

Phylogeny and species delimitation in the group *Rhodocanthopus* of the genus *Passalus* (Coleoptera: Passalidae) inferred from morphological and molecular data, with description of two new species

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Abstract

The genus *Rhodocanthopus* Kaup was originally proposed to include five species; after that, more species were added and different criteria were adopted to delimit it, generating great taxonomic confusion. At present, *Rhodocanthopus* Kaup is considered as a synonym of *Passalus* and is currently named as the group *Rhodocanthopus* without formal taxonomic circumscription. To clarify this, phylogenetic analyses (Bayesian inference and parsimony) were performed using morphological and molecular data with genes 12S, 16S and COI. In both analyses, the group *Rhodocanthopus* resulted monophyletic inside *Passalus* and included seven previously described species, plus *Passalus chocoensis* sp.n. and *Passalus rufiventris* sp.n. Morphologically, the group can be recognized by the presence of secondary internal tubercles over the frontal edge, reduced compound eyes and strong spines on the external edge of the meso- and metatibiae.

Key words

Bess beetles, Mesoamerica, phylogenetic systematics, taxonomy.

1. Introduction

Passalidae is a group of saproxylophagous subsocial beetles that live in rotting logs. The family is mainly pantropical and has preference for humid environments (JIMÉNEZ-FERBANS et al. 2010). In the New World, the family comprises the tribes Passalini and Proculini. Passalini is made up of around 170 species and six genera (JIMÉNEZ-FERBANS & REYES-CASTILLO 2014). The largest genus of Passalini is *Passalus* Fabricius, 1771, with more than 80% of the species of the tribe. LUEDERWALDT (1931) divided *Passalus* in the subgenera *Mitrorhinus*, *Pertinax* and *Passalus*; however, some subgenera of *Passalus* seem to have more affinity with other genera of Passali-

dae. Thus, while *Passalus* (*Passalus*) seems to have more affinity with the genus *Paxillus*, the subgenus *Pertinax* appears to be more related to Proculini (JIMÉNEZ-FERBANS & REYES-CASTILLO 2014). Phylogenetic analyses of Passalidae have focused on general relationships of the family (FONSECA 1987; BOUCHER 2005; FONSECA et al. 2011) or Proculini (GILLOGLY 2005; BOUCHER 2005), while there is no specific study for Passalini or *Passalus*.

The *Rhodocanthopus* species group is placed in *Passalus* (*Pertinax*). Originally, KAUP (1871) proposed the genus *Rhodocanthopus* for five species with the following combination of features: anterior margin of the frons

Table 1. Species cited within *Rhodocanthopus* by various authors. ‘+’ in 2nd column: species analyzed in this study.

Author		Species included
KAUP (1871)	+	<i>R. maillei</i> (Percheron, 1841)
	+	<i>R. caelatus</i> (Erichson, 1847)
	+	<i>R. morio</i> (Percheron, 1835)
	+	<i>R. guatemalensis</i> (Kaup, 1869)
	+	<i>R. punctatostratus</i> (Percheron, 1835)
BATES (1886)	+	<i>R. caelatus</i>
	+	<i>R. guatemalensis</i>
	+	<i>R. maillei</i>
	–	<i>R. inops</i> (Truqui, 1857)
	+	<i>R. punctatostratus</i>
KUWERT (1891)	–	<i>R. anguliferus</i> (Percheron, 1835), transferred to <i>Neleides</i> by KUWERT (1898)
	–	<i>R. brevifrons</i> Kuwert, 1891, transferred to <i>Morosophus</i> by KUWERT (1898)
	+	<i>R. caelatus</i>
	+	<i>R. clypeoneleus</i> Kuwert, 1891
	–	<i>R. depressicornis</i> (Kirsch, 1885), transferred to <i>Morosophus</i> by KUWERT (1898)
	–	<i>R. discrepans</i> Kuwert, 1891, transferred to <i>Trichopleurus</i> by KUWERT (1898)
	–	<i>R. glabristernus</i> Kuwert, 1891, transferred to <i>Aponelides</i> by KUWERT (1898)
	+	<i>R. guatemalensis</i> , transferred to <i>Neleides</i> by KUWERT (1898)
	–	<i>R. mirabilis</i> Kuwert, 1891, transferred to <i>Lophocephalus</i> by KUWERT (1898)
	–	<i>R. inops</i>
	+	<i>R. punctatostratus</i> , transferred to <i>Aponelides</i> by KUWERT (1898)
	–	<i>R. punctulatus</i> (Kaup, 1869), transferred to <i>Trichopleurus</i> KUWERT (1898)
+	<i>R. spiniger</i>	
–	<i>R. stultus</i> Kuwert, 1891, transferred to <i>Microthorax</i> by KUWERT (1898)	
–	<i>R. sulcatipons</i> Kuwert, 1891, transferred to <i>Trichopleurus</i> by KUWERT (1898)	
KUWERT (1898)	–	<i>R. incertus</i> (Percheron, 1841)
	+	<i>R. spiniger</i>
	+	<i>R. spinosus</i> Kuwert, 1898
	+	<i>R. clypeoneleus</i>
PANGELLA (1905)	+	<i>R. caelatus</i>
	–	<i>R. perparvulus</i> (Kuwert, 1898)

cleft, without secondary mediofrontal tubercles, postero-frontal ridges with several internal tubercles (unique in Passalini), abundant punctures on pronotum sides, and with mesosternal scar marked and meso- and metatibiae with several spines (Table 1). Later, BATES (1886) added two new species, and KUWERT (1891) added 10 more, moved *R. morio* (Percheron, 1835) to *Morosophus* Kuwert, 1896 and *R. maillei* (Percheron, 1841) to *Epiphanus* Kaup, 1871. Then, after a re-examination of the species, KUWERT (1898) transferred 12 species of *Rhodocanthopus* into six different genera and narrowed the genus to only four species. Subsequently, PANGELLA (1905) added *P. perparvulus* Kuwert, 1898 (Table 1). GRAVELY (1918) synonymized *Rhodocanthopus* with *Passalus*, which later was accepted by HINCKS & DIBB (1935), who designated *Passalus caelatus* Erichson, 1847 as the type species of the genus *Rhodocanthopus* and included it in *Passalus* (*Pertinax*). Recently, JIMÉNEZ-FERBANS & REYES-CASTILLO (2014) described the genus *Ameripassalus*, in which they incorporated *Passalus guatemalensis* (Kaup, 1869), one of the original five species included by KAUP (1871) in *Rhodocanthopus*, without comments about the four other original species of *Rhodocanthopus*.

Despite the features used by KAUP (1871) for describing the genus *Rhodocanthopus* (syn. *Passalus*), it has not been clearly delimited. Some species such as *Ameripassalus guatemalensis* and *Passalus morio* do not present the features used by KAUP. Another example of the confusion is that *P. maillei*, the species with characters most

similar to the type species (*P. caelatus*) and often hardly distinguishable from it, was not considered to belong to *Rhodocanthopus* by KUWERT. BOUCHER (2005) considered *Rhodocanthopus* as a group of species within the *Passalus* subgenus *Pertinax*, in agreement with HINCKS & DIBB (1935). However, he only mentioned that the group “comprises no less than 15 species, from which three fourths are unpublished”, without indicating which species should be included or which characters group them. In addition, some of the species that KAUP originally included in *Rhodocanthopus* are morphologically so similar that they could well be conspecific. Therefore, it is necessary to include alternative sources of characters that allow for distinction among the taxa studied and for clarifying their relationships.

DNA sequence-based studies have provided insight into phylogenetic relationships in a wide variety of organisms. However, alignment of sequences is still a matter of concern in phylogenetic analyses especially when dealing with homologous sequences of different length (GONZÁLEZ et al. 2006). The alignment is crucially for phylogeny estimation because it must align homologous nucleotide positions. In sequence data, nucleotide positions showing variation such as substitution can be aligned effectively with all available algorithms. However, other mutation events such as duplications, translocations, deletions, insertions and inversions can create complex sequence patterns that defeat such algorithms (MORRISON 2008). Therefore, it has been proposed that

Table 2. Species included in the analysis. MD = morphological data.

Taxa	MD	12S	16S	COI
Proculini				
<i>Heliscus tropicus</i> (Percheron, 1835)	+	+	+	+
<i>Popilius erotylus</i> Reyes-Castillo & Castillo, 1992	+	+	+	+
Passalini				
<i>Ameripassalus guatemalensis</i> (Kaup, 1869)	+	+	+	+
<i>Passalus (Passalus) interstitialis</i> Eschscholtz, 1829	+	+	+	+
<i>Passalus (Passalus) occipitalis</i> Eschscholtz, 1829	+	+	+	+
<i>Passalus (Passalus) punctiger</i> Lepeletier & Serville, 1825	+	+	+	+
<i>Passalus (Pertinax) caelatus</i> Erichson, 1847	+	–	+	+
<i>Passalus (Pertinax) chocoensis</i> sp.n.	+	+	+	+
<i>Passalus (Pertinax) convexus</i> Dalman, 1817	+	+	+	+
<i>Passalus (Pertinax) clypeoneleus</i> (Kuwert, 1891)	+	–	+	+
<i>Passalus (Pertinax) cognatus</i> Truqui, 1857	+	–	+	+
<i>Passalus (Pertinax) epiphanoides</i> (Kuwert, 1891)	+	+	+	+
<i>Passalus (Pertinax) maillei</i> Percheron, 1841	+	+	+	+
<i>Passalus (Pertinax) morio</i> Percheron, 1835	+	–	+	+
<i>Passalus (Pertinax) pertyi</i> Kaup, 1869	+	+	+	+
<i>Passalus (Pertinax) punctatostritatus</i> Percheron, 1835	+	+	+	+
<i>Passalus (Pertinax) rufiventris</i> sp.n.	+	+	+	+
<i>Passalus (Pertinax) spiniger</i> (Bates, 1886)	+	+	+	+
<i>Passalus (Pertinax) spinipes</i> Gravelly, 1918	+	+	+	+

alignment's goal should be to identify the events associated with the homologies for an accurate representation of phylogenetic relationships (e.g. KJER et al. 2009; MARVALDI et al. 2009; MORRISON 2006, 2008; NGUYEN et al. 2015). Thus, development and evaluation of multiple sequence alignment methods is a central field of research in bioinformatics since the mid-1980s (e.g. SUBRAMANIAN et al. 2010; NGUYEN et al. 2015). Currently, there are several alignment approaches that incorporate some features of the DNA molecule such as structural homology (e.g. SMITH et al. 2010; WILL et al. 2007, 2012; WILM et al. 2006). These approaches make use of realistic energy models for RNAs' folding and base pair probabilities during the alignment procedure.

The objective of this study was to use morphological and mitochondrial DNA data to provide the taxonomic circumscription of the group Rhodocanthopus, to test its monophyly, to reconstruct the phylogenetic relationships within the group, and in turn to propose morphological characters that allow its delimitation.

2. Material and methods

2.1. Taxa selection

We studied eight species included in *Rhodocanthopus* by either KAUP (1871), or BATES (1886), or KUWERT (1898) (Table 1). Because there is no consistent or precise indication of which species should be included in the group Rhodocanthopus, species morphologically similar to the type species, *Passalus caelatus*, were included in the

analyses. The outgroup (Table 2) consisted of five species of *Passalus (Pertinax)*, three species of *Passalus (Passalus)* and two species of the tribe Proculini; the latter considered the sister group of Passalini (BOUCHER 2005).

2.2. Morphological study

Adults of all terminal taxa were examined from the following collections: P. Reyes, Instituto de Ecología, A.C. (IEXA), Xalapa, Mexico; Colección Nacional de Insectos, Instituto de Biología de la Universidad Nacional Autónoma de México (IBUNAM), D.F., Mexico; J.C. Schuster, Universidad del Valle of Guatemala, Guatemala (UVGC), Museu de Zoologia, Universidade de São Paulo (MZUSP), and Colección Entomológica Universidad del Magdalena (CBUMAG-ENT), Santa Marta, Colombia. The freshly collected specimens used for DNA analysis also were examined morphologically, and were deposited in IEXA. A morphological data matrix was constructed comprising 19 taxa (including two species newly described herein) and 39 external morphological characters, from which 14 were multistate (Table 3).

2.3. Character descriptions

We use the terminology established by BOUCHER (2005). Characters 1, 3, 9, 10, 13, 14, 15, 17, 19, 23, 24, 26, 28, 31, 36 and 38 were taken from JIMÉNEZ-FERBANS & REYES-CASTILLO (2014), and characters 12, 20, 25 were taken from GILLOGLY (2005) and MARSHALL (2000). **MT** = mediofrontal tubercles.

Table 3. Morphological data matrix. Polymorphisms are indicated with ¥ (0,1).

	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3				
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9
<i>Heliscus tropicus</i>	0	1	1	1	0	-	1	0	1	1	0	1	1	0	2	1	0	0	0	0	2	0	0	0	1	2	1	1	1	1	0	0	1	0	1	1	0	0	1
<i>Popilius erotylus</i>	0	1	1	1	0	-	0	0	1	1	0	1	0	0	2	1	0	0	0	0	2	1	0	0	1	0	1	2	0	1	0	0	1	0	1	1	1	0	1
<i>Ameripassalus guatemalensis</i>	0	2	1	0	0	1	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	1	2	0	0	2	0	0	0	1	1	0	0	0	0	1	1	1	1
<i>Passalus interstitialis</i>	1	0	¥	0	1	0	1	0	1	0	1	0	1	0	1	1	1	1	1	0	1	0	2	0	1	3	1	2	1	0	2	1	0	1	0	1	1	0	¥
<i>Passalus occipitalis</i>	1	0	1	0	1	0	1	0	0	0	1	0	0	0	1	1	1	0	0	1	2	0	2	0	0	1	1	1	1	2	0	0	0	1	1	1	0	1	
<i>Passalus punctiger</i>	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	1	1	1	1	0	0	0	2	0	1	3	1	2	1	0	2	1	0	1	0	1	1	0	1
<i>Passalus convexus</i>	1	0	1	0	0	1	0	0	0	0	1	¥	¥	0	1	1	0	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	1	0	0	1	1	0	0
<i>Passalus epiphanoides</i>	1	0	2	0	0	1	1	0	1	0	1	0	0	0	1	1	0	0	0	0	1	0	1	0	1	1	0	0	1	0	0	1	2	0	1	1	0	1	
<i>Passalus pertyi</i>	1	0	2	0	0	1	2	0	0	0	1	0	0	0	1	1	0	0	0	2	1	0	1	0	2	1	0	0	1	0	0	1	2	0	1	1	0	1	
<i>Passalus caelatus</i>	1	0	0	0	0	1	0	1	1	0	1	0	0	2	1	1	2	1	1	1	0	1	2	1	0	2	2	0	0	1	0	0	1	2	0	0	0	2	
<i>Passalus chocoensis</i> sp.n.	1	0	0	0	0	1	0	1	1	0	1	0	0	2	1	1	2	1	0	1	0	1	2	0	0	2	1	0	0	1	0	0	1	2	0	1	1	0	2
<i>Passalus clypeoneleus</i>	1	0	0	0	0	1	0	1	1	0	1	0	0	2	1	1	2	1	1	1	0	1	2	0	0	2	0	0	0	1	0	0	0	0	0	1	0	0	2
<i>Passalus cognatus</i>	1	0	0	0	0	1	0	1	1	0	1	0	0	2	1	1	2	1	1	1	0	1	2	0	0	2	0	0	0	1	0	0	0	0	0	1	0	0	2
<i>Passalus maillei</i>	1	0	1	0	0	1	0	1	1	0	1	0	0	2	1	1	2	1	0	0	0	1	2	0	0	2	1	0	0	1	0	0	2	2	0	0	0	0	2
<i>Passalus morio</i>	1	0	1	0	0	1	1	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	1	2	0	1	1	0	1
<i>Passalus punctatostratus</i>	1	0	1	0	0	1	0	1	1	0	1	0	0	0	1	1	2	1	1	0	0	1	2	0	0	2	1	0	0	1	0	0	0	2	0	1	1	0	1
<i>Passalus rufiventris</i> sp.n.	1	0	0	0	0	1	0	1	1	0	1	0	0	0	1	1	2	1	0	1	0	1	2	1	0	2	2	0	0	1	0	0	2	2	0	1	1	0	2
<i>Passalus spiniger</i>	1	0	0	0	0	1	0	1	1	0	1	0	0	2	1	0	2	1	1	1	0	1	2	0	0	2	¥	0	0	1	0	0	1	2	0	1	0	1	2
<i>Passalus spinipes</i>	1	0	0	0	0	1	0	1	1	0	1	0	0	2	1	0	2	1	1	1	0	1	2	0	0	2	0	1	0	1	0	0	1	2	0	1	0	1	2

- Clypeus, anterodorsal exposure: (0) present; (1) absent.
- Clypeus, orientation: (0) vertical; (1) horizontal; (2) oblique.
- Infra-anterior angles of clypeus: (0) indistinct; (1) developed, equal in size to MT; (2) strongly developed, bigger than MT.
- Infra-anterior angles of clypeus, position: (0) under the frons; (1) anterior to the frons.
- Secondary MT: (0) absent; (1) present.
- Laterofrontal tubercles relative to MT, position: (0) between MT; (1) fused to MT.
- Internal tubercles, size compared to MT: (0) smaller; (1) subequal; (2) larger.
- Secondary internal tubercles (internal tubercle 2): (0) absent; (1) present.
- Lateroposterior tubercles, distinctness: (0) weak; (1) marked.
- Lateroposterior tubercles, ridge linking to central tubercle: (0) absent; (1) present.
- Frontal ridges, shape: (0) Y-shaped; (1) V-shaped.
- Frontal fossae, pubescence: (0) absent; (1) present.
- Ocular canthus, pubescence: (0) absent; (1) present.
- Eyes, reduction: (0) big eyes, ocular canthus not reaching the middle of the eye; (1) small eyes, ocular canthus reaching the middle of the eyes; (2) very small eyes, ocular canthus extending beyond the middle of the eyes.
- Suprainternal tooth on the left mandible: (0) simple; (1) bifid; (2) trifid.
- Anterior angles of pronotum, shape: (0) sharp (1) right.
- Punctures on pronotum, distribution: (0) restricted to fossae and marginal groove; (1) restricted to lateral area; (2) almost reaching longitudinal prothoracic groove.
- Punctures on pronotum, density: (0) scarce; (1) abundant.
- Rhomboidal prosternellum, shape: (0) acute; (1) truncated.
- Pronotal arms, brightness: (0) shiny; (1) dull.
- Pronotal arms, pubescence: (0) absent; (1) present in posterior half; (2) present everywhere.
- Inferolateral part of pronotum, pubescence: (0) conspicuous; (1) scarce.
- Mesosternal scar, distinctness: (0) absent; (1) present, indistinct; (2) present, markedly developed.
- Posterolateral edge of mesosternum, brightness: (0) dull; (1) shiny.
- Mesepimeron, pubescence: (0) absent; (1) present.
- Metasternal disc, delimitation by punctation: (0) not bounded; (1) bounded in the posterior part, not reaching the middle part; (2) bounded in the posterior part and reaching the middle part; (3) bounded as in 2 and spanning almost the whole disc.
- Punctures on metasternal disc: (0) ≤ 5; (1) absent; (2) ≥ 10.
- Lateroanterior part of metasternum, pubescence: (0) absent; (1) sparse; (2) dense.
- Mestasternal fossae, pubescence: (0) absent; (1) present.
- Mestasternal fossae, width relative to that of mesotibia: (0) greater; (1) smaller.
- Humeri, pubescence: (0) absent; (1) present, sparse at the base; (2) fully pubescent.
- Elytral striae, pubescence: (0) absent; (1) present.
- Elytral interstriae, width relative to that of striae: (0) subequal; (1) smaller; (2) greater.
- Epipleurae, pubescence: (0) absent; (1) present, sparse at the base; (2) densely pubescent until basal third.
- Vertical anterior part of elytra, pubescence: (0) absent; (1) present.

Table 4. Primers used for amplifying DNA.

Primer	Sequence	Reference	Cycle
12S SR-J-14199 SR-J-14594	5'-tactatgttactgacttat-3' 5'-aactaggattagatatacccc-3'	KAMBHAMPATI & SMITH (1995)	94°C 3 min., (94°C 1 min., 51°C 1 min., 72°C 2 min.) × 35 cycles, 72°C 7 min.
16S 16SB 16SH	5'-ccggtttgaactcagatcatgt-3' 5'-tgctgttta(a/t)taaaacatg-3'	HOSOYA et al. (2001)	94°C 3 min., (94°C 1 min., 51°C 1 min., 72°C 2 min.) × 35 cycles, 72°C 7 min.
COI C1-J-2183 TL2-N-3014	5'-caaca(c/t)ttatttgatt(c/t)tt(c/t)gg-3' 5'-t(c/t)ca(a/t)gcacta(a/t)tctgcatatta-3'	SIMON et al. (1994)	94°C 3 min., (94°C 1 min., 51°C 1 min., 72°C 2 min.) × 35 cycles, 72°C 7 min.

36. Marginal groove over anterior ventral edge of the profemur, extension: (0) spans nearly the entire edge (reaching the apical pubescence); (1) spans halfway from base (not reaching the apical pubescence).
37. Marginal groove over anterior ventral edge of the profemur, distinctness: (0) slight; (1) marked.
38. Protibia, shape: (0) with four similar faces; (1) with the external face compressed.
39. External edge of meso- and metatibiae, spines: (0) absent; (1) present, weak; (2) present, strong.

2.4. Molecular study

DNA extraction was made from fresh specimens collected in Bolivia, Colombia, Guatemala and Mexico. One or two legs were removed from each individual for maximizing amount of muscular tissue. Extraction and purification was performed with the “DNeasy blood and tissue kit” (Qiagen, Valencia, CA, USA) by following manufacturer’s instructions.

PCR reactions were performed for amplifying three mitochondrial genes: 12S rRNA, 16S rRNA and COI. These genes have been employed for phylogenetic reconstruction among and within coleopteran genera by several authors (e.g. HOSOYA & ARAYA 2005) and for differentiating species and other groups in Passalidae (e.g. BEZA-BEZA et al. 2011). Reactions were carried out in a thermocycler Eppendorf Mastercycler pro S (Hamburg, Germany) in a standard 25 µl mix containing approximately 100–150 ng of extracted DNA, 5 µl of PCR buffer 5×, 0.2 mM dNTPs, 1.6 µM of both the forward and reverse primers, 2 mM MgCl₂, 0.2 U of Go Taq flexi DNA polymerase (Promega, Madison, WI, USA) and distilled water to adjust volume. Primer sequences and cycle conditions are described in Table 4. Amplified DNA was purified prior to sequencing with the Wizard SV gel and PCR clean-up system kit as described by manufacturers (Promega, Madison, WI, USA), and sequenced using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions. Cycle sequence products were cleaned with an isopropanol precipitation and electrophoresed using an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

For a better representation of the tribe Proculini (out-group taxon), sequences generated by BEZA-BEZA (2013) for genes 12S and COI from *Heliscus tropicus* (Percheron, 1835) and *Popilius erotylus* Reyes-Castillo & Castillo, 1992 were included in the analyses. The edition of resulting sequences was done in the BioEdit software version 7.1.3.0 (HALL 1999; LARKIN et al. 2007). Structural alignment for 12S and 16S was performed with the LocARNA tool for multiple alignment of RNA molecules at <http://rna.informatik.uni-freiburg.de/LocARNA/Input.jsp> (WILL et al. 2007, 2012; SMITH et al. 2010). This program allows for the finding of homology at peripheral regions and conserved structural motifs for predicting secondary structure. Alignment for COI was optimized with the MAFFT program at <http://www.ebi.ac.uk/Tools/msa/mafft/> (KATO & STANDLEY 2013), which allows rapid detection of homologous segments using fast Fourier transform (FFT) through an iterative refinement of an initial alignment. Finally, we used the web server issue of Guidance2 (SELA et al. 2015) to exclude problematic regions of the sequenced genes and the reviewed by eye the alignment, in which we made two changes in positions 200, 201 and 315. The alignment block of the molecular data as it was used for the analyses is presented in the Electronic Supplement file 1.

2.5. Phylogenetic analyses

Morphological data matrix comprised 20 taxa and 39 characters. Sequence data matrices included 25 individuals from 15 species for 12S, and 29 individuals from 19 species for 16S and COI. Phylogenetic analyses were performed in combination with maximum parsimony (MP) and Bayesian inference. In MP we used the ratchet algorithm (NIXON 1999) in Winclada 1.00.08 (NIXON 2002) and NONA (version 2.0, GOLOBOFF 1993). Two hundred iterations were performed holding one tree per iteration and perturbing 10% of the characters. Multistate morphological were treated as non-additive and gaps generated in the alignments as missing data. Branch support values were determined with bootstrap and jackknife with 500 replicates.

Bayesian Phylogenetic Inference (BPI) analysis was conducted using MrBayes 3.1.2 (ALTEKAR et al. 2004;

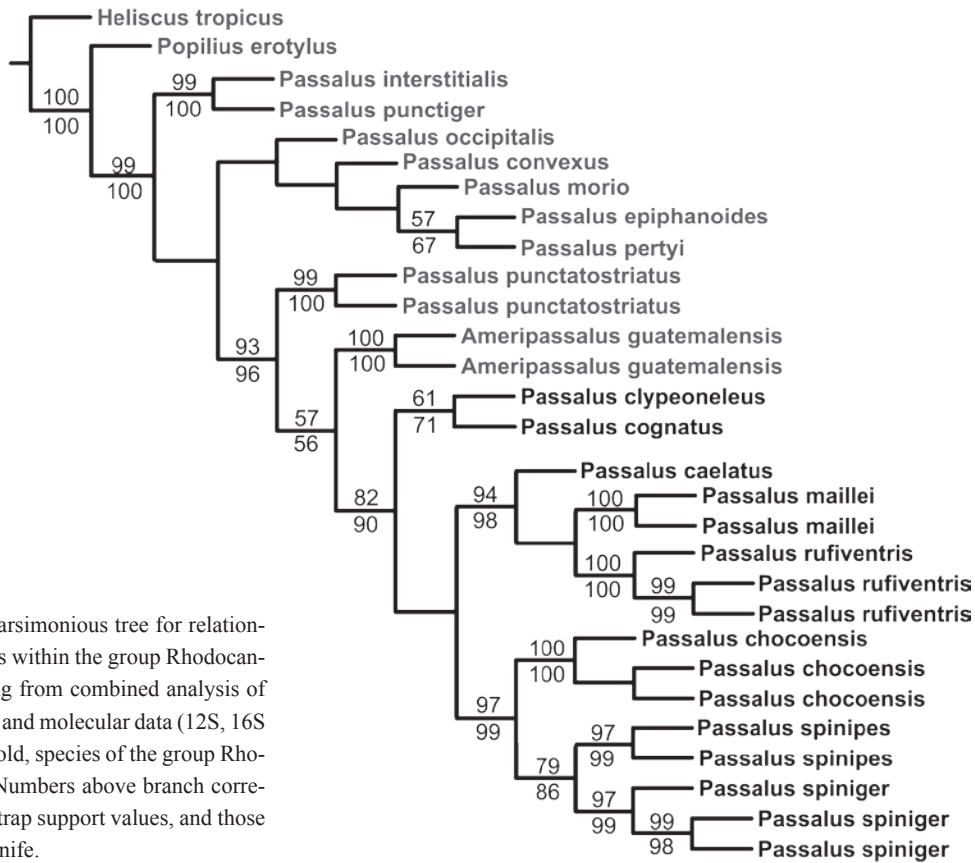


Fig. 1. Most parsimonious tree for relationships of species within the group Rhodocanthopus resulting from combined analysis of morphological and molecular data (12S, 16S and COI). In bold, species of the group Rhodocanthopus. Numbers above branch correspond to bootstrap support values, and those below to jackknife.

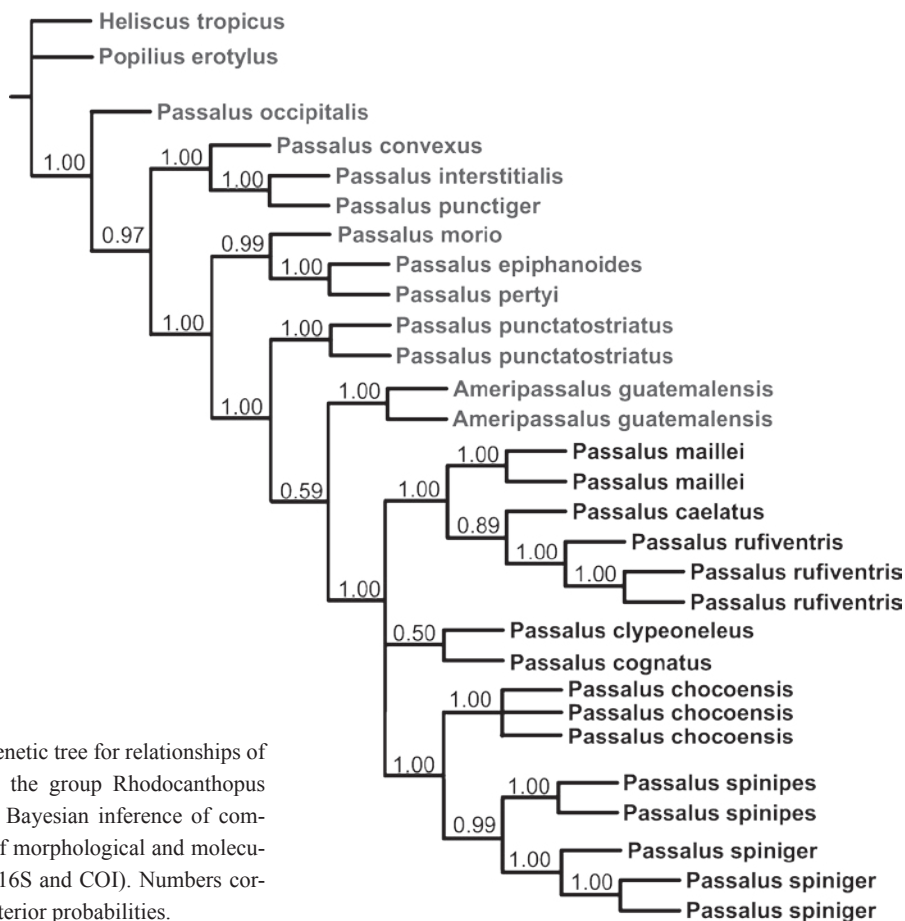


Fig. 2. Phylogenetic tree for relationships of species within the group Rhodocanthopus resulting from Bayesian inference of combined matrix of morphological and molecular data (12S, 16S and COI). Numbers correspond to posterior probabilities.

HUELSENBECK & RONQUIST 2001; RONQUIST & HUELSENBECK 2003). Matrix was partitioned by data type. DNA characters were analyzed with the model GTR + Γ (nst = 6; rates = gamma) and morphological data with the standard discrete model implemented in the program. All trees were given equal weight *a priori*. BPI was performed in two independent 1-million generation runs, with four chains (one cold and three hot) each, until an average standard deviation of split frequencies of 0.01 or less was reached (0.001970). We sampled trees every 100th generation and discarded initial samples applying a “burn-in” value of 25% before calculating the majority consensus tree and posterior probabilities (PP) for clades.

3. Results

Parsimony analysis of morphological and all three gene data sets yielded one shortest tree of L: 1393, CI: 50 and RI: 61 (Fig. 1). The group *Rhodocanthopus* has good support values (Jackknife 90, bootstrap 82); *P. punctato-striatus* formed a well-supported sister group (Jackknife 96 and bootstrap 93) to *A. guatemalensis* and a clade formed by all species of *Rhodocanthopus*. *Rhodocanthopus* species were distributed in three subclades and the majority of internal nodes are statistically well supported (Fig. 1). We conducted an additional parsimony analysis of morphological data including *Passalus spinosus* (a species included by KUWERT 1898 in *Rhodocanthopus*) and the result was similar, with this species as part of *Rhodocanthopus* group (not shown). The results of BPI are similar to MP in showing *Rhodocanthopus* as a monophyletic group (PP: 1.00) related to *Ameripassalus* and *Passalus punctato-striatus*, and all internal nodes are statistically well supported (Fig. 2). As was found in the most parsimony tree, there are three subclades within *Rhodocanthopus*, but in BPI there is a basal polytomy and the relationships of these groups are unresolved.

4. Taxonomy

4.1. *Passalus (Pertinax) chocoensis* sp.n.

Fig. 3

Description. Habitus: Small-sized, 18.9–20.0 mm long, macropterous, body flattened, shiny black.

Head: Anterior border of labrum straight or slightly convex, labrum covered by setae that are less abundant on anterior part. Clypeus hidden below frons, with anterior angles directed downward and located just below latero-frontal + mediofrontal tubercles (sensu BOUCHER 2005). Anterior edge of frons with deep central notch, at the sides of which there are two insinuated tubercles. Latero-

frontal + mediofrontal tubercles large, projected forward. At the base of latero-frontal + mediofrontal tubercles there is a small tubercle (internal tubercle 2) joined by a ridge to the internal tubercle. Internal tubercles developed, located midway between latero-frontal + mediofrontal tubercles and the apex of central tubercle. Frontal ridges “V” shape, emerging from apex of central tubercle. Frontal area punctate ahead of the marked cephalic tumescence (mamelon sensu JIMÉNEZ-FERBANS & REYES-CASTILLO 2014). Central tubercle small, with apex not free; postero-lateral tubercles marked and transverse. Frontal fossae glabrous, with 2 or 3 punctures. Postfrontal groove entire, with small central emargination in inverted “v”. Eyes reduced; canthus glabrous; postocular depression weak. Anterior border of ligula tridentate, central tooth as big as lateral teeth. Maxilla with lacinia bidentate. Hypostomal process glabrous, slightly separated from mentum, reaching the anterior border of median basal region of mentum. Anterior line of gula arched. Antennal club with three short lamellae. Mandible apex tridentate, dorsal tooth slightly bigger; internal inferior tooth bifid in left mandibles and simple in right mandibles; dorsal tooth straight in dorsal view and flat in lateral view; mandibular pubescence only at base, barely visible in dorsal view; mandibular fossae small, not reaching mobile tooth. Middle labial palpomeres as long as distal palpomere and $1.5 \times$ as wide.

Thorax: Pronotum side fully punctate; anterior angles slightly acute; marginal groove on anterior margin occupying 2/3 of pronotum anterior border; median groove well defined; lateral fossae well-defined. Prosternellum rhomboidal, posterior apex truncate. Pronotal arms dull and glabrous. Mesosternum glabrous; mesosternal scar well-defined and elongate. Posterior angle of mesepisternum and mesepimeron glabrous. Metasternal disc with punctures (up to 20) or smooth, bounded by punctate area until middle. Lateroanterior metasternum glabrous; metasternal groove glabrous, narrower than mesotibia.

Elytra: Shiny, anterior border rectangular and glabrous; humeri and epipleura completely glabrous; striae with rounded punctures. **Wings:** Fully developed. **Legs:** Anterior ventral border of profemur with marked and complete groove; meso- and metatibiae with strong spines laterally. **Aedeagus:** Basal piece almost completely separated from parameres; parameres in “V” shape, ventral view; median lobe elongated, being 4/5 of total length of aedeagus.

Variation: Metasternal disc can be smooth, most specimens have 4 punctures and only one has 20 punctures.

Differential diagnosis. *Passalus chocoensis* sp.n. is similar to *P. spiniger*, but is smaller, with abundant punctures on pronotum, and with mesosternal scar dull and weakly developed. *Passalus chocoensis* sp.n. differs from other small-bodied species of *Rhodocanthopus*, such as *P. caelatus* and *P. maillei*, by having strongly protruding cephalic anterior angles, by the abundant punctures on pronotum, which never extend to longitudinal prothoracic groove. The median basal region of mentum of *Passalus chocoensis* sp.n. has a group of setae on each side,

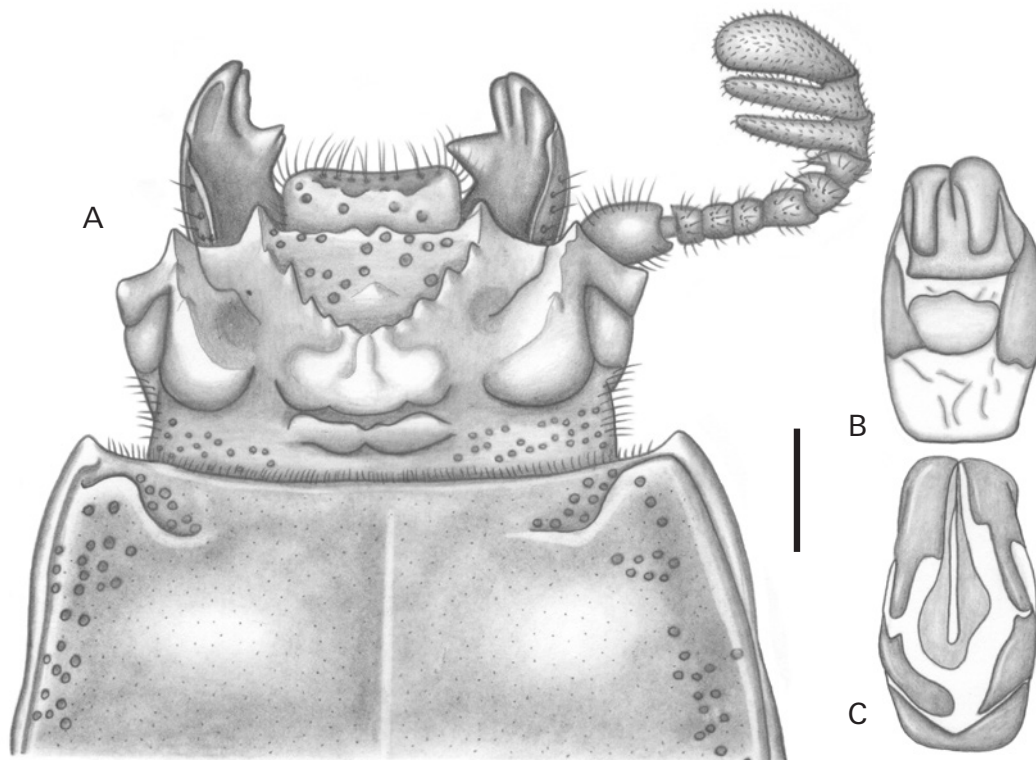


Fig. 3. *Passalus (Pertinax) chocoensis* sp.n. **A:** Head and pronotum, dorsal view. **B:** Aedeagus, dorsal view. **C:** Aedeagus, ventral view. Scale bar: 1 mm

but is completely glabrous in *P. maillei* and *P. caelatus*. Finally, parameres of the aedeagus of *Passalus chocoensis* sp.n. are separated from basal piece, while *P. spiniger*, *P. caelatus* and *P. maillei* have parameres and basal piece completely fused.

Derivatio nominis. Adjective derived from the name of the type locality.

Material. Holotype: ♂, COLOMBIA, Chocó, Tutunendó, Estación Ambiental IAP | 5°44,976'N 76°31,407'W, 60 msnm | 19.vii.2011, Jiménez-Ferbans & Reyes-Castillo (IEXA). – Paratypes 5♀, 4♂, same data as holotype (IEXA, CBUMAG-ENT).

4.2. *Passalus (Pertinax) rufiventris* sp.n.

Fig. 4

Description. Habitus: Small-sized, 14.0–15.5 mm long, macropterous, body flattened, shiny black.

Head: Anterior border of labrum straight, labrum covered by setae that are less abundant on anterior part. Clypeus hidden below frons, with anterior angles directed downward and located just below laterofrontal + mediofrontal tubercles (sensu BOUCHER 2005). Anterior edge of frons with deep central notch, at the sides of which there are two insinuated tubercles. Laterofrontal + mediofrontal tubercles large, projected forward. At the base of laterofrontal + mediofrontal tubercles is a small tubercle (internal tubercle 2) joined by a ridge to the internal tubercle. Internal tubercles developed, located midway

between laterofrontal + mediofrontal tubercles and the apex of central tubercle. Frontal ridges “V” shape, emerging from apex of central tubercle. Frontal area punctate ahead of the marked cephalic tumescence (mamelon sensu JIMÉNEZ-FERBANS & REYES-CASTILLO 2014). Central tubercle small, with apex not free; posterolateral tubercles marked and transverse. Frontal fossae glabrous, with few punctures. Postfrontal groove entire, without central emargination in inverted “v”. Eyes reduced; canthus glabrous; postocular depression weak. Anterior border of ligula tridentate, central tooth slightly longer than lateral teeth. Maxilla with lacinia bidentate. Hypostomal process glabrous, slightly separated from mentum, reaching the anterior border of median basal region of mentum. Anterior line of gula arched. Antennal club with three short lamellae. Mandible apex tridentate, dorsal tooth slightly reduced; internal inferior tooth bifid in left mandibles and simple in right mandibles; dorsal tooth straight in dorsal view and flat in lateral view; mandibular pubescence reaches the base of internal tooth; mandibular fossae small, not reaching mobile tooth. Middle labial palpomere as wide as distal palpomere and $0,8 \times$ as long.

Thorax: Pronotum with abundant punctures, even close to median groove; anterior angles slightly acute; marginal groove on anterior margin occupying 1/2 of pronotum anterior border; median groove well defined; lateral fossae well-defined. Prosternellum rhomboidal, posterior apex truncate. Pronotal arms dull and glabrous. Mesosternal scar well-defined and elongate. Mesepisternum glabrous and mesepimeron glabrous. Metaster-

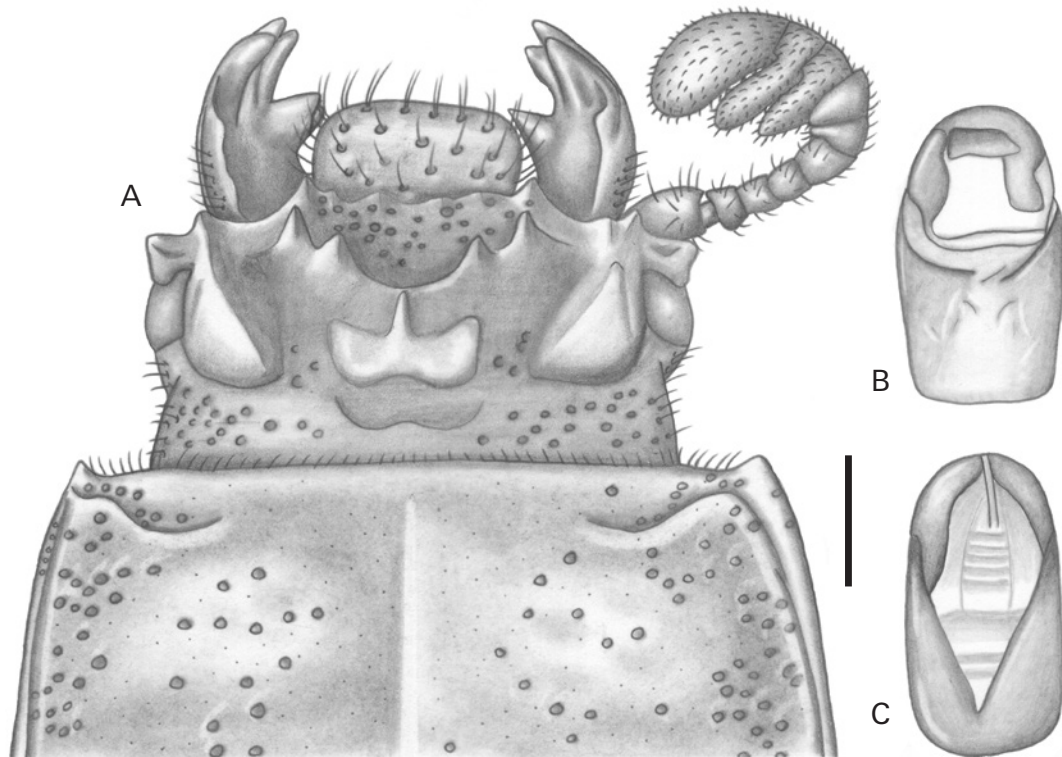


Fig. 4. *Passalus (Pertinax) rufiventris* sp.n. **A:** Head and pronotum, dorsal view. **B:** Aedeagus, dorsal view. **C:** Aedeagus, ventral view. Scale bar: 1 mm

nal disc with abundant punctures, bounded by punctate area until middle. Lateroanterior metasternum glabrous; metasternal groove glabrous, narrower than mesotibia. **Elytra:** Shiny, anterior border rectangular and glabrous; humeri glabrous and epipleura with scarce setae on the base; striae with rounded and marked punctures. **Wings:** Fully developed. **Legs:** Anterior ventral border of profemur with marked and complete (reaching apical pubescence) groove; protibia with dorsal groove incomplete; meso- and metatibiae with strong spines laterally. **Aedeagus:** Basal piece completely fused to parameres; parameres in “V” shape, ventral view; median lobe elongated, being 4/5 of total length of aedeagus.

Variation: Sometimes punctures on frontal area are scarce; pronotal arms can be dull only at the apex and some specimens have mesosternal scar oval.

Differential diagnosis. *Passalus rufiventris* sp.n. is similar to *P. caelatus* (16.4–18.4 mm) and *P. maillei* (16.4–18.7 mm), but is smaller, with body flattened, abdominal tergites reddish in color (even in mature adults) and possesses a strong depression between mesosternal scars (absent or weak in *P. caelatus*).

Derivatio nominis. Adjective, derives from Latin “rufus” for red and “venter” for abdomen.

Material. Holotype: ♂, “COLOMBIA, Chocó, Tutunendó, Estación Ambiental IIAP | 5°44,976'N 76°31,407'W, 60 msnm | 19.vii.2011, Jiménez-Ferbans & Reyes-Castillo” (IEXA). – Paratypes 5♀, 3♂, 3 gender unknown. 4♀, 2♂, same data as holotype (IEXA, CBUMAG-ENT). 1 specimen, gender unknown,

“COLOMBIA, Chocó, Nuquí, El Amargal | 50 m | 27.v.1995, A. Lopera” (IEXA); 1♀, 1♂, “COLOMBIA, Valle [del Cauca], municipio Buenaventura, río Tatabro | 3.xii.1993, 160 msnm | P. Chacón” (UVGC).

4.3. *Passalus (Pertinax) spinipes* Gravelly, 1918

Redescription. Habitus: Small-sized, 20.5–22.7 mm long, macropterous, body slightly flattened of shiny black in color.

Head: Anterior border of labrum straight, labrum covered by setae evenly distributed. Clypeus hidden below frons, with anterior angles directed downward and located just below laterofrontal+mediofrontal tubercles (sensu BOUCHER 2005). Anterior edge of frons with deep central notch, at the sides of which there are two insinuated tubercles. Laterofrontal+mediofrontal tubercles large (twice larger than internal tubercles), projected forward. At the base of laterofrontal+mediofrontal tubercles there is a small tubercle (internal tubercle 2) joined by a ridge to the internal tubercle. Internal tubercles developed, located midway between laterofrontal+mediofrontal tubercles and the apex of central tubercle. Frontal ridges “V” shape, emerging from apex of central tubercle. Frontal area with abundant punctures even on cephalic tumescence (mamelon sensu JIMÉNEZ-FERBANS & REYES-CASTILLO 2014). Central tubercle small, with apex not free; postero-lateral tubercles marked. Frontal fossae glabrous, with few punctures. Postfrontal groove entire, without central emargination in inverted “v”. Eyes

reduced; canthus glabrous; postocular depression weak. Anterior border of ligula tridentate, central tooth as big as lateral teeth. Maxilla with lacinia bidentate. Hypostomal process glabrous, slightly separated from mentum, reaching the anterior border of median basal region of mentum. Median basal region of mentum protruding and pubescent, with lateral fossae pubescent. Anterior line of gula biemarginate. Antennal club with three short lamellae. Mandible apex tridentate; internal inferior tooth bifid in left mandible and simple in right mandible; dorsal tooth straight in dorsal view and flat in lateral view; mandibular pubescence reaching the base of internal tooth; mandibular fossae elongated, reaching mobile tooth. Middle labial palpomeres slightly wider and as long as distal palpomeres.

Thorax: Pronotum side fully punctate; anterior angles slightly acute; marginal groove on anterior margin occupying 1/3 of pronotum anterior border; median groove well defined; lateral fossae well-defined; Prosternellum rhomboidal, posterior apex truncate. Pronotal arms shiny and glabrous on proximal half to prosternellum. Mesosternum glabrous; mesosternal scar oval, well-defined and finely punctate. Posterior angle of mesepisternum and mesepimeron glabrous. Metasternal disc without punctures (smooth), bounded by few punctures in posterior part. Lateroanterior metasternum with scarce and short setae; metasternal groove glabrous, narrower than epipleura. **Elytra:** Shiny, anterior border rectangular and glabrous; humeri with scarce setae on the base; epipleura glabrous; striae with rounded punctures. **Wings:** Fully developed. **Legs:** Anterior ventral border of profemur with weak groove but reaching apical pubescence; protibia with dorsal groove complete; meso- and metatibiae with strong spines laterally. **Aedeagus:** Basal piece completely fused to parameres; median lobe elongated, being more than half of total length of aedeagus, completely sclerotized.

5. Discussion

Our results indicate that Rhodocanthopus represents a monophyletic lineage that includes at least 9 species. Morphologically it can be differentiated by reduced eyes, by the presence of strong spines on the external edge of the meso- and metatibiae and by the presence of secondary internal tubercles on frontal ridges.

Eye reduction is relatively common in Passalidae, usually accompanied by brachypterism in species from high mountains. However, most species of Rhodocanthopus live below 1200 m asl and do not show wing reduction. This allows considering eye reduction as a homologous character among species instead of a convergence enforced by environmental conditions. In the case of strong tibial spines, in Passalini this condition is unique to Rhodocanthopus; although some Proculini seem to have somewhat large spines. Likewise, to the best of our

knowledge, regarding Passalidae, the presence of a secondary internal tubercle is a state unique to Rhodocanthopus.

In both analyses, Bayesian inference and parsimony, a close relationship emerged of *A. guatemalensis* with the group Rhodocanthopus. This could suggest that *A. guatemalensis* should be part of this group. Nevertheless, results of analyses with morphology and sequence data from 12S and 16S independently (not showed) do not relate *A. guatemalensis* with Rhodocanthopus. The relationship obtained in some analyses could be an artifact since it was not possible to include more representatives of *Ameripassalus*, in fact, the node of *Ameripassalus* – Rhodocanthopus has low support values in both BPI and MP. Unfortunately, we were unable to capture other species of the genus despite several field expeditions aimed at collecting fresh material.

The position of *Passalus punctatostratus* could be difficult to interpret. KAUP (1871) included this species within *Rhodocanthopus*, but it lacks the morphological characters proposed in our study for delimiting the group. Besides, all analyses placed this species outside Rhodocanthopus. *Passalus punctatostratus* is one of the most widely distributed species of Passalidae. It can be found from Colombia to the southern United States (SCHUSTER 2002) and from sea level to 2000 m asl (REYES-CASTILLO 2004). Furthermore, its morphology is highly variable. It appears with 13 different synonyms in the catalogue of HINCKS & DIBB (1935). This situation has promoted the view of some specialists that *P. punctatostratus* is a species complex (Schuster pers. comm.). Similarly, nucleotide divergence in the sequence data of our two included specimens was higher than in all other species. There were 97 differences for the 12S and COI, and the rest of the species represented by more than one specimen (even by more than two) had 17 to 30 differences. All this variation reflects that *P. punctatostratus* may comprise a species complex. Therefore, it should be studied including more specimens representing its altitudinal and geographical distribution.

Another species that was originally included in the genus *Rhodocanthopus* but should be excluded from this group is *P. morio*. In our analyses it was related to species of the subgenus *Pertinax* s.str. This is not unexpected, since *P. morio* has morphological characters similar to species of *Passalus (Pertinax)* such as the big size, big eyes, mesosternal scar weak and unarmed tibiae. Also, *P. morio* is distributed in the southern part of South America (JIMÉNEZ-FERBANS et al. 2013), which is in agreement with the South American distribution of subgenus *Pertinax* s.str., but not with the clear Mesoamerican distribution of the group Rhodocanthopus.

Furthermore, the position in the analyses of *P. morio* and other representatives of *Passalus (Pertinax)*, including the type species of the subgenus (*P. convexus* Dalman, 1817), contradicts the monophyly of the subgenus. Therefore, this mixture of representatives of *Passalus (Passalus)* with those of *Passalus (Pertinax)* reminds that a revision of the current classification of the genus *Passalus*

salus is mandatory; moreover, this genus has also been found as a non-monophyletic group in several analyses (FONSECA 1987; GILLOGLY 2005; BOUCHER 2005).

Concluding, the group *Rhodocanthopus* is a monophyletic lineage that can be distinguished both on a morphological and a molecular basis. Probably, it may well be ranked as a subgenus of *Passalus*, or even as a separate genus. This, however, will depend on future developments regarding the “genus” *Passalus*, which may be subdivided in several genera. To address this issue, an increased taxon sampling covering all subgenera (*Passalus*, *Pertinax* and *Mitrorhinus*) and the *Rhodocanthopus* group is necessary. Other aspects of interest are the distribution of the species of *Rhodocanthopus* and the factors of relevance in the species divergence process.

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 (“Contents”)

File 1: jimenezferbans&al-passalus-asp2016-electronicsupplement1.fasta. – The molecular data alignment block as it was used for the analyses.

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