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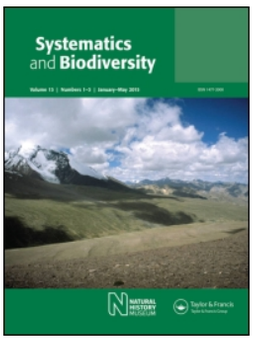

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Research Article

Sequence-based species delineation and molecular phylogenetics of the transitional Nearctic–Neotropical grasshopper genus *Taeniopoda* (Orthoptera, Romaleidae)

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Taeniopoda is a genus of grasshoppers currently represented by 12 species distributed from southern USA to Panama, with most of them occurring along the transitional Nearctic–Neotropical region in central and southern Mexico. Despite being a small group of conspicuous, colourful species, the systematics of *Taeniopoda* has been largely neglected, including its phylogenetic affinity with the morphologically similar, monotypic genus *Romalea*. Here, we assessed the species limits in 11 of the species of *Teniopoda* based on two mitochondrial (mt) markers (COI, cyt *b*). Phylogenetic relationships were reconstructed adding two nuclear gene markers (28S, H3). A relaxed molecular clock analysis was performed based on the mt markers. We detected nuclear mt paralogues (*numts*) and the probable introgression of *T. tamaulipensis* mtDNA in specimens of *T. eques* from central Mexico. Between six and 14 species of *Taeniopoda* were delimited by the sequence-based approaches performed (COI divergence with thresholds of 1 and 2%; General Mixed Yule-Coalescent (GMYC) model). The GMYC and 1% threshold analyses with COI were more congruent with the currently recognized morphology-based taxonomy with 10 and 11 putative species, respectively. Four of these species were regarded as 'stable', since they were supported by at least one of the molecular analyses and by diagnostic morphological features. The species-based phylogeny recovered *Taeniopoda* as paraphyletic with respect to the monotypic genus *Romalea*. Three morphologically and geographically congruent major clades were recovered, two with species having a considerably elevated pronotal crest and one with its members having it less elevated. The origin and subsequent diversification of *Taeniopoda* were estimated to occur from the mid and late Miocene to Pliocene, respectively. The current species diversity in *Taeniopoda* was estimated to occur during the Pleistocene, which was probably influenced by the climatic oscillations that occurred during this period and the uplift of mountain ranges in Central America.

Key words:: DNA barcoding, Insecta, Neotropics, Mexico, phylogeny, species delimitation

Introduction

Species delimitation is defined as the act of identifying species-level biological diversity (Carstens, Pelletier, Reid, & Satler, 2013), and currently is one of the main areas of study in systematics (Hohenegger, 2014; Ruane, Bryson, Pyron, & Burbrink, 2014). Traditionally, the most important source of information to delimit species has been through the examination of morphological features (Abebe, Mekete, & Thomas, 2011; Mutanen & Pretorius, 2007). However, now it is widely accepted that morphology does not always reflects species boundaries, because species could sometimes be indistinguishable morphologically (Bickford et al., 2007; Leavitt, Starrett, Westphal, & Hedin, 2015; Massimino Cocuzza, & Cavalieri, 2014), or their intra- and interspecific variation might be difficult to discern (Gittenberger & Gittenberger, 2011; Katz, Giordano, & Soto-Adames, 2015). For this reason, the simultaneous use of various lines of evidence is now being largely employed to cross-validate species delineation (Berta & Churchill, 2012; Lecocq et al., 2015; Phuong, Lim, Wait, Rowe, & Moritz, 2014).

Mitochondrial (mt) DNA markers currently play a crucial role to complement and corroborate species delimitation based on morphology (Chan et al., 2014; Price et al., 2015; Schmidt, Schmid-Egger, Morinière, Haszprunar, &

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Hebert, 2015; Toussaint et al., 2015). The mt genome has features that make it particularly useful in systematics, since it usually does not recombine and has a higher substitution rate than most nuclear DNA markers (Avise, 2000; Ballard & Whitlock 2004). Mt markers also represent an important source of information used to investigate evolutionary patterns and processes that have sculpted the planet's biodiversity (Joly et al., 2014; Kress, García-Robledo, Uriarte, & Erickson, 2015), including evolutionary processes in complex scenarios between biogeographic areas (e.g., Rodríguez-Gómez & Ornelas, 2015; Tänzler, Toussaint, Suhardjono, Balke, & Riedel, 2014; Teske, Papadopoulos, Barker, Mcquaid, & Beheregaray, 2014). Some factors, however, can affect the performance of mt markers, such as heteroplasmy (White, Wolff, Pierson, & Gemmell, 2008), presence of incomplete lineage sorting and introgression (Choleva, Musilova, Kohoutova-Sediva, Paces, & Janko, 2014; Funk & Omaland, 2003), nuclear mt pseudogenes (*numts*) (Bensasson, Zhang, & Hewitt, 2000; Song, Buhay, Whiting, & Crandall, 2008) or *Wolbachia* infection (Smith et al., 2012).

The Nearctic and Neotropical regions are two adjacent biogeographic areas that are in contact along the central part of the Mexican territory, where there is a transitional confluence of northern and southern biotas (Halffter, 1976; Lomolino, Riddle, & Brown, 2005; Morrone, 2015a, 2015b). This transitional zone comprises the highlands of Mexico and Guatemala (Morrone, 2015b), and is characterized by having a complex geological history and different environments and climates (Ferrusquía, 1998). Various studies have investigated the origin and evolution of the biota that occur in the transitional Nearctic–Neotropical zone, though most of them have focused on vascular plants and vertebrates (e.g., Kobelkowsky-Vidrio, Ríos-Muñoz, & Navarro-Sigüenza, 2014; Munguía-Lino, Vargas-Amado, Vázquez-García, & Rodríguez, 2015; Sanginés-Franco et al., 2015). These studies have revealed the existence of multiple speciation patterns and a vast number of endemisms in the region (Flores-Villela & Gerez, 1994; Miguez-Gutiérrez, Castillo, Márquez, & Goyenechea, 2013; Morrone, 2010).

The genus *Taeniopoda* Stål, 1873 (Orthoptera: Romaleidae) is a group of conspicuous, colourful grasshoppers (Figs 1, 2) distributed from the southern United States to Panama (Fig. 3), with most of its species occurring along the Nearctic–Neotropical transitional zone (Hebard, 1924). Members of *Taeniopoda* are of economic importance because they represent pests in some of the regions where they occur (King & Saunders, 1984; Mariño-Pérez, Fontana, & Buzzetti, 2011). The few studies that have been carried out for members of this genus have mainly focused on their behaviour and physiology (e.g., Bernays, Bright, Howard, Raubenheimer, & Champagne, 1992; Stauffer, Hatle, & Whitman, 2011; Whitman, 1988, 2010), whereas its taxonomy has been largely neglected. The only taxonomic revision for *Taeniopoda* was performed almost a century ago (Hebard, 1924), where a total of 12 species were recognized and divided into three informal groups but without any clear justification.

Romalea microptera (Palisot de Beauvois), of the monotypic genus *Romalea* Serville, is a species restricted to the south-east United States that has been proposed to be closely related to *Taeniopoda* based on external morphology and genitalia (Hebard, 1925; Rehn & Grant, 1959a). Given the overall morphological similarity between *R. microptera* and *T. eques* (Burmeister), which occurs in the southern United States, it was suggested that the former species emerged from the latter by isolation, or both have a common ancestor (Rehn & Grant, 1959b). The genetic diversity of *Romalea* has been studied (Mutun & Borst, 2004), though its relationships with the members of *Taeniopoda* remain to be investigated.

Here we generated sequences of two mt markers to investigate the species boundaries in *Taeniopoda* using two DNA sequence-based approaches. We first searched for potential cases of *numts*, introgression and/or incomplete lineage sorting in the mt sequences in order to exclude them from the analyses. We then evaluated the congruence between the species delimited by the molecular information and the morphology-based taxonomy. For this, we examined the morphological diagnostic features that are employed to distinguish the currently recognized species of *Taeniopoda*, as well as two additional external morphological and six male genitalia characters. We also carried out a species-based phylogenetic analysis for *Taeniopoda* adding two nuclear markers, assessed its phylogenetic affinity with *R. microptera* and estimated the times of origin and subsequent diversification within the group to infer the vicariant events that could have originated its current species diversification.

Materials and methods

Taxon sampling

A total of 211 specimens assigned to 11 of the 12 currently recognized species of *Taeniopoda* were processed for DNA sequencing. Species assignation of specimens was performed following Bruner's (1906) and Hebard's (1924) keys to species. Specimens were collected in localities situated along most of the known geographic distribution of the genus (Fig. 3). The only species that could not be sampled, *T. bicristata* Bruner, is only known from its type material and has ambiguous locality ('Mat, Matamoros, Mexico'). We also generated sequences of five specimens of *R. microptera*, as well as sequences of four species belonging to the romaleid genera *Brachystola* Scudder, *Chromacris* Walker, *Tropidacris* Scudder and

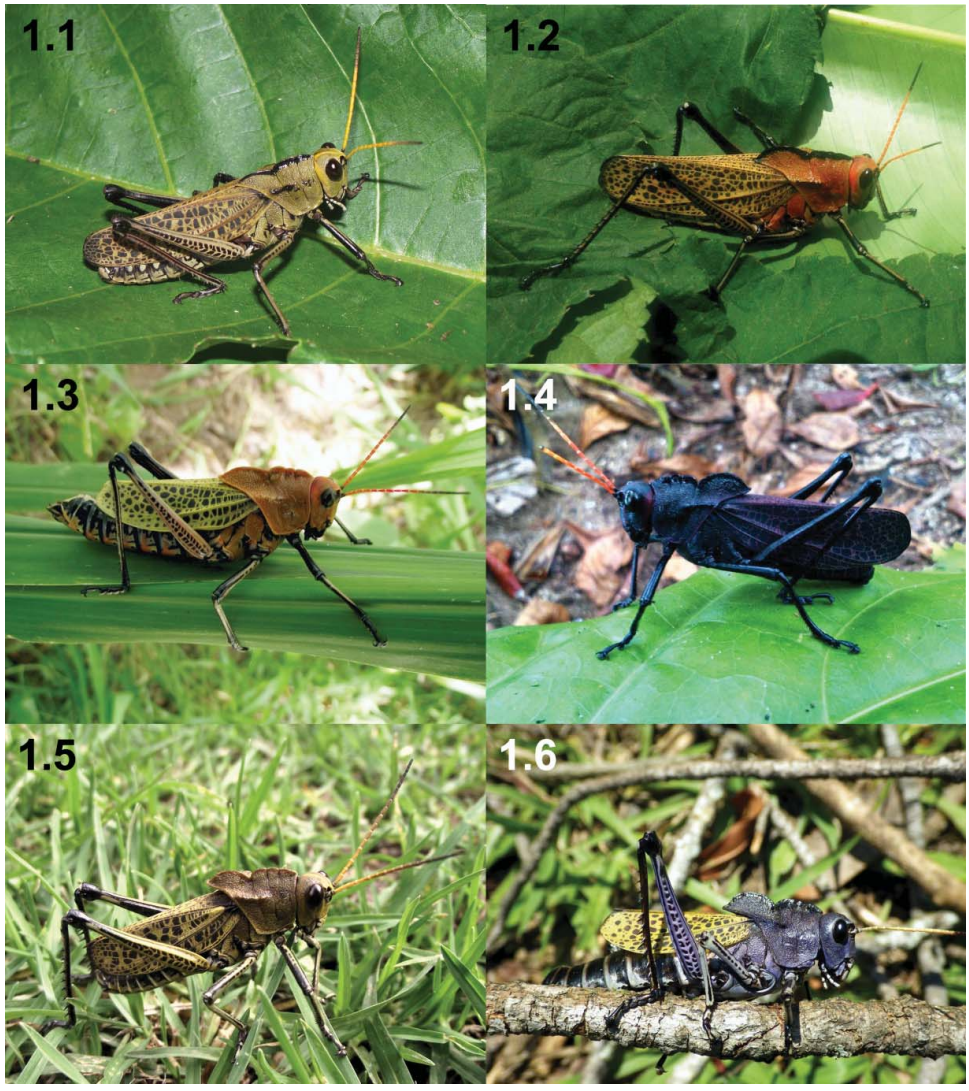

Fig. 1. Photographs of *Taeniopoda* species: (1.1) *T. citricornis* Bruner, (1.2) *T. gutturosa* Bolívar, (1.3) *T. varipennis* Rehn, (1.4) *T. reticulata* (Fabricius), (1.5) *T. auricornis* (Walker), and (1.6) *T. centurio* (Drury).

Cibotopteryx Rehn, using the latter taxon to root all the generated trees.

All sequenced specimens are deposited at the Colección Nacional de Insectos, Instituto de Biología, Universidad Nacional Autónoma de México (IB UNAM). The mounted material that was examined for the morphological part of this work is deposited at IB UNAM, Instituto Tecnológico de Ciudad Victoria, México (ITCV), Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN), the Natural History Museum, London, UK (NHM), Museo de Historia Natural de la Universidad de San Carlos, Guatemala (MUSHNAT), Universidad del Valle de Guatemala (UVG), and Instituto Nacional de Biodiversidad, Costa Rica (InBio). A list with the examined specimens, their species assignment, locality details and DNA voucher and GenBank accession numbers for the four gene markers obtained is provided in Table S1 (see online supplemental material, which is available from the article's Taylor & Francis Online page at https://doi.org/10.1080/14772000.2017.1313792). The mtDNA data generated from this work can also be retrieved from the project file 'Species boundaries in *Taeniopoda*', which is found in the projects section of the Barcode of Life Data Systems (www.boldsystems.org).

Laboratory protocols

Genomic DNA extraction was obtained from a hind leg of each specimen. The genomic DNA was extracted using both the DNeasy Blood & Tissue (QIAGEN®: Austin, EUA) and the EZ-10 Spin Column Genomic

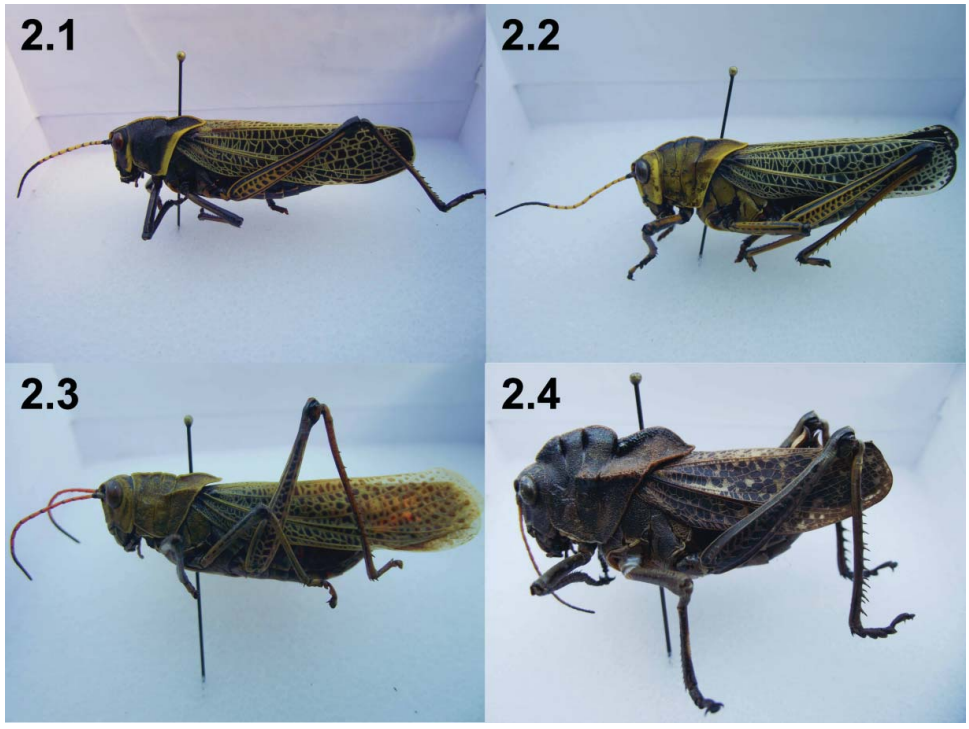

Fig. 2. Photographs of *Taeniopoda* species: (2.1) *T. eques* (Burmeister), (2.2) *T. tamaulipensis* Rehn, (2.3) *T. stali* Bruner, and (2.4) *T. obscura* Bruner.

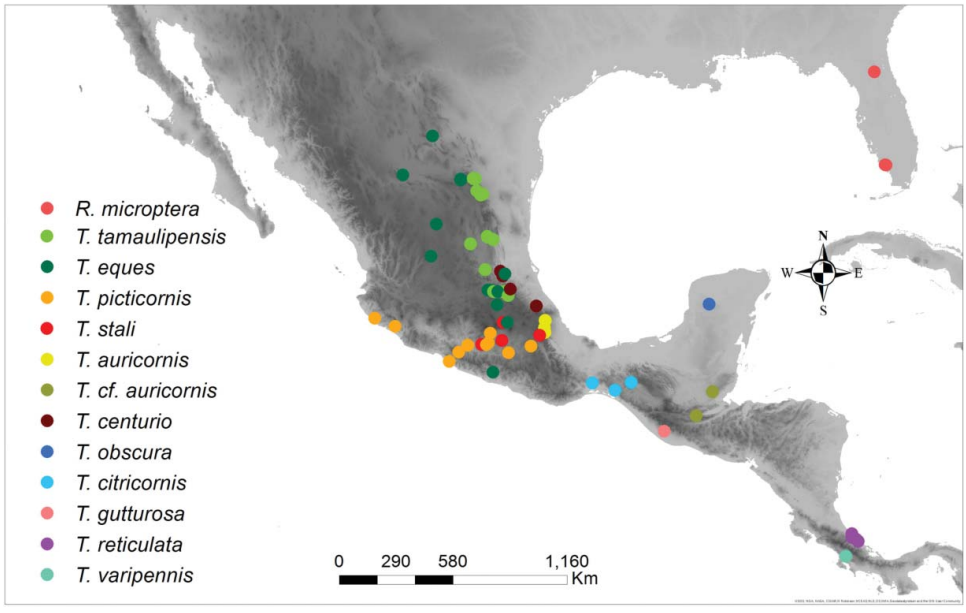

Fig. 3. Map of the sampled localities for the specimens of *Taeniopoda* and *Romalea* examined in this study.

DNA Minipreps (BIO BASIC®; Toronto, Canada) kits following the manufacturers' protocols. Two mt and two nuclear markers were amplified. The mt markers examined included a 626 bp fragment of the Cytochrome Oxidase I and 423 bp of the Cytochrome *b* DNA genes. These two are the most widely employed mt markers for species delimitation analyses (Ceccarelli, Sharkey, & Zaldívar-Riverón, 2012; Grill, Gkiokia, & Alvarez, 2006; Vanhaecke et al., 2012). We also amplified two nuclear DNA markers, a 652 bp fragment belonging to

the 28S ribosomal (r) and 314 bp of the Histone 3 protein DNA genes.

The primers used to amplify the gene fragments were the following: COI.-LCO (5'-GTCAACAAATCATAAAGATATTGG-3') and HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994); cyt *b*.-CB1_5 (5'-TATGTACTACCATGAGGACAAATATC-3)' and CB2_5 (5'-ATTACACCTCCTAATTTATTAGGAAT-3') (Jermiin & Crozier, 1994); 28S.-28SFwd (5'-GCGAACAAGTAACCGTGAGGG- 3') (Belshaw & Quicke, 1997) and 28SRev_Int (5'- GGAGTGCGGAGGCCGCCGCCCMC-3') (this study); and H3.-H3F (5′-ATGGCTCGTACCAAGCAGACVGC-3′) and H3R (5′-ATATCCTTRGGCATRATRGTGAC-3′) (Colgan et al., 1998).

PCRs were carried in 15 μl of total volume containing 2.5 μl of 10× PCR buffer, 1.5 μl of $MgCl_2$ 50mM, 0.5 μ of dNTPS 10mM, 1 μl of each primer 10μM, 0.1 μl of Taq Polymerase (Taq Platinum; Invitrogen®; Carlsbad, EUA), 2 μl of DNA template and ddH_2O to bring volume to 15 μl. The temperature conditions for COI amplification were: 3 minutes of initial denaturation at 95°C; 35 cycles with 1 minute denaturation at 94°C, 1 minute annealing at 50°C and 1 minute of extension at 72°C; and 10 minutes final extension at 72°C. Cyt *b* was amplified using a touchdown with the following temperatures: initial denaturation at 95°C; 10 cycles with 1 minute denaturation at 94°C, 1 min annealing at 61°C with reduction of 0.5°C each cycle and 1 minute of extension at 72°C; followed by 20 cycles with 1 minute denaturation at 94°C, 1 minute annealing at 48°C and 1 minute of extension at 72°C; and a final extension of 10 minutes at 72°C. The PCR conditions for 28S and H3 were the same used for COI, but using 58°C and 55°C for the annealing step, respectively.

Unpurified PCR products were sent for sequencing to the High-Throughput Genomics Center of the University of Washington (www.htseq.org) and to the genomics unit at IB UNAM. All sequences were edited with Sequencher 4.14 (Genecodes®, USA) and aligned with the program ClustalW (Larkin et al., 2007) implemented in Bioedit 7.1.3.0 (Hall, 1999).

Detection of numts, mt introgression and incomplete lineage sorting

Species delineation analyses based on mtDNA sequence data could be biased by the presence of mt introgression or incomplete lineage sorting (Leaché, 2009). Moreover, species boundaries based on mtDNA sequence data are particularly difficult to assess in orthopterans due to the frequent presence of *numts* within this order (Bensasson et al., 2000; Song, Moulton, & Whiting, 2014). We therefore carried out two different approaches to detect sequences that could represent the above three phenomena.

We first searched for *neonumts* (Song et al., 2014), i.e., recent duplications that did not have time to accumulate enough mutations, in the mt datasets based on the presence of stop codons, indels, polymorphism (double peaks) in chromatograms, codon position substitution bias and variation in rates of evolution (Calvignac, Konecny, Malard, & Douady, 2011; Song et al., 2014). We also carried out separate Bayesian analyses for the two mt markers to detect incorrect phylogenetic placement of taxa that could potentially represent *paleonumts*, i.e., ancient paralogues with long branch lengths nested in separate clades distantly related to the orthologue (Song et al., 2014), or possible cases of introgression or incomplete lineage sorting (Leaché, 2009). Incorrect phylogenetic placement was established based on our morphological examination. All sequences that potentially represented cases of incomplete lineage sorting, mt introgression or *numts* were excised from the species delimitation analyses.

Bayesian phylogenetic analyses were carried out separately for the two mt and the two nuclear markers with MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). Two databases were analysed for each mt marker, one including and the other one excluding the detected *numts* and potential cases of introgression or incomplete lineage sorting. The nuclear markers only included a subset of the specimens sequenced for the mt genes (Table S1, see supplemental material online). Three partitions were each considered for COI and cyt *b*. Substitution models for each partition were calculated with jModeltest (Darriba, Taboada, Doallo, & Posada, 2012) using the Bayesian criterion. Each analysis consisted of two simultaneous runs of 100 million generations each, sampling trees every 1000 generations and saving branch lengths. We discarded the 25% of the sampled trees as burn-in based on convergence of the two simultaneous runs, according to the values of the average standard deviation of split frequencies (a value < 0.01 was considered as convergence of the two simultaneous runs). The remaining trees were employed to build a phylogram with posterior probabilities of clades, considering them as significantly supported if they had a posterior probability value ≥ 0.95. The separate and concatenated matrices analysed in this study can be retrieved from Table S2 (see supplemental material online).

DNA sequence-based species delimitation

Two DNA sequence-based methods for species delineation were performed for specimens of 11 of the described species of *Taeniopoda* and for *R. microptera*. We first

used the 2% genetic divergence criterion for the Barcoding locus, which comprises a fragment of the COI gene (Hebert, Cywinska, Ball, & DeWaard, 2003). This has been shown to be a reliable approach for the exploration of putative species in various groups of animals (Kerr, Lijtmaer, Barreira, Hebert, & Tubaro, 2009). We also employed the threshold of 1% genetic divergence for the above marker, which represents a more conservative approach to delimit species (Ratnasingham & Hebert, 2007). Corrected genetic distances for COI were calculated using the Kimura 2-parameter (K2P) model with MEGA 7.0 (Kumar, Secher & Koichiro, 2016).

The Generalized Mixed Coalescent Yule (GMYC) model also was implemented separately for the COI and cyt *b* markers and for the concatenated mt dataset. An ultrametric tree, required for the GMYC model to distinguish a branching pattern of intraspecific coalescence and speciation events (Pons et al., 2006), was generated for each matrix with BEAST version 2.2.1 (Bouckaert et al., 2014). Duplicated haplotypes were excised with Collapse version 1.2 (Posada, 2004). Analyses were run for 50 million generations, saving trees every 1,000 generations, using a lognormal distribution relaxed clock and the Yule prior. The resulting maximum credibility ultrametric trees were employed to delimit species with the GMYC method implemented in the SPLITS package in the R programming environment (http://r-forge.r-project.org/projects/splits), using the single threshold optimization. We did not perform a multi-threshold GMYC analysis because it has been shown to have a poor performance both with simulations and empirical data (Fujisawa & Barraclough, 2013; Kekkonen, Mutanen, Kaila, Nieminen, & Hebert, 2015; Monaghan et al., 2009).

Morphological examination

We evaluated the congruence between the species delineation approaches based on mtDNA sequence data and the species currently recognized by the morphology-based taxonomy. For this, we recorded the morphological diagnostic features reported by Hebard (1924) to distinguish species in *Taeniopoda* in all our examined specimens. These include nine colour, shape and sculpture adult features. Moreover, we recorded two additional external morphological characters that were potentially informative to delimit species in the group (male and female adult body size, pronotal crest sculpture).

We also recorded six internal male genitalia characters for a subsample of the two to four specimens for each of the putative species delimited by any of the DNA sequence-based species delineation approaches performed. Internal male genitalia was dissected for each specimen immersing the distal part of the abdomen in 10% KOH for 10 minutes, and subsequently removing it with a hook, immersing it again in 10% KOH for 20 minutes and then removing the muscle tissue. These features were observed with a ZEISS® Stemi DV4 stereomicroscope and photographed in a Leica Z16 APO. The morphological features examined in this work are listed in Table S3 (see supplemental material online). Digital pictures of male genitalia of representative species of *Taeniopoda* are shown in Fig. S1 (see supplemental material online).

Phylogenetic relationships and molecular clock estimates

The phylogenetic relationships among specimens belonging to the putative species that were delimited by at least one of the species delineation approaches based on molecular evidence were reconstructed carrying out a concatenated Bayesian analysis using the mt and nuclear markers with MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). The analysis employed the above parameters, as well as the same partitions and evolutionary models for the mt markers. Three partitions were considered for the nuclear H3 gene according to their codon positions, whereas the nuclear 28S rDNA marker was regarded as a single partition. The terminal taxa included in the Bayesian analysis only included a subset of the specimens sequenced for the mt genes.

A relaxed molecular clock analysis was also performed for a concatenated mt dataset with BEAST version 2.2.1 (Bouckaert et al., 2014). We did not include the two nuclear markers since their scarce variation does not have an impact in the derived topology but considerably affects the molecular divergence time estimates. The analysis was run for 100 million generations, sampling trees every 10,000 generations, using a Death-Birth tree prior, an uncorrelated relaxed lognormal rate, considering one partition for each gene marker (GTR+G for both mt genes) and burn-in was established after 10 million generations. Absolute node ages (percentage of change per million years) were obtained using the insect mutation rates reported by Pons and Vogler (2005) and Papadopoulou, Anastasiou, and Vogler (2010) for COI (3.36%) and cyt *b* (4.22%) [COI ucld.mean (subst/site/my): lognormal distribution in real space, with initial value = 0.0168; Log (Mean) = 0.0168; Log (Stdev) = 0.2; cyt *b* ucld.mean (subst/site/my): lognormal distribution in real space, with initial value = 0.0211; Log (Mean) = 0. 0211; Log (Stdev) = 0.17]. Effective sample size (ESS) $\geq$ 200 for each parameter was confirmed with Tracer version 1.6 (Drummond & Bouckaert, 2015). A maximum clade credibility tree with the associated Bayesian 95% High Probability Density Interval was then built with TreeAnnotator version 1.8.1 contained in the BEAST Package.

Results

Detection of *numts* and gene genealogies

A total of 212, 194, 42, and 66 sequences were generated for COI (626 bp), cyt *b* (423pb), 28S (652 bp), and H3 (314 bp), respectively. The evolutionary models selected for each partition and the main characteristics of the four gene markers are provided in Table S4 (see supplemental material online).

The Bayesian analyses performed with all the generated sequences for the two mt markers showed that 15 and 13 COI and cyt *b* sequences had an apparent incorrect phylogenetic placement, respectively (Fig. S2, see supplemental material online). Of these, five COI and three cyt *b* sequences had highly polymorphisms in their chromatograms. Four cyt *b* sequences were considered as *paleonumts* because they were placed at the base of a clade with all members of *Taeniopoda*. The two mt phylograms had a clade with specimens of *T. eques* from Central Mexico intermingled with those of *T. tamaulipensis* Rehn, two clades containing specimens of *T. stali* Bruner and *T. picticornis* (Walker), and a clade with *T. varipennis* Rehn, *T. reticulata* (Fabricius), *T. citricornis* Bruner, and *T. gutturosa* Bolívar. We consider that the sequences of *T. eques* from Central Mexico represent a case of mt introgression with *T. tamaulipensis* based on the consistent morphological differences that exist between these two species. The above sequences of *T. eques* therefore were excised from the species delineation analyses.

The lack of consistent morphological differences between *T. stali* and *T. picticornis*, and *T. citricornis* and *T. gutturosa* (see below) on the other hand led us to maintain all their sequences. The generated sequences of *T. varipennis* and *T. reticulata* shared single COI and cyt *b* haplotypes or had considerably low variation between them; however, they are morphologically distinct and we thus maintained all their sequences for the subsequent analyses.

The Bayesian phylograms obtained from separate COI and cyt *b* analyses excluding all *numts* and presumable cases of mt introgression are congruent in their significantly supported relationships (Fig. 4). The monophyly of *Taeniopoda* was not recovered in the COI phylogram, since *R. microptera* appeared deeply nested within it. Only two species of *Taeniopoda* were significantly recovered as exclusive with the two mt markers: *T. tamaulipensis* and *T. centurio* (Drury). *Taeniopoda obscura* Bruner and *T. eques* were also recovered as exclusive in the COI topology (PP = 0.98 and 0.99).

The above two mt genealogies recovered the specimens assigned to *T. picticornis* and *T. stali* intermingled in a significantly supported clade, each having at least two of the following three groups: group (A) composed by specimens of *T. picticornis* from the Pacific Mountain Ranges subprovince (Sierra Madre del Sur Province); group (B) containing species of both species from the Pacific Coastal Plains, Balsas Depression and Septentrional Balsas highlands (Sierra Madre del Sur province) and the Meridional Extension subprovince [Trans-Mexican Volcanic Belt (TMBV) province]; and group (C) comprising specimens assigned to both species from the Septentrional Balsas highlands, Oaxaca and Puebla Highlands and Balsas Depression subprovinces in the Sierra Madre del Sur province, and the Eastern Portion subprovince in the TMBV province.

The specimens assigned to *T. auricornis* (Walker) appeared divided into two separate, geographically congruent clades, one with specimens from Veracruz and the other one from Guatemala. The specimens of *T. reticulata*, *T. varipennis*, *T. citricornis*, and *T. gutturosa* formed a single, largely unresolved clade (COI, cyt *b*: PP = 1.0 and 1.0).

The phylograms derived from the two nuclear markers, though largely unresolved, recovered a paraphyletic *Taenipoda* with respect to *R. microptera* (Fig. S3, see supplemental material online). The 28S phylogram recovered two main clades, one with *T. citricornis*, *T. gutturosa*, and *T. reticulata* (PP = 0.62), and the other with the remaining species of the genus and *R. microptera* (PP = 0.79). The H3 phylogram recovered the specimens of *Romalea* forming a clade with *T. guttorosa* and one specimen of *T. eques* (PP = 0.74).

DNA sequence-based species delimitation

The threshold of 2% for species delimitation using the COI corrected distances delimited six species of *Taeniopoda* (Table S5, see supplemental material online; Fig. 5). COI corrected distances among specimens assigned to *T. gutturosa*, *T. reticulata*, *T. varipennis*, and *T. citricornis*, and between specimens of *T. obscura* and *T. auricornis*, were lower than the above threshold. Use of the 1% threshold of genetic divergence on the other hand delimited 11 species, of which only three were concordant with the 2% threshold. Under the 1% threshold, the specimens assigned to *T. picticornis* and *T. stali* appear divided into the same three groups mentioned above.

A total of 10 and 14 species were delimited by the separate COI and cyt *b* GMYC analyses, respectively (Fig. 5; Fig. S4, see supplemental material online). The concatenated mt GMYC analysis showed a considerable species oversplitting (Fig. S4, see supplemental material online). We therefore only considered the results obtained by the separate mt markers.

Only two of the species delimited by the GMYC analyses with COI and cyt *b* corresponded to currently recognized species, *T. tamaulipensis* and *T. centurio*. The

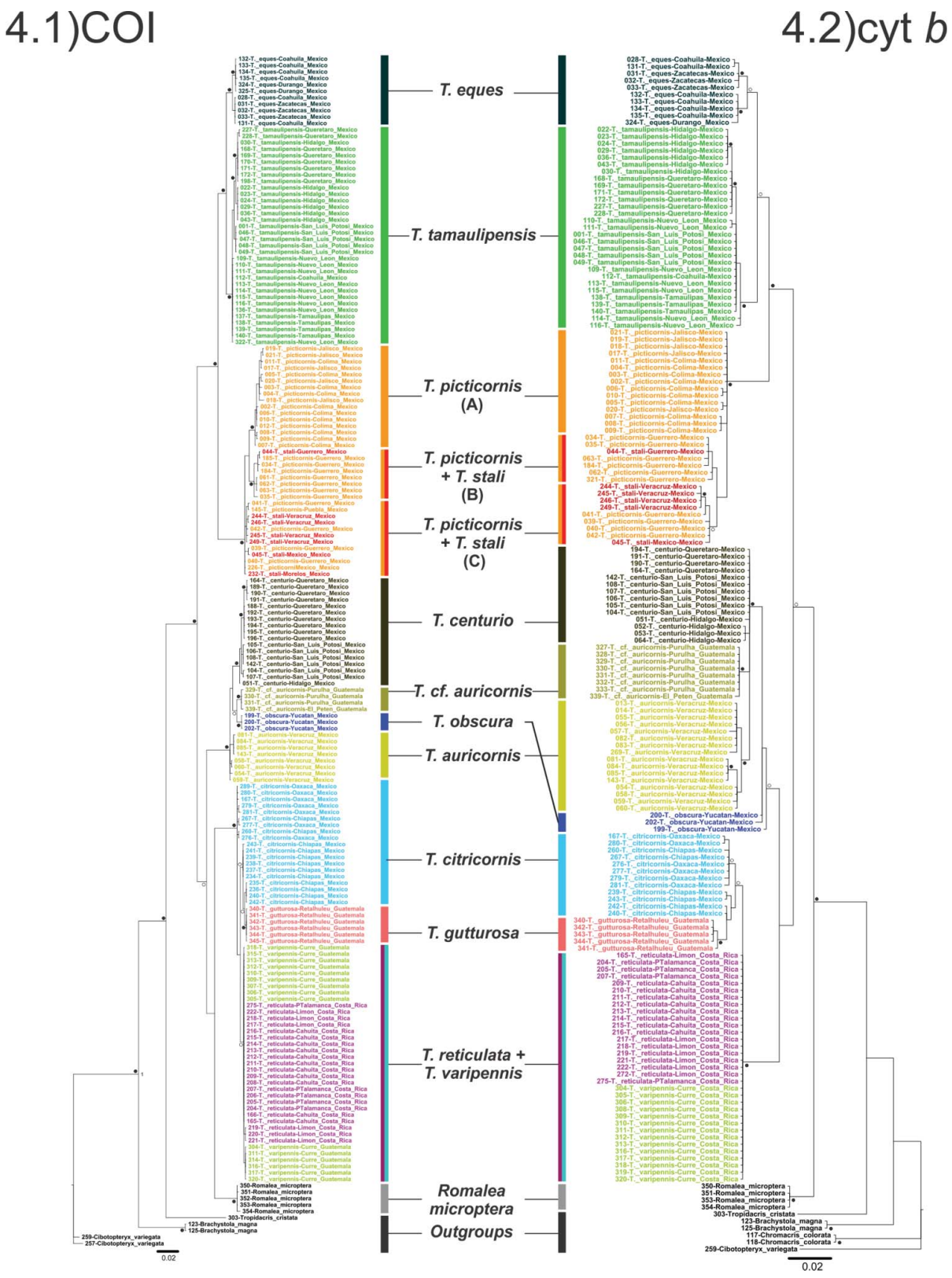

Fig. 4. Phylograms reconstructed with the Bayesian analysis based on the (4.1) COI and (4.2) cyt *b* mitochondrial markers excluding probable cases of introgression/incomplete lineage sorting events and *numts*. Black and white circles near branches indicate Bayesian posterior probabilities ≥ 0.95 and ≥ 0.90 ≤ 0.94, respectively.

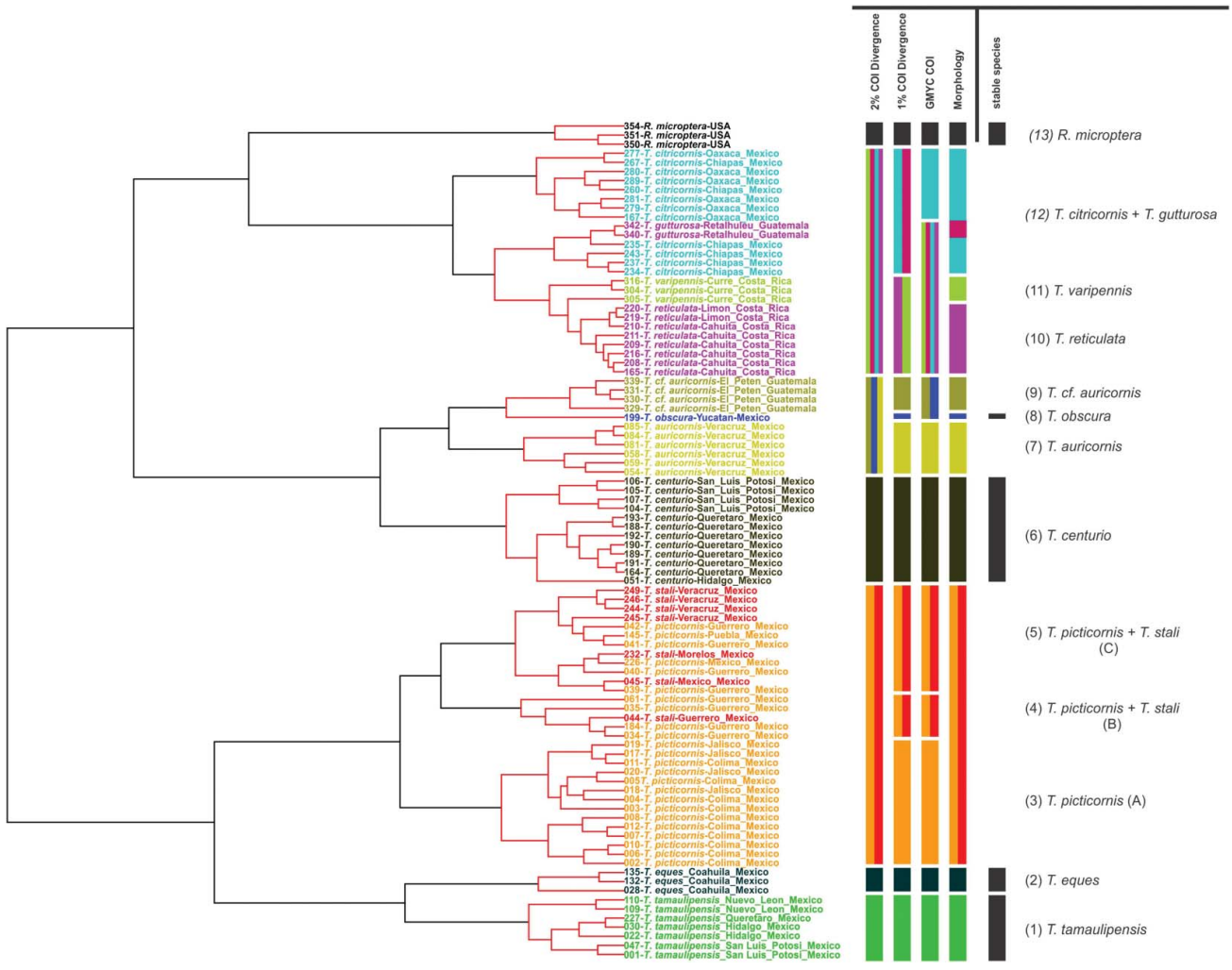

Fig. 5. Ultrametric tree constructed with the COI dataset used for the species delimitation with the GMYC method. Bars beside the tree summarize the results obtained for the species delineation analyses. Black bars indicate the stable species (*sensu* Padial et al., 2009); i.e., species delimited by the molecular analyses and the species-based taxonomy.

specimens assigned *T. picticorns* and *T. stali* were split into the same three species delimited by the COI 1% divergence threshold, whereas those of *T. reticulata* and *T. varipennis* appeared fused as a single GMYC species.

The GMYC analysis with COI was more congruent with the morphology-based taxonomy. This analysis clustered the specimens of *T. eques* as a single evolutionary unit. *Taeniopoda auricornis* was divided into two GMYC species, one with members from Veracruz and the other one with specimens from Guatemala. Four currently recognized species were on the other hand intermingled in two GMYC species with COI, one comprising specimens of *T. citricornis* from Chiapas and Oaxaca, and the other one with specimens of *T. citricornis* from Chiapas, specimens of *T. gutturosa* from Guatemala, and the included specimens of *T. reticulata* and *T. varipennis*.

Morphological confirmation of species boundaries

The morphological features and the states recorded for each taxon are shown in Table S3 (see supplemental material online). The male genitalia features had considerable intraspecific variation and thus were not informative to delimit species in the group. The colour of pronotum and pronotal crest sculpture help to distinguish the three species delimited by the two genetic distance thresholds and the GMYC model, *T. eques*, *T. tamaulipensis*, and *T. centurio*. *Taeniopoda obscura*, on the other hand, can be distinguished from the remaining species of the genus by two exclusive external morphological features, pronotum black and lateral carina of pronotum distinctly prominent, though it was only recovered as a distinct species by the COI 1% threshold.

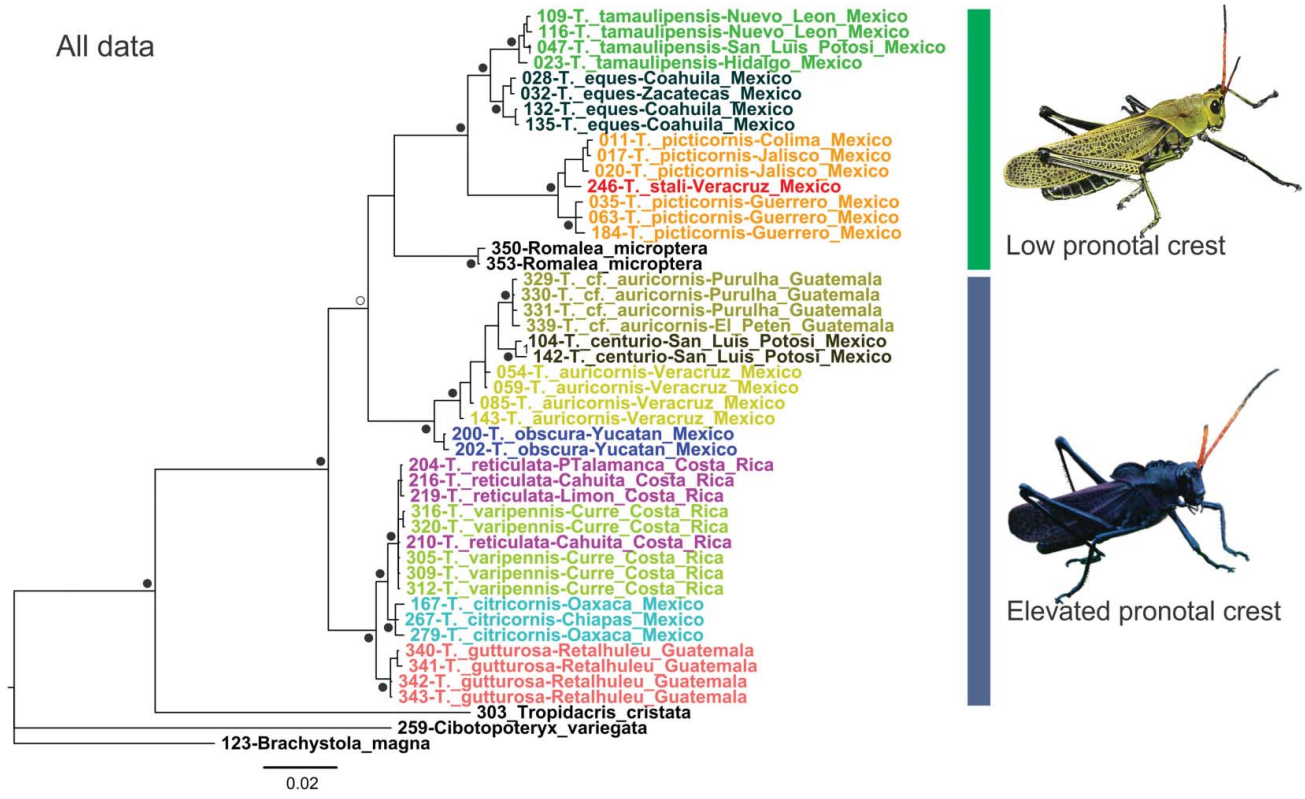

Fig. 6. Bayesian phylogram derived from the concatenated COI + cyt *b* + 28S + H3 datasets. Black circles near branches indicate Bayesian posterior probabilities ≥ 0.95.

Taeniopoda reticulata and *T. varipennis* are morphologically distinguishable from each other; however despite the DNA sequence-based species delineation analyses fused them as a single evolutionary lineage. The two geographically isolated populations assigned to *T. auricornis* that appeared as separate species by the GMYC and COI 1% threshold analyses could only be morphologically distinguished from each other by the female and male body size.

There were no consistent morphological features that helped to distinguish the specimens assigned to *T. picticornis* from those of *T. stali*. The only character that was proposed to distinguish these two taxa, colour of the antenna, was found to be highly variable even among individuals from same localities. All but one (see Discussion section) of the specimens assigned to *T. gutturosa* and *T. citricornis* could be distinguished from each other by two features, colour of antenna and pronotum.

Phylogenetic relationships and divergence-time estimates

The phylogram derived from the Bayesian concatenated analysis employing four genetic markers and including representatives of the putative species recovered by the different species delineation approaches based on mt sequence data is shown in Fig. 6. *Taeniopoda* was significantly supported as paraphyletic with respect to *R. microptera* (PP = 1.0). A clade with *T. varipennis*, *T. reticulata*, *T. citricornis*, and *T. gutturosa* (PP = 1.0) was recovered as sister to two clades (PP = 0.99), one with the two lineages assigned to *T. auricornis*, *T. obscura*, and *T. centurio* (PP = 1.0), and the other one with *T. eques*, *T. tamaulipensis*, *T. picticornis*, *T. stali*, and with *R. microptera* at the base (PP = 0.88). The former two clades are represented by taxa distributed from central Mexico to Central America, and are characterized by having a considerably elevated pronotal crest (at least 0.3 times total height of pronotum). The remaining clade, on the other hand, has taxa that occur from the southern United States to central Mexico and have a lower pronotal crest elevation (≤ 0.25 times total height of pronotum).

The chronogram derived from the relaxed molecular clock analysis (Fig. S5, see supplemental material online) indicates that *Taeniopoda* could have originated during the mid Miocene to Pliocene, 12.66–2.71 MYa (mean = 6.81 MYa). In this analysis the genus was divided into two main clades, one with the species with elevated pronotal crest including *R. microptera* at the base, and the

other one with species lacking this morphological feature. These two clades were estimated to have diverged during the late Miocene to Pliocene, 7.48–2.40 MYa, and started to diversify from the late Miocene to early Pleistocene, 6.12–1.35 MYa. The species diversification that resulted in the currently recognized species of *Taeniopoda* was on the other hand estimated to occur during the early to mid Pleistocene, 2.35–0.29 MYa.

Discussion

Detection of *numts*, introgression and incomplete lineage sorting

Species delineation methods based on mtDNA sequence data are widely employed in systematics to draw taxonomic inferences along with morphological evidence (e.g., Álvarez-Presas & Riutort, 2014; Cruz-Barraza, Vega & Carballo, 2014; Jiruskova & Bocak, 2015). However, *numts* represent a potential source of error in this kind of studies, and its presence has been documented in several animal groups at intra- and interspecific levels (Ahmed & Jaffar Ali, 2015; Haran, Koutroumpa, Magnoux, Roques, & Roux, 2015; Jordal & Kambestad, 2014; Song et al., 2008).

Here we found that some cyt *b* sequences of specimens assigned to *T. centurio*, *T. picticornis*, *T.citricornis*, and *T. tamaulipensis* could represent *paleonumts*, since they were recovered at the base of a clade containing the remaining sequences of *Taeniopoda* (Song et al., 2014). We also report the existence of a number of putative *neonumts* in our COI and cyt *b* datasets, which were detected based on the presence of polymorphisms in chromatograms. Our study therefore notes the importance of carrying out approaches to detect *numts* in phylogenetic studies in order to avoid the inclusion of paralogues that lead to the reconstruction of wrong evolutionary relationships as well as species overestimation, especially in groups like Orthoptera, which have been reported to contain a large number of mt pseudogenes.

Our gene genealogies including all the generated sequences recovered four cases of non-reciprocal monophyly, one in the clade with sequences of *T. eques* and *T. tamaulipensis*, another one represented by two clades with members of *T. picticornis* and *T. stali*, and the remaining two in the clade containing *T. citricornis*, *T. reticulata* and *T. varipennis.* Non-reciprocal monophyly can be explained by recent divergence leading to incomplete differentiation and incomplete lineage sorting (Funk & Omland, 2003; Mao, Zhang, Nakamura, Guan, & Qiu, 2014; Welch, Yoshida, & Fleischer, 2011), introgression, which implies the movement of genes from one species into the genome of another one (Peters, Zhuravlev, Fefelov, Logie, & Omland, 2007; Rheindt & Edwards, 2011; Wang et al., 2014), or taxonomic error, where species are incorrectly identified (Funk & Omland, 2003).

In the case of the non-reciprocal monophyly between *T. eques* and *T. tamaulipensis*, we propose that this could be due to mt introgression, since the two species are morphologically distinguishable from each other (completely black pronotum and slightly crushed antennal segments in *T. eques*; pronotum laterally green and never completely black and antennal segments not crushed in *T. tamaulipensis*). For *T. picticornis* and *T. stali*, their non-reciprocal monophyly is probably due to their taxonomic uncertainty, since the only feature that distinguishes them, the colour of antenna, is highly variable. Further studies including additional markers and populations will also help to understand the low mt variation between *T. reticulata* and *T. varipennis*, which are morphologically well differentiated and appear to be allopatric.

Performance of species delineation approaches

Species delimitation based on genetic distances has been mainly criticized because the proposed thresholds are not linked to any biological phenomenon and are not universal (Cognato, 2006; Fregin, Haase, Olsson, & Alström, 2012). However, it has been shown that it represents an efficient approach as a first assessment of species richness, especially in megadiverse, poorly studied taxa (Zaldívar-Riverón et al., 2010; Ceccarelli et al., 2012; Gutiérrez-Arellano, Gutiérrez-Arellano, & Zaldívar-Riverón, 2015). Moreover, it is a practical tool to reassess and confirm delimitations proposed by traditional taxonomy (Schmidt et al., 2015). We found discordant results between the species limits recovered with the COI 1 and 2% thresholds and those obtained by the GMYC method, though the COI 1% threshold was more congruent with the latter approach and with our morphospecies discrimination.

The methods for species delimitation based on coalescence involve a particular biological process and have the advantage of having statistical support (Fujita, Leaché, Burbrink, McGuire, & Moritz, 2012). Currently, the GMYC model is one of the most widely used approaches based on coalescence due to its stability regarding the proportion of unique haplotypes, ultrametric tree reconstruction conditions and taxonomic sampling (Ceccarelli et al., 2012; Talavera, Dincă, & Vila, 2013). This approach, however, tends to overestimate the number of species in the presence of high population structure or considerably high values of effective population size (Esselstyn, Evans, Sedlock, Khan, & Heaney, 2012; Tänzler, Sagata, Surbakti, Balke, & Riedel, 2012). Here we found that the concatenated mt dataset overestimated the number of species with the GMYC method, probably due to the increase of genetic structure in the data.

Taxonomic inferences

Establishing species limits in *Taeniopoda* has been hampered by the lack of consistent diagnostic morphological features. Most of the diagnostic morphological features employed within the genus are differences in colour pattern, which were proposed in the only taxonomic revision of the genus carried out almost a century ago (Hebard, 1924). Some of these colour pattern features are known to be highly variable, which highlights the taxonomic uncertainty of some of the species involved. Here we employed mtDNA sequence data and two DNA sequence-based species delineation approaches to assess the number of species diversification events that occurred within this Nearctic–Neotropical group of grasshoppers, and compared our results with the main diagnostic morphological features that are currently employed for its recognized species. This molecular study has helped to clarify the actual number of species within this morphologically conserved group of romaleids, and will serve as a robust basis to carry out further studies using additional molecular markers and morphological information from different character systems.

We summarized the species of *Taeniopoda* that are delimited by the molecular evidence (Fig. 5). Between six and 14 species of *Taeniopoda* were discriminated depending on the molecular approach performed. The GMYC and the 1% threshold analyses with COI were more congruent with the currently recognized morphology-based taxonomy with 10 and 11 putative species, respectively. We regard four of these delimited species as 'stable' (*sensu* Padial et al., 2009), since they were supported by at least one of the molecular species delineation approaches and by consistent diagnostic morphological features. Below we list the four stable species together with their known geographic distribution based on examined museum material (De Jesús-Bonilla et al., unpubl. data): (1) *T. tamaulipensis*, distributed along the Sierra Madre Oriental and the Mexican Plateau in the states of Coahuila, Nuevo León, Tamaulipas, San Luis Potosí, Hidalgo, and Queretaro; (2) *T. eques*, occurring from Arizona, Nuevo Mexico and Texas in southern USA to central Mexico; (3) *T. centurio*, recorded for southern Sierra Madre Oriental in the states of San Luis Potosí, Queretaro, Hidalgo and Puebla, Mexico, to Nicaragua in Central America; and (4) *T. obscura*, with most of its records restricted to the Yucatán Peninsula and Guatemala, but with an unconfirmed record in San Luis Potosí in central Mexico.

The population groups assigned to *T. auricornis* that were recovered by some of the molecular analyses as two separate evolutionary lineages could only be morphologically distinguished from each other by the male and female adult body size. As in other orthopteran taxa, adult body size in *Taeniopoda* has been reported to be considerably variable intraspecifically (Hebard, 1924; Rehn and Grant, 1959b). We therefore maintained these taxa as a single species. *Taeniopoda auricornis* had so far been reported for the Mexican states of Hidalgo, Veracruz, and the southern portion of Tamaulipas (Hebard, 1924). The specimens from Guatemala reported here therefore represent the first confirmed record of the species for this country.

The DNA sequence-based approaches for species delineation did not recover the exclusivity of *T. citricornis* with respect to *T. gutturosa*. Only two diagnostic features, antennal and pronotal colour, distinguish these two species. In *T. gutturosa* these structures have been reported to be scarlet red to orange, whereas in *T. citricornis* they are olivaceous green (Bruner, 1906; Hebard, 1924). In our morphological examination we assigned the specimens from Santiago Ixtaltepec, Oaxaca, to the latter species based on their colour features. However, one specimen (DNA voucher no. R167), had a scarlet red to orange pronotum and antenna. The actual status of these two taxa therefore needs to be further investigated.

Taeniopoda reticulata and *T. varipennis* are morphologically distinguishable from each other, and apparently have a disjunct geographic distribution separated by mountain ranges of recent formation (Abratis & Wörner, 2001; Bergoeing, 2006). However, all the species delineation analyses performed fused them as a single species. *Taeniopoda reticulata* occurs from the eastern side of the Central Mountainous Axis of Costa Rica to the Atlantic Coast of Costa Rica and Panama, whereas *T. varipennis* is mainly distributed along the Pacific coastal regions in Costa Rica and Nicaragua. Additional studies are needed to investigate whether the lack of mtDNA sequence variation in these two species is due to their recent divergence or due to mt introgression.

The specific status of the three separate groups containing the specimens assigned to *T. picticornis* and *T. stali* requires to be assessed in more detail. These groups were consistently delimited as separate species in some of our molecular analyses, and they are geographically congruent with respect to each other, since they are exclusively composed of specimens from separate localities along the Trans-Mexican Volcanic Belt (TMVB) and the Sierra Madre del Sur provinces. However, we did not find any consistent diagnostic morphological feature that helps to distinguish them from each other. In his revision of the genus, Hebard (1924) questioned the validity of *T. picticornis*, since he found that the only feature that distinguished it from *T. stali*, yellow to orange antenna in the former one and scarlet in the latter, is highly variable. In this study we observed that the scarlet colour of the antenna in the specimens assigned to *T. stali* appears to decolour after they are mounted or preserved in ethanol, changing to yellow or orange.

Phylogenetic relationships and biogeographic inferences

Our concatenated phylogenetic analysis based on four gene markers significantly supports the paraphyly of *Taeniopoda* with respect to *R. microptera*, though the relationships of this species within the group remain to be clarified. Previous studies based on genitalia, external morphology and physiological features had suggested a close relationship between the members of these two genera (Hebard, 1925; Rehn & Grant, 1959a, 1959b; Roberts, 1941; Stauffer et al., 2011; Stauffer & Whitman, 2007). Thus, the synonymy of *Taeniopoda* with *Romalea* needs to be formally established.

The relationships recovered in this study within *Taeniopoda* do not correspond with the three species-groups mentioned by Hebard (1924), who proposed them without any morphological justification. One of these groups contained *T. eques*, *T. picticornis*, and *T. stali*, another one only comprised *T. obscura*, and the third had the remaining species. Our best estimate of phylogeny instead recovered three morphologically and geographically congruent major clades, two with species having an elevated pronotal crest and with a Mesoamerican distribution, and another one with species having a lower elevated pronotal crest and distributed from the southern United States to central Mexico.

The chronogram reconstructed shows that *Taeniopoda* probably originated during the Miocene, with its subsequent diversification occurring from the late Miocene to Pliocene. During these geological periods there was an intense geological activity in Mesoamerica, which shaped its current physiography (Castillo, 1991; Ferrusquia, 1998). Based on these time estimates, we suggest that the recent stages of formation of the TMVB (7.5–3 My) could have influenced the origin of the major clades within *Taeniopoda*, since the species having or lacking a considerably elevated pronotal crest are mostly distributed to the south and north of this mountain range, respectively. The TMBV began its formation 20 million years ago, and it has been in change until recent times (Ferrari, Orozco-Esquivel, Manea, & Manea, 2012). This province has been associated with the formation and/or diversification of various groups of plants (Gándara & Sosa, 2014), reptiles (Bryson, García-Vázquez, & Riddle, 2012a, 2012b), fishes (Kallman & Kazianis, 2006; Mateos, 2005; Ornelas-García, Domínguez-Domínguez, & Doadrio, 2008), and even other orthopterans (Pedraza-Lara, Barrientos-Lozano, Rocha-Sánchez, & Zaldívar-Riverón, 2015).

The current species of *Taeniopoda* appear to have diversified during the Pleistocene. This period was characterized by having a global climate change composed by a series of glacial and interglacial periods (Ehlers & Gibbard, 2008), which promoted the expansion-contraction and/or the isolation-reconnection of the biota (Hewitt, 2000). These climatic oscillations also have been suggested as the main speciation event that led to the current species diversity in other groups of insects of recent divergence in North and Central America (Callahan & McPeek, 2016; Knowles & Alvarado-Serrano, 2010; Pedraza-Lara et al., 2015).

The probable recent speciation event that led to the origin of *T. reticulata* and *T. varipennis* on the other hand could have been promoted by recent, rapid mountain uplifts of the Talamanca, Tilarán, Guanacaste, and Central mountain ranges that occurred in the Costa Rican and Panamanian territory. These mountain uplifts have been estimated to have started from mid to recent Pleistocene, and thus agree with the low genetic differentiation that has been observed between the above two species (Bergoeing, 2006; Castillo, 1991).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

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