565274	[23]
<i>Naupactus sulfuratus</i> Champion	<i>N_sulfuratus</i>	ME, Oaxaca, Salina Cruz	GU565270	[23]
<i>Naupactus tremolerasi</i> Hustache	<i>N_tremolerasi</i>	BR, RG do Sul, Santa María	MH537937	
<i>Naupactus tucumanensis</i> Hustache	<i>N_tucumanensis</i>	AR, Tucumán	MH537938	
<i>Naupactus verecundus</i> Hustache	<i>N_verecundus</i>	AR, La Pampa, Santa Rosa	AF211490	[23]
<i>Naupactus versatilis</i> Hustache	<i>N_versatilis1</i>	AR, CABA	MH537939	
	<i>N_versatilis2</i>	AR, Misiones, Teyú Cuaré	MH537916	IBOL MLPCU0117
<i>Naupactus xanthographus</i> (Germar)	<i>N_xanthographus</i>	AR, Buenos Aires, Punta Lara	AY790880.1	[23]
<i>Pantomorus auripes</i> Hustache	<i>Pantomorus_aur</i>	AR, Córdoba	AY770383	[23]
<i>Pantomorus cinerosus</i> (Boheman)	<i>Pantomorus_cin</i>	AR, Córdoba	AY770384	[23]
<i>Pantomorus postfasciatus</i> (Hustache) (misidentified as <i>N. ambiguus</i> [23])	<i>Pantomorus_pos1</i>	AR, Chaco, Resistencia	MH537917	
	<i>Pantomorus_pos2</i>	BR, RG de Sul, Santa Maria	MH537918	
<i>Pantomorus ruizi</i> (Brèthes)	<i>Pantomorus_rui1</i>	AR, Chubut, Trelew	MH537925	[23]
	<i>Pantomorus_rui2</i>	AR, La Pampa	AY770385	[23]
<i>Pantomorus viridisquamosus</i> (Boheman)	<i>Pantomorus_vir</i>	AR, Buenos Aires	AY770386	[23]
<i>Parapantomorus fluctuosus</i> (Boheman)	<i>Parapantomorus_flu</i>	BR, São Paulo	MH537941	
<i>Phacepholis albicans</i> (Sharp)	<i>Phacepholis_alb</i>	ME, Guerrero, Tecpan	GU565278	[23]
<i>Phacepholis globicollis</i> (Pascoe)	<i>Phacepholis_glo</i>	ME, Oaxaca, Salina Cruz	GU565273	[23]
<i>Phacepholis viridicans</i> (Sharp)	<i>Phacepholis_vir</i>	ME, Jalisco, Chamela	GU565277	[23]
* <i>Symmathetes setarius</i> (Boheman)	<i>N_setarius</i>	BR, Mato Grosso	MH537923	[24]
<i>Symmathetes setulosus</i> Hustache	<i>Symmathetes_setu</i>	AR, Catamarca, Las Esquinas	MH537924	[24]
<i>Teratopactus nodicollis</i> (Boheman)	<i>Teratopactus_nod</i>	BR, São Paulo	AY770387	[15]

For each terminal, we recorded data for 49 discrete morphological characters, of which 37 correspond to external morphology and 12 to female and male genitalia; 35 characters are coded as double state (binary) and 14 as multistate. The list of morphological characters is given in Table 2 and the data matrix is shown in Table S1. When genitalia could not be examined (mainly because males are unknown), character states were scored with '?' and treated as missing data. For the illustrations of several characters, particularly those of male and female genitalia, see Lanteri and del Rio [2]. The acronyms used to describe the shape of the rostrum are as follows: WF, maximum width of forehead; WR, width of rostrum at apex excluding borders of scrobes.

The combined data matrix includes 60 terminal units by 812 characters (49 morphological and 763 molecular).

Table 2. List of the 49 morphological characters, character states and codes.

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0. Rostrum, lateral margins: subparallel to slightly convergent anteriorly (WF/WR less than 1.25×) (0); moderately convergent anteriorly (WF/WR 1.25–1.50×) (1); strongly convergent anteriorly (WF/WR more than 1.50×) (2).
 1. Rostrum, lateral carinae: absent (0); present (1).
 2. Mouthparts, prementum, long setae on external face: present (0); absent (1).
 3. Rostrum, anteocular impression: distinct (0); indistinct (1).
 4. Head, eyes: flat (0); convex (1); strongly convex (2); conical (3).
 5. Head, post-ocular constriction: absent to very slight (0); present (1).
 6. Antennae, shape of scape: clavate, broad (0); slightly capitate, slender (1).
 7. Antennae, length of scape: not reaching hind margin of eye (0); reaching to exceeding hind margin of eye (1).
 8. Antennae, relative length of funicle antennomeres 1 and 2: funicle antennomere 2 about as long as antennomere 1 (0); funicle antennomere 2 more than 1.5× longer than antennomere 1 (1).
 9. Antennae, length of funicle antennomeres 4 to 7: distinctly longer than wide (0); about as long as to slightly longer than wide (1).
 10. Pronotum, shape: subconical (0); subcylindrical (1).
 11. Pronotum, convexity of disc (males): flat to slightly convex (0); strongly convex (1).
 12. Pronotum, lateral longitudinal impressions: present (0); absent (1).
 13. Pronotum, lateral tubercles: absent (0); present (1).
 14. Pronotum, macrosculpture of surface: irregularly shaped and connected foveae (0); granulate (1); slightly granulate to smooth (2).
 15. Scutellum, vestiture: present (0); absent (1).
 16. Elytra, shape of scales: rounded (0); oval (1); piliform (2).
 17. Elytra, setae: recumbent (0); erect (1).
 18. Elytra, white, obliquely ascending stripes, on sides on posterior third: absent (0); present (1).
 19. Elytra, white stripes along intervals 6 to 8: absent (0); present (1).
 20. Elytra, brown, rectangular maculae on middle length of interval 3: absent (0); present (1).
 21. Elytra, outline of base: strongly bisinuate (0); slightly bisinuate (1); straight (2).
 22. Elytra, development of humeri: well-developed (0); reduced (1); absent (2).
 23. Elytra, humeral tubercle: absent (0); present (1).
 24. Elytra, declivity of disc: slightly to strongly ascending towards declivity (0); elytral disc not ascending towards declivity (1).
 25. Elytra, height in lateral view: high (0); flat (1).
 26. Elytra, proximity of striae 9 and 10: striae 9 and 10 confluent along posterior 2/3 (0) striae 9 and 10 slightly closer to each other along posterior 2/3 (1).
 27. Elytra, presence of apical tubercles: absent (0); present (1).
 28. Legs, separation of front coxae from each other (females): contiguous (0); separated from each other (1).
 29. Legs, width of front femora less than 1.5× as wide as hind femora (0); more than 1.5× as wide as hind femora (1).
 30. Legs, denticle on front femora: absent (0); present (1).
 31. Legs, row of denticles on inner edge of tibiae: absent to indistinct in all tibiae (0); present in front tibiae only (1); present in the three pairs of tibiae (2).
 32. Legs, mucro of tibiae: present only on front tibiae (0); present on front and middle tibiae (1).
 33. Legs, metatibial apex: not to slightly widened (0); distinctly widened (apex about 1.5–2× as wide as minimum width of tibia) (1).
 34. Legs, corbel at metatibial apex: well-developed (0); indistinct, metatibial apex thickened (1); absent (=metatibial apex simple) (2).
 35. Legs, relative length of combs at metatibial apex: dorsal comb distinctly longer than distal comb (0); dorsal and distal comb about same length (1); dorsal comb shorter than distal comb (2).
 36. Venter, denticles on ventrite 2 of male: absent (0); present (1).
 37. Female terminalia, shape of sternite VIII (plate): subrhomboidal, not elongate (0); subrhomboidal, very elongate (1); suboval (2); subpentagonal (3).
 38. Female terminalia, length of ovipositor (distal plus proximal gonocoxites): ovipositor shorter than abdominal length (0); equal to slightly longer than abdominal length (1).
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Table 2. Cont.

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|-----|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 39. | Female terminalia, setae along each side of baculi, in their posterior half: absent (0); with three pairs of long setae (1); with rows of several setae (2). |
| 40. | Female terminalia, sclerotization of distal coxites: slightly sclerotized (0); strongly sclerotized, projected in a short nail-shaped piece (1); strongly sclerotized, projected in a long nail-shaped piece (2). |
| 41. | Female terminalia, styli: present (0); absent (1). |
| 42. | Spermathecal duct: straight (0); undulate to spiraled (1); curled (2). |
| 43. | Spermatheca, shape of corpus: subcylindrical (0); rounded (1). |
| 44. | Spermatheca, walls of corpus: slightly thickened at proximal portion (0); strongly thickened at proximal portion (1). |
| 45. | Spermatheca, shape and length of collum (=duct lobe): conical, short (0); subcylindrical, long (1); subcylindrical, long and with basal prominence (2). |
| 46. | Aedeagus, length of median lobe relative to its apodemes (=temones): median lobe about as long as its apodemes (0); about twice as long as its apodemes (1). |
| 47. | Aedeagus, shape of apex of median lobe: acute to rounded (0); arrow-shaped (1). |
| 48. | Aedeagus, sclerites of internal sac: absent or not <i>Cyrtomon</i> type (0); sclerites consisted of a pair of lateral struts on each side of a pyriform piece connected with ejaculatory duct = <i>Cyrtomon</i> type (1). |
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2.2. Molecular Data: DNA Assay and Sequencing

Cytochrome Oxidase I (COI) was chosen as molecular marker because is the most commonly used in Naupactini for analyses at species level. COI sequences derived from different sources. Most of them were obtained at the Instituto de Ecología, Genética y Evolución, Buenos Aires (IEGEB-CONICET/UBA), or at the Biodiversity Institute of Ontario, University of Guelph, Canada, and they are available at the Barcode of Life Data Systems (BOLD) [23], Invertebrates from Argentina project, with the participation of A. Lanteri and M.G. del Río. Other sequences have already been published by some of us [15,16,24–27], and one sequence was downloaded from GenBank (see Table S1).

The DNA assayed at the IEGEB-CONICET/UBA was extracted following the protocol of Sunnucks and Hales [28]. The COI mitochondrial gene was amplified using the following primers designed by Normark [29]: S1718 (5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3') and A2442 (5'-GCT AAT CAT CTA AAA ATT TTA ATT CCT GTT GG-3').

Amplification was carried out in a total volume of 50 μ L with 50–100 ng of DNA used as template, 0.5 μ M of each primer (Thermo Scientific, Rockford, IL, USA), 0.1 mM of each dNTP (Promega, Madison, WI, USA), 1.0 unit of Taq polymerase, 3.0 mM MgCl₂ and 1 \times reaction buffer (Thermo Scientific, Rockford, IL, USA). Amplifications were performed in a thermal cycler GeneAmp PCR System 2700 (Applied Biosystems, Inc., Gaithersburg, MD, USA) under the following conditions: 94 °C for 1 min, 35 cycles at 94 °C for 1 min, 46 °C for 1.5 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. Each series of amplifications included a negative control with no template DNA. Double-stranded PCR products were separated by electrophoresis on a 1% agarose gel with TAE buffer containing GelRed TM (GenBiotech, Buenos Aires, Argentina). The PCR products were purified using an AccuPrep purification kit (Bioneer, Daejeon, Korea). DNA was sequenced using a 3130-XL Automatic Sequencer (Applied Biosystems, Inc., Foster City, CA, USA) at the Unidad de Secuenciación y Genotipificado (FCEyN, UBA, Buenos Aires, Argentina). The sequences obtained have been entered into GenBank under the accession numbers MH537908–42.

To avoid amplification of COI pseudogenes [30], sequences were translated according to the invertebrate mitochondrial genetic code in MEGA v. 5 [31] and examined using as reference amino acid sequences obtained for several insect orders [32]. A copy containing no frame-shifts or stop codons was assumed to be mitochondrial [33,34]. Sequence alignment was done using CLUSTAL W [35].

The molecular data matrix included 763 bp of the mtDNA COI gene corresponding to positions 210–973.

2.3. Phylogenetic Analyses

The combined and molecular data sets were analyzed using Maximum Parsimony (MP) and Bayesian approaches. For the MP method, a heuristic search with TBR branch swapping was applied to a series of 500 random addition sequences, retaining 30 trees per replicate, using TNT v1.5 [36]. Clade stability was evaluated by 1000 parsimony bootstrap replications [37] and support values over 40% were mapped onto internal nodes of the tree. All characters were considered as un-weighted and non-additive. For the MP trees we provided the total length (L), consistency index (CI) [38] and retention index (RI) [39].

The Bayesian analysis was performed using BEAST2 v2.4.8 [40] on Cipres Science Gateway (<http://www.phylo.org>) [41] with random starting trees without constraints. The optimal substitution model was selected using the jModeltest software v2.0 [42], on the basis of the corrected Akaike Information Criterion, as suggested by Burnham and Anderson [43]. We applied the substitution models GTR + I + G and Lewis MK for COI and morphological data, respectively. 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 [40]. The analyses were run for a total of 30 million generations with sampling every 10,000 generations. The convergence of the runs was evaluated by accessing log files in TRACER v1.6 [44]. We generated a maximum clade credibility tree in TreeAnnotator v2.4.8 [40], using a burn-in of 10% (3000 trees) and visualized in FigTree v1.4.3 [45].

The trees obtained from both analyses were rooted with *Cyrtomon inhalatus* (Germar) (Naupactini clade II sensu Lanteri and del Rio [2]).

3. Results

3.1. Bayesian Analysis

The tree obtained from the Bayesian analysis is shown in Figure 1. Clades with posterior probabilities ≥ 0.95 are indicated in boldface. *Litostylus* was recovered as the sister genus of the remaining taxa (PP 0.8). The species groups of *Naupactus* are spread into four main clades of the tree, with PP values ≥ 0.50 : clade A includes the pair *N. stupidus*–*N. sulfuratus* (PP 1); clade B includes the majority of species, with *N. tucumanensis* to *N. versatilis* (PP 1) as sister group of *Hoplopactus*–*anterius*–*N. inermis*; clade C includes the pair *N. cinereidorsum*–*N. cyphoides* related to *Teratopactus*; and clade D includes the species usually classified as belonging to genera other than *Naupactus*, except for the group ((*N. dissimilis*–*N. xanthographus*) *N. navicularis*) (PP 1), *N. cervinus*–*N. dissimulator* (PP 1), and *N. setarius*.

The combined approach provided strong evidence for the monophyly of the genera *Aramigus*, *Eurymetopus* and *Phacepholis*, but this would not be the case for *Pantomorus*. In addition, well-supported nodes proved intergeneric relationships for *Galapaganus*–*Phacepholis*, and *Eurymetopus*–*Floresianus* to be robust. The best supported group within *Pantomorus* is *P. auripes*–*P. ruizi* (PP 0.98).

The terminal units regarded as the same species are recovered in the same groups, despite their different geographic origins, sex or morphotypic variation. For example, the two females of *Lanterius micaceus* belonging to the *micaceus* morphotype (originally described as *Mimographus micaceus*) are grouped with the male of *villosipennis* morphotype (originally described as *M. villosipennis*) (synonymy by Lanteri, 1985 [46]). Similarly, the two morphotypes of *Atrichonotus taeniatulus* (*taeniatulus* and *pictipennis*) [18], originally described as different species, are recovered as conspecific.

The length of the branches of the Bayesian tree indicate a very high infraspecific variation in the parthenogenetic species *Aramigus tessellatus*, *A. conirostris* and *N. cervinus*, which show several divergent lineages and/or cryptic species [26,47].

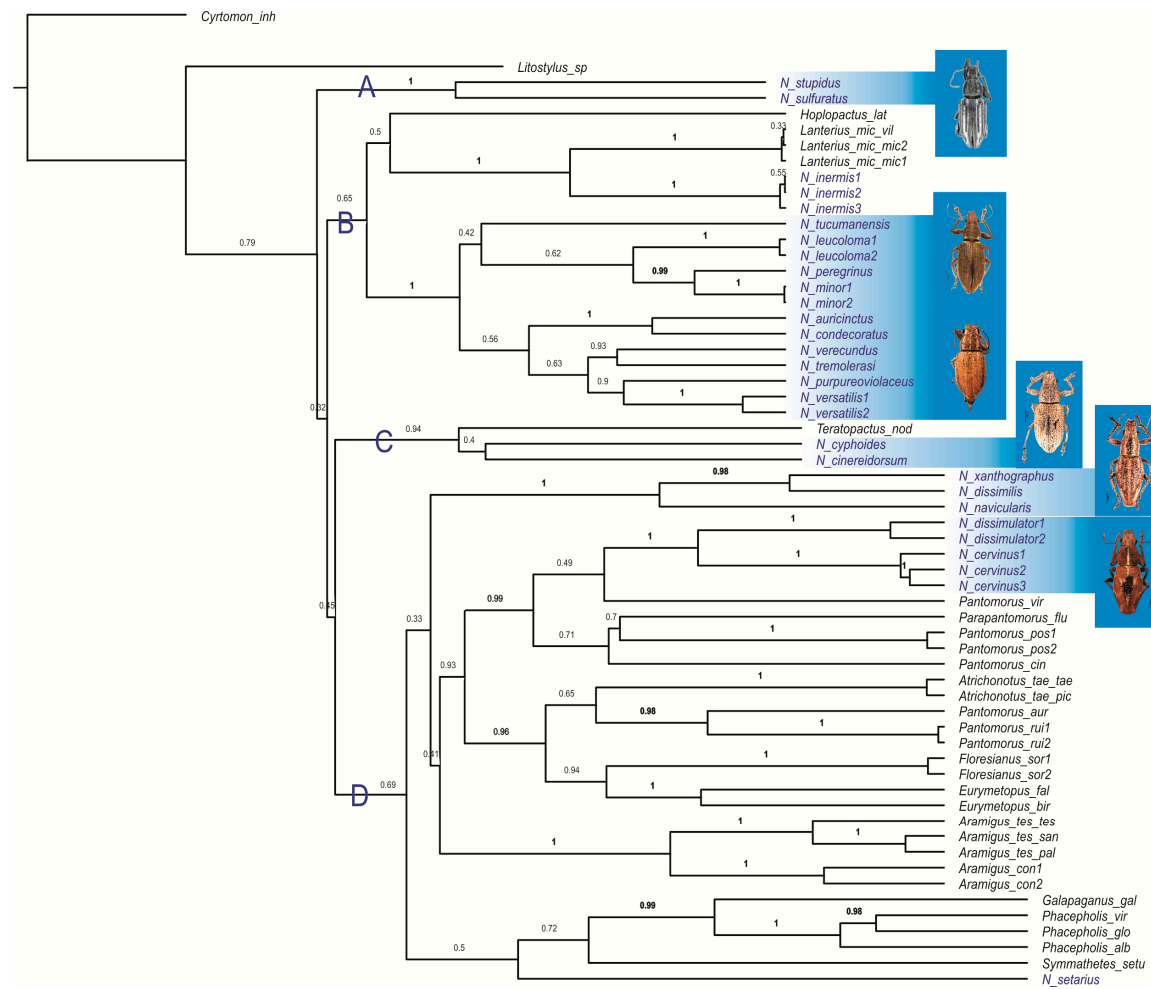


Figure 1. Tree obtained by Bayesian analysis (MCC) from a data matrix of 60 terminal units of Naupactini by 812 characters. Posterior probabilities ≥ 0.95 are indicated in boldface. The groups of *Naupactus* are highlighted in blue and illustrated.

3.2. Parsimony Analysis

The Parsimony analysis of the combined data set yielded four most parsimonious trees (L = 2457 steps; CI = 0.52; RI = 0.27) (Figure 2), which best support the same monophyletic groups as the Bayesian tree. There are changes in the relationships among some weakly supported groups and unstable species: (1) the pair *N. sulfuratus*–*N. stupidus* is recovered in the same clade as *Galapaganus*–*Phacepholis*; (2) the group *N. auricinctus* to *N. tucumanensis* is strongly supported, but the interspecies relationships within it are slightly different from those in the Bayesian tree; (3) *N. cinereidorsum*, *N. cyphoides* and *Teratopactus* are recovered in the same clade as *N. navicularis* (*N. xanthographus*–*N. dissimilis*), and *Aramigus*; (4) *Naupactus setarius* + *S. setulosus* are sister species within a large clade that includes the pair *N. dissimulator*–*N. cervinus* and species of other genera (*Pantomorus*, *Parapantomus*, *Atrichonotus*, *Floresianus* and *Eurymetopus*).

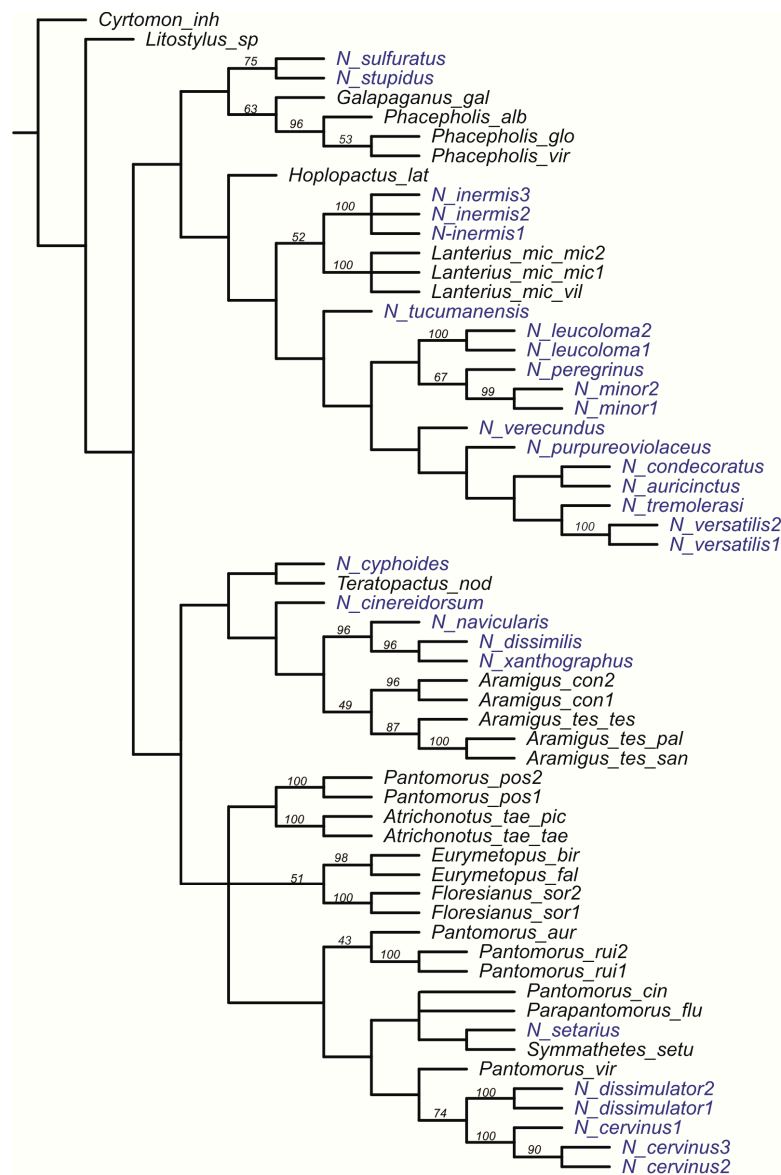


Figure 2. Strict consensus tree obtained by parsimony analysis from a data matrix of 60 terminal units of Naupactini by 812 characters. Bootstrap values ≥ 40 are indicated onto branches. The species of *Naupactus* are highlighted in blue.

4. Discussion

Naupactus is recognized by a particular combination of characters, which is useful for the generic identification but misses out exclusive synapomorphies, e.g., the presence of rows of setae along the ovipositor is a derived character for Naupactini, but also occurs in other genera such as *Lanterius*. Moreover, this feature is lacking in some species of the same genus, e.g., the Central American *N. sulfuratus* and *N. stupidus*. The most diagnostic characters of *Naupactus* are: parallel-sided rostrum, orientated anteriorly and with strong lateral carinae; long antennae with funicular antennomere 2 distinctly longer than antennomere 1; wide pronotum, elevated over the mesothoracic peduncle, lacking tubercles; squamose scutellum; moderately bisinuate to straight elytral base; well-developed to reduce humeri, lacking tubercles; fully-developed to reduce hind wings; slightly separate to contiguous front coxae; front femora distinctly wider than hind femora, lacking large denticles or spines; mucro and row of denticles usually present on inner margin of front tibiae; metatibial apex having broad to slender squamose corbel or lacking corbel; penis without flagellum; proximal half of spermatheca with strongly thickened walls, collum (=duct lobe) usually short and ramus indistinct; ovipositor usually not exceeding length of abdomen, bearing styli and rows of long setae along its posterior two-thirds.

Clade A corresponds to the sister species *N. sulfuratus* and *N. stupidus*, which are the only *Naupactus* from Central America included in our taxon sampling. In the MP tree these species are closer to other Central American or Northern South American naupactines (e.g., *Phacepholis* and *Galapaganus*). Additional information suggests that they might be related to some *Naupactus* from northern South America not included in our analyses, e.g., *N. instabilis* Boheman (from Colombia and Venezuela) and *N. litoris* Bordón (from Venezuela).

Naupactus of clade B are more closely related to *Lanterius* and *Hoplopactus* than to other *Naupactus* in both analyses. Indeed, *N. inermis* was recovered as a sister species of *Lanterius micaceus*. Consequently, we decided to transfer *N. inermis* to *Lanterius*, a genus that mainly differs from *Naupactus* in its smaller body size, slender pronotum, not elevated over the thoracic peduncle, and the 9 and 10 elytral striae separated along their posterior two-thirds. *Hoplopactus* differs from *Lanterius* and *Naupactus* by a distinct apomorphic character, namely the presence of one to three spines on the inner margin of front femora. Neither *Lanterius* nor *Hoplopactus* have yet been taxonomically revised.

The group *N. tucumanensis* to *N. versatilis* is well supported by the combined evidence and includes two weakly supported subgroups: *N. auricintus* to *N. versatilis* is mainly characterized by the undulate to spiraled spermathecal duct, and *N. tucumanensis* to *N. minor* is recognized by a particular color pattern of white stripes along sides of pronotum and elytra, and the penis about $\frac{1}{2}$ longer than its apodemes. The second subgroup corresponds to *N. leucoloma* species group *sensu* Lanteri and Marvaldi [10] described for the white-fringed weevils *N. leucoloma*, *N. minor*, *N. peregrinus*, *N. tucumanensis* and *N. albolateralis*. The relationship *N. peregrinus*–*N. minor* is strongly justified (PP 0.99). Scataglini et al. [15] recovered the sister relationship *N. leucoloma*–*N. minor*, but the species *N. peregrinus* and *N. tucumanensis* were not available for that analysis.

Clade C includes the sister species *N. cinereidorsum*–*N. cyphoides* and *Teratopactus*. The type species of *Naupactus*, *N. rivulosus*, would belong to this group [48]. *Teratopactus* occurs in similar environments (woodlands and savannas) and mainly differentiates from the typical *Naupactus* by the apomorphies of the tubercles at the humeri and, in some cases, on the sides of pronotum; the front coxae separated from each other; the styli of the ovipositor usually lacking, and the distal coxites transformed into strong nail-like pieces adapted to oviposition of isolated eggs in the soil [49].

Clade D includes the species of *Naupactus* assigned to the *N. xanthographus* species group, mainly characterized by the presence of one pair of tubercles at the apex of the elytra [21], *N. cervinus* and *N. setarius*, plus those of some genera other than *Naupactus* (*Aramigus*, *Atrichonotus*, *Pantomorus*, *Parapantomorus*, *Floresianus*, *Eurymetopus*, *Galapaganus*, *Phacepholis* and *Symmalthetes*). Within the *N. xanthographus* species group Lanteri & del Río [21] recognized two subgroups: one comprising *N. xanthographus*, *N. navicularis*, *N. dissimilis* and *N. mimicus*, having well-developed, squamose corbels at the metatibial apex, and the other composed of *N. dissimulator* and *N. marvaldiae*, without corbels.

In our trees, these subgroups are recovered as independent lineages. The former subgroup shows unstable relationships, as evidenced by the different results from the Bayesian and MP trees. *Naupactus dissimulator* is always recovered as sister species of *N. cervinus* based on molecular data and some synapomorphies of the female and male genitalia (e.g., shape of spermatheca and sclerites of the internal sac of the penis). The latter species lacks the pair of tubercles at the apex of the elytra, typical of the *N. xanthographus* species group, suggesting that these tubercles evolved independently at least twice in the genus *Naupactus* and were lost in *N. cervinus*.

Naupactus cervinus is a species complex containing divergent parthenogenetic lineages and cryptic species [26]. It has been classified in *Naupactus* [3], *Pantomorus* [13,14] and *Asynonychus* (type species *A. godmanni* Crotch, junior synonym of *N. cervinus*) [12,50] and according to previous analyses its phylogenetic position is uncertain. It was placed close to *Aramigus* when only morphological characters were used [2], while it was recovered as the sister species of *N. dissimulator* when molecular information was added [15]. In this work we confirm its relationship with *N. dissimulator*, although additional taxonomic information suggests that might be closer to some species not included in our analysis, such as *N. marvaldiae* [21] and other undescribed naupactines close to *Alceis*, considered as a synonym of *Naupactus* in some old classifications [51].

The South American *Pantomorus* herein analyzed (classified as *Naupactus* in Morrone [12]) do not form a monophyletic group. The pair *P. auripes* + *P. ruizi* is recovered in the same group as *Atrichonotus*, *Floresianus* and *Eurymetopus*, and the remaining *Pantomorus* and *Parapantomorus*, in the group that includes *N. cervinus*–*N. dissimulator*. As in the case of *N. cervinus*, we guess that several naupactines from South America alternatively classified in *Pantomorus*, *Parapantomorus* or *Naupactus*, belong to or are more closely related to *Alceis*. Unfortunately, the available molecular information and the taxon sampling are insufficient to take a definite decision about the correct placement of these taxa.

Aramigus (South America) and *Phacepholis* (Central and North America), considered subjective synonyms of *Pantomorus* in some classifications (e.g., [13,51] and later revalidated [4,52]), are monophyletic genera, which is in agreement with previous phylogenetic analyses [16,47]. They are grouped neither together nor with other *Pantomorus*, thus supporting the hypothesis that *Pantomorus sensu* Wibmer and O'Brien [13] is not monophyletic. *Aramigus* is not close to any particular group of South American *Pantomorus* or *Naupactus*, included in our analysis, whereas *Phacepholis* is related to *Galapaganus* [53,54]; however, in previous analysis [2,16], *Phacepholis* is more related to the Central American *Pantomorus* (the type species *P. albosignatus* Boheman from Mexico). We believe that the latter hypothesis is more plausible and that it was retrieved closer to *Galapaganus* because of the absence of species from that area.

Symmathetes was also considered as a synonym of *Pantomorus* in earlier classifications [13,51] and latter revalidated [12]. In our MP tree and in the MP tree using four molecular markers [24], *N. setarius* was recovered as sister species of *Symmathetes setulosus*, consequently we propose to transfer the former species to *Symmathetes* and to establish the new combination *Symmathetes setarius* (Boheman). This species is very similar to the type species *S. kollari* Schoenherr except for its flat eyes. *Symmathetes* mainly characterizes by the expanded metatibial apex, split off in *S. kollari* and *S. setarius*.

Eurymetopus is monophyletic and related to *Floresianus*, based on several morphological synapomorphies and also supported by molecular evidence [15]. Although grouped within the same clade, *Atrichonotus* is not recovered as sister taxon of the pair *Eurymetopus*–*Floresianus* as in Lanteri and del Río [2]. The fact that some species show intermediate characters between *Atrichonotus* and *Eurymetopus*, e.g., *Atrichonotus whiteheadi* Lanteri [55], suggests that the three genera are related.

4.1. Taxonomic Implications of Phylogenetic Analyses

The *Pantomorus*-*Naupactus* complex includes several lineages with derived characters, such as shorter and more conical rostrum, shorter antennae, reduced to absent hind wings and parthenogenetic reproduction, which might have evolved several times, thus obscuring phylogenetic signal and leading to high degrees of homoplasy [2,15,56]. *Pantomorus sensu lato* (including species from different areas

of North, Central and South America) is an example of a non-monophyletic genus diversified in new adaptive zones or marginal areas within the range of this complex. Other groups undergoing diversification in marginal areas would have acquired exclusive synapomorphies, allowing the recognition of monophyletic genera, e.g., *Phacepholis* would have diversified along the western coast of Central America and the Great Plains of North America, and it is recognized by the particular shape of the spermatheca and the presence of a series of small denticles on the second ventrite of the male [4,16,20]; *Aramigus* and *Eurymetopus* have acquired several synapomorphies in the female genitalia (particular shape of spermatheca, sternite VIII or ovipositor) and would have diversified in grasslands and steppes of South America [18,52,57]; and *Galapaganus*, which displays synapomorphies in the male genitalia (setae around the ostium), would have diversified along the western coast of South America and the Galapagos Islands [24,53,54,58,59].

The result obtained herein raises the dilemma that the recognition of several genera within the *Pantomorus-Naupactus* complex leads *Naupactus* to be non-monophyletic. Morrone [12] made an attempt to solve this problem by transferring all the South American species of *Pantomorus* to *Naupactus*, in a checklist based on neither revisionary nor phylogenetic studies. However, these nomenclatural changes did not solve the problem of the monophyly of *Naupactus*, but instead they created a large genus very difficult to circumscribe. According to our analysis, if *Naupactus* is monophyletic, it may include not only the South American *Pantomorus* but also *Hoplopactus*, *Lanterius*, *Teratopactus*, *Aramigus*, *Eurymetopus*, *Floresianus*, *Parapantomorus*, *Galapaganus*, *Phacepholis* and *Symmalthetes*. Moreover, the phylogeny of Naupactini [2] suggests that the naupactine genera diversified in the High Andes, Paramos and Puna (*Amitrus* Schoenherr, *Amphideritus* Schoenherr, *Asymmalthetes* Wibmer and O'Brien, *Leschenius* del Río, *Melanocyphus* Jekel, *Obrieniolus* del Río and *Trichocyphus* Heller) also belong to the *Pantomorus-Naupactus* complex, and might be classified in *Naupactus*.

We conclude that so far there is no satisfactory solution for the classification of the highly diversified weevil genus *Naupactus* and its relatives. One more comprehensive taxon sampling and new molecular evidence will contribute to essential information for a more definite conclusion. Until then, we prefer to maintain *Naupactus* as non-monophyletic and to accommodate the remaining species in species groups, subgroups or genera useful for further evolutionary or biogeographic studies (see [1]), thereby avoiding the creation of unnecessary generic names.

Classification should serve as a general reference system, endowed with explanatory, predictive and heuristic properties providing foundation for all comparative studies in biology [60]. The field of Phylogenetic Systematics [61] has greatly benefited from the use of molecular markers and, more recently, of genomic data, all of which have given rise to novel hypotheses on the evolution of animals and plants [62–65]. However, there is an increasing gap between phylogenetic analyses and classifications based on Linnaean nomenclature, probably because of the difficulties in translating monophyletic groups inferred from molecular phylogenetic signals into words [66]. In addition, there are many other issues affecting final results, such as the poorly known morphology of several taxa that are yet to be revised, genealogies resulting from insufficient taxon samplings, and the effect that the absence of some terminal taxa may have on phylogenetic hypotheses; the fact that a single tree may result in more than one classification, even if it represents a robust phylogenetic hypothesis; and a potential conflict between the dynamic nature of phylogenetic analysis and the desirable stability of the Linnaean Classification and Nomenclature. In the case of hyperdiverse groups of animals, such as weevils, the reduction of the gap between Phylogeny and Classification will take a time. Meanwhile, we attempt to shed light on the evolution of particularly complex taxa, such as the genus *Naupactus*, while being conscious of nomenclatural decisions.

4.2. Taxonomic Amendments

In order to address the taxonomic implications of our phylogenetic results, we propose the following nomenclatural changes, relative to Wibmer and O'Brien [13] and Alonso-Zarazaga and

Lyal [3]: (i) to transfer the species *Naupactus inermis* to the genus *Lanterius*; (ii) to transfer the species *Naupactus setarius* to the genus *Symmalthetes*.

Lanterius inermis (Hustache), new combination.

Symmalthetes setarius (Boheman) new combination.

Supplementary Materials: The following data is available online at <http://www.mdpi.com/1424-2818/10/3/59/s1>, Table S1: Morphological data matrix.

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