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Title: Paraphyletic species no more – genomic data resolve a Pleistocene radiation and validate morphological species of the *Melanoplus scudderi* complex (Insecta: Orthoptera)

Running head: Genomic and genitalic concordant delimitation signals

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Abstract:

Rapid speciation events, with taxa generated over a short time period, are among the most investigated biological phenomena. However, molecular systematics often reveals contradictory results compared with morphological/phenotypical diagnoses of species under scenarios of recent and rapid diversification. In this study, we used molecular data from an average of over 29,000 loci per sample from RADseq to reconstruct the diversification history and delimit the species boundary in a short-winged grasshopper species complex (*Melanoplus* Scudderi group), where Pleistocene diversification has been hypothesized to generate more than 20 putative species with distinct male genitalic shapes. We found that based on a maximum likelihood molecular phylogeny that each morphological species indeed forms a monophyletic group, contrary to the result from a previous mitochondrial DNA sequence study. By dating the diversification events, the species complex is estimated to have diversified during the Late Pleistocene, supporting the recent radiation hypothesis. Furthermore, coalescent-based species delimitation analyses provide quantitative support for independent genetic lineages, which corresponds with the morphologically-defined species. Our results also showed that male genitalic shape may not be predicted by evolutionary distance among species, indicating that this trait is not only labile, but also implying that selection may play a role in character divergence. Additionally, our findings suggest that the rapid speciation events in this flightless grasshopper complex might be associated primarily with the fragmentation of their grassland habitats during the Late Pleistocene. Collectively, our study highlights the importance of integrating multiple sources of information to delineate species, especially for a species complex that diversified rapidly, and whose divergence may be linked to ecological processes that create geographic isolation (i.e., fragmented habitats), as well as selection acting on characters with direct consequences for reproductive isolation (i.e., genitalic divergence).

Key words: genitalic shape, Pleistocene, RADseq, species complex, species delimitation

Introduction:

Rapid diversification events, or radiations, are among the most investigated biological phenomena because they can be useful to infer the proximate causes and constrains that generate and maintain biodiversity (Losos & De Queiroz 1997; Rundell & Price 2009). Yet, the study of ecological and phenotypic divergence among closely related taxa that are of primary interests under such scenarios is often confounded by the extreme difficulty of not only confidently identifying species boundaries using molecular data (i.e., many putative species are either polyphyletic or paraphyletic), but also obtaining a robust reconstruction of the underlying history (i.e., conflicting gene tree topologies can complicate phylogenetic estimation)(e.g., the Lake Victoria cichlids: Wagner et al. 2013; Darwin's finches: Farrington et al. 2014; Cadena et al. 2018). Moreover, without the statistic confidence from molecular or phenotypic data to support inferred putative species, the proposition of a radiation and high species diversity itself may be questionable (e.g., artifacts of taxonomic inflation; Cadena et al. 2018). That is, the underlying mechanisms leading to speciation can be confounded with those that are responsible for population subdivision (Smith et al. 2013; Sukumaran and Knowles 2017). When different phylogenetic histories are reconstructed because of choices of different data sets and the criteria for identifying taxa, it contributes to further conflicting inferences of diversification processes and history.

Such challenges, including conflicting results across data types, are exemplified in the short-wing grasshoppers belonging to the *Melanoplus scudderi* species complex (Uhler, 1964) (Fig. 1). There are more than 20 recognized putative species in the *M. scudderi* species complex according to a recent revision (Hill 2015). The complex is geographically widespread. However, most species have restricted geographic distributions (Fig. 1). They are recognized by a unique genitalic shape (Fig. 2), a key diagnostic character in the genus *Melanoplus* (e.g., Knowles and Otte 2000), and in Orthoptera more generally (e.g., Cohn et al. 2013), whose divergence by sexual selection may play a role in speciation (e.g., Eberhard 1996), including in grasshoppers (e.g., Marquez and Knowles 2007; Knowles et al. 2016). However, the putative species are not

monophyletic, based on analyses of mitochondrial loci (Hill 2015). It has been hypothesized that there has been insufficient time for lineage sorting to result in reciprocal monophyly of described taxa. For example, if the reductions of grasslands and expansion of forests in the Late Pleistocene facilitated the rapid diversification in the species group (see Hill 2015), the lack of monophyly would be expected. On the other hand, it is possible that the divergences among these morphologically distinct taxa represent the incipient stages of speciation (see Huang and Knowles 2016), without the reproductive isolation necessary for sustained evolutionary independence over time.

The study of the systematics of *M. scudderi* species complex is also more generally representative of the challenges that arise when the biology of the group seems incongruent with the taxon's traits. For example, prior to the recent thorough revision (Hill 2015), only one morphological species was described. Such a systematic proposal is suspect on several fronts. First, biogeographically it would certainly be a puzzling observation that a flightless species would be so widely distributed (see Knowles and Otte 2000), not to mention the questions that such a range raises about dispersal mechanisms given it seem unlikely that a flightless species would traverse major physical barriers (e.g., the Mississippi river; Fig. 1) thereby preventing the initiation of species differentiation. Moreover, the lack of morphological divergence (or potential "cryptic" diversity) largely seems to reflect an exclusive focus on easily visible external characters (e.g., wing patterns and body coloration). In fact, when internal characters, and specifically the male genitalia were examined (Hill 2015), a diversity of potential taxa were revealed. As such, the "cryptic" species diversity in the *M. scudderi* species complex, as with other studies, has a direct impact on biodiversity estimates, with downstream implications for ecological and evolutionary studies (Bickford et al. 2007). On the other hand, whether distinct genitalic shapes correspond to species boundaries is not always clear or rarely empirically evaluated (e.g., tests of correlated evolution of the genitalic complex, Marquez and Knowles, 2007; tests of lock-and-key hypothesis, Masly 2012). Likewise, not only in *Melanoplus*, but in other insects, whether genital divergence reflects taxonomic over-splitting has been a persistent

concern (Masly 2012). Note that when two species are morphologically and/or ecologically indistinguishable and genitalic differentiation is the only diagnostic character to separate different species, it is conventionally assumed that divergence in male genitalia may function as a reproductive barrier.

In this study, we harnessed the power of next generation sequencing (McCormack et al. 2013; Andrews et al. 2016) to generate thousands of nuclear loci for estimating phylogenetic relationships and testing hypothesized species boundaries in the *M. scudderi* species complex. Specifically, we (1) estimated the phylogenetic relationships and times of diversification in the species complex, and (2) tested for a correspondence between morphologically identified species boundaries and genetic lineages detected under a coalescent-model. Lastly, we used this framework to (3) evaluate the evolution of male genitalia, and specifically, whether divergence in these characters are correlated with phylogenetic distance, where deviations could suggest the action of selection during the divergence of the putative taxa. We discuss our results with respect to what they highlight in particular about species boundaries and the evolutionary diversification of the *M. scudderi* species complex, as well as more general issues surrounding the reconciliation and interpretation of discordance between data types when studying speciation and species delimitation.

Materials and Methods:

Species sampling and genomic data

Genomic data were collected from 96 individuals, with 3 to 5 representatives per putative species sequenced for each of 20 putative species from the *M. scudderi* species complex (collection and voucher information in supplementary Table S1); only males were sequenced so that individuals could be assigned to putative taxa based on the diagnostic genitalic characters (see Hill 2015). Specifically, we applied a reduced-representation library to contend with large genomes of *Melanoplus* grasshoppers (about 4 GB). Likewise, we applied analyses that are robust to missing data (i.e., SVDQuartets) or carefully selected variable and complete molecular

data sets among individual samples for analyses that may (or may not) be sensitive to missing data (i.e., bpp analyses). Detailed methods are provided below.

Genomic DNA was extracted from tissues stored in absolute alcohol using Qiagen DNeasy following the manufacturer's Animal Tissue Protocol (Qiagen, Germany). Two reduced representation libraries were constructed following a double digest restriction-site associate DNA sequencing protocol(ddRADseq) (for details see Peterson et al. 2012); each library contained a total of 48 individuals from all 20 putative species. Briefly, for each library, individuals (with a total genomic DNA of *ca*. 160 ng) were double-digested using restriction enzymes *Eco*RI and *Mse*I and uniquely tagged with a 10bp barcode. The digested and tagged products were then pooled and size-selected for 350-450bp fragments using a Pippin Prep (Sage Science, MA, USA). Size-selected fragments were PCR amplified with iProof™ High-Fidelity DNA Polymerase (BIO-RAD, CA, USA). DNA quantification and cleaning with Agencourt AMPure XP (Beckman Coulter, CA, USA) occurred after every step in the library construction procedure. Each genomic library was sequenced on an Illumina HiSeq2000 at the Centre for Applied Genomics (Toronto, ON) to generate 150bp single-end reads.

The sequence data was processed using pyRAD v3.0.6 pipeline (Eaton 2014) to identify SNPs. Specifically, after de-multiplexing, low quality sites (quality score > 20) were converted to Ns, and reads with a minimum coverage depth (m = 5) were assembled into putative alleles. Loci with more than 2 alleles per individual sample were discarded, given they violate diploid expectations. A *de novo* assembly and alignment of different loci across all the sampled individuals were then performed for sequences with a minimum similarity of 88% and a maximum of two low quality sites per locus (assemblies using minimum sequence similarities of 85% and 90% were also generated and did not differ significantly). Downstream sites after site 115 were excluded for all aligned clusters to remove low-quality bases near the 3' ends (see Figs. S1 and S2). Additionally, we excluded loci containing one or more heterozygous sites shared across more than 10 individuals (or approximately two putative species) to account for incomplete lineage sorting and/or potential interspecific gene flow between closely related taxa

while avoiding indiscriminate clustering of paralogs (i.e. shared heterozygous sites across too many samples most likely represent clustering of paralogs with a fixed difference rather than true heterozygous sites; Eaton and Ree, 2013). A concatenated unlinked SNP file (i.e., with one randomly selected SNP from each locus) and a file with individually aligned loci in separate blocks were generated for loci that were present in at least 10 individuals.

A total of 167,078,647 raw reads passed the initial quality control, with an average of 1,740,403 good reads per sample (S.E. = 81,147). Clustering analyses within samples identified an average of 59,615 clusters (S.E. = 1,710) with a minimum of 5× and mean 18× per cluster sequencing depth (Table S2). The final dataset contained 93,720 variable loci with an average of 29,644 (S.E. = 830) loci per individual; a summary of pre- and post-processing read numbers is given in Supplement Table S2. The number of loci shared between individuals is not correlated with their phylogenetic relatedness, indicating that the amount of missing data between individuals was not caused by mutations in the restriction sites (Huang and Knowles 2016b; see Fig. S7 and the result section below), and instead reflects the finite number of reads available for two lanes of sequencing on a Illumina HiSeq2000.

Phylogenetic and divergence time estimation

Phylogenetic relationships were estimated from the genomic data using two approaches: one in which the data across loci were concatenated, and one in which potential genealogical discord across loci is accommodated (i.e., using a coalescent-based analysis; Knowles and Kubatko 2010). An unlinked SNP file from pyRAD pipeline was used for phylogenetic reconstruction (a total of 94,846 loci).

For the analyses based on concatenation of the data, one SNP, which was shared across most of the analyzed individuals, was randomly chosen for each locus to build a concatenated unlinked SNP dataset. A Maximum likelihood (ML) tree was estimated using the GTRGAMMA model implemented in RAxML v. 8.1.17 (Stamatakis 2014). We also performed a fast bootstrapping analysis with 100 replicates in RaxML with the model GTRCAT to assess nodal

support and the reconstructed ML-tree was visualized in FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) using the midpoint rooting option.

An estimate of phylogenetic relationships based on the unlinked SNP dataset were generated using the coalescent-based analysis of SVDQuartets (Chifman & Kubatko 2014) implemented in PAUP* v. 4.0a (Swofford 2002). Specifically, an exhaustive search of all possible quartets option was specified for estimating a bifurcating tree from the sampled quartets using Quartet FM (Fiduccia & Mattheyses 1982). Each SNP was treated as an independent locus and 1,000 bootstrapping replicates were analyzed based on the same search options as described above to estimate branch support. The estimated tree was visualized using FigTree; note the same node as in the ML phylogenetic analysis based on concatenated data was used to root the tree.

Times of divergence among the 20 putative species of the *M. scudderi* species complex were estimated using the program bpp v. 4 (Flouri et al. 2018). The species tree estimated with SVDQuartets with species (not individuals) representing operational taxonomic units (OTUs) was used as a fixed species tree topology to estimate divergence times (i.e., applying the A00 model in bpp). A series of R scripts were applied to edit and convert the data file from pyRAD (.loci file) analysis into a bpp input file (see Huang 2016, 2018). For the data used in this analysis, (1) only sequence clusters, or loci, present in a at least one representative per taxon were retained, and (2) the maximum pairwise sequence divergence between samples was set to 8% following Hill (2015) based on analyses of mitochondrial sequence data; the common threshold was chosen to facilitate comparison across studies. The resulting dataset contained 4,132 variable loci (Fig. S2). Branch lengths were estimated using the full set of 4,132 loci, and using four sets of 1,000 randomly chosen loci, where the loci were mutually exclusive among the four 1,000 loci data sets. The bpp analyses were run for 10^5 generations with a sampling frequency of 2 and a burn-in period of 10^4, and we specified the diploid variable to indicate that all the sequences from each individual were un-phased. We also estimated the species tree topology (i.e., using the A01 model in bpp) based on the full 4,132 loci data set using bpp. The

distributions of θ and τ priors were informed from point estimates based on the number of segregation site per site (Figs. S2 and S3).

Species delimitation

Three recently diversified clades (Fig. 1), each with 4-6 morphologically well-differentiated putative species, were selected for species delimitation analyses. It is theoretically possible to perform species delimitation with 20 species using the program bpp (version 4; Flouri et al. 2018), given the compatibility with short reads of unlinked neutral loci from ddRADseq libraries (see Flouri et al. 2018). However, such an analyses would be challenging because parallel computation was not available when we conducted the analyses. Furthermore, given that the main idea of our study is to test whether recently diverged lineages can be supported by genomic data as distinct species, the exclusion of distantly related lineages with respect to each focal clade of interest is reasonable (Fig. 1).

For bpp analyses, we randomly chose 100 loci from the 4,132 loci data set for each of the separate analyses of the different subsets of taxa (see Fig. 1 for the identities of putative taxa included for each subset); the analyses were repeated five times for each subset using randomly chosen, and mutually exclusive, sets of 100 loci each. Priors for θ and τ values were informed from point estimates based on the completed 4,132 loci data set (Fig. S4). To account for phylogenetic uncertainty among putative species (see Results section; Fig. 1), we used the unguided option in bpp to estimate the supports of evolutionary independence for each analysis (A11 model; Yang and Rannala 2014). The bpp analyses were run for 5×10^5 generations with a sampling frequency of 10 and a burn-in period of 5×10^4.

Morphological analyses of male genitalia

Outlines of the lateral and ventral view of the male genitalia, specifically the shape of the apical valves of the aedeagus, were collected from digital images of specimens (data available as supplementary data in Dryad; see also Hill 2015); for details regarding outline data collection see

protocol detailed in Marquez and Knowles (2007). We analyzed one individual per species, an standard approach applied in similar comparative phenotypic and phylogenetic studies on grasshoppers and crickets (e.g., Marquez and Knowles 2007; Oneal and Knowles 2013; Knowles and Cohn 2016), as well as in other animal groups (e.g., catfish [Silva et al. 2016] and turtles [Dickson and Pierce 2019]). Shape analyses were performed on the outline coordinates using the R package *momocs* (Bonhomme et al. 2014). Specifically, elliptical Fourier analyses were performed (Kuhl and Giardina 1982) and the best number of harmonics was chosen based on the harmonic power that described the analyzed shapes. Principle component analyses (PCA) were applied to quantitatively investigate the differences in the outlines of male genitalic shapes among putative species.

To examine the evolution of the male genitalia, we tested whether there is a significant correlation between phylogenetic distance and morphological difference in male genitalic shapes. Specifically, phylogenetic distance between pairs of putative species was calculated using the *cophenetic* function implemented in *ape* (Paradis and Schliep 2018) based on the branch lengths in the reconstructed species tree, where the branch lengths were estimated using bpp (Fig. 1). The difference in male genitalic shape between pairs of species was represented by Euclidean distance calculated based on the PC1 and PC2 coordinates from the PCAs. The phylomorphospace function implemented in *phytools* (Revell 2012) was used to visualize the correlation between phylogenetic distance and shape difference in the male genitalia. Similarly, the PC1 and PC2 coordinates from PCAs were used as continuous phenotypic data sets for generating a phylomorphospace plot, and visualized using the *Phenogram* function implemented in *phytools* for each of the four retained PCs.

We also tested whether the divergence in male genitalic shape among the putative species was consistent with a Brownian motion model. For these analyses, we extracted the first two PCs from the PCAs of the lateral and ventral shape variation of the male genitalia. Multi-dimensional phylogenetic signal tests using the extracted PCs with 1,000 permutations were used to test a Brownian motion model with the *fast.SSC* function in the R package *Rphylopars* (Goolsby et al.

2016). The function uses a fast ancestral state reconstruction algorithm to calculate the sum of square changes between ancestral and descendant nodes/tips based on a model described in Klingenberg and Gidaszewski (2010). Specifically, we tested (1) all four extracted PCs, (2) the two PCs from lateral shape, and (3) the two PCs from ventral shape, as well as (4) each of the individual PCs. A scaled sum of squared changes equals to 1 indicates the evolution of the continuous trait data can be explained by Brownian motion. A larger than 1 value indicates the evolution of the trait data is more conserved than expected given their phylogenetic distance, while a smaller than 1 value implies the traits evolution is more labile between taxa than expected by their phylogenetic distance.

Results:

Monophyly of putative species and divergence time estimates

Estimates of the phylogenetic relationships among individuals based on the analysis of the concatenated data (Fig. S5) show morphological identified putative species were monophyletic with high bootstrap support (> 90%). The species tree estimated using the coalescent-based approach of SVDQuartets were generally consistent with the relationships inferred from the concatenated data (Fig. 1). Moreover, the estimated relationships correspond to the geography of putative species distributions. For example, taxa east of the Mississippi River (i.e., *Melanoplus quercicola* (Hebard 1918), *Melanoplus davisi* (Hebard 1918) and *Melanopolus chattahoochee* (Hill 2015) form a well-supported (bootstrap values = 100) monophyletic group. Among the 17 remaining putative species, which are west of the Mississippi River, clear geographic structure (especially, among *Melanoplus irwinorum* (Hill 2015), *Melanoplus optimus* (Hill 2015), *Melanoplus ottei* (Hill 2015), *Melanoplus taurus* (Hill 2015), and *Melanoplus texensis* (Hart 1906)) is evident based on the phylogenetic relationships, with relatively recent divergences (see Fig. 1).

A species tree topology search using bpp resulted in similar species tree topology with high posterior probability support (Fig. S6), with few minor differences (compare with Fig. 1).

The most probable species tree topology from bpp analysis (Fig. S6) was observed in 86.7% of the post burn-in species trees. The subject data set did not show biased sampling effect of shared loci between closely related species (Fig. S7), because we specifically selected loci that do not have any missing taxon (see materials and methods section for details).

The divergence time estimates were consistent across four independent subsets of 1000 loci (Figs. S8 & S9). Assuming a generation time of one year, the common ancestor of the 17 western species may have originated 333.3 thousand years ago (kya) (based on the genomic mutation rate of 2.9×10^{-9} estimated for *Heliconius melpomene*; Keightley et al. 2015) or 62.5 kya (based on a genomic mutation rate of 1.6×10^{-8} estimated for *Aedes aegypti*; Sherpa et al. 2018) ($\tau = 2\mu t$, where τ is the estimated population divergence in substitutions per site, μ is the per site mutation rate per generation, and t is the absolute divergence time in years). The time to the most recent common ancestor for the species from the *M. scudderi* species group – i.e. the common ancestor of *M. scudderi*, *M. mississippi*, *M. coreyi*, *M. muscogee*, *M. relictus*, and *M. folkertsi* – that dispersed across the Mississippi River is estimated around 0.0006 τ units, or about 100 to 18.8 kya, depending on the different mutation rate calibrations applied. The most recent speciation event occurred between *M. mississippi* and *M. scudderi* (τ = 0.0003), or around 50 or 9.4 kya, whereas the initial split between the eastern (3 species) and the western (17 species) lineages is around 0.0048 τ units (Fig. 1), or 150 or 700 kya depending on which calibrated mutation rate is applied, both of which are consistent with divergence during the Mid-Pleistocene (126 to 781 kya).

Morphologically diagnosed species boundaries correspond to those delimited genetically

All morphologically diagnosed species boundaries are supported by genetic-based delimitation analyses in bpp analyses with high posterior probability support (posterior probabilities > 0.99). The results were consistent across five replicate data sets with 100 mutually exclusive and randomly chosen loci (see Materials and Methods section) for each of the analyzed subsets of taxa (see Fig. 1 for lineages analyzed in each subset). Specifically, from all the post

burn-in samples of the MCMC searches, < 1% of the species delimitation results lumped species. The only exception involved analyses of the subset *M. mississippi*, *M. scudderi*, *M. coreyi*, *M. folkertsi*, *M. muscogee*, and *M. relictus*, in which 16% of the species delimitation results lumped *M. scudderi* and *M. mississippi* into one species. Note that these are the only two species that dispersed across the Mississippi River, and represent the most recent speciation event (see Fig. 1). Although support for the delimited taxa in this group was lower, it was nevertheless still relatively high; the probability of all six of them are independently evolving lineages is around 84%.

Evolution of male genitalia

Morphological dissimilarity was not correlated with phylogenetic distance among species (Fig. 3). When visualizing the phenotypic data and the phylogenetic relationship among species using ventral and lateral genitalic shapes (Fig. 3), there is no detectable phylogenetic structure on phenotypic trait values. For example, sister species *M. scudderi* and *M. mississippi* have similar genitalic shapes, while another sister species pair *M. folkertsi* and *M. muscogee* exhibit very different genitalic shapes. On the other hand, distantly related species such as *M. davisi* and *M. seltzerae* have very similar shaped genitalia (Figs 2, 3, S10 and S11).

Tests of the four PC values (PC1 and PC2 from both the lateral and ventral genitalic shape analyses) suggests evolution of genitalic shape is more labile than expected by their phylogenetic relationships among species (total sum of squared changes = 0.38017; scaled sum of squared changes = 0.54303); however, this deviation from a Brownian motion model is not statistically significant ($P = 0.158$). Analyses of the lateral and ventral genitalic shape variables showed similar results. Specifically, a total sum of squared changes = 0.62396 and 0.38017, and a scaled sum of squared changes = 0.55729 and 0.54303, were estimated for the lateral and ventral shape variables, respectively, neither of which are significant ($P = 0.57$ and 0.17, respectively). Analyzing each individual PCs also resulted in the same inference; all the tests resulted in values of scaled sum of squared changes around 0.5 and P values > 0.1.

The shape analyses (Figs. S12) showed that > 20 harmonics are sufficient to describe lateral and ventral shape variation in the genitalia (Figs. S13). The first two axes of the PCA explained > 50% of the shape variation across putative species (Figs 2, S14); all shapes were correctly centered with a common point of origin (Fig. S13).

Discussion:

The study of recent biological radiation events has been shown extremely insightful into the origin and structuring of global biodiversity patterns (e.g., the adaptive radiations in the Darwin's finches and the Lake Victoria cichlids), but rampant conflicts among data types can obscure the systematic frameworks upon which such study are reliant upon. Our study of the recent radiation of a grasshopper species complex demonstrates how with the resolution provided by genomic data and powerful analytical approaches, we can move beyond the confusion surrounding phylogenetic relationships and species boundaries when diversification occurs rapidly in the recent past. Specifically, we not only confirm the validity of utilizing variation in genitalic shape to delimit recently diverged evolutionary entities, by showing distinct male genitalic shapes correspond to independently evolving lineages identified by the genomic data, our analysis also shows divergence in these characters occurred rapidly and recently. Below, we discuss potential factors that may have contributed to the diversification of taxa in the *M. scudderi* species complex, as well as importance of integrating phenotypic and genomic data in insect systematics, and for delimiting species of *Melanoplus* grasshoppers in particular.

Multiple evolutionarily independent lineages radiated in late Pleistocene

The Pleistocene glacial cycles have promoted speciation in many North American faunas, including grasshoppers (Knowles 2000; Knowles and Richards 2005; Carstens and Knowles 2007). However, many such studies have focused on speciation in montane taxa, where the effect of glacial cycles and repeated are manifests in shifts in the altitudinal distribution of suitable habitats. For members of the *Melanoplus scudderi* species complex from the eastern and central

regions of southern North America, the geography of species divergence occurred on a fundamentally different landscape. Yet, diversification in many ways mirrors that of montane *Melanoplus* grasshoppers – that is, rapid and recent speciation (Fig. 1).

With regards to geographic isolation, our study may fit with others that have invoked the fragmentation, or the horizontal restructuring of habitat types from the great plains during the Late Pleistocene as a potentially important factor in the divergence of species from southeastern United States (e.g., Satler and Carstens 2016). Fossil pollen records indicate that during the change from a dry to a wet climate during the late Pleistocene to early Holocene, including during the last glacial maximum (LGM), the southeastern United States was dominated by prairie and savannah habitat types (Russel et al. 2009). However, this once continuously distributed grassland was subsequently fragmented into multiple patches after the LGM by expanding forest habitats. Furthermore, with the wetter climate, potential increased water flow in the rivers and their drainages (Bentley Sr. et al. 2016) may have created additional physical barriers that further contributed to the isolation of grassland inhabitants (Hill 2015). As such, and as grassland dependent species, populations of taxa from the *Melanoplus scudderi* species complex may have become recently fragmented into local grassland patches, contributing to the divergence into multiple species as past widespread ancestral populations became locally isolated (Hill 2015). Such a scenario is consistent with several aspects of the history of diversification inferred from the genomic data. For example, the reconstructed species tree reveals an initial divergence between an eastern lineage – which is composed of *M. davisi*, *M. quercicola,* and *M. chattahoochee* – and a western lineage – which contains the remaining 17 species from the complex (Fig. 1). Subsequent to the isolation of the eastern and western lineages, a western lineage, but not an eastern one, dispersed across the Mississippi river in late Pleistocene (ranging from 100 to 18.8 kya; see results section), consistent with the hypothesis that previously expansive areas of grassland on the North American Coastal Plain formed in the Mississippi River delta because of the lowered sea level in the Pleistocene (Bentley Sr. et al. 2016), possibly facilitating dispersal of the flightless *Melanoplus* grasshoppers across a main

geographic barrier, the Mississippi river. Many terrestrial organisms from the Appalachian mountains and the Southeastern coastal region of the United States have retreated into hypothesized climatic refugia in the glacial periods during the Pleistocene (e.g., Walker et al. 2009). This climatic constraint may help explain why the eastern lineage did not expand westward, while the western lineage expanded eastward (Fig. 1). Note that the two geographically widespread lineage *M. scudderi* and *M. mississippi* that are distributed on both sides of the Mississippi River (Fig. 1; Hill 2015) originate from western ancestors.

Evolution of genitalia variation and applications for delimiting species

Genitalic morphology has been an important, although sometimes debated, character in taxonomic studies in Orthoptera (e.g., Cohn et al. 2013). With differentiation in size, shape, and genitalic structures, evolutionary changes in genitalic morphology between species have been hypothesized to play an important role in intra-specific recognition during copulation, and may be under strong sexual selection (e.g., Eberhard 1996; Masly 2012; Oneal and Knowles 2015; Fujisawa et al. 2019). The genetic distinctiveness of the species revealed from the coalescent-based delimitation analyses indicates little (if any) gene flow between species with different male genitalic shapes, where genetic distinctiveness is maintained despite recent species divergence. Specifically, species with different male genitalic shapes form reciprocal monophyletic groups in *M. scudderi* species complex (Fig. S5) and their evolutionary independence is statistically supported by delimitation analysis based on the genomic data.

The corresponding between phenotypic and genetic divergence not only corroborates hypotheses about species boundaries, but it also implies that divergence in genitalic shapes reflect selectively driven divergence. For example, when considering past analyses based on mitochondrial genes, it is clear that the evolution of male genitalic shape in the *M. scudderi* species complex outpaces the sorting of shared ancestral mitochondrial nucleotide variation after species divergence (cf. Figs. 49 and 50 in Hill 2015). Specifically, different genetic groups (Figs. 1 & S5) can be unambiguously distinguished by examining the male genitalic shape, while

mitochondrial sequence data failed to identify the different evolutionary entities (Hill 2015). Moreover, some recent speciation events (< 10 kya), such as is the case of the sister taxa *M. mississippi* and *M. scudderi*, can be inferred based on male genitalic shape (Hill 2015). Furthermore, these sister species have extensive geographic overlap (Fig. 1), while their genetic uniqueness is still well-maintained (as supported by bpp results). That is, the lack of genetic distinctiveness in mitochondrial genetic variation reflects insufficient time for sorting, and as such not in conflict with a recent divergence history because mitochondrial DNA simply cannot effectively discriminate species of rapid diversification origin (Hickerson et al. 2006; Wagner et al. 2013). Such species diversity can, however, be successfully identified by carefully examining male genitalia variation or using genomic data from hundreds of thousands of loci, as our results show.

Conflicting results in systematic studies happen frequently when different data types are used to delimit species or to reconstruct their evolutionary history (Hillis 1987; Sites and Marshall 2004; Cohen 2018). However, when the basis of this conflict is examined, the conflict itself, while inconvenient for species delimitation, can be informative about the speciation process and help make robust, biologically meaningful inferences about taxonomy (Yang and Rannala 2014; Huang and Knowles 2016; Sukumaran and Knowles 2017). Specifically, because morphological traits subject to strong natural or sexual selection, such as the genitalic morphology (Eberhard 1996; Masly 2012), can evolve rapidly, species specific phenotypes may become evident before neutral genetic markers show differentiation (e.g., Sites and Marshall 2004; Wagner et al. 2013; Solis-Lemus et al. 2014). Likewise, the conflicts among data types and between molecular data sets revealed in the systematics of the *M. scudderi* species complex have collectively provided insights into its diversification history and, thus, such differences should not hamper taxonomic decisions.

Determining whether changes in genitalic shape is the cause or a consequence of speciation is difficult (e.g., Oneal and Knowles 2013). The geographic distribution pattern of species in the *M. scudderi* species complex implies a dominant role for allopatric speciation,

potentially in association with the rapid and simultaneous fragmentation of grassland habitats during late Pleistocene (see above). Currently, there is not much geographic overlap between species (Fig. 1), which indicates that secondary contacts between diverged lineages have been rare. It is possible that species split may have simply resulted from geographic isolation and observed changes in genitalic shape between species have accumulated after speciation. However, the differences in genitalic shape are not a simple function of the phylogenetic distance among species (Figs. 3 and S15). Furthermore, although not statistically significant, comparison to expectations under a Brownian motion model indicate a pattern of evolutionary lability in genitalic shapes across species of the *M. scudderi* complex; note the lack of significance does not mean genitalia are evolving neutrally (see Harmon 2019). Together, the analyses suggest the genitalia may have played a significant role in promoting speciation in the study system. Irrespective of whether differences in male genitalic shape are a proximate cause of speciation in *M. scudderi* species complex, the genitalic shape is still a good diagnostic character.

Are we oversplitting geographic/morphological taxa into "species?"

The boundary between geographic/ecological forms and species has been blurry in taxonomic and evolutionary studies, with recent advances in obtaining and analyzing genetic data stirring more controversy (Freudenstein et al. 2017; Sukumaran and Knowles 2017). Genomic data have become a commonly used data type in systematics and evolutionary biology, where new models and associated computer programs have been rapidly developed. However, recent simulation-based as well as empirical studies show the limitations of relying upon genetic data alone for delimiting species (Sukumaran and Knowles 2017; Huang 2018). For example, the commonly used multi-species coalescent model in species delimitation confounds genetic structure within species with genetic divergence associated with species boundaries. By showing that phenotypic and genetic boundaries delimit the species lineages, our study avoids the misleading inferences that can occur when relying only on genetic data.

Lastly, with regards to concerns of potential over-splitting of taxa based on differences in the genitalia, our work validates the identity of the species diagnosed by genitalic differences (see also Knowles 2001b for another example in *Melanoplus*). The only case in which there was some suggestion that species may be oversplit, and what might be considered as sibling species (Mayr 1942), involves the divergence between *M. scudderi* and *M. mississippi* (see Fig 1). *Melanoplus scudderi* and *M. mississippi* are morphologically similar, but their geographic distributions largely overlap (Fig. 1). Although, we have only genotyped one population for each taxon and we do not know if these two putative sibling species are cohesive across their respective geographic distributions, our results suggest a possible example of cryptic divergence, especially given that our molecular data show statistically significant genetic divergence between the two species, which form two reciprocally monophyletic genetic groups of individuals (Fig. S5). Consequently, we conclude that species status should be conferred to all the genetically differentiated lineages, which also correspond to unique male genitalic shapes in the *M. scudderi* species complex.

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Data availability:

All raw sequence data are archived in the NCBI sequence read archive database under bioproject PRJNA574680. R scripts and input data for SVDQuartets, bpp, and shape analyses are available in the Dryad data repository (doi: 10.5061/dryad.dncjsxkvg).

Author contributions:

L. L. Knowles conceived the study. J. Hill collected the samples and took the image data. J. Ortego prepared the ddRADseq libraries. J-P. Huang processed and analyzed the data. All authors wrote and read the manuscript.

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Conflict of Interest statement:

The authors declare no conflict of interest.

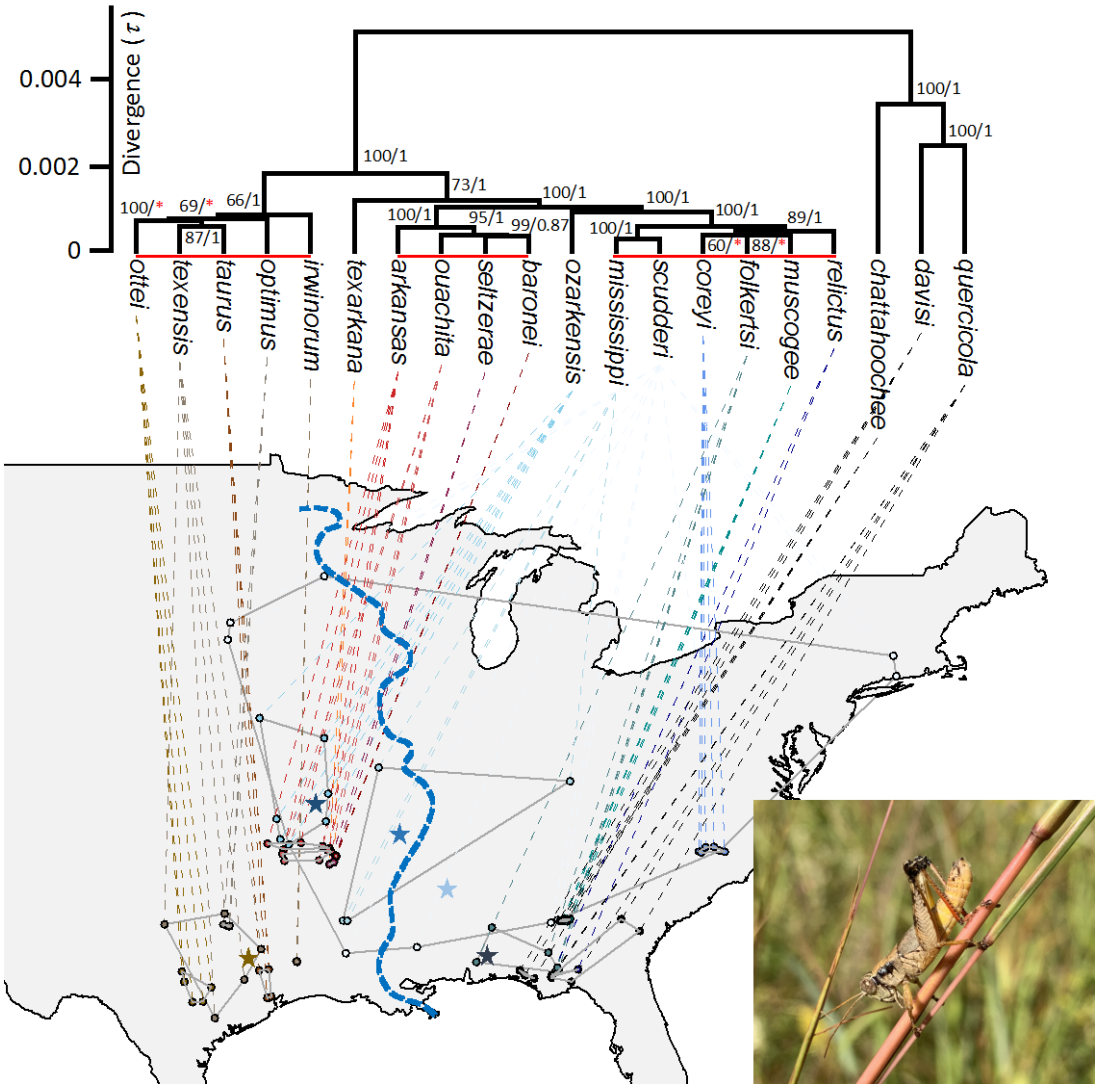


Fig. 1. An estimated species tree of 20 species from the *M. scudderi* species group and their approximate geographic distribution, as marked by the differently colored areas based on geographical coordinates from Hill (2015). The sampling sites for geographically widespread species were indicated with asterisks. Numbers on nodes of the tree are bootstrapping support values from SVDQuartets analysis (before slash) and posterior support values from the bpp analysis (after slash). Red asterisks indicate conflicting nodes between species tree topologies reconstructed using SVDQuartets (shown in this figure) and bpp (available in supplementary

data; Fig. S6). Divergence time is expressed in substitutions per site (τ). The groups of putative species lineages tested in separate species delimitation analyses are marked by red bars at the tree tips. The Mississippi river is identified by the thick blue dash line. An adult *M. relictus* on *Schizachyrium scoparium*. Thomas Co., Georgia, USA (image taken by J. Hill).

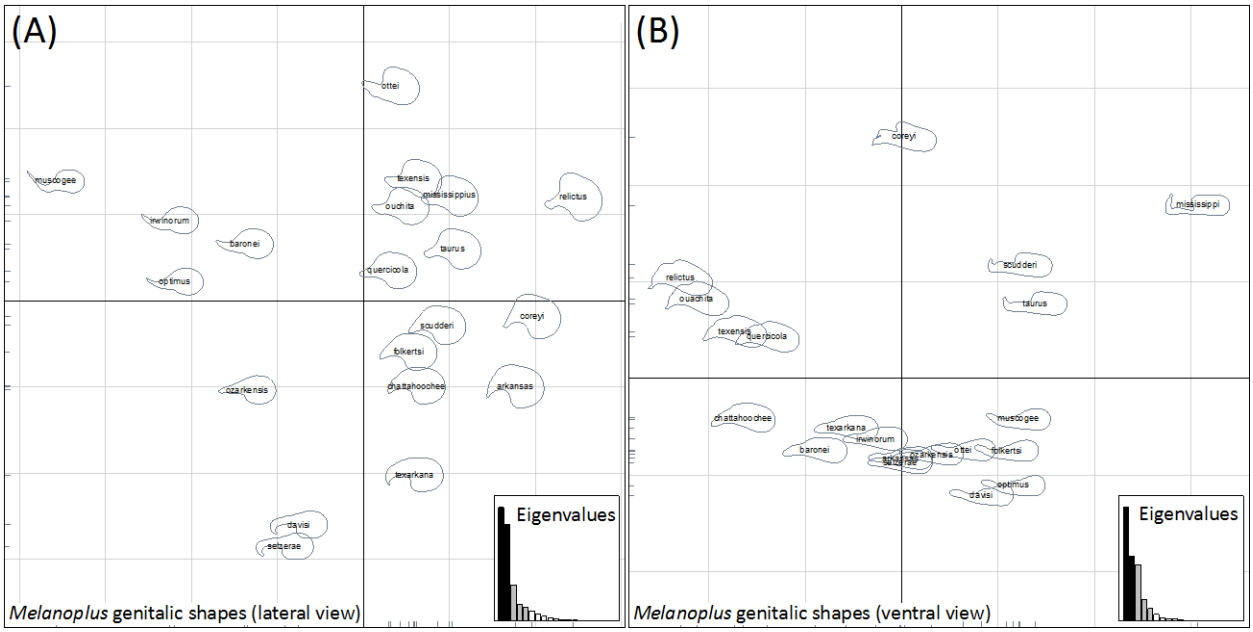


Fig. 2. The position of putative species in the morphospace based on the PCA results of the shape analyses of the (A) lateral and (B) ventral views of the male genitalia (see Figs. S10 and S11 for the original shape outlines and Figs. S13 and S14 for a characterization the mean and variance of shape differences associated with each axis).

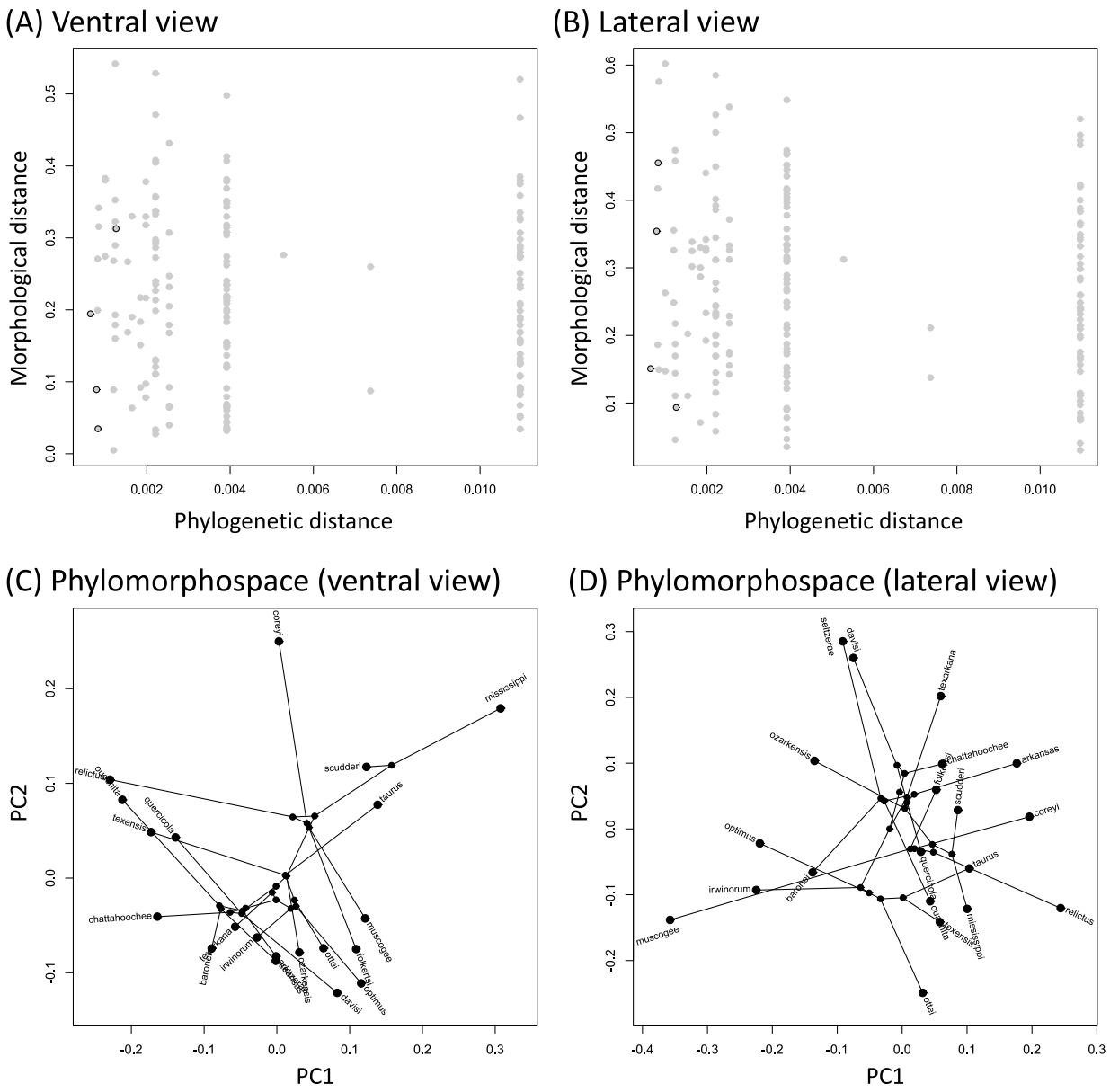


Fig. 3. Plots for association between phylogenetic relationships and dissimilarities between male genitalic shapes. (A) and (B) are plots of phylogenetic distance between species against the shape dissimilarity between species calculated based on the Euclidean distance derived from PC1 and PC2 coordinates. (C) and (D) are phylomorphospace plots derived using the PC1 and PC2

values from the shape analyses and the reconstructed species tree (Fig. 1) using the *phylomorphospace* function in *phytools*.

Short figure legends:

Fig. 1. A reconstructed phylogeny and geographic distribution of species from the *M. scudderi* species complex.

Fig. 2. PCA results from analyzing male genitalic shapes.

Fig. 3. Plots for association between phylogenetic relationships and dissimilarities between male genitalic shapes.

Supporting information:

Table S1. Collection and voucher information of samples from putative species in the *Melanoplus* scudderi complex

Table S2. Summary of pyRAD filtering results

Fig. S1. The (A) distributions of site variation and (B) the position of the variable site of the retained loci after processing.

Fig. S2. Summaries of the genetic data of retained sequences after filtering the ddRADseq data.

Fig. S3. The distribution of estimated theta values from the 4,132 retained variable loci used in phylogenetic and species delimitation analyses.

Fig. S4. The distributions of estimated theta values and maximum pairwise genetic differences from each of the three groups of lineages (see Fig. 1) for each of the separate species delimitation analyses.

Fig. S5. A maximum likelihood phylogenetic estimate of the 96 sampled individuals based on a concatenated data set of SNPs.

Fig. S6. The best species tree topology from bpp 4 analyses.

Fig. S7. A pairwise estimation of shared loci between samples from a 4132 loci data set.

Fig. S8. The estimated species tree and divergence times using a total of 4,132 variable loci.

Fig. S9. The estimated species tree and divergence times using four different sets of 1000 variable loci.

Fig. S10. Outlines of the lateral view of the male genitalia shapes.

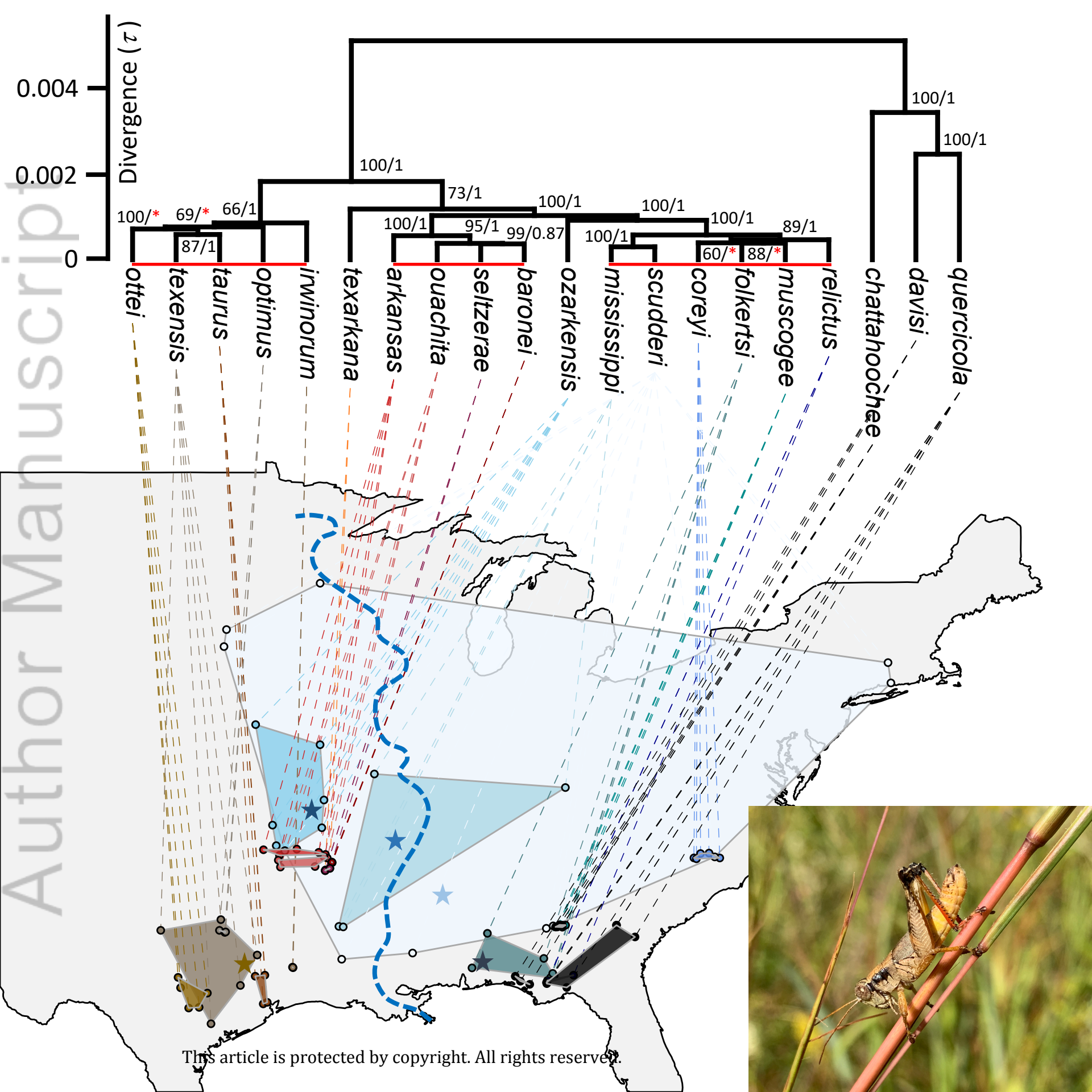
Fig. S11. Outlines of the ventral view of the male genitalia shapes.

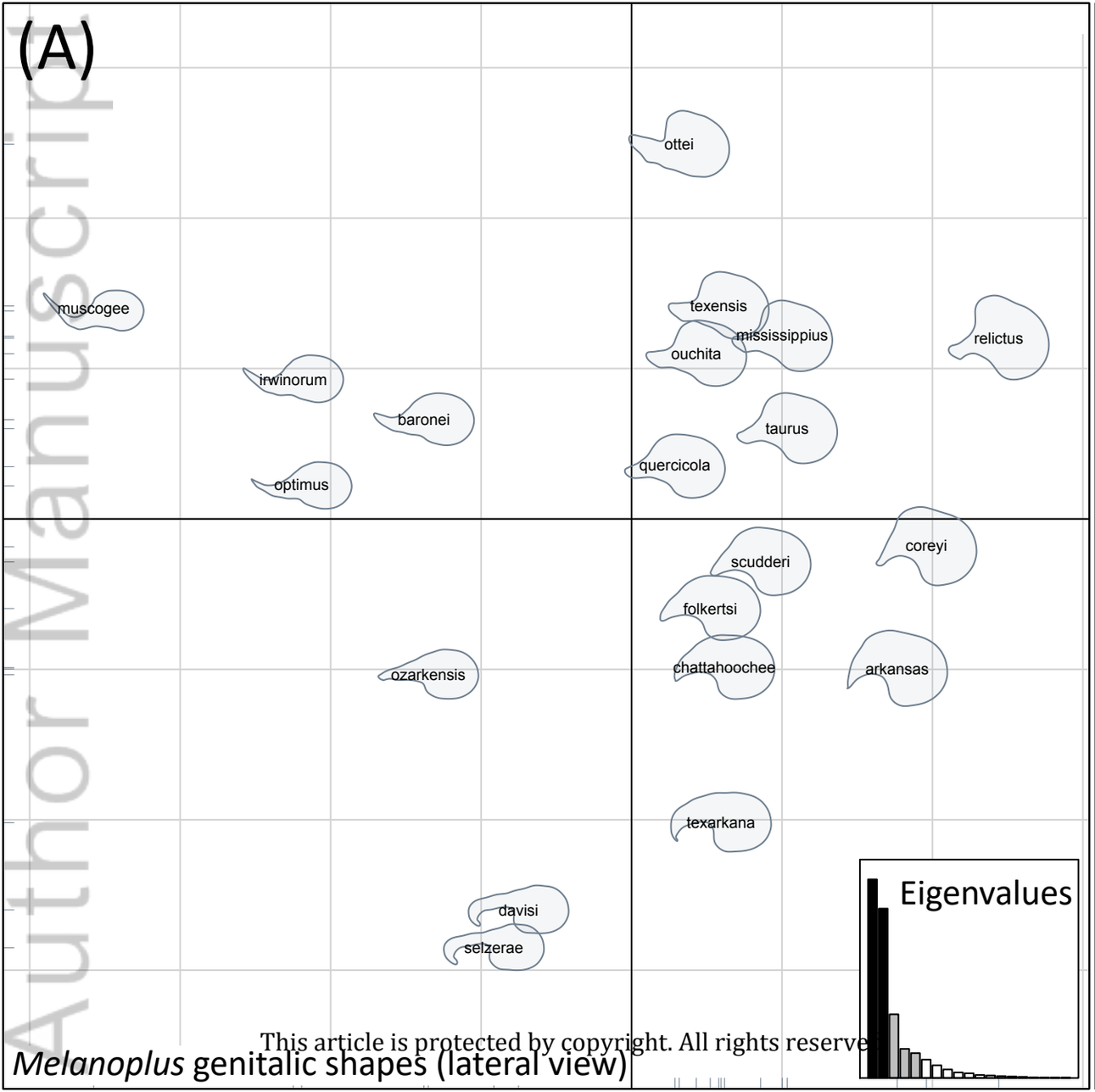
Fig. S12. Results from elliptical Fourier analyses using different number of harmonics and the shape data for the (A) lateral and (B) ventral view of the male genitalia.

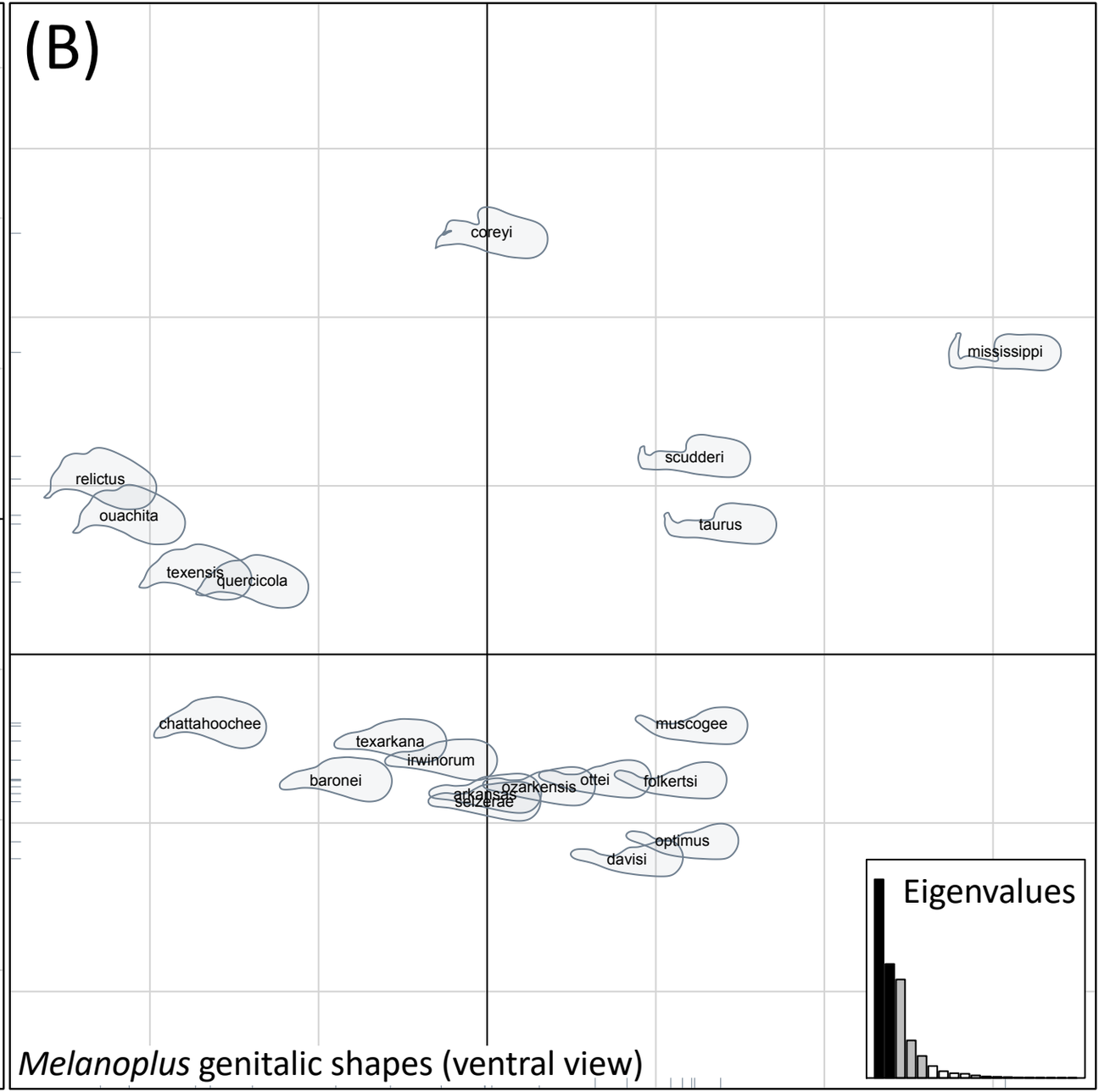
Fig. S13. A overlay of all the outlines of the (A) lateral and (B) ventral male genitalia shape variation across individuals.

Fig. S14. The first five PCs from shape analyses based on the (A) lateral and (B) ventral views of the male genitalia shape.

Fig. S15. Phenograms based on individual PCs and the reconstructed species tree.



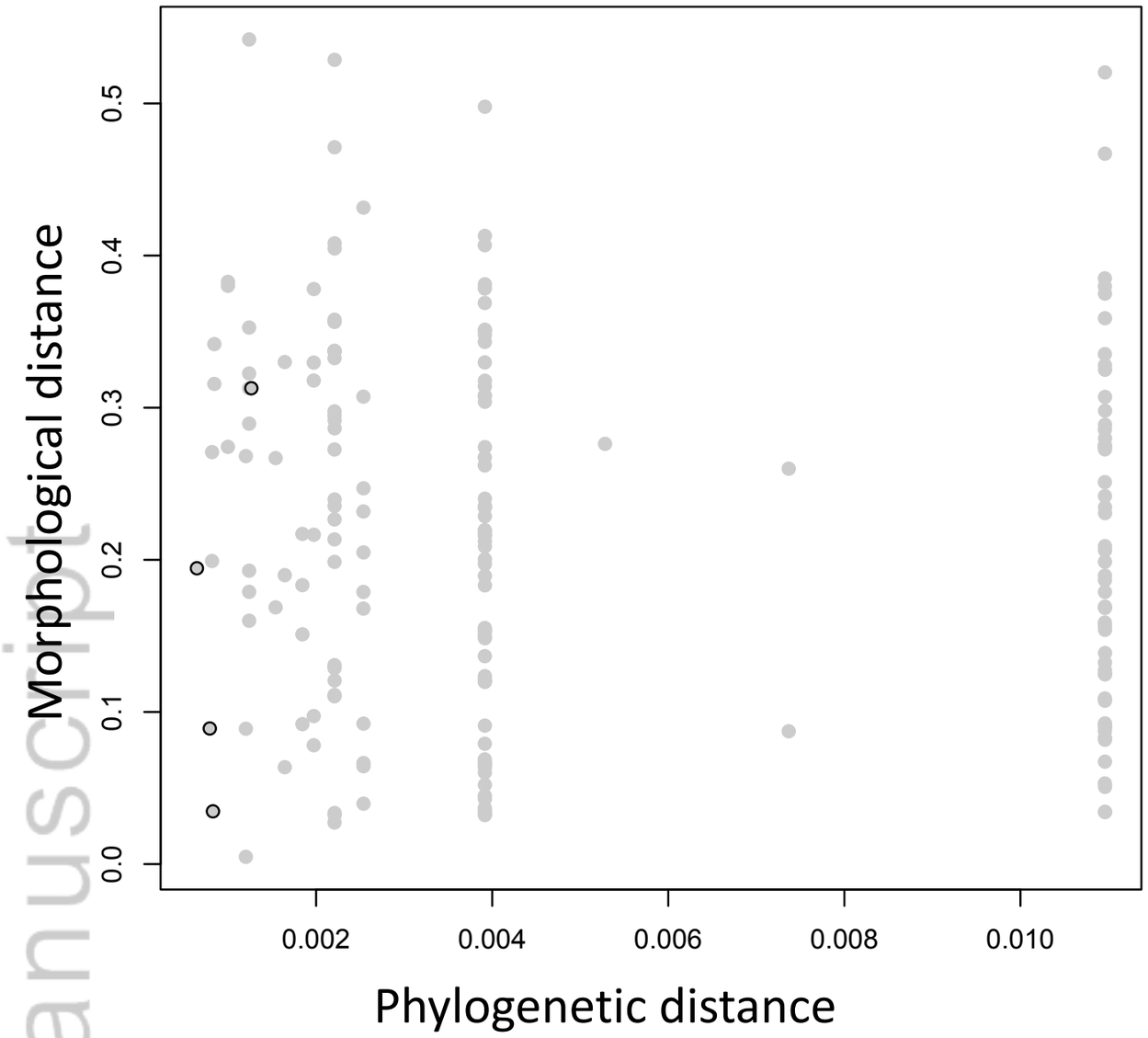




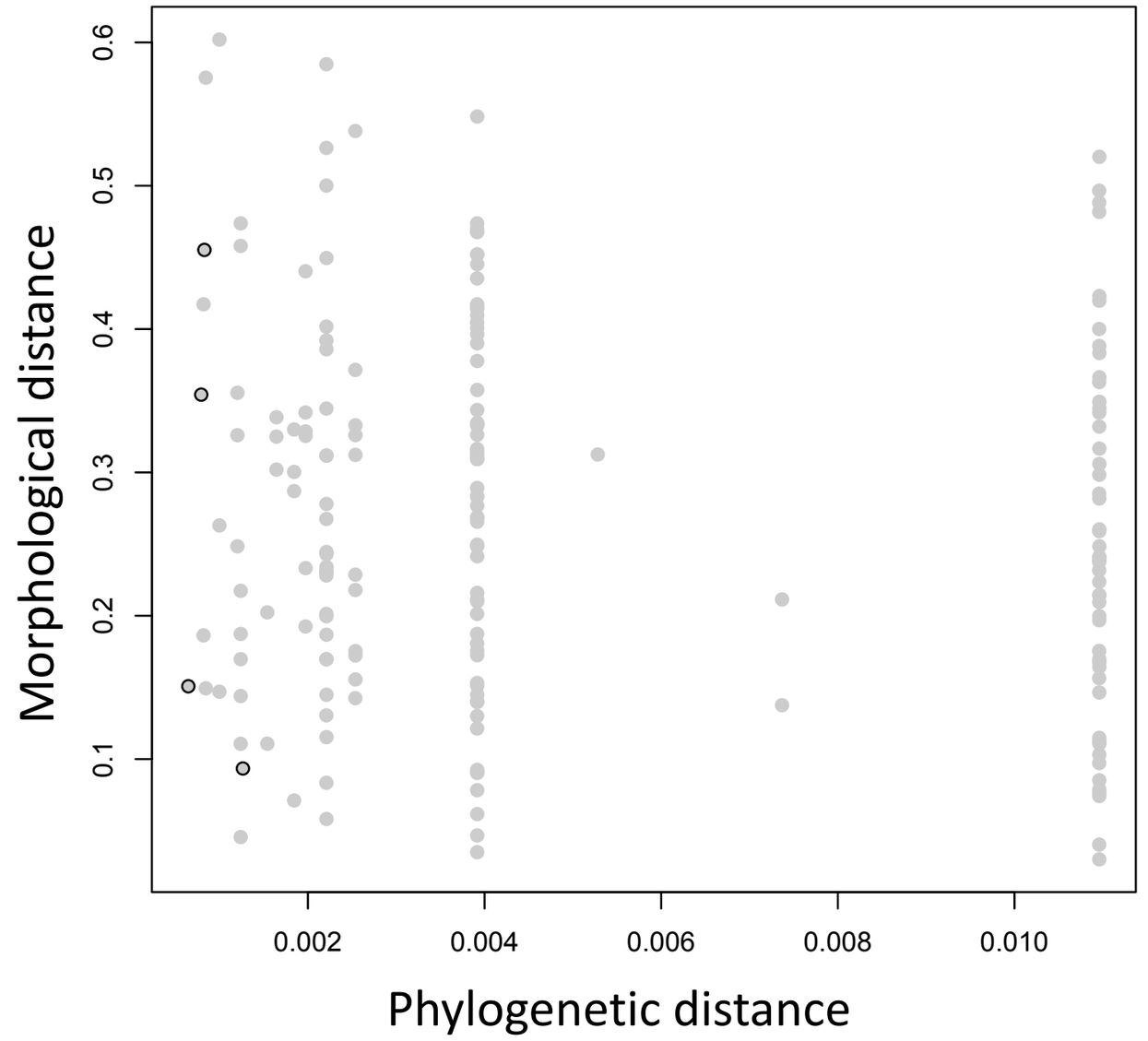
Melanoplus genitalic shapes (lateral view)

Melanoplus genitalic shapes (ventral view)

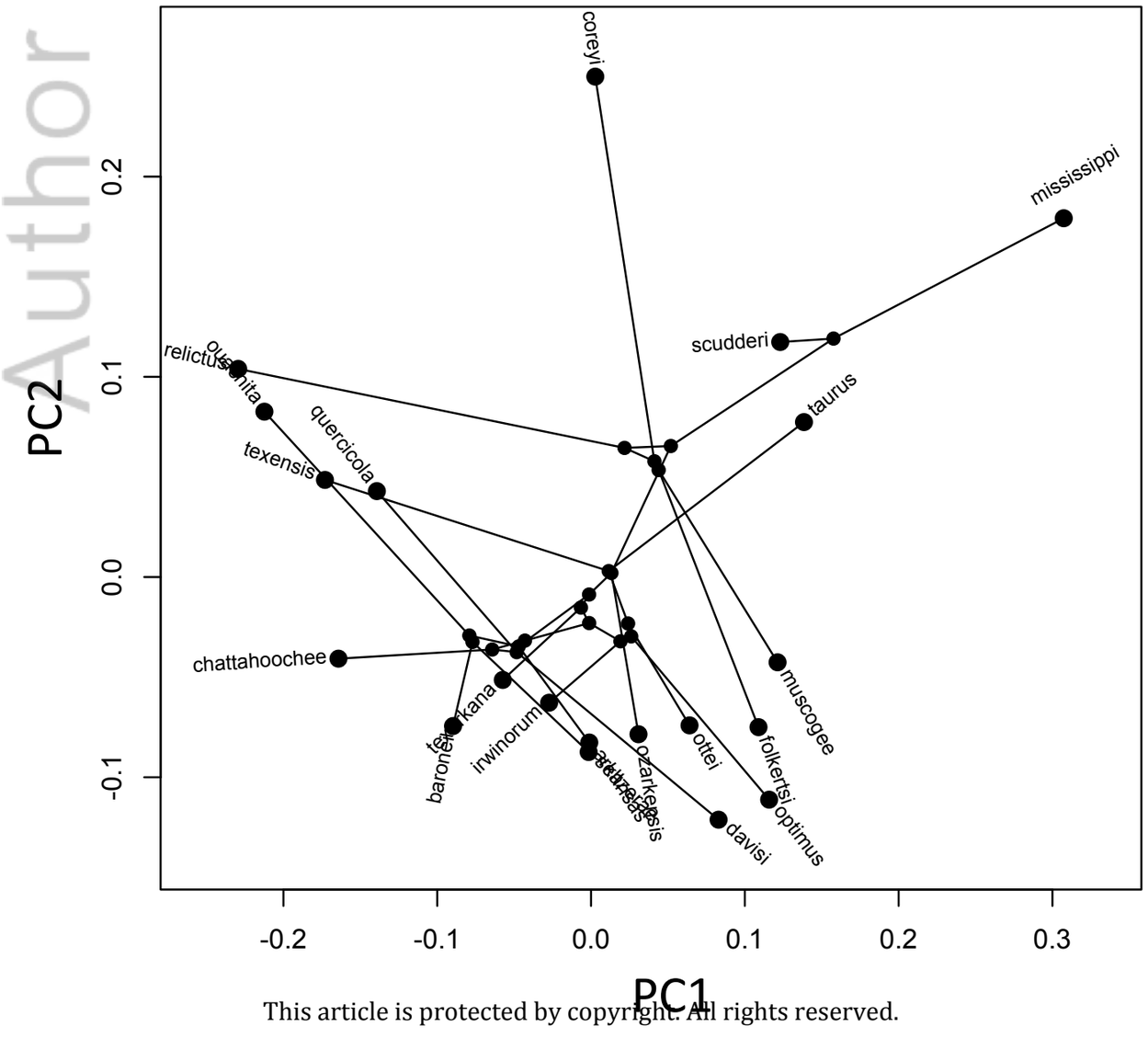
(A) Ventral view



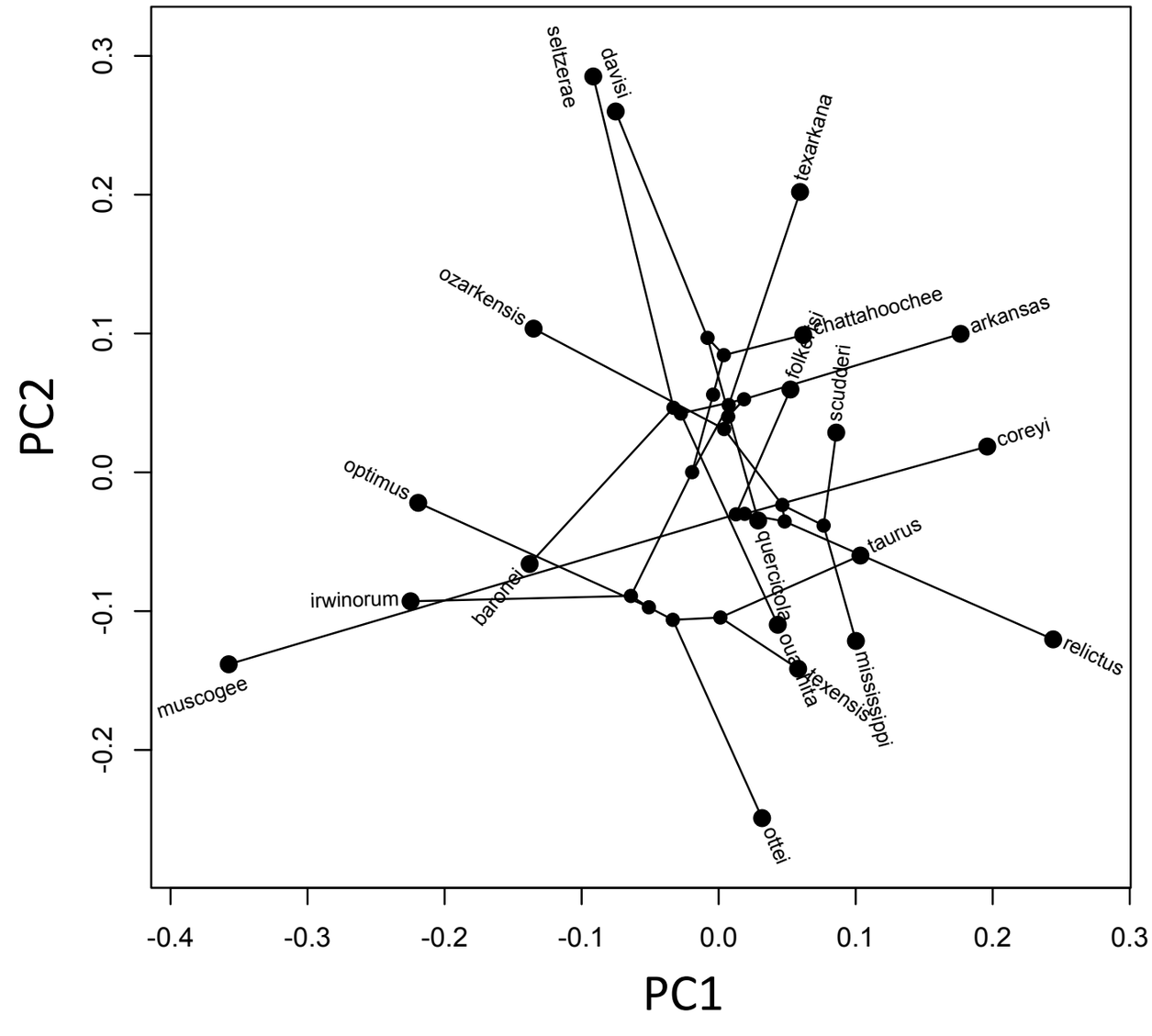
(B) Lateral view

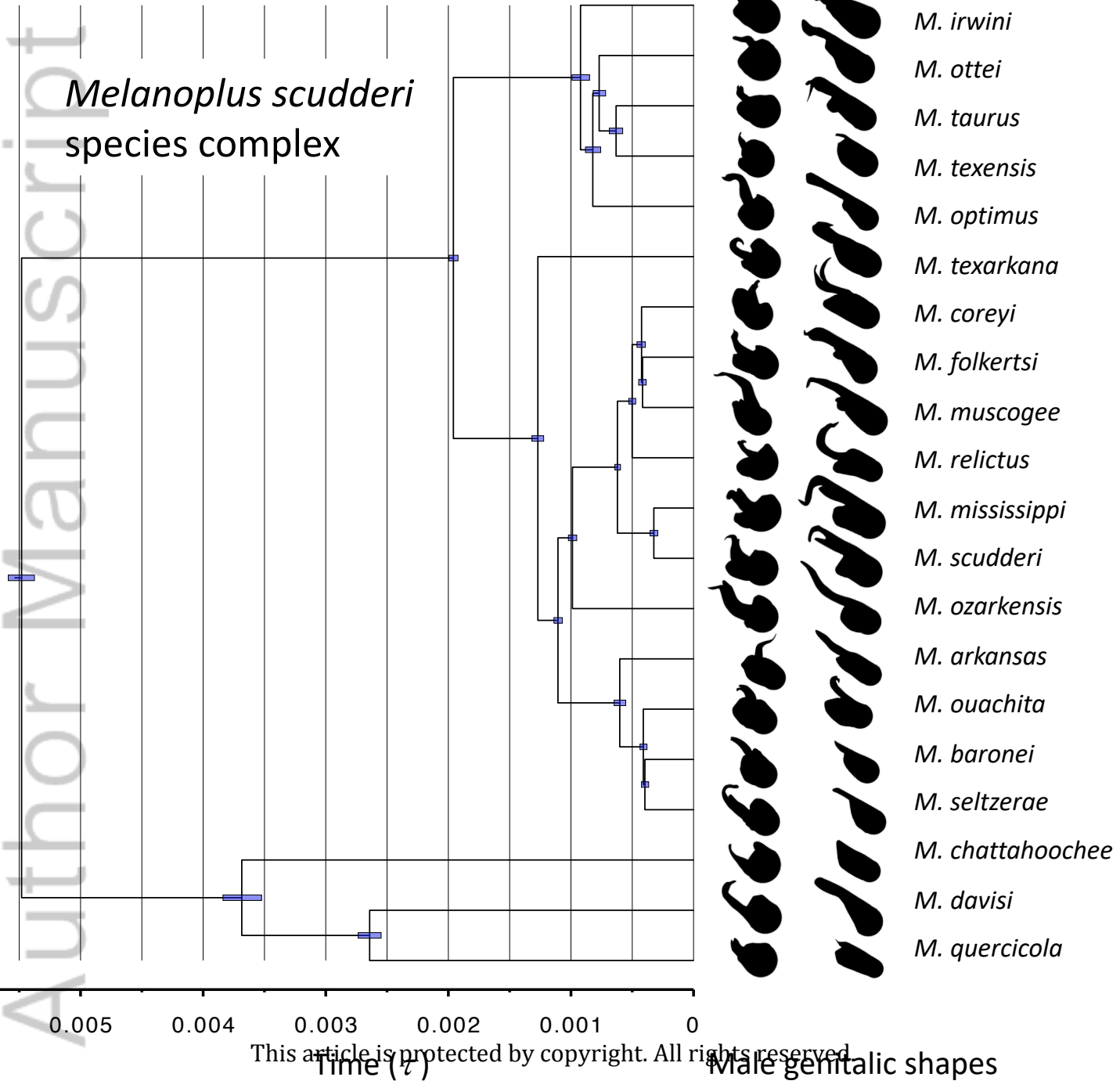


(C) Phylomorphospace (ventral view)



(D) Phylomorphospace (lateral view)





Title: Paraphyletic species no more – genomic data resolve a Pleistocene radiation and validate morphological species of the *Melanoplus scudderi* complex (Insecta: Orthoptera)

Author list: Jen-Pan Huang, JoVonn G. Hill, Joaquín Ortego, L. Lacey Knowles*

1. Genomic data validate morphological species designations in *Melanoplus* grasshoppers from southeastern United States
2. The difference in male genitalic shape, which is evolutionarily labile, can be a good diagnostic character to distinguish species of recent and rapid diversification origin
3. Fragmentation of grassland habitats in Late Pleistocene may have facilitated rapid speciation events in the flightless *Melanoplus* grasshoppers