Taxonomic reassessment of the genus *Dichotomius* (Coleoptera: Scarabaeinae) through integrative taxonomy

Carolina Pardo-Diaz[1], Alejandro Lopera Toro[2], Sergio Andres Peña Tovar[3], Rodrigo Sarmiento-Garcés[4], Melissa Sanchez Herrera[1], Camilo Salazar[1]

[1] Biology Program, Faculty of Natural Sciences and Mathematics, Universidad del Rosario, Bogota D.C., Colombia
[2] Fundacion Ecotropico, Bogota D.C., Colombia
[3] Universidad Distrital Francisco José de Caldas, Bogota D.C., Colombia
[4] Universidad Nacional de Colombia, Bogota D.C., Colombia

Corresponding Author:
Carolina Pardo-Diaz[1]
Cra. 24 No 63C-69, Bogotá D.C. 111221, Colombia

Email address: geimy.pardo@urosario.edu.co

Deleted: Programa de Biología, Facultad de Ciencias Naturales y Matemáticas

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Abstract

Dung beetles of the subfamily Scarabaeinae are widely recognised as important providers of multiple ecosystem services and are currently experiencing revisions that have improved our understanding of higher-level relationships in the subfamily. However, the study of phylogenetic relationships at the level of genus or species is still lagging behind. In this study we investigated the New World beetle genus *Dichotomius,* one of the *richest within the New World* Scarabaeinae, using the most comprehensive molecular and morphological dataset for the genus to date (in terms of number of species and individuals). Besides evaluating phylogenetic relationships, we also assessed species delimitation through a novel Bayesian approach (iBPP) that enables morphological and molecular data to be combined. Our findings indicate that *Dichotomius* is a monophyletic genus and support the existence of the subgenera *Selenocopris* and *Dichotomius* sensu stricto (s.s), but not the recent synonymy of *Selenocopris* with *Luederwaldtinia*. Some species-groups within the genus were also recovered, and particularly within *Dichotomius* s.s., species-groups seem associated with elevational distribution. Our species delimitation analyses were largely congruent irrespective of the set of parameters applied, but the most robust results were obtained when molecular and morphological data were combined. Although our current sampling and analyses were not powerful enough to make definite interpretations on the validity of all species evaluated, we can confidently recognise *D. nisus, D. belus* and *D. mamillatus* as valid and well differentiated species. Overall, our study provides new insights into the phylogenetic relationships and classification of dung beetles and has broad implications for their systematics and evolutionary analyses.

Introduction

Scarabaeinae dung beetles are one of the most morphologically diverse groups of animals (Philips 2011) comprising more than 6000 species and 200 genera worldwide (Tarasov & Génier 2015). Within this dung-feeding subfamily, *Dichotomius* Hope, 1838 constitutes one of the richest genera endemic to the Americas, with 171 described species (Schoolmeesters 2019). Compared to other regions, its diversity is highest in South America where more than 100 species can be found (Bohórquez & Montoya 2009; Vulinec 1999). Species in this genus vary in size (5-38 mm), show strong sexual dimorphism and have colours usually ranging from dark brown to black (Nunes 2017; Sarmiento-Garcés & Amat-García 2014; Vaz-de-Mello et al. 2011). Furthermore, *Dichotomius* species are typically nocturnal, more abundant in the rainy season and prevalent in several Neotropical terrestrial habitats where they play multiple ecological roles (López-Guerrero 2005; Maldaner et al. 2015; Vulinec 1999). For example, they promote bioturbation, remove faeces from forests and pastures, bury seeds, stimulate seed germination and even act as intermediate hosts of swine parasites (Almeida et al. 2014; Nichols et al. 2008; Vulinec 1999).

The taxonomy of these beetles, which is entirely based on morphological characters, is still not sufficiently resolved despite them being ubiquitous and ecologically relevant. The genus was divided into four subgenera by Luederwaldt (1929): *Dichotomius* sensu stricto (s.s.), *Selenocopris*, *Homocanthonides* and *Cephagonus* (Luederwaldt 1929). Since then there have been few changes, the most relevant done by Martinez (1951) that keeps *Dichotomius* s.s. and *Homocanthonides*, but changes *Selenocopris* to *Luederwaldtinia* and *Cephagonus* to *Selenocopris* (Martínez 1951). The most recent revision of the genus *Dichotomius* differentiates the four subgenera based mainly on variations of the clypeo-genal angle (Nunes 2017)

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supporting the initial division by Luederwaldt in 1921: *Dichotomius* s.s. (70 spp); *Homocanthonides* (1 spp); *Selenocopris* (75 spp) and *Cephagonus* (16 spp). These subgenera are further divided into species groups, each one containing multiple species (Luederwaldt 1929; Martínez 1951; Nunes 2017; Nunes & Vaz-de-Mello 2013; Nunes & Vaz-de-Mello 2016). Although there has been a recent interest in revising these subgenera and species groups, their definition is still problematic due to relying on morphological traits alone (Maldaner et al. 2015; Nunes 2017; Nunes & Vaz-de-Mello 2013; Nunes & Vaz-de-Mello 2016). This problem also applies to species delimitation in the genus because some species such as *Dichotomius satanas* display a spectacular range of morphological variability, which suggests the possibility of distinct species being misclassified as a single one (Sarmiento-Garcés & Amat-García 2014). In fact, some authors consider *D. satanas* as a species complex in need of revision (Nunes 2017). For example, specimens of *D. satanas* from Central America have been reported to look different from those from Colombia (with the type being from this country), and within Colombia, females of *D. satanas* from the Eastern Cordillera have two or four protuberances on the pronotum while females from the Western and Central cordillera have only two (Fig. S1) (Sarmiento-Garcés & Amat-García 2014).

Molecular taxonomy constitutes an alternative to accurately delimit and identify species that lack useful morphological characters (Dayrat 2005; Dupuis et al. 2012; Schlick-Steiner et al. 2009; Schwarzfeld & Sperling 2014). This approach has been primarily used in Scarabaeinae beetles to resolve deep relationships (Gunter et al. 2016; Tarasov & Génier 2015). However, the ~~molecular study of the~~ relationships at the genus or species level in this subfamily remains understudied. For this reason, there is currently no molecular phylogeny available for *Dichotomius*. Recent studies on deep phylogenies for Coleoptera and dung beetles, however,

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indicate that the genus is likely paraphyletic (although this result is based on a small number of species of *Dichotomius* and only one individual per species) (Bocak et al. 2014; Monaghan et al. 2007).

In recent years taxonomists have begun to integrate different lines of evidence to discover and delimit species, which is often referred to as "integrative taxonomy" (Padial & De La Riva 2010; Schlick-Steiner et al. 2009). The application of this approach, usually done through the combination of molecular and morphological information, has improved taxonomic rigor yielding a more precise biodiversity inventory (both reducing or increasing species numbers) (Sturaro et al. 2018). In this study we implemented an integrative taxonomy approach that combines morphological and molecular data (both mitochondrial and nuclear) to make a preliminary assessment of the species diversity and phylogenetic relationships in the genus *Dichotomius*. The information derived from this research is crucial to further characterise species' richness as well as to understand patterns of adaptation, speciation and biogeography in these dung beetles.

Materials & Methods

Sampling

Our total sample set consisted of 304 individuals of *Dichotomius* (31 species). The morphological analysis of male genitalia included 208 individuals from 28 species (Table S1), whereas the genetic analysis consisted of 145 specimens from 16 species; 52 of these sequences were obtained from GenBank (Table S1). This is representative of 14 species-groups and three subgenera in *Dichotomius*. All specimens for which we obtained data (DNA or morphology) came from the following biological collections: (i) Colección Alejandro Lopera-Toro (CALT-

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ECC, Colombian Collection ID 2), (ii) Museo de Historia Natural Universidad Distrital (MUD, Colombian Collection ID 46), and (iii) Colección de Artrópodos de la Universidad del Rosario (CAUR, Colombian Collection ID 229). These individuals were identified by experts or using most recent taxonomical keys (Nunes 2017; Sarmiento-Garcés & Amat-García 2014; Vaz-de-Mello et al. 2011).

Morphometric analyses

Because shape of the male genitalia is considered one of the most informative morphological characters in the classification of *Dichotomius* species (López-Guerrero 2005; Sarmiento-Garcés & Amat-García 2014), we analysed the quantitative variation of the aedeagus in 208 individuals (28 species; Table S1). Male genitalia preparation followed a standard procedure: we detached the last two abdominal segments, soaked them in 10% KOH at 60ºC - 70ºC for 12 hours and neutralized them in 1% acetic acid to finally store them in glycerine (Sarmiento-Garcés & Amat-García 2014). Then, we cleaned and dissected the aedeagus. Finally, we photographed the aedeagus in dorsal view and using a Leica DFC320 digital camera coupled to a Leica S6 stereoscope at 4X magnification.

We applied landmark-based geometric morphometrics to these photographs in order to analyse genital shape. We used tpsDig v.2.31 (Rohlf 2004) to digitise 33 landmarks per individual that describe the outline of the aedeagus, all of them were placed on the parameres (Fig. S2a). This landmark dataset was subjected to superimposition using a Generalized Procrustes Analysis (GPA) in the R package 'geomorph' (Adams & Otárola-Castillo 2013). For this, the software aligns, scales and rotates the configurations to line up the corresponding landmarks as closely as possible, minimizing differences between landmark configurations

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without altering shape. Then, we obtained partial warps (or shape variables) that indicate partial contributions of hierarchically scaled vectors spanning a linear shaped space. With this information we generated a consensus shape that summarises the aedeagus' shape variation among all *Dichotomius* species included (Fig. S3). In this way, each specimen's shape is quantified by the deviation of its landmark configuration from the average landmark configuration (i.e. consensus shape), which allows to visualise differences between groups. Differences in aedeagus' shape among species were tested using a Procrustes MANOVA applied to the aligned landmark configurations. This was done using the *procD.lm* function in the 'geomorph' R package (Adams & Otárola-Castillo 2013).

We implemented a principal component analysis (PCA) on the procrustes aligned data using the *plotTangentSpace* function in the 'geomorph' R package (Adams & Otárola-Castillo 2013). Of the 66 PCs produced, the first two cumulatively accounted for ~92% of the total shape variance; therefore, further analyses were performed on these PCs. We used the function *plotRefToTarget* from the same package to generate the deformation grids representing the extremes (maximum and minimum) of shape variation along the principal components 1 and 2 (PC1 and PC2). We then applied a discriminant analysis of principal components (DAPC) using the R package 'adegenet' (Jombart 2008).

We also applied a model-based hierarchical clustering using the R package 'mclust' (Scrucca et al. 2016) in order to identify groups of individuals that resemble each other, independent of other evidence or *a priori* assignments. This method uses expectation maximization (EM) to estimate the Maximum Likelihood (ML) of alternative multivariate mixture models that describe shape variation in the data and estimates the optimal number of clusters based on the Bayesian Information Criterion (BIC). All models were evaluated for a

predefined number of 1 to the maximum number of morphospecies studied (28 in our case, i.e. those for which morphology data was available).

Molecular analyses

We extracted DNA from legs of 95 specimens of *Dichotomius* using the DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions with minor modifications: 40 μL of proteinase K were used, protein digestion lasted for at least 2 hours and the final elution was made in 100 μL of warm AE buffer. Then, we amplified the 3' and 5' ends of the cytochrome c oxidase I gene (*COI*), and the nuclear gene 28S. All PCR reactions were performed in a final volume of 10 μL containing 1μL of 10X Buffer, 0.6 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTP mix (10 mM), 0.5 μL of each primer (10 µM), 0.05 μL of DNA polymerase (5U/µl; QIAGEN) and 5.85 μL of dH_2O. To amplify the 3' end of the COI gene we used the primers C1-J-2183 (Jerry: 5'-CAACATTTATTTTGATTTTTTGG-3') and TL2-N-3014 (Pat: 5'-TCCAATGCACTAATCTGCCATATTA-3') (Simon et al. 1994). The amplification PCR profile consisted of an initial denaturation step of 94ºC for 5 minutes, 7 cycles of denaturation at 94ºC for 1 minute, annealing at 48ºC for 45 seconds and extension at 72ºC for 1 minute, followed by 33 cycles of denaturation at 94ºC for 45 seconds, annealing at 52ºC for 45 seconds and extension at 72ºC for 1.5 minutes, with a final extension at 72 ºC for 10 minutes. The 5' end of the COI gene (the barcode) was amplified with the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG -3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA -3') (Folmer et al. 1994), using the following PCR conditions: 94ºC for 5 minutes, 35 cycles of 94ºC for 30 seconds, 45ºC for 30 seconds, 72ºC for 1.5 minutes and a final extension at 72ºC for 7 minutes. To amplify the 28S gene we used the

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primers 28SFF (5'-TTACACACTCCTTAGCGGAT-3') and 28SDD (5'-GGGACCCGTCTTGAAACAC-3') (Monaghan et al. 2007). PCR cycling was 94ºC for 5 minutes, 38 cycles of 94ºC for 30 seconds, 53ºC for 30 seconds, 72ºC for 45 seconds and a final extension of 72ºC for 10 minutes.

All PCR products were purified with ExoSAP and their bidirectional sequencing was carried out by ELIM Biopharmaceuticals Inc. (Hayward, CA). Forward and reverse sequences from each amplicon were verified and assembled into a single consensus contig based on a minimum match of 80% and a minimum overlap of 50 bp using CLC main workbench.

Sequences of each genetic marker were aligned independently using MUSCLE (Edgar 2004) in MESQUITE v3.04 (Maddison & Maddison 2011); poorly aligned regions were corrected manually. Protein coding sequences were translated into amino acids to confirm the absence of stop codons and anomalous residues in MESQUITE v3.04 (Maddison & Maddison 2011). Additional sequences of *Dichotomius* available in GenBank (Table S1) were downloaded and integrated into the alignments. All sequences generated by us were deposited in GenBank and their accession numbers are listed in Table S1.

We estimated a phylogenetic tree based on the sequence information from the 3' COI, 5' COI, 28S and 16S. All sequences from the latter marker were obtained from GenBank and correspond to the species *D. nisus*, *D. yucatanus*, *D. parcepunctatus* and *D. boreus* (Table S1). We concatenated all genes into a single alignment (2,546bp) that included 16 species of *Dichotomius* and nine outgroups: *Deltochilum larseni*, *Neateuchus proboscideus*, *Ontherus diabolicus*, *Pedaria* sp., *Panelus* sp., *Australammoecius occidentalis*, *Euphoresia* sp., *Brindalus porcicollis*, *Pleurophorus caesus* (Table S1). We calculated a ML tree using IQ-TREE using the entire haplotype set derived from all species and individuals (Nguyen et al. 2015) with 1,000

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ultrafast bootstrap replicates. This was done based on the substitution model showing the smallest AIC score for each partition (i.e. COI, 28S and 16S), which was also selected using IQ-TREE ((Nguyen et al. 2015); Table S2).

To test whether *D. satanas* exhibits genetic clustering associated to the Colombian Cordilleras of the Andes as previously suggested (Sarmiento-Garcés & Amat-García 2014), we also estimated a ML topology using all sequences available for the Colombian specimens of this species (COI and 28S) and using the conditions aforementioned. The sequences were all concatenated into a single alignment of 2,145bp that included one individual of *D. boreus, D. quinquelobatus* and *D. protectus* (outgroups) and 60 individuals of *D. satanas:* 8 from the Central Cordillera of Colombia, 14 from the West Cordillera of Colombia and 38 from the East Cordillera of Colombia.

Finally, we used DnaSP version 6.12.01 (Rozas et al. 2003) to calculate diversity parameters (i.e. number of haplotypes (H), haplotype diversity, genetic diversity (π and θ) and Tajima's D) for all species and for *D. satanas,* as well as summary statistics of population differentiation among populations of *D. satanas*.

Species delimitation analyses

We implemented a joint Bayesian inference based on genetic and phenotypic data to delimit species using iBPP (Solís-Lemus et al. 2014). This was done using two independent data sets: (i) all species, and (ii) *D. satanas* from Colombia only. In both cases, we ran the program for three different datasets: (i) morphological and molecular data combined, (ii) morphological data alone, (iii) molecular data alone. In all cases, we used the species-tree topology from IQ-tree as the guide tree. The morphological character matrix used as input included the values of PC1 and

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PC2 from the geometric morphometric analyses. The molecular matrix included all sequences available for the markers 3' COI, 5' COI, 16S and 28S. We specified nine combinations of the prior distribution for the ancestral population size (θ) and the root age of the tree (τ) ranging from scenarios that represent large population sizes and a deep divergence time (θ =G (1, 10) and τ =G (1, 10)) to those representing small population sizes and a shallow divergence time (θ = G (2, 2000) and τ =G (2, 2000)) as previously used (Eberle et al. 2016; Olave et al. 2017). We used default values of σ^2 and $\kappa = 0$, thus these priors are non-informative and the program estimates them. The MCMC analysis was run over 50,000 generations, sampling every 1,000 steps and using a 10% burn-in. We confirmed the robustness of the results by running the analysis with both the algorithms 0 and 1 for rjMCMC searches. As results were very similar, we present those of algorithm 1. The parameters of the locus-specific rates of evolution were fine-tuned using an auto option.

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Results

Morphological analyses

When we tested for aedeagus shape variation in the entire Procrustes shape space, we found differences among all categories tested (i.e. subgenera, species-groups and species; Procrustes MANOVA $p < 0.001$ in all cases). The PCA of the aedeagus shape revealed that most of its variation is contained in few dimensions. The first two PCs accounting for 91.9% of the total variance. PC1 explained 84.16% of the aedeagus shape and was driven by the width of the lateral outer margins in the apex of the parameres, ranging from being broad to narrow (Fig. 1a and Fig. S2). PC2 explained 7.7% of morphological of the aedeagus shape variation and describes the shape formed by the sides of the parameres (Fig. 1a and Fig. S2). The DAPC suggests the

existence of four discrete genitalia morphology groups within *Dichotomius* (Fig. 1b and Fig. S4). The first group (depicted in red tones) was composed mostly by members of the subgenus *Selenocopris* sensu (Nunes 2017) from the species-groups Agenor, Batesi and Inachus (i.e. *D. agenor, D. batesi, D. belus, D. deyrollei, D. favi, D. fortestriatus,* and *D. yucatanus*). This group also contained individuals of the subgenus *Dichotomius* s.s., exclusively those in the species-group Carolinus (i.e. *D. amicitiae* and *D. coenosus*). Finally, the species *D. fonsecae* (subgenus *Cephagonus,* species group Fissus) also clustered in this first group. The second group (depicted in green tones) was mainly formed by species that belong to the subgenus *Dichotomius* s.s. from the species-groups Boreus, Buqueti and Mamillatus (i.e. *D. boreus, D. compresicollis, D. mamillatus, D. podalirius, D. riberoi* and *D. robustus*); the species *D. inachoides* (subgenus *Selenocopris*, species-group Agenor) also grouped here. The third group (yellow) consisted exclusively of individuals from *D. nisus* (isolated species in the *Selenocopris* subgenus). The fourth group comprised only species from the subgenus *Dichotomius* s.s., species-group Mormon, namely: *D. alyattes, D. andresi, D. ohausi, D. protectus, D. divergens, D. quinquelobatus, D. quinquedens* and *D. satanas* (blue tones). Although the species *D. costaricensis* and *D. worontzowi* (both of the *Dichotomius* s.s. subgenus) appeared well differentiated from any other species or group, we only have one sample for each of them, preventing us from making strong inferences. Consistently, mclust identified four clusters entirely coincident with the groupings obtained above (Fig. 1c). This variation is best explained by a model with 'diagonal distribution, variable volume and equal shape' (VEI; BIC=1152.184).

In summary, genitalia morphology supported the existence of the subgenus *Selenocopris* (red group) but excluded *D. nisus* (yellow) from it, placing it as an independent entity. Also, species in the Carolinus group, currently classified as members of *Dichotomius* s.s., fall into

Selenocopris. The subgenus *Dichotomius* s.s. is divided into two clusters, one that contains lowland species (green group) and the other composed only by highland Andean species (blue).

Molecular analyses

We found *Dichotomius* as a monophyletic genus with two well-supported deep clades (Fig. 2, Fig S5). The first clade only contains species from the subgenus *Dichotomius* s.s. plus, as its sister, *D. (Selenocopris) nisus*. The second clade is almost exclusively composed of species from the *Selenocopris* subgenus, except for *D. carolinus*, which is currently included within *Dichotomius* s.s. (Nunes 2017).

Within the subgenus *Dichotomius* s.s we observed a further well-supported subgrouping of species by species-group, with the Mormon, Boreus and Mamillatus groups forming each a monophyletic cluster (Fig. 2; Fig S5). Within each of these species-groups most species appeared as monophyletic, except for *D. satanas*. This species formed two monophyletic clades, one consisting of Colombian specimens and the other composed by Central American individuals (Fig. 2; Fig S5).

Within the *Selenocopris* subgenus all species were recovered as monophyletic but this was not the case for species-groups (Fig. 2; Fig S5). For example, although we recovered a monophyletic clade composed by most members of the Agenor species-group (i.e. *D. agenor*, *D. deyrollei* and *D. amplicollis*), *D. belus*, which is also a member of the Agenor species-group, did not fall into this clade (Fig. 2; Fig S5). In contrast, *D. yucatanus* and *D. parcepunctatus* formed a monophyletic and well supported cluster despite belonging to different species groups (group Inachus and Batesi, respectively; Fig. 2; Fig S5). Finally, the position of *D. carolinus* (and the

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Carolinus species-group), is not well supported (Fig. 2; Fig S5). In general, mtDNA showed higher haplotype diversity than the 28S nuclear gene (Table 1).

When populations of *D. satanas* from Colombia were analysed separately to evaluate whether this species displays genetic clustering associated with geography or phenotype (Sarmiento-Garcés & Amat-García 2014), we mainly observed clustering and genetic differentiation associated to the three Cordilleras of the north of the Andes (Fig. 3, Table 2). Individuals from the Central and the Western Cordilleras were reciprocally monophyletic, and both were sister to the Western Cordillera clade. Interestingly, this phylogenetic pattern associates to morphological differences in the females: the Central and Western clusters contain females with only two protuberances in the pronotum, while the cluster of the Eastern Cordillera includes females with two and four protuberances. At the same time, the latter cluster separates into two inner groups, one that contains only females with four protuberances and the second, where females of two and four protuberances are found (Fig. 3).

Species delimitation

The total-evidence (morphology and DNA) approach to Bayesian species delimitation (iBPP) did not support the *a priori* morphospecies assignment (Fig. 4). In most θ and τ scenarios tested, the posterior probability for the existence of the 16 morphospecies evaluated was lower than 50%. The only *a priori* defined species that consistently presented high support for all prior combinations were *D. belus*, *D. nisus* and *D. mamillatus*. All other species were better supported only when modelling small population sizes (θ =0.01) and medium to deep divergence time (τ =0.05 and τ =0.1), but never when modelling a shallow divergence time (τ = 0.01; Fig. 4).

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Consistent with the phylogenetic tree, the haplotype networks showed a clear separation between the *Selenocopris* and *Dichotomius* s.s. subgenera. This was more evident in the mitochondrial markers, which provided better resolution than the ribosomal 28S. Specifically, both mitochondrial markers separated the *Selenocopris* and *Dichotomius* s.s. by at least 50 mutational steps, while the nuclear 28S did so by two mutational steps (Fig. 3). In general, mtDNA showed high haplotype diversity (Table 1) and, within the *Selenocopris* subgenus, most mitochondrial haplotypes were exclusive to single species. Interestingly, there were no shared haplotypes between the *D. nisus* and the subgenera *Selenocopris* and *Dichotomius* s.s. as both mitochondrial markers showed *D. nisus* having a unique haplotype separated from any other by at least 20 mutational steps (Fig. 3). In contrast, within the *Dichotomius* s.s. subgenus we observed some species having unique and well differentiated mitochondrial haplotypes, but in general, all these haplotypes derived from *D. satanas* (Fig. 3). This pattern was more evident in the 3' end of the COI gene.¶

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In contrast, the existence of the subgenera *Selenocopris* and *Dichotomius* s.s. was strongly supported, regardless of the θ and τ *priors* used (Fig. 4). In the subgenus *Dichotomius* s.s, the species-groups Mormon, Boreus and Mamillatus showed strong support, but the existence of species within these groups was less supported. In the Mormon group, the separation of *D. quinquelobatus* from other members of this group showed high posterior probability values in most scenarios, except for those with $\tau = 0.01$. However, the separation of *D. protectus* from *D. andresi*, or Colombian *D. satanas* from *D. alyattes* was rarely supported (Fig. 4). This was also observed in the Boreus species-group, where the delimitation between *D. boreus* and *D. podalirius* always had low posterior probabilities (Fig. 5).

Within the subgenus *Selenocopris* the monophyly of species-groups was far less supported. For example, the non-monophyly of the Agenor group always showed high posterior probabilities (Fig. 4). Similarly, the delimitation between *D. yucatanus* and *D. parcepunctatus* (currently considered as members of different species-groups) consistently received low support under almost all scenarios tested.

The species delimitation based on molecular or morphological data alone were consistent with the total-evidence approach at the level of subgenera (Fig. S6). However, the results of these independent data types tended to provide stronger supports to species-groups and some species, especially the molecular data.

Finally, the total-evidence analysis of species delimitation done in *D. satanas* failed to identify any of the phylogenetic clusters associated to geography as separate species (in most θ and τ scenarios tested the support for these clusters was lower than 60%, Fig. S7a). This suggests that *D. satanas* is likely a single species with phenotypic polymorphism. However, just as before,

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436 the analyses with only molecular data presented stronger supports while the analysis based on
437 morphological data provided very poor support Fig. S7b and c).
438
439 ## Discussion
440 Since the first description of *Dichotomius* by Hope in 1838 (Hope 1838) about 170 species have
441 been described in the genus using morphology as the only diagnostic tool, and although there
442 have been recent morphological revisions, *Dichotomius* remains a challenging taxonomic puzzle
443 (Nunes 2017; Nunes & Vaz-de-Mello 2013; Nunes & Vaz-de-Mello 2016; Nunes et al. 2012).
444 Here we used aedeagus morphology and phylogenetic analyses to assess the validity of some
445 species in this dung beetle genus. Our study suggests it is necessary to make a comprehensive
446 revision of the number of species within the genus that combines DNA sequence and
447 morphological data.
448 Despite what previous deep phylogenies of the subfamily Scarabaeinae had suggested
449 (Bocak et al. 2014; Monaghan et al. 2007), we found *Dichotomius* as a monophyletic genus. This
450 is likely because our study is the first to include a more extensive sampling of species and
451 individuals in this genus. We also confirmed the existence of the subgenera *Dichotomius* s.s. and
452 *Selenocopris* in the molecular phylogeny and, to a lesser extent, in the morphology of the
453 aedeagus. This separation also seems consistent with distributional patterns, where according to
454 our current sampling, *Selenocopris* species occur in both Central and South America, but
455 *Dichotomius* s.s. is restricted to South America with only one exception: *D. satanas*.
456 However, the position of *D. nisus* outside *Selenocopris* and the inclusion of the Carolinus
457 group inside this subgenus was unexpected. Until recently, *D. nisus* was recognised as the type
458 species for the *Luederwaldtinia* subgenus (Martínez 1951) but because both *Luederwaldtinia* and
459 *Selenocopris* subgenera include described species that have clypeal teeth but lack clypeo-genal

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Commented [MOU7]: This is all a convoluted explanation. You can't 'confirm the monophyly' of something and then say that not all of the species in that group belong there. It's internally contradictory. Needs to be rephrased here and elsehwere.

462 angle, Nunes synonymised *Luederwaldtinia* with *Selenocopris* (Nunes 2017). Even so, Nunes
463 still recognised *D. nisus* as unique within *Selenocopris*, leading to its classification in a separate
464 species-group as an "isolated species" (Nunes 2017; Nunes & Vaz-de-Mello 2013). However,
465 our data does not agree with this synonymisation as neither the aedeagus morphology nor the
466 molecular data support the placing of *D. nisus* within *Selenocopris* and, in fact, both data types
467 show this species more closely related to members of *Dichotomius* s.s. Also, *D. nisus* has a
468 unique distribution and ecology that differentiates it from other *Dichotomius,* being a common
469 species that is restricted to Orinoquia lowlands, pastures and open environments (França et al.
470 2016; Louzada & Carvalho E Silva 2009). Therefore, the resurrection of *Luederwaldtinia* with
471 *D. nisus* as type species needs to be evaluated by studying the morphology and DNA variation of
472 other species previously under this subgenus. On the other hand, species in the Carolinus
473 species-group (currently classified within *Dichotomius* s.s.) grouped within the *Selenocopris*
474 subgenus in both data types, suggesting that this species-group should be re-classified.
475 Considering Carolinus species as part of *Selenocopris* also makes sense in the light of geographic
476 distribution since species in this species-group are restricted to Central America, where to our
477 knowledge the subgenus *Dichotomius* s.s. occurs with only one species.
478 The subgenus *Selenocopris* was recovered by the molecular and morphological data,
479 although only DNA data allowed to explore inner relationships. In this way, the molecular
480 phylogeny and the total-evidence delimitation analysis supported the existence of the Agenor
481 species group (i.e. *D. agenor, D. deyrollei* and *D. amplicollis*), but strongly supported the
482 exclusion of *D. belus* from it, contradicting its current classification. This separation may reflect
483 differences in ecology or distribution of *D. belus* from the other members of the Agenor species-
484 group. For instance, while all these species occur in xerophytic forests, *D. belus* is the only of

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them that can reach elevations up to 2200 masl (Arellano et al. 2008; Giraldo et al. 2018). This suggests that elevation and/or humidity variables may have contributed to the differentiation of *D. belus*, possibly acting as a barrier between this species and other lowland species in the Agenor group. In addition, *D. belus* falls much less frequently in pitfall traps compared to *D. agenor*, even though it is abundant when manually collected in cattle dung pads; this may indicate the existence of differences in behaviour or at least in food preferences.

Also within the subgenus *Selenocopris* we recovered *D. yucatanus* and *D. parcepunctatus* as closely related sister species. In consequence, the total-evidence species delimitation analysis failed to recognise them as different species despite they belonging to different species-groups (Inachus and Batesi) and having a very distinct geographic distribution. This finding is consistent with a previous molecular phylogeny built for the tribe Scarabaeidae that recovered *D. yucatanus* and *D. parcepunctatus* as sister species across all the 9008 ML trees sampled (Borrow 2011). Unfortunately, the existing information on these species is insufficient to explain this pattern and more studies about the ecology and/or distribution of these species are needed.

Within the subgenus *Dichotomius* s.s. our data strongly supported the existence of the species-groups Mamillatus, Mormon and Boreus, and overall, this grouping coincides with differences in elevational distribution. For example, aedeagus morphology grouped the species-groups Mamillatus and Boreus in a single cluster that contains only lowland species with Amazonian distribution (green in Fig. 1), while the Mormon group is composed only by highland species restricted to the Andes (blue in Fig. 1). The molecular phylogeny separated the lowland cluster in the corresponding Mamillatus and Boreus groups, but these were not reciprocally monophyletic since both *D. podalirius* and *D. boreus* (Boreus group) are more closely related to

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the highland species. Also, the total-evidence species delimitation found strong support for the separation of *D. podalirius* and *D. boreus,* which can be partially explained by the ability of *D. boreus* to reach higher elevations (100-1000 masl) than *D. podalirius* (100-350 msal) in the foothills of the Eastern Cordillera of Colombia (Medina et al. 2001).

In contrast, species in the Mormon species-group clustered all together and were hardly distinguishable at the molecular level. Even so, *D. satanas* split in two monophyletic clusters that correspond to Central American and Colombian individuals, suggesting they are different entities. Nonetheless, the species delimitation method applied was not able to discriminate these taxa as independent entities (except for *D. quinquelobatus*). Interestingly, while all species in the Mormon group are found in elevations between 1000 and 2000 masl only *D. quinquelobatus* goes down and reaches the foothills of the Eastern Colombia Cordillera (120-2200 masl (Sarmiento-Garcés & Amat-García 2014)), thus receiving some influence from the Orinoquia and Amazonia. Our phylogeny suggests that the highland clade derives from lowland species, although this needs further confirmation.

Additionally, while Colombian *D. satanas* showed population structure associated with the Andean Cordilleras, and under morphological based taxonomic studies these populations would be identified as two species, none of our delimitation analyses discriminated these populations as separate species. Therefore, the currently available data indicates that Colombian *D. satanas* is a single species that displays a remarkable phenotypic variation in the number of protuberances (two and four) on the pronotum of females. This is a unique condition in the Scarabaeinae subfamily, and this variation is associated with geography to some extent. At present it is not possible to pinpoint the factors contributing to the maintenance of this variation although processes such as sexual selection, known to drive horn polymorphism in multiple

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534 species of beetles (Emlen et al. 2007; Kijimoto et al. 2013; Simmons & Watson 2010), may be
535 implicated. Also, the fact that the four-protuberances morph is collected only in open and
536 disturbed habitats whilst the two-protuberances morph is mostly found in forested habitats
537 suggests that variables such as temperature variation, vegetation coverage and/or food
538 availability, that drastically differ between the two habitats, may be promoting the differentiation
539 between these morphs, at least in females.

540 In general, the results of our total-evidence species delimitation analyses under different
541 scenarios of population size and divergence time were remarkably congruent. However, when
542 the delimitation analysis was based on molecular or morphological data alone the results were
543 much more sensitive to the *priors* used, either supporting most the *a priori* morphospecies
544 assignments (molecular data) or almost none at all (morphology data). This pattern has been
545 previously observed in other studies of species delimitation in beetles, where only the
546 combination of morphological and molecular data resulted in robust estimates by reducing the
547 sensitivity to *prior* parameter choice (Eberle et al. 2016). Our current sampling (in terms of taxa
548 and genes) does not permit us to make definite interpretations on the validity of all species of
549 *Dichotomius*, but we can confidently recognise *D. nisus, D. belus* and *D. mamillatus* as valid and
550 well differentiated species. Although it would have been ideal to reach a final conclusion for all
551 species evaluated here, species delimitation methods are extremely sensitive to multiple biases
552 such as insufficient or unbalanced sampling, incomplete lineage sorting, population structure
553 and/or hybridisation (Astrin et al. 2012; Carstens et al. 2013; Meyer & Paulay 2005; Petit &
554 Excoffier 2009; Sukumaran & Knowles 2017). In our study, we used the morphology of male
555 genitalia as diagnostic trait but other traits used for the identification of *Dichotomius* (Nunes
556 2017) need to be considered. Also, we had an unbalanced representation of species in our

dataset, which also needs to be corrected in future studies. Despite these limitations, this is the first time an integrative species delimitation approach is implemented in *Dichotomius* and we feel that our analytical procedures were adequate enough to reveal the ambiguous taxonomic position of several taxa.

Altogether, our findings indicate the need to revise the current taxonomic classification of *Dichotomius* in the light of both morphological and molecular data. Only such an integrative approach will allow a comprehensive characterisation of the diversity, ecology and distribution of species in this genus, to ultimately understand the mechanisms and processes involved in their adaptation, diversification and speciation.

Conclusions

Dichotomius is a rich and diverse dung beetle genus (Nunes & Vaz-de-Mello 2016) that belongs to the tribe Dichotomini, one of the most problematic tribes in Scarabaeinae (Tarasov & Dimitrov 2016). Therefore, the validation of its taxonomy and evolutionary relations constitutes a step towards a reassessment of the systematic and phylogenetics of New World dung beetles as a whole. Our implementation of a total-evidence species delimitation approach that integrates genetic and phenotypic information provided a powerful tool to accurately delineate lineages in *Dichotomius* and suggest the existence of fewer species in the genus. We recommend including additional species as well as to sample more loci and phenotypic traits to further improve the taxonomy and biogeography of *Dichotomius*. However, we highlight the importance of our findings in the understanding of the biogeographical and evolutionary processes influencing this genus, as well as their significance for taxonomy and conservation.

Acknowledgements

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We would like to thank Rodrigo Sarmiento and Santiago Montoya for helping with the identification of some species and providing valuable opinions. We also thank Camila Ruiz for her help processing some samples of *D. satanas*. All specimens used came from the following collections: CALT-ECC (Colombian Collection ID 2), MUD (Colombian Collection ID 46), and CAUR (Colombian Collection ID 229).

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