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

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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.

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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 ~~of~~ more than 6000 species and 200 genera worldwide (Tarasov & Génier 2015). Within this dung-feeding subfamily, *Dichotomius* Hope, 1838 constitutes one of the richest ~~genus~~ 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)

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,

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 specimens 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-

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 male genitalia are 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

139 without altering shape. Then, we obtained partial warps (or shape variables) that indicate partial
140 contributions of hierarchically scaled vectors spanning a linear shaped space. With this
141 information we generated a consensus shape that summarises the aedeagus', shape variation
142 among all *Dichotomius* species included (Fig. S3). In this way, each specimen's shape is
143 quantified by the deviation of its landmark configuration from the average landmark
144 configuration (i.e. consensus shape), which allows to visualise differences between groups.
145 Differences in aedeagus' shape among species were tested using a Procrustes MANOVA applied
146 to the aligned landmark configurations. This was done using the *procD.lm* function in the
147 'geomorph' R package (Adams & Otárola-Castillo 2013).
148 We implemented a principal component analysis (PCA) on the procrustes aligned data
149 using the *plotTangentSpace* function in the 'geomorph' R package (Adams & Otárola-Castillo
150 2013). Of the 66 PCs produced, the first two cumulatively accounted for ~92% of the total shape
151 variance; therefore, further analyses were performed on these PCs. We used the function
152 *plotRefToTarget* from the same package to generate the deformation grids representing the
153 extremes (maximum and minimum) of shape variation along the principal components 1 and 2
154 (PC1 and PC2). We then applied a discriminant analysis of principal components (DAPC) using
155 the R package 'adegenet' (Jombart 2008).
156 We also applied a model-based hierarchical clustering using the R package 'mclust'
157 (Scrucca et al. 2016) in order to identify groups of individuals that resemble each other,
158 independent of other evidence or *a priori* assignments. This method uses expectation
159 maximization (EM) to estimate the Maximum Likelihood (ML) of alternative multivariate
160 mixture models that describe shape variation in the data and estimates the optimal number of
161 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 (Patt: 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

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). When additional sequences of *Dichotomius* were available in GenBank (Table S1), these 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 (Table S1). Because the species and specimens sequenced were not necessarily the same in all markers (Table S1), we reduced the data set in such a way that each species was represented in at least two loci. To this end, we first combined the haplotypes of all individuals from the same species into a consensus haplotype by coding polymorphic sites with their corresponding IUPAC ambiguity code. This was done for each of the four genetic markers. Then, we concatenated all genes into a single alignment (2,546bp) that included 16 species of *Dichotomius* and nine

208 outgroups: *Deltochilum larseni*, *Neateuchus proboscideus*, *Ontherus diabolicus*, *Pedaria* sp.,
209 *Panelus* sp., *Australammoecius occidentalis*, *Euphoresia* sp., *Brindalus porcicollis*,
210 *Pleurophorus caesus* (Table S1). We calculated a ML tree using IQ-TREE (Nguyen et al. 2015)
211 with 1,000 ultrafast bootstrap replicates. This was done based on the substitution model showing
212 the smallest AIC score for each partition (i.e. each locus), which was also selected using IQ-
213 TREE ((Nguyen et al. 2015); Table S2). Relationships among species in the 3' COI, 5' COI and
214 28S were also estimated with TCS haplotype networks using PopART with default parameters
215 (Leigh & Bryant 2015) and using the entire haplotype set derived from all species and
216 individuals.

217 To test whether *D. satanas* exhibits genetic clustering associated to the Colombian
218 Cordilleras of the Andes as previously suggested (Sarmiento-Garcés & Amat-García 2014), we
219 also estimated a ML topology using all sequences available of the 3' COI, 5' COI and 28S for
220 this species and using the conditions aforementioned. The sequences were all concatenated into a
221 single alignment of 2,145bp consisting of one individual of *D. agenor* (outgroup) and 79
222 individuals of *D. satanas:* 25 from Central America, 7 from the Central Cordillera of Colombia,
223 11 from the West Cordillera of Colombia and 36 from the East Cordillera of Colombia.

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

228

229 *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 for all species and also for *D. satanas* 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 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.

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-group 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-group 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

277 entirely coincident with the groupings obtained above (Fig. 1c). This variation is best explained
278 by a model with 'diagonal distribution, variable volume and equal shape' (VEI; BIC=1152.184).
279 In summary, genitalia morphology supported the existence of the subgenus *Selenocopris*
280 (red group) but excluded *D. nisus* (yellow) from it, placing it as an independent entity. Also,
281 species in the Carolinus group, currently classified as members of *Dichotomius* s.s., fall into
282 *Selenocopris*. The subgenus *Dichotomius* s.s. is divided into two clusters, one that contains
283 lowland species (green group) and the other composed only by highland Andean species (blue).
284
285 *Molecular analyses*
286 We found *Dichotomius* as a monophyletic genus with three well-supported deep clades (Fig. 2).
287 The first clade only contains species from the subgenus *Dichotomius* s.s. The second, sister to the
288 previous one, is solely composed of *D. nisus*. The third clade, sister to the other two, is almost
289 exclusively composed of species from the *Selenocorpis* subgenus, except for *D. carolinus,*
290 currently included within *Dichotomius* s.s. (Nunes 2017). Within the subgenus *Dichotomius* s.s
291 we observed a further well-supported subgrouping of species by species-group, with the
292 Mormon, Boreus and Mamillatus groups forming each a monophyletic cluster (Fig. 2). Within
293 the *Selenocopris* subgenus, most members of the Agenor species-group clustered together (i.e. *D.*
294 *agenor, D. deyrollei* and *D. amplicollis*), except for *D. belus* (Fig. 2). In contrast, *D. yucatanus*
295 and *D. parcepunctatus* are monophyletic and well supported despite belonging to different
296 species groups (group Inachus and Batesi, respectively; Fig. 2). Finally, the position of *D.*
297 *carolinus* (and the Carolinus species-group), is not well supported (Fig. 2).
298 Consistent with the phylogenetic tree, the haplotype networks showed a clear separation
299 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.

When populations of *D. satanas* 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. 4, Table 2). Individuals from the Central and the Eastern Cordilleras were reciprocally monophyletic, and both were sister to the Central American clade. In contrast, individuals from the Western Cordillera were not monophyletic although most of them clustered together. 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.

323

324 *Species delimitation*

325 The total-evidence (morphology and DNA) approach to Bayesian species delimitation (iBPP) did
326 not support the *a priori* morphospecies assignment (Fig. 5). In most θ and τ scenarios tested, the
327 posterior probability for the existence of the 16 morphospecies evaluated was lower than 50%.
328 The only *a priori* defined species that consistently presented high support for all prior
329 combinations were *D. belus, D. nisus* and *D. mamillatus.* All other species were better supported
330 only when modelling small population sizes (θ =0.01) and medium to deep divergence time (τ
331 =0.05 and τ =0.1), but never when modelling a shallow divergence time (τ = 0.01; Fig. 5).
332 In contrast, the existence of the subgenera *Selenocopris* and *Dichotomius* s.s. was
333 strongly supported, regardless of the θ and τ *priors* used (Fig. 5). In the subgenus *Dichotomius*
334 s.s, the species-groups Mormon, Boreus and Mamillatus showed strong support, but the
335 existence of species within these groups was less supported. In the Mormon group, the separation
336 of *D. quinquelobatus* from other members of this group showed high posterior probability values
337 in most scenarios, except for those with τ = 0.01. However, the separation of *D. protectus* from
338 *D. andresi,* or *D. satanas* from *D. alyattes* was rarely supported (Fig. 5). This was also observed
339 in the Boreus species-group, where the delimitation between *D. boreus* and *D. podalirius* always
340 had low posterior probabilities (Fig. 5).
341 Within the subgenus *Selenocopris* the existence of species-groups was far less supported.
342 For example, the non-monophyly of the Agenor group always showed high posterior
343 probabilities (Fig. 5). Similarly, the delimitation between *D. yucatanus* and *D. parcepunctatus*
344 (currently considered as members of different species-groups) consistently received low support
345 under almost all scenarios tested.

346 The species delimitation based on molecular or morphological data alone were consistent
347 with the total-evidence approach at the level of subgenera (Fig. S5). However, the results of
348 these independent data types tended to provide stronger supports to species-groups and some
349 species, especially the molecular data.
350 Finally, the total-evidence analysis of species delimitation done in *D. satanas* failed to
351 identify any of the phylogenetic clusters associated to geography as separate species (in most θ
352 and τ scenarios tested the support for these clusters was lower than 60%, Fig. S6a). This suggests
353 that *D. satanas* is likely a single species with phenotypic polymorphism. However, just as before,
354 the analyses with only molecular data presented stronger supports while the analysis based on
355 morphological data provided very poor supports Fig. S6b and c).
356
357 ## Discussion
358 Since the first description of *Dichotomius* by Hope in 1838 (Hope 1838) about 170 species have
359 been described in the genus using morphology as the only diagnostic tool, and although there
360 have been recent morphological revisions, *Dichotomius* remains a challenging taxonomic puzzle
361 (Nunes 2017; Nunes & Vaz-de-Mello 2013; Nunes & Vaz-de-Mello 2016; Nunes et al. 2012).
362 Here we used aedeagus morphology and phylogenetic analyses to assess the validity of some
363 species in this dung beetle genus. Our study suggests it is necessary to make a comprehensive
364 revision of the number of species within the genus that combines DNA sequence and
365 morphological data.
366 Despite what previous deep phylogenies of the subfamily Scarabaeinae had suggested
367 (Bocak et al. 2014; Monaghan et al. 2007), we found *Dichotomius* as a monophyletic genus. This
368 is likely because our study is the first to include a more extensive sampling of species and
369 individuals in this genus. We also confirmed the existence of the subgenera *Dichotomius* s.s. and

370 *Selenocopris* in the molecular phylogeny and, to a lesser extent, in the morphology of the
371 aedeagus. This separation also seems consistent with distributional patterns, where according to
372 our current sampling, *Selenocopris* species occur in both Central and South America, but
373 *Dichotomius* s.s. is restricted to South America with only one exception: *D. satanas*.
374 However, the position of *D. nisus* outside *Selenocopris* and the inclusion of the Carolinus
375 group inside this subgenus was unexpected. Until recently, *D. nisus* was recognised as the type
376 species for the *Luederwaldtinia* subgenus (Martínez 1951) but because both *Luederwaldtinia* and
377 *Selenocopris* subgenera described species that have clypeal teeth but lack clypeo-genal angle,
378 Nunes synonymised *Luederwaldtinia* with *Selenocopris* (Nunes 2017). Even so, Nunes still
379 recognised *D. nisus* as unique within *Selenocopris*, leading to its classification in a separate
380 species-group as an "isolated species" (Nunes 2017; Nunes & Vaz-de-Mello 2013). However,
381 our data does not agree with this synonymisation as neither the aedeagus morphology nor the
382 molecular data support the placing of *D. nisus* within *Selenocopris* and, in fact, both data types
383 show this species more closely related to members of *Dichotomius* s.s. Also, *D. nisus* has a
384 unique distribution and ecology that differentiates it from other *Dichotomius,* being a common
385 species that is restricted to Orinoquia lowlands, pastures and open environments (França et al.
386 2016; Louzada & Carvalho E Silva 2009). Therefore, the resurrection of *Luederwaldtinia* with
387 *D. nisus* as type species needs to be evaluated by studying the morphology and DNA variation of
388 other species previously under this subgenus. On the other hand, species in the Carolinus
389 species-group (currently classified within *Dichotomius* s.s.) grouped within the *Selenocopris*
390 subgenus in both data types, suggesting that this species-group should be re-classified.
391 Considering Carolinus species as part of *Selenocopris* also makes sense in the light of geographic

392 distribution since species in this species-group are restricted to Central America, where to our
393 knowledge the subgenus *Dichotomius* s.s. occurs with only one species.
394 The subgenus *Selenocopris* was recovered by the molecular and morphological data,
395 although only DNA data allowed to explore inner relationships. In this way, the molecular
396 phylogeny and the total-evidence delimitation analysis supported the existence of the Agenor
397 species group (i.e. *D. agenor, D. deyrollei* and *D. amplicollis*), but strongly supported the
398 exclusion of *D. belus* from it, contradicting its current classification. This separation may reflect
399 differences in ecology or distribution of *D. belus* from the other members of the Agenor species-
400 group. For instance, while all these species occur in dry forests, *D. belus* is the only of them that
401 can reach elevations up to 2200 masl and be found in xerophytic conditions (Arellano et al.
402 2008; Giraldo et al. 2018). This suggests that elevation and/or humidity variables may have
403 contributed to the differentiation of *D. belus*, possibly acting as a barrier between this species
404 and other lowland species in the Agenor group. In addition, *D. belus* falls much less frequently in
405 pitfall traps compared to *D. agenor,* even though it is abundant when manually collected in cattle
406 dung pads; this may indicate the existence of differences in behaviour or at least in food
407 preferences.
408 Also within the subgenus *Selenocopris* we recovered *D. yucatanus* and *D.*
409 *parcepunctatus* as closely related sister species. In consequence, the total-evidence species
410 delimitation analysis failed to recognise them as different species despite they belonging to
411 different species-groups (Inachus and Batesi) and having a very distinct geographic distribution.
412 This finding is consistent with a previous molecular phylogeny built for the tribe Scarabaeidae
413 that recovered *D. yucatanus* and *D. parcepunctatus* as sister species across all the 9008 ML trees
414 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 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. Consistently, 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 *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 *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 species of beetles (Emlen et al. 2007; Kijimoto et al. 2013; Simmons & Watson 2010), may be implicated. Also, the fact that the four-protuberances morph is collected only in open and disturbed habitats whilst the two-protuberances morph is mostly found in forested habitats suggests that variables such as temperature variation, vegetation coverage and/or food availability, that drastically differ between the two habitats, may be promoting the differentiation between these morphs, at least in females.

In general, the results of our total-evidence species delimitation analyses under different scenarios of population size and divergence time were remarkably congruent. However, when the delimitation analysis was based on molecular or morphological data alone the results were much more sensitive to the *priors* used, either supporting most the *a priori* morphospecies assignments (molecular data) or almost none at all (morphology data). This pattern has been previously observed in other studies of species delimitation in beetles, where only the combination of morphological and molecular data resulted in robust estimates by reducing the sensitivity to *prior* parameter choice (Eberle et al. 2016). Our current sampling (in terms of taxa

and genes) does not permit us to make definite interpretations on the validity of all species of *Dichotomius*, but we can confidently recognise *D. nisus, D. belus* and *D. mamillatus* as valid and well differentiated species. Although it would have been ideal to reach a final conclusion for all species evaluated here, species delimitation methods are extremely sensitive to multiple biases such as insufficient or unbalanced sampling, incomplete lineage sorting, population structure and/or hybridisation (Astrin et al. 2012; Carstens et al. 2013; Meyer & Paulay 2005; Petit & Excoffier 2009; Sukumaran & Knowles 2017). In our study, we used the morphology of male genitalia as diagnostic trait but other traits used for the identification of *Dichotomius* (Nunes 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. Even more, our results are indicative of the existence of fewer species in *Dichotomius* than currently recognised based on qualitative morphological traits. The latter is supported by the fact that species delimitation methods are known to overinflate (but not underestimate) the number of species (Sukumaran & Knowles 2017; Yang et al. 2019).

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 Deltochilini, 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.

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674

Table 1(on next page)

Genetic diversity indices for all species and for *D. satanas*

Table 1. Genetic diversity indices for all species and for *D. satanas*

Gen		Number of haplotypes (H)	Haplotype diversity	Nucleotide diversity (π)	Substitution rate (θ)	Tajima's D
3' COI	*D. satanas*	21	0.94	0.03595	0.02930	0.8262 (ND)
	All species	74	0.98	0.08341	0.06133	1.19252 (ND)
5' COI	*D. satanas*	29	0.97	0.02355	0.02361	-0.0084 (ND)
	All species	48	0.98	0.06589	0.06897	-0.1526 (ND)
28S	*D. satanas*	3	0.59	0.00248	0.00229	0.268 (ND)
	All species	10	0.84	0.02057	0.01225	2.249*

ND: Non-different from zero. *Significance <0.05

Table 2(on next page)

Population differentiation among populations of *D. satanas*

WC: Western Cordillera; CC: Central Cordillera; EC: Eastern Cordillera. Central America was not included because its sequences were only available for one fragment. NA: not computable. **0.001 < p < 0.01; ***p<0.001

Table 2. Summary statistics of population differentiation among populations of *D. satanas*.

	WC - CC			WC - EC			CC - EC		
	N_{ST}	D_{XY}	D_a	N_{ST}	D_{XY}	D_a	N_{ST}	D_{XY}	D_a
5' COI	0.24**	0.03784	0.00948	0.14**	0.04250	0.00623	0.49**	0.02877	0.01399
3' COI	0.41**	0.05559	0.02281	0.22**	0.06536	0.01392	0.55***	0.03722	0.02073
28S	NA	0.000001	0.000001	0.66**	0.00332	0.00218	0.56**	0.00382	0.00214

WC: Western Cordillera; CC: Central Cordillera; EC: Eastern Cordillera. Central America was not included because its sequences were only available for one fragment. NA: not computable. **$0.001 < p < 0.01$; ***$p<0.001$

Figure 1

Shape variation of the aedeagus in 28 species of *Dichotomius*

(a) Principal Component Analysis (PCA) and deformation grids showing the shape change of the aedeagus associated with PC1 and PC2. **(b)** Scatter plot of the DAPC analysis with species identity as prior information; ellipses correspond to the 95% confidence interval around the centroid. **(c)** Model based clustering showing the best fitting cluster model by BIC; bars below represent the reassignment probabilities to the clusters with individuals ordered by cluster (top bar) and by *a priori* defined morphospecies (bottom bar).

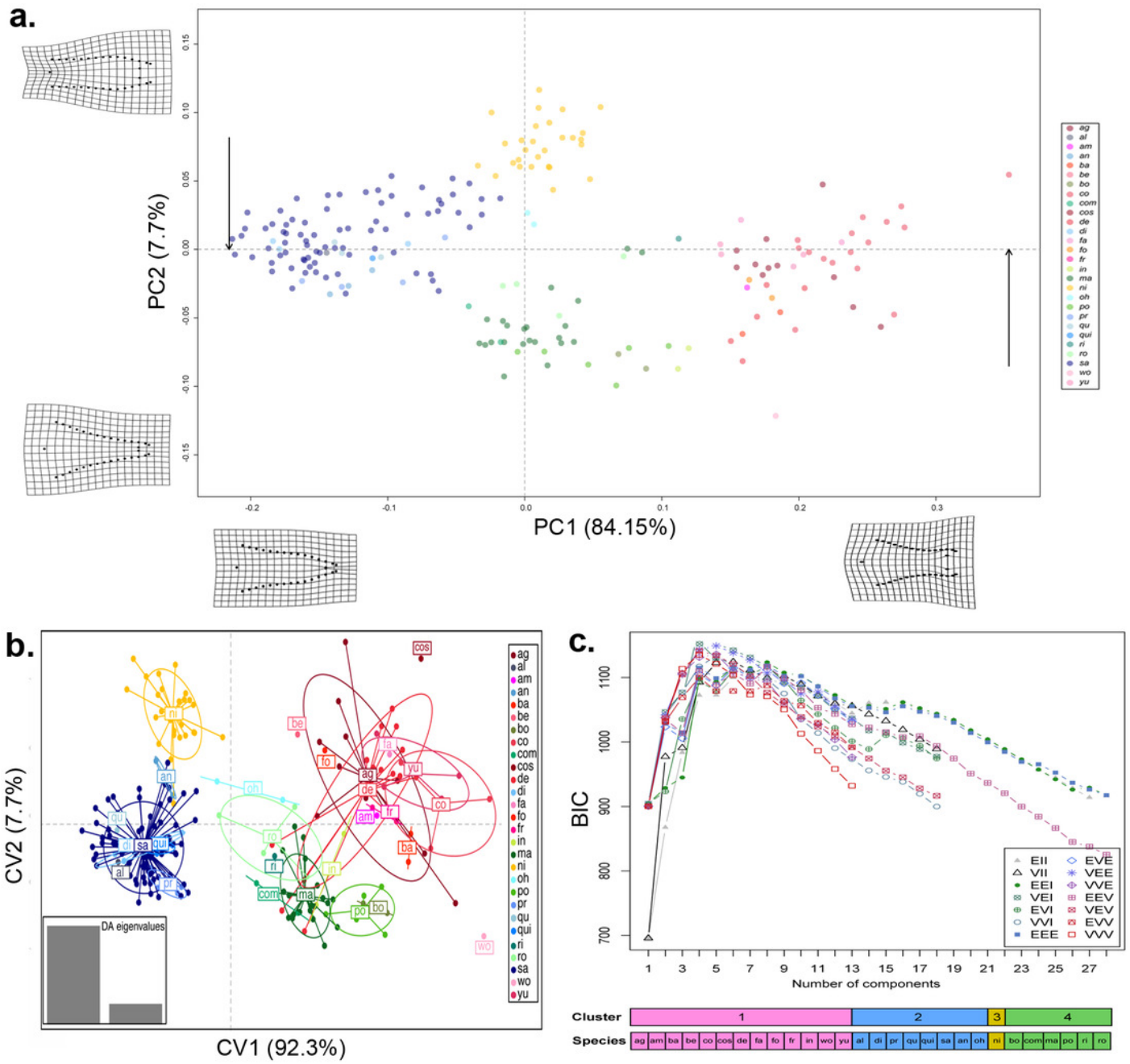
a.
PC2 (7.7%)
PC1 (84.15%)
b.
CV2 (7.7%)
CV1 (92.3%)
DA eigenvalues
c.
BIC
Number of components
EII
VII
EEI
VEI
EVI
VVI
EEE
EVE
VEE
VVE
EEV
VEV
EVV
VVV
Cluster
Species

Figure 2

Phylogenetic relationships of *Dichotomius* species

ML tree of 16 species of *Dichotomius* and nine outgroup species. This tree is based on the combination of the COI and 28S genes and has a single sequence representative for each species. Squares mapped onto branches, in the following order, indicate: subgenus, species-group, and habitat/ecosystem. Circles on nodes indicate bootstrap support.

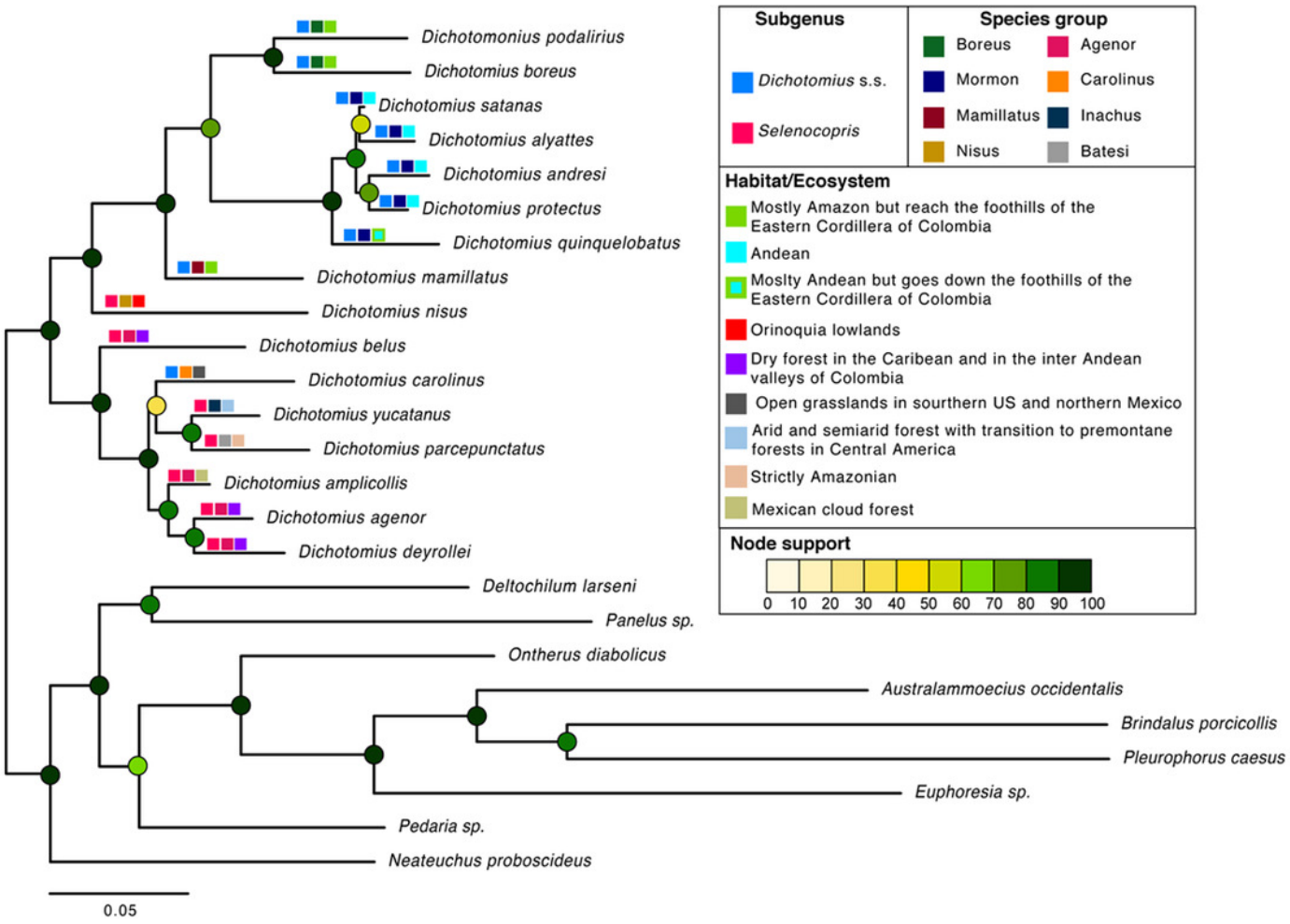

Figure 3

Haplotype networks

The 5’COI network (top) was built for 80 individuals from 13 species, the 3’COI network (middle) contains 116 individuals from 15 species, and the 28S network (bottom) comprises 31 individuals from 11 species. Each circle represents a unique haplotype and it is coloured according to species; the size of circles is proportional to the number of individuals sharing the same haplotype. Numbers on branches indicate mutational steps.

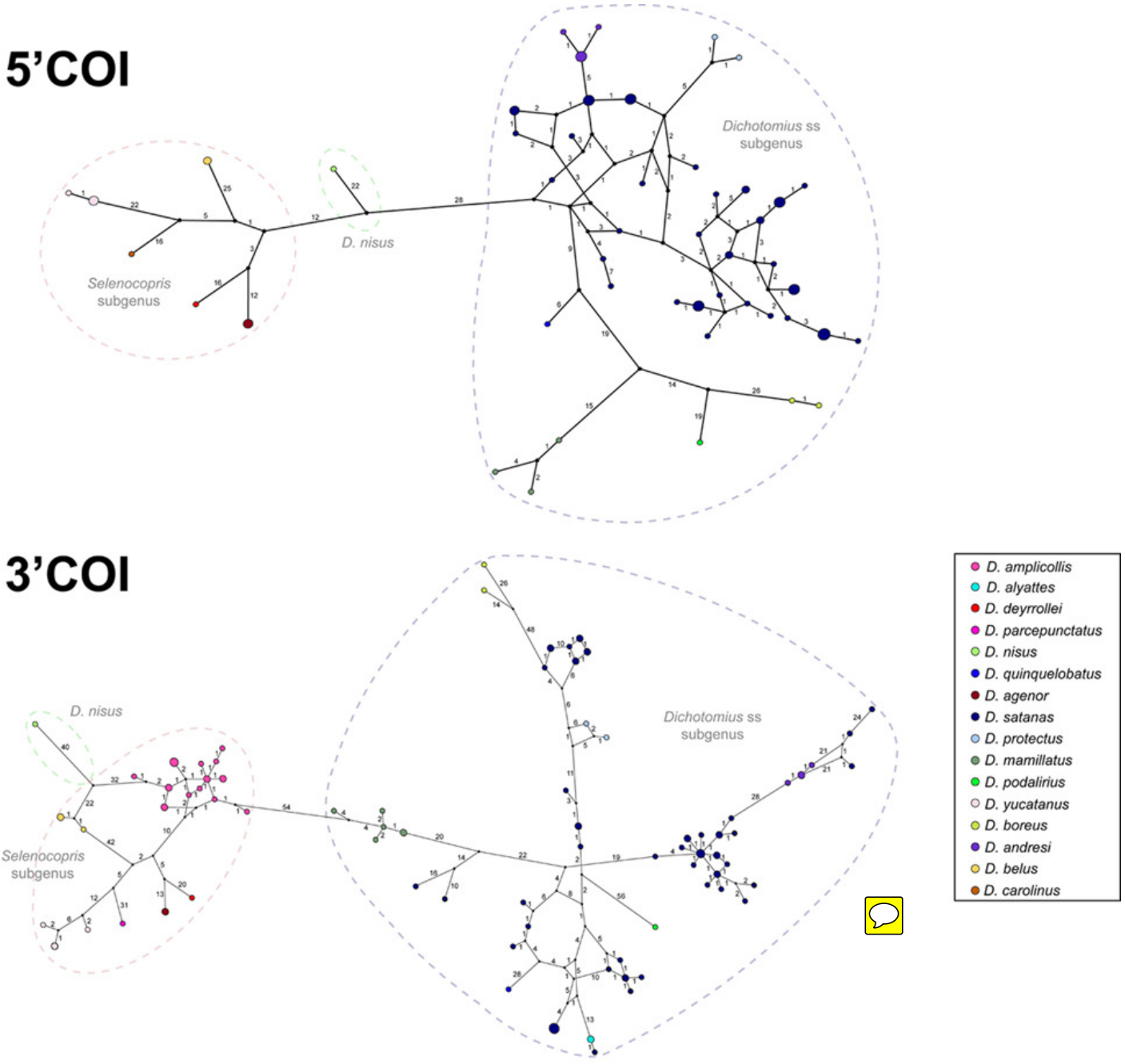
5'COI
Dichotomius ss subgenus
D. nisus
Selenocopris subgenus
3'COI
D. nisus
Dichotomius ss subgenus
Selenocopris subgenus
D. amplicollis
D. alyattes
D. deyrrollei
D. parcepunctatus
D. nisus
D. quinquelobatus
D. agenor
D. satanas
D. protectus
D. mamillatus
D. podalirius
D. yucatanus
D. boreus
D. andresi
D. belus
D. carolinus
28S
Dichotomius ss subgenus
Selenocopris subgenus

Figure 4

Phylogenetic relationships among populations of *D. satanas*

ML tree based on the concatenation of the 5'COI, 3'COI and 28S gene fragments. Circles on nodes indicate bootstrap support. Coloured squares highlight geographic clusters.

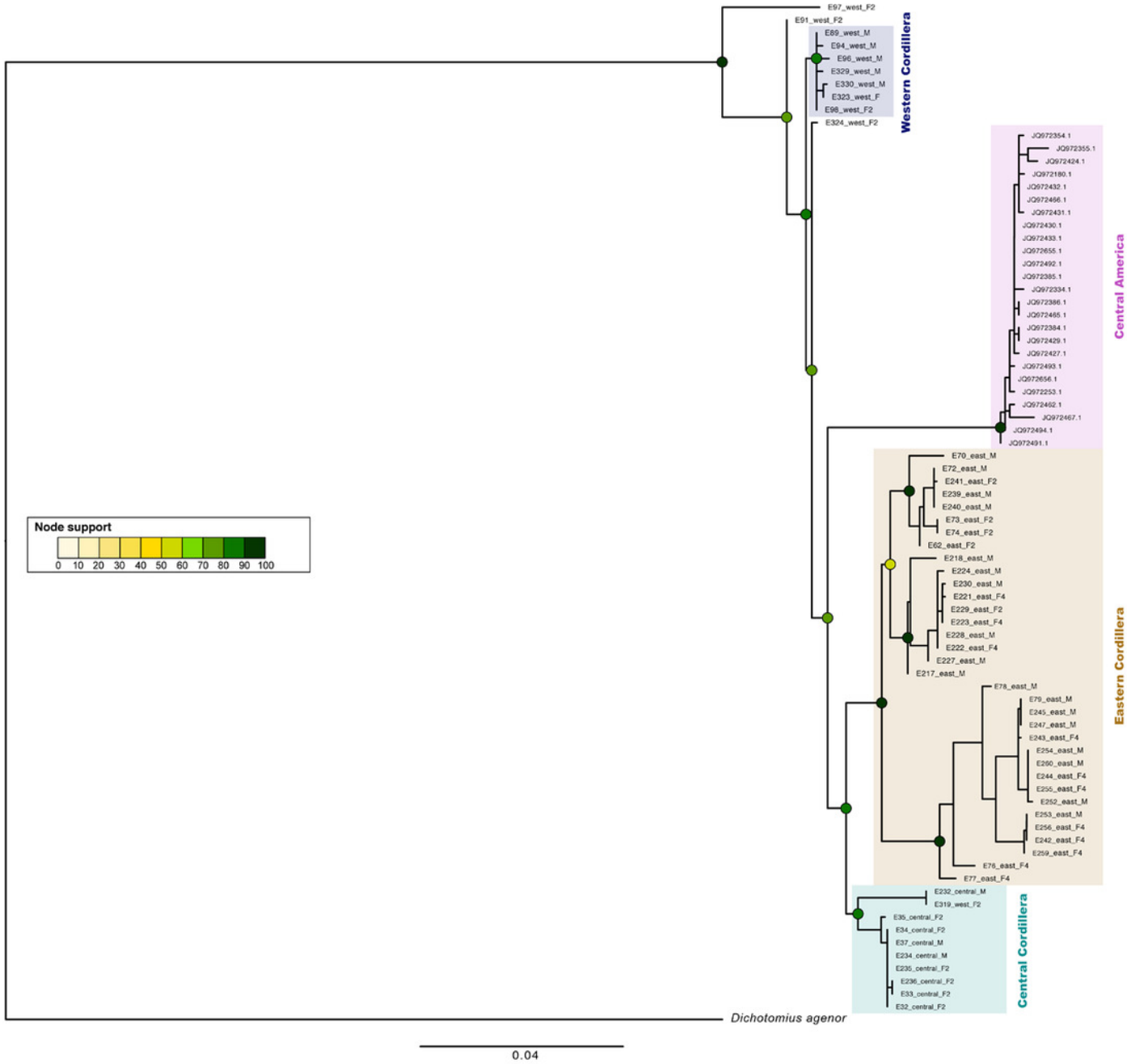

Figure 5

Total-evidence Bayesian species delimitation

Mean posterior probabilities of Bayesian species delimitations were inferred under 9 different