MOLECULAR ECOLOGY**Integrating genomic and phenotypic data to evaluate alternative phylogenetic and species delimitation hypotheses in a recent evolutionary radiation of grasshoppers**

Journal:	<i>Molecular Ecology</i>
Manuscript ID	MEC-17-1092.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	16-Dec-2017
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Keywords:	Speciation, Integrative species delimitation, Evolutionary radiation, Phylogenomic inference, ddRADSeq

**Integrating genomic and phenotypic data to evaluate alternative
phylogenetic and species delimitation hypotheses in a recent
evolutionary radiation of grasshoppers**

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Running title: Phylogenomic inference in a recent radiation

Abstract

Although resolving phylogenetic relationships and establishing species limits is a primary goal of systematics, these tasks remain challenging at both conceptual and analytical levels. Here, we integrated genomic and phenotypic data and employed a comprehensive suite of coalescent-based analyses to develop and evaluate competing phylogenetic and species delimitation hypotheses in a recent evolutionary radiation of grasshoppers (*Chorthippus binotatus* group) composed of two species and eight putative subspecies. To resolve the evolutionary relationships within this complex, we first evaluated alternative phylogenetic hypotheses arising from multiple schemes of genomic data processing and contrasted genetic-based inferences with different sources of phenotypic information. Second, we examined the importance of number of loci, demographic priors, number and kind of phenotypic characters, and sex-based trait variation for developing alternative species delimitation hypotheses. The best-supported topology was largely compatible with phenotypic data and showed the presence of two clades corresponding to the nominative species groups, one including three well-resolved lineages and the other comprising a four-lineage polytomy and a well-differentiated sister taxon. Integrative species delimitation analyses indicated that the number of employed loci had little impact on the obtained inferences but revealed the higher power provided by an increasing number of phenotypic characters and the usefulness of assessing their phylogenetic information-content and differences between sexes in among-taxa trait variation. Overall, our study highlights the importance of integrating multiple sources of information to test competing phylogenetic hypotheses and elucidate the evolutionary history of species complexes representing early-stages of divergence where conflicting inferences are more prone to appear.

KEYWORDS: ddRADSeq, evolutionary radiation, integrative species delimitation, phylogenomic inference

INTRODUCTION

Elucidating evolutionary relationships, establishing species boundaries, and discovering new taxa are paramount goals of systematics (Coyne & Orr, 2004; Wiens, 2007). These tasks are also of great importance for ecological, evolutionary and biodiversity conservation studies of endangered and taxonomically problematic species groups (Agapow et al., 2004; Huang & Knowles, 2016a; Weir, Haddrath, Robertson, Colbourne, & Baker, 2016). On the one hand, delimiting and naming species is necessary to ensure that **both the scientific community and conservation agencies refer** to the same biological entity when using a consensual name (Agapow et al., 2004). **On the other hand, determining the phylogenetic relationships among lineages and characterizing their ecological and phenotypic variation is fundamental to define and prioritize conservation units at different taxonomic and evolutionary levels (Moritz, 2002).** This takes a decisive importance under the ongoing biodiversity crisis **resulting** from the severe impacts of human activities and global change, which are expected to lead numerous species to extinction before they are discovered (Costello, May, & Stork, 2013), including taxa from remote areas but also cryptic species inhabiting well-surveyed regions (Hotelling et al., 2016; Weir et al., 2016). Despite the relevance of delineating species boundaries, the available approaches are not exempt from limitations and controversial aspects, primarily because they rely on **alternative species concepts that were defined considering different biological properties to recognize taxa (e.g. reproductive isolation, monophyly, phenotypic cohesion, etc.)** (Agapow et al., 2004; de Queiroz, 2007; Freudenstein, Broe, Folk, & Sinn, 2017).

The ability to discover new taxa **and reconstructing the evolutionary history of species** was strongly **enhanced** by the application of molecular tools almost three decades ago (Avice et al., 1987; Hebert, Cywinska, Ball, & DeWaard, 2003; Nieto-Montes de Oca et al., 2017). Yet, the advance of **molecular systematics** has been historically limited by the amount of genetic data that can be obtained for non-model organisms using Sanger sequencing (Carstens, Lemmon, & Lemmon, 2012; Olson, Hughes, & Cotton, 2016; Rokas & Carroll, 2005). For example, recent literature reviews indicate that **phylogenetic** and DNA-based species delimitation studies have generally employed

fewer than ten loci (Carstens, Pelletier, Reid, & Satler, 2013; Caviedes-Solis, Bouzid, Banbury, & Leaché, 2015; see also Harris, Carling, & Lovette, 2014). The advent of high-throughput sequencing technology substantially improved our capability to sample hundreds to thousands of loci from non-model organisms (Leaché & Oaks, 2017; Lemmon & Lemmon, 2013; e.g. Emerson et al., 2010; Pyron et al., 2014). In parallel with the capability of generating genomic information, different model-based methods have been developed to improve **phylogenetic** and species delimitation inference (Camargo, Morando, Avila, & Sites, 2012; Chifman & Kubatko, 2009; Knowles & Carstens, 2007; O'Meara, 2010; Vachaspati & Warnow, 2015; see also Chou et al., 2015; Fujita, Leaché, Burbrink, McGuire, & Moritz, 2012). In particular, recently developed approaches based on multispecies coalescent (MSC) models that incorporate the estimation of demographic parameters and **accommodate gene tree-species tree conflicts have become broadly popular** (Bryant, Bouckaert, Felsenstein, Rosenberg, & RoyChoudhury, 2012; Edwards et al., 2016; Yang & Rannala, 2010, 2014, 2017). This has opened the door for exploring species-levels of genetic variation at an unprecedented resolution (Leaché et al., 2015; Potter, Bragg, Peter, Bi, & Moritz, 2016; Yoder et al., 2016) and improving our understanding on the mechanisms underlying speciation processes (Huang, 2016; Papadopoulou & Knowles, 2015; Weir et al., 2016).

Despite its power and advantages, high-throughput sequencing presents major challenges emerging from the lack of consensual criteria for assembling and filtering sequence data, an aspect that can impact the robustness and accuracy of phylogenetic inferences (Takahashi, Nagata, & Sota, 2014) and, ultimately, the outcomes of species delimitation analyses (Leaché & Fujita, 2010; Olave, Sola, & Knowles, 2014). These uncertainties generate competing phylogenetic hypotheses that need to be tested and, ideally, contrasted with non-genetic sources of information (e.g. phenotypic or ecological data) within an integrative framework (Harrington & Near, 2012; Lee & Palci, 2015). Thus, the integration of genomic and phenotypic information can contribute to accurately establish species limits and evaluate to what extent the inferred evolutionary relationships are compatible with morphological evidence (Lee & Palci, 2015; Solís-Lemus, Knowles, & Ané, 2015). This is particularly important in recent evolutionary radiations,

which usually show considerable ancestral polymorphism and different magnitudes in genomic and phenotypic axes of divergence, and when species divergence occurs with gene flow or is selectively driven (Degnan & Rosenberg, 2009; Satler, Carstens, & Hedin, 2013; Solís-Lemus et al., 2015). However, although defining independently evolving lineages (i.e. species, *sensu de* Queiroz, 2007) can be strongly impacted by the nature of the employed data (e.g. genetic, ecological, phenotypic, behavior, etc.), most delimitation methods currently available are exclusively based on molecular information (Edwards & Knowles, 2014; Hedin, Carlson, & Coyle, 2015; McKay et al., 2013; Lamanna et al., 2016). Recently, Solís-Lemus et al. (2015) developed a unified statistical approach for species delimitation that enables the joint analysis of multisequence DNA data and quantitative traits in a Bayesian framework (e.g. Dornburg, Federman, Eytan, & Near, 2016; Olave, Avila, Sites, & Morando, 2017). However, this integrative approach does not incorporate a model of trait evolution accommodating sexual dimorphism (Solís-Lemus et al., 2015), a problem that has been eluded by only considering male phenotypes (Eberle, Warnock, & Ahrens, 2016; Huang & Knowles, 2016a; Solís-Lemus et al., 2015) or ignoring sexually dimorphic traits (Pyron, Hsieh, Lemmon, Lemmon, & Hendry, 2016). Beyond sex-based trait variation, the importance of within-to-between lineage trait variance and of using different number of characters or contrasting sources of phenotypic information to elucidate the evolutionary history of species has not been yet empirically addressed (Solís-Lemus et al., 2015).

Here, we integrate extensive genomic (ddRADSeq) and phenotypic data (linear and geometric morphometric analyses) and employ a comprehensive suite of coalescent-based methods to understand the evolutionary history of the *Chorthippus binotatus* group species complex (Orthoptera: Acrididae) (Defaut, 2011, 2015), a recent evolutionary radiation of grasshoppers belonging to the highly speciose acridid subfamily Gomphocerinae (> 1,000 species; Cigliano, Braun, Eades, & Otte, 2017). This complex is distributed through southwest Europe (France, Spain and Portugal) and northwest Africa (Morocco) and includes species with wide distributions and narrow endemic taxa restricted to different mountain ranges (Figure 1). Currently, this group comprises two species (*Chorthippus binotatus* and *Chorthippus saulcyi*) that in turn

encompass a total of eight subspecies representing a continuum of ecological and phenotypic differentiation (Defaut, 2011, 2015; Lluçà-Pomares, 2002; Figs. S1). Since the description of the main nominal species in the 19th century, the taxonomy of the group has undergone important changes on the basis of morphological studies, but the phylogenetic relationships among the putative species within this complex remain unresolved (Defaut, 2011). The different stages of genetic and phenotypic divergence represented within this species complex make it an excellent system for evaluating the power and drawbacks of currently available approaches to resolve the evolutionary relationships and establish species limits in recent radiations where conflicting inferences are more likely to appear (Shaffer & Thomson, 2007; Takahashi, Nagata, & Sota, 2014). In particular, we first evaluated competing phylogenetic hypotheses arising from multiple schemes of ddRADSeq data processing and contrasted genomic-based inferences with different sources of phenotypic information. Second, we employed an integrative approach that accommodates genetic and phenotypic data in a Bayesian framework (Solís-Lemus et al., 2015) to determine the importance of number of loci, demographic priors, number and kind of phenotypic characters, and sex-based trait variation for developing and testing alternative species delimitation hypotheses.

MATERIALS AND METHODS

Sample collection

Between 2013 and 2014, we sampled the eight taxa that constitute the *Chorthippus binotatus* group species complex (subgenus *Glyptobothrus*) (Cigliano et al., 2017; Defaut, 2011, 2015; Table S1). In total, we sampled and performed genetic and morphological analyses for 80 individuals (5 males and 5 females per taxon) (Table S1). One specimen from *Chorthippus apricarius*, a species also belonging to the subgenus *Glyptobothrus* (Mayer, Berger, Gottsberger, & Schulze, 2010; Nattier et al., 2011), was used as outgroup in phylogenomic analyses. Subspecies codes and further information on sampling locations are given in Table S1.

Linear and geometric morphometric analyses

We took length measurements of left hind femur, left forewing and its median area, prozone, and pronotum following the procedure described in Nogueras, García-Navas, Cordero, & Ortego (2016). Afterwards, we calculated ratios between these morphological traits that were previously considered of taxonomic value (Defaut, 2011): forewing length relative to femur length (FWL/FL), median area length relative to forewing length (MAL/FWL), and prozone length relative to pronotum length (PZ/PR; Figure 2). Differences among taxa in these three traits were analyzed separately for each sex using one-way ANOVAs.

We took digital images of forewings and pronota **to characterize their shape variation by geometric morphometric analyses**. We used TPSDIG to digitize ten and eleven homologous landmarks for forewings and pronota, respectively (Rohlf, 2015; Figure 2). Trait shape was characterized separately for each sex by a procrustes fit aligned by principal axes using MORPHOJ v.1.05d (Klingenberg, 2011). **Following Chazot et al. (2016), allometry was examined** by pooling the dataset by subspecies and carrying out a multivariate regression of shape on centroid size. Centroid size significantly explained an important proportion of variance of forewing (σ^2 : 9.44%, $P = 0.009$; σ^2 : 22.63%, $P < 0.001$) and pronotum (σ^2 : 5.21%, $P < 0.043$; σ^2 : 5.49%, $P < 0.038$) shape. **Given that phenotypic plasticity accounts for a high proportion of size variation in grasshoppers (Butlin & Hewitt, 1986; Whitman 2008) and the fact that our geometric morphometric analyses were intended to analyze pure shape variation (Chazot et al., 2016; Sasakawa, 2016), we removed allometric effects by calculating new covariance matrices based on the residuals of the multivariate regressions before performing subsequent analyses (see Klingenberg, 2016).**

Afterwards, we used MORPHOJ to examine shape variation for each trait and sex using a Principal Component Analysis (PCA) on the size-corrected residuals. We retained the first two principal components (PC), which explained a high proportion of forewing (σ^2 : 76.04%; σ^2 : 82.21%; Fig. S2) and pronotum (σ^2 : 57.67 %; σ^2 : 62.11%; Fig. S3) shape variation. Principal component scores were used separately by sex and trait in subsequent species delimitation analyses (PC1_{FW}, PC2_{FW} and PC1_{PR},

PC2_{PR}). Forewing and pronotum shape variation were visually displayed using thin-plate spline diagrams as implemented in TPSSPLIN (Rohlf, 2015). **Finally, we conducted Canonical Variates Analyses (CVA) using MORPHOJ to maximize variance between subspecies, find the axes along which they are best discriminated, and calculate between-taxa Mahalanobis distances (D_2). Significance of Mahalanobis distances were determined using permutation tests with 10,000 replicates.**

Library preparation and sequencing

For genomic analyses, we selected 5 individuals from each taxon ($n = 40$ individuals) and one individual from *C. apricarius*. We employed a salt extraction protocol to purify genomic DNA from a hind femur of each specimen (Aljanabi & Martinez, 1997). Genomic DNA from each specimen was individually barcoded and processed into a genomic library using the double-digestion restriction-fragment-based procedure (ddRADSeq) described in Peterson, Weber, Kay, Fischer, & Hoekstra (2012) with some minor modifications as detailed in Lanier, Massatti, He, Olson, & Knowles (2015) and Massatti, & Knowles (2016). Briefly, DNA was double-digested using *EcoRI* and *MseI* restriction enzymes (New England Biolabs), followed by the ligation of Illumina adaptors and unique 7-base-pair barcodes. Ligation products were pooled, size-selected between 475 and 580 base pairs (bp) using a Pippin Prep (Sage Science) machine, and amplified by iProofTM High-Fidelity DNA Polymerase (BIO-RAD) with 12 cycles. Single-read 151 bp sequencing was performed on an Illumina HiSeq2500 platform at The Centre for Applied Genomics (Hospital for Sick Children, Toronto, Canada).

Bioinformatics pipeline

We followed a multi-approach to filter raw sequences and perform data quality controls (Herrera, Watanabe, & Shank, 2015). Firstly, we used the program *process_radtags* distributed as part of the STACKS v.1.35 pipeline (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013) to demultiplex and quality-filter sequence reads. We retained reads with a Phred score > 10 (using a sliding window of 15%), no adaptor contamination, and that had an unambiguous barcode and restriction cut site.

Afterwards, raw sequence data quality was checked in FASTQC v.0.11.5 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and sequences were trimmed to 129 bp using SEQTK (Heng Li, <https://github.com/lh3/seqtk>) in order to remove low-quality reads near the 3' ends. Secondly, reads retained after *process_radtags* were further quality-filtered using the program PYRAD v.3.0.66 (Eaton, 2014) in order to convert base calls with a Phred score < 20 into *Ns* and discard reads with > 2 *Ns* (Fig. S4).

As in Takahashi et al. (2014), we used PYRAD to cluster retained reads within- and across samples considering different clustering thresholds of sequence similarity ($W_{\text{clust}} = 0.85$ and 0.90). Clusters with a minimum coverage depth < 5 were discarded ($d = 5$). Loci containing one or more heterozygous sites across more than 15% of individuals were excluded ($\text{maxSH} = p.15$) because a shared heterozygous site across many samples likely represents clustering of paralogs with a fixed difference rather than a true heterozygous site (Eaton, 2014). Following to Hipp et al. (2014), the maximum number of polymorphic sites in a final locus was set to 20 ($\text{maxSNPs} = 20$). In a final filtering step, we generated three datasets by using different values for the minimum taxon coverage in a given locus discarding loci that were not present in at least 4, 10 or 20 samples ($\text{minCov} = 4, 10$ and 20, which represent the 10, 25 and 50% of samples, respectively). This procedure was repeated to generate datasets including or excluding *C. apricarius*, which was used as outgroup in phylogenomic analyses. Finally, following the approach described in Huang (2016), we checked our clustering and filtering output from PYRAD and trimmed our aligned clusters to 110 bp due to a systematic increase in sequence variation after this position (R scripts available in https://github.com/airbugs/Dynastes_introgression; Huang, 2016).

Phylogenomic inference - Species tree estimation

Several studies have highlighted that phylogenetic methods based on concatenation of genomic data can yield incongruent phylogenetic inferences (Kubatko & Degnan, 2007; Song, Liu, Edwards, & Wu, 2012; Xi, Liu, Rest, & Davis, 2014; see also Edwards et al., 2016; Springer & Gatesy, 2016).

Therefore, we built phylogenies using different SNP datasets and two coalescent-based methods of species tree estimation. **Firstly, for each dataset we generated a species tree using SVDQUARTETS** (Chifman & Kubatko, 2014) as implemented in PAUP* v.4.0a152 (Swofford, 2002). It has been documented that SVDQUARTETS exhibits a good performance under many simulated conditions compared to alternative approaches for species tree estimation (Chou et al., 2015). SVDQUARTETS uses SNP data to infer phylogenetic relationships between quartets of taxa under the **multispecies coalescent (MSC) model** and then assembles these quartets into a species tree. Species trees were constructed by exhaustively evaluating all possible quartets from the dataset. Uncertainty in relationships was quantified using nonparametric bootstrapping with 100 replicates. For SVDQUARTETS analyses, we analyzed the six SNP matrices that included *C. apricarius* as outgroup and were obtained by setting different values of clustering thresholds ($W_{\text{clust}} = 0.85$ and 0.90) and minimum taxon coverage in a given locus ($\text{minCov} = 4, 10$ and 20). This allowed us to assess the impact of different proportions of missing data and number of loci on the recovered species tree (Huang & Knowles, 2016b; Leaché et al., 2015; Takahashi et al., 2014).

Additionally, we generated a species tree using SNAPP v.1.3.0 (Bryant et al., 2012) plug-in for BEAST2 v.2.4.3 (Bouckaert et al., 2014). SNAPP uses biallelic SNPs to infer phylogenetic relationships and branch lengths and estimate current and ancestral population sizes. We ran analyses using different gamma prior distributions (gamma, α , β) for the ancestral population size parameter (θ). The priors used were $G(2, 200)$, $G(2, 2,000)$ and $G(2, 20,000)$, which would define different scenarios ranging from small to large effective population sizes. The forward (u) and reverse (v) mutation rates were set to be calculated by SNAPP. We used the log likelihood correction and sampled the coalescent rate. The remaining parameters were left at default values. Due to computational burden, SNAPP analyses were only run using the SNP matrix generated in PYRAD using $W_{\text{clust}} = 0.90$ and $\text{minCov} = 10$. We used the R package *phrynomics* (Barb Banbury, <http://github.com/bbanbury/phrynomics>) to remove non-binary and invariant SNPs, code heterozygotes, and format input files for SNAPP. We ran two independent runs for each prior using different starting seeds for ≥ 2 million Markov chain Monte

Carlo (MCMC) generations, sampling every 1,000 steps. We used TRACER 1.6 to check stationarity and convergence of the chains and confirm that effective sampling sizes (ESS) for all parameters were > 200 (Rambaut, Suchard, Xie, & Drummond, 2014). We removed 10% of trees as burn-in and combined tree and log files for replicated runs using LOGCOMBINER v.2.4.1. We used TREEANNOTATOR v.1.8.3 to obtain maximum credibility trees. The full set of likely species trees was displayed with DENSITREE v.2.2.1 (Bouckaert, 2010), which is expected to show fuzziness in parts of the tree due to gene flow or other causes of phylogenetic conflict (e.g. Zarza et al., 2016).

Genomic-based species delimitation

Initially, we tested competing species delimitation hypotheses using the Bayes Factor species delimitation method (BFD^{*}; Leaché, Fujita, Minin, & Bouckaert, 2014) as implemented in SNAPP. This method allows the comparison of alternative species delimitation scenarios in an explicit MSC framework by calculating and comparing marginal likelihood estimates (MLE) for each model. We conducted a path sampling analysis of fourteen steps each consisting of 100,000 MCMC generations with 10,000 pre-burning generations, sampling each 100 steps and using an α -value of 0.3. These settings were sufficient to ensure convergence and obtain ESS > 200 . The Bayes Factor (BF) test statistics ($2 \times \ln \text{BF}$) was calculated, where BF is the difference in MLE between two competing models (*base scenario* - *alternative scenario*). Six competing species delimitation hypotheses were defined based on current taxonomy (Defaut, 2011), geographic distribution of putative species and phylogenomic analyses (Table 1). Given that BFD^{*} analyses are computationally highly demanding, we only ran them using $G(2, 2,000)$ as prior distribution for the ancestral population size parameter (θ) (i.e. the 'intermediate population size' scenario used for SNAPP analyses). It has been shown that estimates of effective population sizes and divergence times obtained by SNAPP strongly depend on the choice of priors, however, BFD^{*} analyses seen to be robust to prior misspecification (Leaché et al., 2014; Rittmeyer & Austin, 2015). In addition, SNAPP runs for species tree estimation using alternative priors for θ parameter as $G(2, 200)$ and $G(2, 20,000)$ yielded the same topology (see 'Results' section).

The species delimitation hypotheses were tested using the same SNP dataset and parameters employed for species tree estimation in SNAPP.

We also delimited taxa using the BAYESIAN PHYLOGENETICS & PHYLOGEOGRAPHY program (BPP v.3.3; Yang & Rannala, 2010, 2014), which has been shown to be more accurate than alternative model-based methods for establishing species limits (Camargo et al., 2012). BPP analyzes multilocus sequence data under the MSC model, employing a reversible-jump MCMC (rjMCMC) to estimate the posterior probability for different delimitation models and species trees. An earlier version of BPP (v.2.x) was reliant on user-specified guide trees and only evaluated those potentials models that were generated by collapsing or failing to collapse nodes on such predefined topology (option A10, Rannala & Yang, 2013; Yang & Rannala, 2010; see also Olave et al., 2014; Zhang, Rannala, & Yang, 2014). The recently developed BPP version (v.3.x) jointly performs species tree estimation and species delimitation (option A11; Yang, 2015; Yang & Rannala, 2014). This version includes the nearest-neighbor interchange (NNI) algorithm, which is able to significantly change the topology of an input species tree and circumvents the need of specifying a fixed input guide tree. **In this study, we used both guided (option A10) and unguided (option A11) implementations of BPP.** For guided analyses (option A10) we used as input guide trees the five different topologies yielded by SVDQUARTETS and SNAPP analyses. **In turn, we performed all BPP analyses separately for *C. binotatus* and *C. saulcyi* clades (Zhang, Zhang, Zhu, & Yang, 2011). In this case, we also employed both guided and unguided BPP options and considered as guide trees the seven clade-specific topologies emerging from SVDQUARTETS species tree analyses.**

We assessed the impact of different demographic scenarios on species delimitation inference considering several combinations of gamma priors for ancestral population size (θ) and root age (τ_0) (Leaché & Fujita, 2010). Following Huang & Knowles (2016a), we considered four prior combinations: $\theta = G(1, 10)$, $\tau = G(1, 10)$, which would correspond to a large population size and deep divergence scenario (prior A); $\theta = G(1, 10)$, $\tau = G(2, 2,000)$, large population size and recent divergence (prior B); $\theta = G(2, 2,000)$, $\tau = G(1, 10)$, small population size and deep divergence (prior C); and $\theta = G(2, 2,000)$, $\tau = G(2, 2,000)$, small population size and recent divergence (prior D). A

Dirichlet prior was assigned to other divergence time parameters (Yang & Rannala, 2010). We used a uniform rooted tree-prior on the species tree topology (prior 1). **We let an automatic adjustment of the finetune parameters, allowing swapping rates to range between 0.30 and 0.70** (Yang, 2015). Also, our runs were replicated employing the two species delimitation algorithms (0 and 1) to ensure that our results were consistent between different searching algorithms. **Specific parameters tuning both species delimitation algorithms were adjusted to $\varepsilon = 2$ (for algorithm 0), and $\alpha = 2$ and $m = 1$ (for algorithm 1)** (Yang & Rannala, 2010).

Each analysis was run three times to confirm consistency among runs using different starting trees. Two of these runs were always initiated using as starting trees either one-species model (all internal nodes are collapsed) and a fully resolved tree (all internal nodes split) to ensure that the chains were mixing adequately. The third run was started from a randomly selected starting tree considering a partially resolved tree. Because BPP could suffer MCMC mixing problems when using large datasets (Rannala & Yang, 2013, 2017; Yang & Rannala, 2010), we explored the impact of the number of loci employed on species delimitation inference. Accordingly, we performed all guided analyses (option A10) using three genomic datasets consisting of 25, 50 and 200 sequence loci. Moreover, we used two additional larger genomic datasets of 500 and 1,000 loci for performing jointly species tree estimation and species delimitation (A11 option). We generated these different subsets by randomly selecting loci from a dataset originally containing 32,317 sequences. **To this end, we used a custom R script written by J-P. Huang and available at https://github.com/airbugs/Dynastes_delimitation.** The original dataset was generated in PYRAD excluding **the outgroup** and using $W_{\text{clust}} = 0.90$ and $\text{minCov} = 10$. We ran each analysis for 100,000 generations, sampling every 10 generations (10,000 samples), after a burning of 100,000 generations. Lineages delimited with a posterior probability (PP) of >0.95 in all analyses were considered to be well supported.

Integrative species delimitation

iBPP v.2.1.2 is a recently developed program intended to delimit species by combining phenotypic and genetic data into a MSC model (Solís-Lemus et al., 2015). iBPP was built upon the architecture of

the early version of BPP v.2.1 (Rannala & Yang, 2013; Yang & Rannala, 2010) and incorporates models of evolution for continuous quantitative traits under a Brownian motion (BM) process (Solís-Lemus et al., 2015). We used non-informative priors for the BM control parameters ($v_0 = 0$; $\kappa_0 = 0$). All iBPP analyses were run considering the same demographic scenarios, settings, **species trees and clade-specific topologies**, number of replicates, and subsets of loci described in the previous section for guided BPP analyses (option A10). To explore the influence of different phenotypic data on species delimitation inference, we ran iBPP using (i) only linear morphological data (FWL/FL, MAL/FWL and PZ/PR), (ii) only geometric morphometric data ($PC1_{FW}$, $PC2_{FW}$ and $PC1_{PR}$, $PC2_{PR}$) and (iii) a combination of the two datasets. **iBPP independently estimates the phylogenetic signal (λ) for each trait and we used this parameter to evaluate their respective informativeness for species delimitation (Lynch, 1991; Pagel, 1999; Solís-Lemus et al., 2015).** As for above guided BPP analyses, all iBPP runs were conducted using three genomic datasets consisting of 25, 50 and 200 sequence loci. Given that Orthoptera show a remarkable sexual dimorphism (Hochkirch & Gröning, 2008; Laiolo, Illera, & Obeso, 2013), we performed our analyses separately for each sex in order to assess the impact of sex-based trait variation on species delimitation. Finally, we also replicated all the above described analyses only considering phenotypic data (i.e. without genomic data) (Dornburg et al., 2016; Eberle et al., 2016; Huang & Knowles, 2016a; Solís-Lemus et al., 2015). Each analysis was run 2-4 times to confirm consistency among runs performed using different random starting trees. We ran each analysis for 100,000 generations, sampling every 10 generations (10,000 samples), after a conservative burning of 300,000 generations. Lineages delimited with $PP \geq 0.95$ in all analyses were considered to be well supported.

RESULTS

Sequencing and genomic datasets

We obtained 104.29 million sequence reads, of which 88.87 million were retained after different filtering steps. On average, we retained $2.16 (\pm 0.4 \text{ SD})$ million reads per sample. All individuals were

retained in the final analyses with a range of 1.57-3.13 million reads (Fig. S4). Clustering within samples using two values of clustering thresholds ($W_{\text{clust}} = 0.85/0.90$) yielded an average of 56,733 ($\pm 7,933$ SD) and 67,420 ($\pm 9,309$ SD) loci per sample, respectively. After clustering among samples using three different values for the *minCov* parameter, the resulting genomic datasets including outgroup contained 76,966, 24,961, 6,443 SNPs (using $W_{\text{clust}} = 0.85$, and *minCov* = 4, 10 and 20, respectively), and 97,680, 31,706, 7,939 SNPs (using $W_{\text{clust}} = 0.90$, and *minCov* = 4, 10 and 20, respectively).

Linear and geometric morphometrics analyses

ANOVAs showed that all three linear morphological traits differed significantly among taxa in both sexes (all $P_S < 0.001$) (see Fig. S1). **Complementary non-parametric Kruskal-Wallis tests confirmed this result (all $P_S < 0.016$).** Forewing shape variation analyses showed that individuals from the same subspecies were mostly clustered in the morphospace (Fig. S2). The two putative species (*C. binotatus* and *C. saulcyi*) were well separated in the morphospace, while the different subspecies within them partially overlapped for the two sexes (Fig. S2). Pronotum shape variation analyses revealed a similar clustering pattern between species (Fig. S3), although there was more overlap between putative subspecies, particularly in females (Fig. S3). **Mahalanobis distances (D_2) were significantly different between all subspecies for the two traits and sexes (Table S2).**

Phylogenomic inference - Species tree estimation

Species tree estimation analyses using SVDQUARTETS provided five slightly different topologies depending on the SNP matrix used (Figure 3). **The major topological difference among species trees was the phylogenetic position of the taxon from Massif Central (*C. s. algoaldensis*), which was strongly supported as an external sister lineage of either the *C. binotatus* group or the *C. saulcyi* group (Figure 3).** The phylogenomic relationships within the *C. saulcyi* group also exhibited considerable uncertainty. The lineages from the Pyrenees (*C. s. moralesi* and *C. s. vicdessossi*) and the lineages from northeastern Iberia and the Maritime Alps (*C. s. saulcyi* and *C. s. daimeii*, respectively)

generally clustered into two different clades, but the split nodes were not always well supported. Conversely, phylogenomic relationships within the putative *C. binotatus* group (*C. b. binotatus*, *C. b. armoricanus*, and *C. b. atlati*) were well-resolved and nodes presented a very high support, either when *C. s. algoaldensis* was included or not as its sister lineage (Figure 3). The SVDQUARTETS species tree showing the highest overall support across all nodes was the one generated with the SNP matrix (31,706 SNPs) obtained by setting PYRAD parameters to $W_{\text{clust}} = 0.90$ and $\text{minCov} = 10$ (Figure 3d). This species tree inferred two major clades according to the current taxonomy of the group (*C. binotatus* and *C. saulcyi* groups). The *C. s. algoaldensis* subspecies was well-resolved as an external lineage with respect to the *C. saulcyi* group and the relationships within this group showed high support (Figure 3d).

SNAPP recovered a final dataset containing 3,245 biallelic SNPs. SNAPP results did not provide a single well-supported topology and confirmed the uncertainty detected for the *C. saulcyi* group with SVDQUARTETS analyses (Figure 4a). We used TREE SET ANALYSER as implemented in BEAST2 package to determine the proportion of trees supporting each topology. The most frequent topology (~41%) was similar to that recovered by SVDQUARTETS when analyzing the SNP matrix obtained with $W_{\text{clust}} = 0.90$ and $\text{minCov} = 10$ (Figure 3d, Figure 4). The second and third most frequent topologies (~23% and ~20%), which were similar to that yielded by SVDQUARTETS when using the matrix obtained with $W_{\text{clust}} = 0.90$ and $\text{minCov} = 4$, also supported *C. s. algoaldensis* as an external lineage of the *C. saulcyi* group (Figure 3c). Both SNAPP topologies differed on the phylogenetic position of *C. s. saulcyi* and *C. s. daimei* subspecies within the *C. saulcyi* group and, consequently, the support of such split was very low in the consensus tree (Figure 4b). SNAPP runs using $G(2, 200)$ and $G(2, 20,000)$ as priors for θ parameter converged on the same topology (results not shown).

Species delimitation

Species delimitation analyses with the BFD* method strongly supported an eight species model (H_1 , Table 1). The second best-supported scenario (H_2) considered *C. s. moralesi* and *C. s. vicdessossi* as the same taxon but received much lower support (Table 1). **Additional hypotheses testing less**

plausible species delimitation scenarios according to our phylogenomic analyses yielded non converging runs and, thus, they were discarded (results not shown). Both guided-by tree (option A10) and unguided (option A11) species delimitation analyses using BPP provided always a very high support for divergence (PP = 1.00) of all lineages regardless of guide tree topologies, demographic prior combinations, number of loci or delimitation algorithms. Similarly, integrative species delimitation analyses in iBPP combining genomic and morphological data supported all splitting events (PP = 1.00). This result was consistent irrespective of guide trees, prior combinations, number of loci, delimitation algorithms or morphological matrices based on distinct traits and sexes. Nonetheless, results from iBPP only based on morphological data provided contrasting node supports across analyses and were sensitive to guide tree topology, sex-specific trait variation and the morphological matrix (Figure 3). Low support was found for the most recently diverged nodes from either *C. binotatus* or *C. saulcyi* groups regardless of the phylogenetic scale considered (i.e. all taxa vs. clade-specific analyses; Figure 3, Fig. S5). In general, female-based morphological data increased the support for closely related lineages compared with male-based morphological datasets (Figure 3). When employing male-based morphology, the usage of only geometric morphometric data (4 traits) tended to provide slightly higher support for divergence than only using data based on linear morphology (3 traits) (Figure 3, Fig. S5). Conversely, linear morphological traits provided a higher support for species split than geometric morphometric data in females (Figure 3). Analyses based on the combined morphological dataset (7 traits) consistently provided a higher support for divergence than analyses based on specific data matrices (Figure 3, Fig. S5). In analyses conducted only using morphological data, the usage of priors corresponding to large population sizes (priors A and B) reduced the support for splitting lineages, as opposed to priors corresponding to small population sizes (priors C and D; Figure 3, Fig. S5).

DISCUSSION

Genome-wide data and a suite of coalescent-based methods allowed us to infer the phylogenomic relationships among the closely-related taxa forming the *Chorthippus binotatus* group species complex. Although the relationships among some taxa were unresolved or inconsistent across different genomic datasets, the most supported topology was largely congruent with phenotypic data. Our analyses also showed considerable differences in phylogenetic information content among the employed characters and revealed the impact of sex-based trait variation on the ability of integrative species delimitation methods to detect species limits, which offers important insights for future studies aimed to develop and test competing taxonomic hypotheses (Solís-Lemus et al., 2015; e.g. Eberle et al., 2016; Harrington & Near, 2012).

Reconstructing evolutionary relationships

Genome-wide ddRADSeq data have been proven to yield considerable power to elucidate evolutionary relationships at different phylogenetic scales (Beheregaray et al., 2017; Bryson, Savary, Zellmer, Bury, & McCormack, 2016; Yoder et al., 2016). However, our results are also in agreement with previous studies documenting the impacts that ddRADSeq data filtering and assembling can have on phylogenomic inference (Herrera & Shank, 2016; Leaché et al., 2015; Takahashi et al., 2014). The most robust topology in terms of node supports was recovered using an inclusive genomic dataset including a high proportion of missing sites, which is in concordance with the findings from previous empirical studies (Jiang, Chen, Wang, Li, & Wiens, 2014; Rubin, Ree, & Moreau, 2012; Wagner et al., 2013). We found that using lower clustering thresholds of sequence similarity decreased about 20% the number of loci retained in the final genomic dataset, which may lead to the misidentification of orthologous loci and, consequently, generate conflicting topologies even for deep branches and varying branch support values (Takahashi et al., 2014). Thus, this and previous studies indicate that phylogenetic inference using ddRADSeq data requires to routinely explore the potential impacts of different settings during sequence assembling and data filtering (Huang & Knowles, 2016b; Razkin et al., 2016).

479 **The best-supported topology yielded** two well-resolved clades that correspond to the main
 480 nominative species groups (*C. binotatus* and *C. saulcyi*), two lineages whose divergence has been
 481 dated about 3 Ma (García-Navas, Nogueras, Cordero, & Ortego, 2017). This topology indicates that
 482 putative *C. binotatus* and *C. saulcyi* species groups are monophyletic, which is in concordance with
 483 the most recent morphology-based taxonomy (Defaut, 2011). **Notwithstanding, species trees**
 484 **generated with genomic matrices obtained using lower W_{clust} values highly supported *C. s.***
 485 ***algoaldensis* as a sister lineage of the *C. binotatus* group.** This finding is in line with the taxonomic
 486 classification proposed by Chopard (1952), who described this subspecies and placed it within the *C.*
 487 *binotatus* species group. Beyond genomic data, the intermediate position of the subspecies *C. s.*
 488 *algoaldensis* is corroborated by both morphological and ecological traits. **Although iBPP analyses**
 489 **based on morphological data suggest a closer relationship of *C. s. algoaldensis* with the *C. saulcyi***
 490 **clade (Figure 3),** all our linear and geometric morphometric analyses indicate that this subspecies is
 491 placed at an intermediate position along the morphological differentiation axes separating *C. binotatus*
 492 and *C. saulcyi* species groups (Fig. S1-S3). While all members from the *C. saulcyi* group exhibit
 493 gramineous feeding requirements, the subspecies *algoaldensis* also feeds on scrub-legume species
 494 (tribe *Genisteae*), an ecological specialization characterizing the *C. binotatus* group (Defaut, 2011;
 495 V.N. and J. O., personal observation). The phylogenomic relationships among the other putative
 496 subspecies of the ***C. saulcyi* group were not well resolved (Figure 4), which is in agreement with**
 497 **previous morphological-based studies** (Defaut, 2011; Lluçà-Pomares, 2002). The low support for
 498 the phylogenetic relationships of these lineages and their similar branch lengths suggest a hard
 499 polytomy resulted from a simultaneous split event or, alternatively, the lack of resolution of our
 500 genomic dataset for resolving the evolutionary history of this clade (Campagna, Gornau, Silveira,
 501 Siepel, & Lovette, 2015; Hoelzer & Melnik, 1994; Shaffer & McKnight, 1996). The split of the *C.*
 502 *saulcyi* group has been estimated to take place during the Pleistocene (~1.5 Ma), likely driven by
 503 glacial cycles (Hewitt, 1999), which is congruent with the rapid speciation characterizing the recent
 504 radiation of the Gomphocerinae subfamily (García-Navas et al., 2017; Mayer et al., 2010; Nattier et
 505 al., 2011).

While our analyses revealed considerable uncertainty in the phylogenomic relationships within the *C. saulcyi* clade, we found consistently well-resolved branches in the *C. binotatus* group. Phylogenomic analyses also indicated that the recently described French subspecies (*C. b. armoricanus*) and the Iberian one (*C. b. binotatus*) constitute two well-supported lineages (Defaut, 2015; Figure 4). **Recent phylogeographic studies on this species have shown that it exhibits a strong population genetic structure at mtDNA and nuclear microsatellite markers (Noguerales, Cordero, & Ortego, 2017, 2018), a pattern in line with the relatively deep lineage divergence found in the present study (Figure 4). The large distribution range of *C. binotatus* together with its montane character and narrow feeding requirements could have promoted long-term population isolation in different regions and, ultimately, the formation of cryptic lineages (Defaut, 2011, 2015; Noguerales et al., 2017, 2018).**

Genomic-based species delimitation

Genetic-based approaches of species delimitation (BFD* and BPP) provided congruent results and supported all lineages as distinct species. Despite literature on systematics is increasingly encouraging the employment of large genome-wide data for taxonomic delimitation (Lemmon & Lemmon, 2013; McCormack, Hird, Zellmer, Carstens, & Brumfield, 2013), the power of BPP for establishing species boundaries has been highlighted even when using a few loci (Camargo et al., 2012; Caviedes-Solís et al., 2015). Accordingly, we obtained virtually identical results using both small (25 loci, 2,750 bp) and large (1,000 loci, 110,000 bp) genomic datasets (see also Hime et al., 2016). However, **although genetic-based analyses of species delimitation consistently suggest that all lineages should be considered as distinct taxa, these inferences must be interpreted with extreme caution. On the one hand, we cannot discard that the most supported species delimitation scheme yielded by BFD* and BPP analyses is biased by the limited geographical extent of our sampling. In this sense, analyzing only one population per putative taxon does not allow accounting for potential intra-lineage variation, which might have hampered our capability to confidently designate species within the group (Lohse, 2009). On the other hand, a**

recent simulation study has brought the attention on the fact that MSC approaches may not be able to discriminate between species boundaries and genetic structure resulting from population-level processes (Sukumaran & Knowles, 2017; see also Barley, Brown, & Thomson, 2017). This suggests that a remarkable intra-specific population genetic structure could be misidentified as inter-specific diverging lineages that may not represent full species (Pyrón et al., 2016; Sukumaran & Knowles, 2017). In turn, overestimation of species-level diversity could be exacerbated when using vast genome-wide data due to its power to detect fine-grain population structuring even at small geographic scales (Weir et al., 2016). As suggested by Sukumaran & Knowles (2017), species delimitation inferences resulting from genetic-based MSC approaches should be treated as hypothesis to be subsequently tested under an integrative framework incorporating non-genetic sources of information (Edwards & Knowles, 2014; Schlick-Steiner et al., 2010; Yeates et al., 2011). **Consequently, given our sampling design and the propensity of MSC models to overestimate species numbers, our species delimitation results must be interpreted as working hypotheses to be tested in more detail in the future. Further studies combining a more exhaustive sampling and a comprehensive suite of model-based species delimitation approaches will help to accurately determine the true number of species composing the studied group (Fujisawa, Aswad, & Barraclough, 2016; Jackson, Carstens, Morales, & O'Meara, 2017; Solís-Lemus et al., 2015).**

Integrative species delimitation

To date, integrative species delimitation approaches have been based on sequential analyses employing genetic and non-genetic sources of data (Andujar, Arribas, Ruiz, Serrano, & Gómez-Zurita, 2014; Wachter et al., 2015). However, recognizing species boundaries under this framework rely on qualitative and comparative analyses that do not provide a statistical confidence parameter to formally test alternative hypotheses, hindering its repeatability and objectivity (Schlick-Steiner et al., 2010; Yeates et al., 2011). In order to overcome this issue, **we evaluated species boundaries** using jointly genomic and phenotypic information under a quantitative and statistically unified framework (iBPP,

Solís-Lemus et al., 2015). Conversely to our expectations, integrating these two kinds of data did not result in a fewer number of inferred species and, thus, we were not able to reject the notion that genomic-based inference overestimates the number of taxa (Eberle et al., 2016; Pyron et al., 2016). Results from iBPP analyses combining genetic and phenotypic information were consistent regardless the number of employed loci, demographic scenario, morphological dataset or sex-based trait variation, indicating that these factors had little impact on species identification. Nevertheless, analyses only based on morphological data revealed the impacts that sex-based trait variation, number and kinds of phenotypic characters, and guide tree specification can potentially have on species delimitation inferences. For instance, the support of *C. s. algoaldensis* as a distinct species varied depending on its phylogenetic position in relation to *C. binotatus* and *C. saulcyi* clades (Figure 3), illustrating the consequences of guide tree misspecification (Leaché & Fujita, 2010; Olave et al., 2014). We also found that female-based morphological information generally provided higher posterior probabilities in node support in comparison to male-based data, which could be explained by the fact that both linear and geometric morphometric data from females exhibited on average a higher phylogenetic signal ($\lambda \sim 0.69 - 0.62$, respectively) compared to that obtained for males ($\lambda \sim 0.63 - 0.49$). This finding is particularly interesting taking into account that most studies aiming to infer species boundaries have exclusively considered morphological variation in males (Eberle et al., 2016; Huang & Knowles, 2016a). However, we should note that our results are not likely transferable to other organisms, particularly to those groups in which between-species phenotypic variation is explained by male traits modelled by sexual-driven selection (Huang & Knowles, 2016a). Our results highlight the importance of incorporating sex-based phenotypic variability when delineating species boundaries and indicate the need for developing new species delimitation methods integrating trait evolution models that account for sexual dimorphism (Solís-Lemus et al., 2015).

Beyond the relevancy of the different methods by which we measure and summarize phenotypic variation, the examination of the phylogenetic signal (λ) for each morphological trait provided valuable information on their respective usefulness for future species delimitation

studies (Solís-Lemus et al., 2015). In this sense, variables describing the morphological variation of tegmina such as forewing length, median plate relative length or the first axis of forewing shape exhibited the highest between-species variation for both sexes ($\lambda > 0.75$). Forewings are involved in several biological functions in Orthoptera such sound production during courtship, flight and thermoregulation (Noguerales et al., 2016; Petit, Picaud, & Elghadraoui, 2006; Thomas et al., 2001), therefore natural and sexual selection can be responsible of strong among-species variation in this structure (Klingenberg, Debat, & Roff, 2010). Conversely, prozone length and pronotum shape showed on average low phylogenetic signal ($0.30 < \lambda < 0.60$), indicating the lower informativeness of these traits for delimiting taxa (Solís-Lemus et al., 2015). Apart from these differences among traits, we found that the cumulative information contained in an increasing number of traits provided the highest support for all nodes regardless of sex. This indicates that the use of a broad suite of well-known traits exhibiting different degrees of variation could be beneficial when aiming at determining species boundaries, particularly at early-stages of divergence where conflicting inferences are more prone to appear (see Solís-Lemus et al., 2015).

CONCLUSIONS

Our study corroborates the power of genome-wide data to unravel evolutionary relationships among recently diverged taxa and highlights the importance of integrating genetic and phenotypic information to test competing phylogenomic and species delimitation hypotheses. In addition, our findings indicate the importance of using multiple sources of phenotypic information from the two sexes to capture subtle patterns of differentiation characterizing recent evolutionary radiations (Rannala & Yang, 2017; Solís-Lemus et al., 2015). Future integrative species delimitation approaches should consider developing trait evolution models accommodating phenotypic variation resulting from sexual dimorphism (Solís-Lemus et al., 2015) and resolve the proneness of currently available methods to confound divergence patterns promoted by species vs. population processes when defining taxa limits (Sukumaran & Knowles, 2017).

ACKNOWLEDGEMENTS

We wish to thank to Bernard Defaut for providing valuable information about sampling locations, Amparo Hidalgo-Galiana for library preparation, Anna Papadopoulou for her valuable help in data analysis and Sergio Pereira (The Centre for Applied Genomics) for Illumina sequencing. **Stephen F. Spear, R. Alexander Pyron and two anonymous referees provided valuable comments on an earlier draft of this manuscript.** Logistical support was provided by Laboratorio de Ecología Molecular from Estación Biológica de Doñana (LEM-EBD) and Laboratorio de Genética from Instituto de Investigación en Recursos Cinegéticos (IREC). We also wish to thank to Centro de Supercomputación de Galicia (CESGA) for access to computer resources. This work was supported by Ministerio de Economía y Competitividad and European Social Fund (grant numbers: CGL2011-25053, CGL2014-54671-P, CGL2016-8742-R), Junta de Comunidades de Castilla-La Mancha - European Social Fund (grant numbers: POII10-0197-0167, PEII-2014023-P) and European Regional Development Fund (grant number: UNCM08-1E-018). VN was supported by a FPI pre-doctoral fellowship (fellow number: BES-2012-053741) from Ministerio de Economía y Competitividad. JO was supported by a Ramón y Cajal (fellow number: RYC-2013-12501) and Severo Ochoa (fellow number: SEV-2012-0262) research fellowships.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

DATA ACCESSIBILITY

All morphological and genomic data and associated files for analyses are stored and accessible through the Dryad Digital Repository (doi: <https://doi-org/xxxxx/xxxxx>).

AUTHOR CONTRIBUTIONS

VN and JO conceived and designed the study. VN analysed the data supervised by JO. VN, PJC and JO collected the samples. VN wrote the manuscript with help of JO and inputs of PJC. All authors read and approved the final manuscript.

TABLE 1 Results of BFD* analyses testing the support of competing species delimitation hypotheses. The table shows the clustering scheme defining each alternative species delimitation hypothesis (H_i). For each hypothesis, we show marginal likelihood estimates (MLE), their Bayes Factors (calculated as $2 \times \ln \text{BF}$) and their rank. Hypothesis 1 (H_1) was considered as base scenario. Also, we present the number of SNPs recovered by SNAPP from the original biallelic SNP matrix for testing a given hypothesis. The original matrix contained 31,700 SNPs and was generated in PYRAD without outgroup and by setting a clustering threshold value of $W_{\text{clust}} = 0.90$ and a minimum taxon coverage value in a given locus of $\text{minCov} = 10$. Subspecies codes as in Figure 1 and Table S1.

Species delimitation hypothesis (H_i)	Species	SNPs	MLE	BF	Rank
H_1 : (ATL) (BIN) (ARM) (ALG) (SAU) (DAI) (MOR) (VIC)	8	3,245	-29,721.61	-	1
H_2 : (ATL) (BIN) (ARM) (ALG) (SAU) (MOR+VIC) (DAI)	7	4,243	-39,643.74	1.98×10^4	2
H_3 : (ATL) (BIN) (ARM) (ALG) (SAU+MOR+VIC) (DAI)	6	6,412	-57,940.66	5.64×10^4	3
H_4 : (ATL) (BIN) (ARM) (ALG) (SAU+DAI) (MOR+VIC)	5	9,364	-83,359.68	10.72×10^4	4
H_5 : (ATL) (BIN, ARM) (ALG) (SAU+DAI+MOR+VIC)	4	11,739	-105,638.43	15.18×10^4	5
H_6 : (ATL) (BIN+ ARM) (ALG+SAU+DAI+MOR+VIC)	3	16,647	-153,962.18	24.8×10^4	6

FIGURE 1 Map displaying the approximate distribution range of each of the eight subspecies from the *Chorthippus binotatus* group species complex. White dots indicate the geographic location of sampling sites for each taxon. Topographic background from NASA Shuttle Radar Topographic Mission (SRTM Digital Elevation Data, <http://srtm.csi.cgiar.org/>). Detailed information for each taxon and sampling site is given in Table S1.

FIGURE 2 Positions of the landmarks used to characterize pronotum (panel a) and forewing (panel b) shape in the different subspecies of the *Chorthippus binotatus* group species complex. The scale bar was used to standardize landmark distances to the same absolute scale across all images. We also indicate the main traits considered for defining the position of the forewing and pronotum landmarks, as well as the length of prozone and metazone.

FIGURE 3 Mean posterior probabilities (PP) of species delimitation across runs using four gamma prior combinations (γ , α , β) for ancestral population size (θ) and root age (τ). Panels a-e represent five alternative guide trees recovered from SVDQUARTETS using different genomic datasets generated in PYRAD by setting different values of clustering thresholds ($W_{\text{clust}} = 0.85$ and 0.90) and minimum taxon coverage in a given locus ($\text{minCov} = 4, 10$ and 20). **Node support in terms of bootstrapping values is reported in parentheses for each node.** Results of species delimitation are based on analyses only using morphological data in iBPP. These analyses were performed employing three different morphological datasets: (i) only linear morphology, (ii) only geometric morphometrics and (iii) a combined dataset of both matrices. For each guide tree, species delimitation results are shown for males (left) and females (right). Color-coded boxes at each speciation split represent the mean PP for different combinations of demographic priors and morphological datasets (legend at bottom left). Subspecies codes as in Figure 1 and Table S1.

FIGURE 4 Species tree inferred by SNAPP using biallelic SNPs (panel a). The original SNP matrix ($n = 31,700$ SNPs) was generated in PYRAD by setting a clustering threshold value of $W_{\text{clust}} = 0.90$ and a minimum taxon coverage value in a given locus of $\text{minCov} = 10$. The number of biallelic SNPs recovered by SNAPP is detailed at the left bottom of the figure. The species tree was obtained using a gamma prior distribution (gamma, α , β) of $G(2, 2,000)$ for the ancestral population size parameter (θ value). Runs performed using $G(2, 200)$ and $G(2, 20,000)$ as priors yielded similar topologies. The first (blue), second (red) and third (green) most supported topologies are shown with different colors. Posterior probabilities for the most supported topology are indicated on the nodes of a maximum credibility tree (panel b). Subspecies codes as in Figure 1 and Table S1.