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Genomic footprints of an old affair: Single nucleotide polymorphism data reveal historical hybridization and the subsequent evolution of reproductive barriers in two recently diverged grasshoppers with partly overlapping distributions

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Abstract

Secondary contact in close relatives can result in hybridization and the admixture of previously isolated gene pools. However, after an initial period of hybridization, reproductive isolation can evolve through different processes and lead to the interruption of gene flow and the completion of the speciation process. *Omocestus minutissimus* and *O. uhagonii* are two closely related grasshoppers with partially overlapping distributions in the Central System mountains of the Iberian Peninsula. To analyse spatial patterns of historical and/or contemporary hybridization between these two taxa and understand how species boundaries are maintained in the region of secondary contact, we sampled sympatric and allopatric populations of the two species and obtained genome-wide single nucleotide polymorphism data using a restriction site-associated DNA sequencing approach. We used Bayesian clustering analyses to test the hypothesis of contemporary hybridization in sympatric populations and employed a suite of phylogenomic approaches and a coalescent-based simulation framework to evaluate alternative hypothetical scenarios of interspecific gene flow. Our analyses rejected the hypothesis of contemporary hybridization but revealed past introgression in the area where the distributions of the two species overlap. Overall, these results point to a scenario of historical gene flow after secondary contact followed by the evolution of reproductive isolation that currently prevents hybridization among sympatric populations.

KEYWORDS

coalescent-based simulations, ddRAD-seq, hybridization, introgression, reproductive isolation

1 | INTRODUCTION

Elucidating the processes that generate and maintain species diversity is a main ambition of evolutionary research (Fitzpatrick, Fordyce, & Gavrilets, 2009; Graham, Ron, Santos, Schneider, & Moritz, 2004; Grant, Grant, Markert, Keller, & Petren, 2004). The formation and persistence of species often depends on the evolution of reproductive isolation mechanisms that prevent interbreeding with other recently diverged taxa or closely related lineages (Marques, Draper, Riofrio, & Naranjo, 2014; Soltis & Soltis, 2009). In the context of allopatric populations, long-term geographical isolation can facilitate the development of reproductive barriers through genetic drift or divergent selection pressures, which can ultimately lead to population divergence and speciation (Hoskin, Higgie, McDonald, & Moritz, 2005; Maguilla, Escudero, Hipp, & Luceno, 2017; Schenk, Kontur, Wilson, Noble, & Derryberry, 2018). However, lineages or species often

come into secondary contact and, if barriers to gene flow are lacking or incomplete, hybridization will take place. This phenomenon can have different outcomes with important evolutionary consequences (Abbott et al., 2013; Mallet, 2005; Mayr, 1963). At one extreme, barriers to gene exchange may break down upon secondary contact and lead to the collapse of formerly distinct species in a hybrid swarm characterized by extensive admixture of parental genotypes (i.e., speciation reversal or lineage fusion; Kearns et al., 2018; Taylor et al., 2006). At the opposite extreme, reduced fitness of hybrids and strong selection against them will favour the rapid evolution of barriers to gene flow (i.e., prezygotic isolation), which can ultimately lead to total reproductive isolation and culminate in the completion of the speciation process (i.e., reinforcement of isolation; Butlin, 1995; Dobzhansky, 1937; Lemmon & Juenger, 2017; Servedio & Kirkpatrick, 1997). An intermediate scenario is the formation of tension zones with a variable geographical width determined by the equilibrium between dispersal and selection against hybrids (Barton & Hewitt, 1985; Key, 1968).

Hybrid zones have been defined as "windows on the evolutionary processes" (Harrison, 1990; Hewitt, 1988) and their study through space (Barton & Hewitt, 1989; Howard, Waring, Tibbets, & Gregory, 1993) and time (Britch, Cain, & Howard, 2001; Buggs, 2007) has provided important insights into the evolution of reproductive isolation and the formation of new species. Past climate changes, such as the glacial cycles of the Pleistocene, have resulted in recurrent range expansions and contractions in many organisms, often putting into geographical contact closely related species and linages that had remained geographically isolated for large periods of time. Alpine and montane species represent a paradigmatic example of species experiencing dramatic distributional shifts in response to Pleistocene glacial cycles, descending to lower altitudes and expanding their distributions in cold periods and shrinking their ranges to high-elevation areas during interglacials (Schmitt, 2009; Seddon, Santucci, Reeve, & Hewitt, 2001; Tzedakis, Emerson, & Hewitt, 2013). Under these conditions, closely related species that evolved in isolation during interglacial periods can recurrently come into secondary contact and hybridize. In other cases, interbreeding species share a large portion of their respective ranges but only hybridize in certain areas with specific environmental conditions or scattered patches where they meet, forming a mosaic hybrid structure rather than a well-defined cline limited to narrow contact zones (Barton & Hewitt, 1985). Independently of their nature and origin, contact zones offer the opportunity to study in real time the process of reproductive isolation or, if completed, to obtain indirect evidence about when (tempo) and how (modes) it might have evolved. However, testing alternative hypotheses about the evolution of reproductive isolation can be extremely challenging, making it necessary to integrate multiple sources of analytical evidence (e.g., phylogenetics and population genetics) and to develop model-based approaches considering the biogeographical context of gene flow (Payseur & Rieseberg, 2016). The high power of genomic data to resolve historical events of hybridization and test complex scenarios of gene flow in virtually any organismal model and biogeographical setting has exponentially increased our capacity to quantify the magnitude and timing of interspecific gene flow and distinguish among alternative demographic scenarios (e.g., de Manuel et al., 2016; Lohse, Clarke, Ritchie, & Etges, 2015; Ortego, Gugger, & Sork, 2018).

Here, we use as a study system two recently diverged grasshopper species with partly overlapping distributions to illustrate the potential of integrating different analytical approaches for inferring the tempo and mode of evolution of reproductive isolation (or its lack thereof) and gain insights into the speciation process. Grasshoppers (Orthoptera: Caelifera) are an interesting system to study hybridization and its evolutionary consequences, as many species have very recently evolved in allopatry (Mayer, Berger, Gottsberger, & Schulze, 2010; Ragge & Reynolds, 1998) and present incomplete barriers to gene flow (e.g., Saldamando, Tatsuta, & Butlin, 2005). In turn, their distributions often overlap across large geographical areas (Hill, 2015), show a mosaic distribution (Rohde et al., 2017) or have recurrently come into secondary contact as a consequence of range shifts driven by past climate changes (Bridle, Baird, & Butlin, 2001), providing ideal biogeographical scenarios for the study of hybridization and the evolution of reproductive isolation (Butlin, Ritchie, & Hewitt, 1991; Virdee & Hewitt, 1994). In this study we focus on two grasshopper species of the subgenus *Dreuxius* (genus *Omocestus*), a species complex comprising nine taxa distributed in the Iberian Peninsula and northwest Africa (Cigliano, Braun, Eades, & Otte, 2019; García-Navas, Noguerales, Cordero, & Ortego, 2017). Most species of this complex are distributed in allopatry, isolated at high elevation in different mountain ranges. One exception are the taxa *Omocestus minutissimus* (Brullé 1832) and *O. uhagonii* (Bolivar 1876), which show partially overlapping distributions in the Central System Mountains of the Iberian Peninsula. As with other species of the subgenus, both taxa are brachypterous, present a low dispersal capacity and have a similar annual life cycle, with an adult breeding phase from the end of July to the beginning of October (Clemente, Garcia, & Presa, 1991). The two species are predominantly graminivorous and occupy open habitats tightly linked to cushion and thorny shrub formations that they use as refuge (Clemente et al., 1991). However, both species differ on the extent of their distributions and elevational ranges. While *O. minutissimus* presents a wider distribution, with patchy populations distributed in eastern and central Iberia from sea level to 2,500 m elevation, *O. uhagonii* is restricted to the Central System and altitudes over 1,600–1,800 m (Figure 1). The two taxa partially co-occur across the distribution range of *O. uhagonii*, with several sympatric populations at high elevations in which adult individuals of the two species coexist at high numbers in the same microhabitats (J. Ortego, personal observation). Therefore, this system provides an interesting case study to analyse the presence of contemporary and past hybridization and understand the maintenance of species boundaries in two closely related taxa that might have weak or recently evolved reproductive isolation mechanisms.

We extensively sampled sympatric and allopatric populations of *O. minutissimus* and *O. uhagonii* across their respective

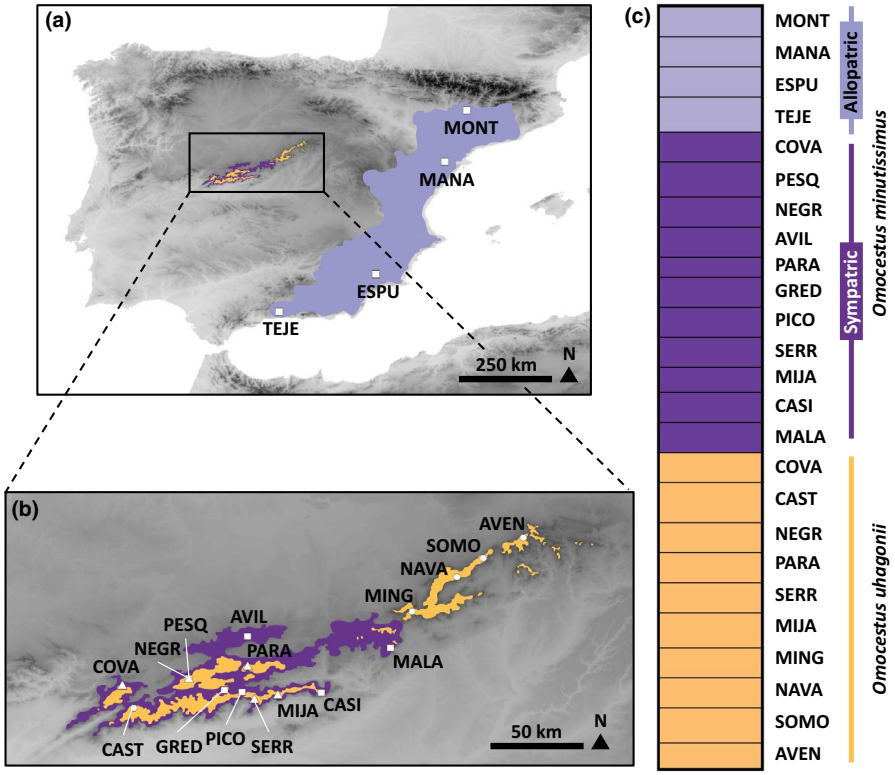

FIGURE 1 Biogeographical setting of the study system. (a,b) Maps showing the sampled populations and the distribution range of the two studied taxa based on our own species records (*Omocestus minutissimus*: purple areas and squares; *O. uhagonii*: light orange areas and dots). *O. minutissimus* presents a partially overlapping distribution with *O. uhagonii* in the Central System (deep purple) and allopatric populations (light purple) in eastern Iberia. Triangles indicate sampling localities where the two species were found living in sympatry. (c) Genetic assignment of individuals based on the results of FASTSTRUCTURE. Individuals are partitioned into *K* coloured segments representing the probability of belonging to the cluster with that colour and thin vertical black lines separate individuals from different populations. Population codes as in Table S1 [Colour figure can be viewed at wileyonlinelibrary.com]

distribution ranges and genotyped them via restriction-site-associated DNA sequencing (ddRAD-seq; Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) to infer historical and contemporary interspecific gene flow and elucidate the evolutionary outcomes of such processes. Specifically, we first used Bayesian clustering analyses to determine the genetic ancestry of individuals and test the hypothesis of contemporary hybridization in sympatric populations of the two taxa. Second, we employed a suite of phylogenomic approaches and a coalescent-based simulation framework to evaluate alternative scenarios of historical hybridization and estimate the timing, magnitude and directionality of interspecific gene flow. Our genomic data rejected the hypothesis of contemporary hybridization but revealed the presence of past genetic introgression, pointing to a scenario of historical hybridization after secondary contact followed by the evolution of reproductive barriers that today prevent gene flow among sympatric populations of the two species.

2 | MATERIALS AND METHODS

2.1 | Population sampling

Between 2011 and 2015, we sampled *O. minutissimus* (88 individuals, 15 populations) and *O. uhagonii* (64 individuals, 10 populations) from a total of 25 populations that cover the entire distribution range of the two taxa (Table S1; Figure 1). Eleven sampling populations of *O. minutissimus* are located in central Iberia and partly overlap with the distribution range of *O. uhagonii* (hereafter referred as sympatric populations). Among these populations of *O. minutissimus*, five were strictly sympatric with *O. uhagonii* and the two species were collected from the same localities (Table S1; Figure 1). The rest of the sampled populations of *O. minutissimus* are located in eastern Iberia and separated >300 km from the nearest population of *O. uhagonii* (hereafter referred to as allopatric populations). We stored specimens in 2-ml vials with 96% ethanol and preserved them at −20°C until needed for DNA extraction. Detailed information on sampling populations is presented in Table S1.

2.2 | Genomic library preparation and data processing

We used NucleoSpin Tissue kits (Macherey-Nagel) to extract and purify total genomic DNA from a hind leg of each individual. We processed genomic DNA in house following the ddRAD-seq procedure described in Peterson et al. (2012), with some minor modifications as detailed in Lanier, Massatti, He, Olson, and Knowles (2015). Briefly, we digested DNA with the restriction enzymes *Eco*RI and *Mse*I (New England Biolabs) and ligated Illumina adaptors including unique 7-bp barcodes to the digested fragments of each individual. We pooled ligation products into four different libraries, size selected for fragments between 475 and 580 bp using a Pippin Prep machine (Sage Science), and amplified them by polymerase chain reaction (PCR) with 10–12 cycles using the iProof High-Fidelity DNA Polymerase (Bio-Rad). We sequenced the libraries in single-read 151-bp lanes on an Illumina HiSeq2500 platform at The Centre for Applied Genomics (Hospital for Sick Children).

We demultiplexed raw sequences using PROCESS _ RADTAGS, a program distributed as part of the STACKS pipeline (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). We only retained reads with Phred scores ≥10 (using a sliding window of 15%), no adaptor contamination and unambiguous barcode and restriction cut sites. We checked read quality in FASTQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed sequences to 130 bp using a SEQTK script (Heng Li, https://github.com/lh3/seqtk) in order to remove low-quality reads near the 3′ ends. As an additional quality-filtering step, we used PYRAD version 3.0.66 (Eaton, 2014) to convert base calls with a Phred score <20 into Ns and discard reads with >2 Ns. We assembled retained reads into de novo loci using PYRAD, considering parameter values for clustering threshold of sequence similarity (W_{CLUST} = 0.85), minimum coverage depth (d = 5), maximum number of individuals with shared heterozygous sites (*maxSH* = p.10) and maximum number of polymorphic sites in a final locus (*maxSNPs* = 20) based on suggestions from the literature (Eaton, 2014; Eaton & Ree, 2013; Takahashi, Nagata, & Sota, 2014). Finally, we generated final data sets for subsequent analyses discarding loci that were not present in at least ~25% of the samples (*minCov* = ~25%).

2.3 | Genetic structure and hybrid identification

We identified hybrids and introgressed individuals between *O. minutissimus* and *O. uhagonii* using the Bayesian clustering methods implemented in the programs FASTSTRUCTURE version 1.0 (Raj, Stephens, & Pritchard, 2014) and STRUCTURE version 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). First, we used the highly efficient algorithm implemented in FASTSTRUCTURE to analyse the entire data set including all populations. Second, we used both FASTSTRUCTURE and classic STRUCTURE to perform more detailed analyses focused on the Central System, the region where the ranges of the two taxa partly overlap, with some populations living in sympatry and, thus, the two species currently have the opportunity to hybridize. We ran FASTSTRUCTURE analyses using a simple prior, considering a convergence criterion of 1×10^{-7} and conducting 25 independent runs for each value of K (from K = 1 to K = 10). Following Raj et al. (2014), we used the *chooseK.py* script to assess model complexity by estimating the metrics $K^*_{\substack{\neq \\ \varepsilon}}$, the value of K that maximizes the log-marginal likelihood lower bound (LLBO) of the data, and K^*_{ε}, the smallest number of model components explaining at least 99% of the cumulative ancestry contribution. We plotted individual co-ancestry coefficients for the most likely K value using DISTRUCT version 1.1 (Rosenberg, 2004). We ran STRUCTURE assuming correlated allele frequencies and admixture, and without using prior population information (Hubisz, Falush, Stephens, & Pritchard, 2009; Pritchard et al., 2000). We conducted 15 independent runs for each value of K (from K = 1 to K = 10) to estimate the optimal number of genetic clusters with 200,000 Markov chain Monte Carlo (MCMC) cycles, following a burn-in step of 100,000 iterations. We used STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to assess the number of genetic clusters that best describes our data according to log probabilities of the data (LnPr(X|K) for each value of K (Pritchard et al., 2000) and the ΔK method (Evanno, Regnaut, & Goudet, 2005). We used CLUMPP version 1.1.2 and the Greedy algorithm to align multiple runs of STRUCTURE for the same K value (Jakobsson & Rosenberg, 2007) and DISTRUCT to visualize as bar plots the individual's probabilities of population membership.

2.4 | Phylogenomic analyses and inference of historical hybridization

To determine the presence of historical hybridization (i.e., introgression), we employed four-taxon ABBA/BABA tests based on the D-statistic (Durand, Patterson, Reich, & Slatkin, 2011) and TREEMIX analyses (Pickrell & Pritchard, 2012). Assuming that the sister taxa P1 and P2 diverged from P3 and an outgroup species O, the D-statistic is used to test the null hypothesis of no introgression (D = 0) between P3 and P1 or P2. D values significantly different from zero indicate gene flow between P1 and P3 (D < 0) or between P2 and P3 (D > 0). We assigned sympatric populations of *O. minutissimus* to P1 (64 individuals, from localities COVA, PESQ, NEGR, AVIL, PARA, GRED, PICO, SERR, MIJA, CASI and MALA; hereafter, MS), allopatric populations of *O. minutissimus* to P2 (24 individuals, from localities MONT, PORT, ESPU and TEJE; hereafter, MA) and populations of *O. uhagonii* to P3 (64 individuals from all sampling localities for this taxon; hereafter, US). Note that the sympatric and allopatric populations of *O. minutissimus* are located in the central and west portions of the species distribution range, respectively, and correspond to the two well-defined genetic clusters identified by Bayesian clustering analyses for this taxon (see Section 3 and Figure 1). We used as the outgroup (O) the taxon *O. antigai* (Bolívar, 1897), a species also belonging to the subgenus *Dreuxius* (Cigliano et al., 2019; García-Navas et al., 2017). Specifically, we used sequences from 94 individuals of this species available at the NCBI Sequence Read Archive (SRA) under BioProject PRJNA543714 (Tonzo, Papadopoulou, & Ortego, 2019). We performed ABBA/BABA tests in PYRAD and used 1,000 bootstrap replicates to obtain the standard deviation of the D-statistic (Eaton & Ree, 2013; e.g., Huang, 2016).

We also analysed the potential presence of introgression and determined the direction of gene flow with TREEMIX version 1.12 (Pickrell & Pritchard, 2012). We used TREEMIX to construct a tree-based model of population genetic relationships and infer events of genetic admixture using single nucleotide polymorphism (SNP) frequency data. TREEMIX fits a population graph (i.e., a phylogenetic tree that incorporates admixture) on the basis of allele frequencies and a Gaussian approximation to genetic drift, allowing patterns of splits and mixtures in multiple populations to be inferred. To perform TREEMIX analyses, we pooled populations into the same three groups used to run ABBA/BABA tests. In a first step, we estimated a maximum-likelihood tree rooted with *O. antigai*. Then, we tested

the existence of a range of migration events (m = 0–5, with three replicated runs each) and calculated the proportion of the variance in population covariances explained by the population graph with different numbers of admixture events to determine the model best fitting the data (e.g., Gompert et al., 2014). We assumed the independence of all SNPs and used a window size of one SNP (k = 1; e.g., Vera, Díez-del-Molino, & García-Marín, 2016).

2.5 | Testing alternative models of gene flow

As TREEMIX only models migration as discrete events and does not consider continuous gene flow (Pickrell & Pritchard, 2012), we applied the coalescent-based modelling approach implemented in FASTSIMCOAL2 (Excoffier, Dupanloup, Huerta-Sanchez, Sousa, & Foll, 2013) to statistically test the relative fit of more complex historical demographic models to our genomic data. We used FASTSIMCOAL2 and the site frequency spectrum (SFS; Excoffier et al., 2013) to test seven alternative models of gene flow (Figure 2). These models considered the same three population groups used in ABBA/BABA tests and TREEMIX analyses. All scenarios considered an early split between the two species followed by the divergence between sympatric (MS) and allopatric (MA) populations of *O. minutissimus*. The tested scenarios considered (a) total absence of post-divergence gene flow (Model 0, not shown in Figure 2); (b) gene flow only between the two population groups of *O. minutissimus* (i.e., absence of interspecific gene flow; Models 1 and 2); (c) historical gene flow between sympatric populations of *O. uhagonii* and *O. minutissimus* (i.e., interspecific gene flow) but absence of post-divergence gene flow between the two population groups of *O. minutissimus* (Models 3 and 4); and (d) gene

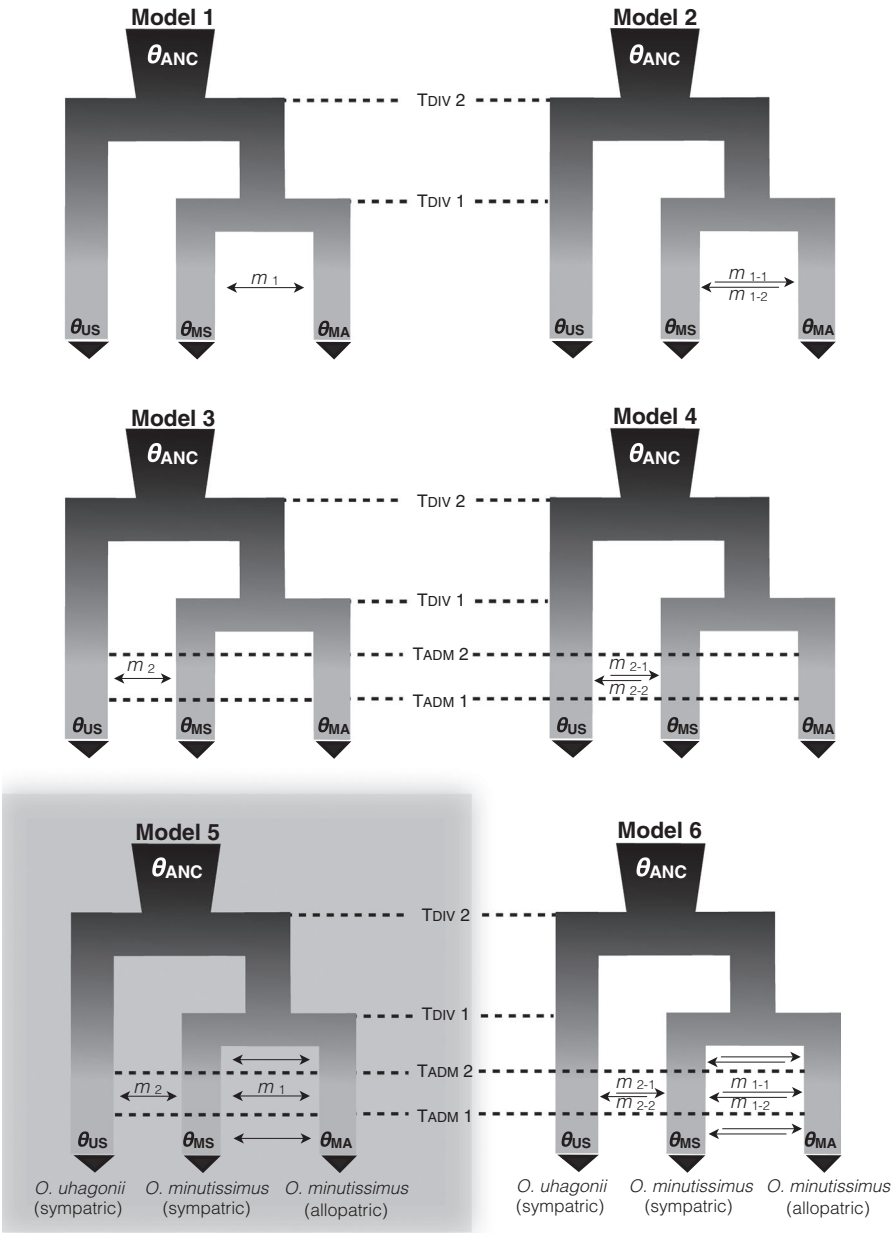

FIGURE 2 Alternative migration models tested using FASTSIMCOAL2. Parameters include ancestral (θ_{ANC}) and contemporary (θ_{US}, θ_{MS}, θ_{MA}) effective population sizes, timing of population split (T_{DIV}) and admixture (T_{ADM}), and migration rates (m) between different pairs of populations. Grey background highlights the best-supported model

flow between the two population groups of *O. minutissimus* and historical gene flow between sympatric populations of *O. uhagonii* and *O. minutissimus* (Models 5 and 6) (Figure 2). These scenarios were tested considering both symmetric (Models 1, 3 and 5) and asymmetric (Models 2, 4 and 6) gene flow (Figure 2). Note that detailed Bayesian clustering analyses performed across multiple populations of the two taxa in the region where their distribution ranges overlap did not show any evidence of ongoing interspecific gene flow (see Section 3) and, for this reason, we did not consider models incorporating contemporary interspecific gene flow.

We calculated a folded joint SFS considering a single SNP per locus to avoid the effects of linkage disequilibrium. Because we did not include invariable sites in the SFS, we fixed the effective population size for one population group (US) to enable the estimation of other parameters in FASTSIMCOAL2 (e.g., Lanier et al., 2015; Papadopoulou & Knowles, 2015). The effective population size (N_e) fixed in the models was calculated from the level of nucleotide diversity (π) and estimates of mutation rate per site per generation (μ), according to the equation $N_e = \pi/4\mu$ (Lynch & Conery, 2003). Nucleotide diversity for US (π = 0.005) was estimated from polymorphic and nonpolymorphic loci using DNASP version 6.11.01 (Librado & Rozas, 2009) and the.*allele* file generated by PYRAD. We considered the average mutation rate per site per generation of 2.80×10^{-9} estimated for *Drosophila melanogaster* (Keightley, Ness, Halligan, & Haddrill, 2014). To remove all missing data for the calculation of the joint SFS and minimize errors with allele frequency estimates, each population group was down-sampled to 25% of individuals (32, 12 and 32 genes for MS, MA and US, respectively) using a custom Python script written by Isaac Overcast and available at GitHub (https://github.com/isaacovercast/easySFS). The final SFS contained 2,071 variable SNPs. Each of the seven models was run 100 replicated times using the computing resources provided by CESGA (Galician Supercomputer Center) considering 100,000–250,000 simulations for the calculation of the composite likelihood, 10–40 expectation-conditional maximization (ECM) cycles, and a stopping criterion of 0.001 (Papadopoulou & Knowles, 2015). We used an information-theoretic model selection approach based on Akaike's information criterion (AIC) to determine the probability of each model given the observed data (Burnham & Anderson, 2002; e.g., Abascal et al., 2016; Thome & Carstens, 2016). After the maximum likelihood was estimated for each model in every replicate, we calculated the AIC scores as detailed in Thome and Carstens (2016). AIC values for each model were rescaled (ΔAIC) calculating the difference between the AIC value of each model and the minimum AIC obtained among all competing models (i.e., the best model has ΔAIC = 0). Point estimates of the different demographic parameters for the best-supported model were selected from the run with the highest maximum composite likelihood. Finally, we calculated confidence intervals of parameter estimates from 100 parametric bootstrap replicates by simulating SFS from the maximum composite likelihood estimates and re-estimating parameters each time (Excoffier et al., 2013; e.g., Papadopoulou & Knowles, 2015).

3 | RESULTS

3.1 | Genomic data

Illumina sequencing returned an average of 2.74×10^6 reads per sample. After quality control, an average of 2.33×10^6 reads per sample was retained (Figure S1). The data sets obtained with PYRAD for all populations and only those from the Central System retained 15,219 and 20,350 unlinked SNPs, respectively.

3.2 | Genetic structure and hybrid identification

The model complexity value that maximized the marginal likelihood in FASTSTRUCTURE analyses for the data set including all populations was K = 3 across all replicates and the number of model components used to explain structure in the data was K = 3 in 15 replicates and K = 4 in 10 replicates. FASTSTRUCTURE analyses for K = 3 split populations of *O. uhagonii* and sympatric and allopatric populations of *O. minutissimus* into different genetic clusters in which all individuals showed high probabilities of population membership (q > 0.99; Figure 1c). Assignment values to additional genetic clusters in FASTSTRUCTURE for analyses with K > 4 were extremely low in all cases (q < 0.001) and, thus, their respective bar plots were virtually identical to those obtained for K = 3 (for a similar result, see Baiz, Tucker, & Cortes-Ortiz, 2019; Tonzo et al., 2019).

The model complexity value that maximized the marginal likelihood in FASTSTRUCTURE analyses of the Central System populations was equal to K = 2 in all replicates and the number of model components used to explain structure in the data was K = 2 in 16 replicates and K = 3 in nine replicates. FASTSTRUCTURE analyses for K = 2 split populations of the two species in different genetic clusters and all individuals showed high probabilities of population membership (q > 0.99; Figure 3a). Again, assignment values to additional genetic clusters in FASTSTRUCTURE for analyses with K > 2 were extremely low in all cases (q < 0.001) and, thus, their respective bar plots were virtually identical to those obtained for K = 2. Classic STRUCTURE analyses also yielded an "optimal" clustering value for K = 2 according to the ΔK criterion (Figure S2). The two inferred genetic groups supported a clear separation of the two species (Figure 3b). STRUCTURE analyses showed that populations of *O. uhagonii* from the eastern Central System present no signal of introgression from *O. minutissimus*. However, several populations of *O. minutissimus* and *O. uhagonii* from the western Central System, where the distribution of the two taxa overlap and five sampling localities present sympatric populations, showed signals of reciprocal genetic introgression (Figure 3b). These results suggest that FASTSTRUCTURE is less likely to reveal small proportions of admixed ancestry in comparison with STRUCTURE, which has been also suggested in previous studies (Stift, Kolar, & Meirmans, 2019; Tonzo et al., 2019).

STRUCTURE analyses indicate that populations of *O. uhagonii* and *O. minutissimus* present significant differences in the degree of introgression from the other species (one-way ANOVAs, introgression

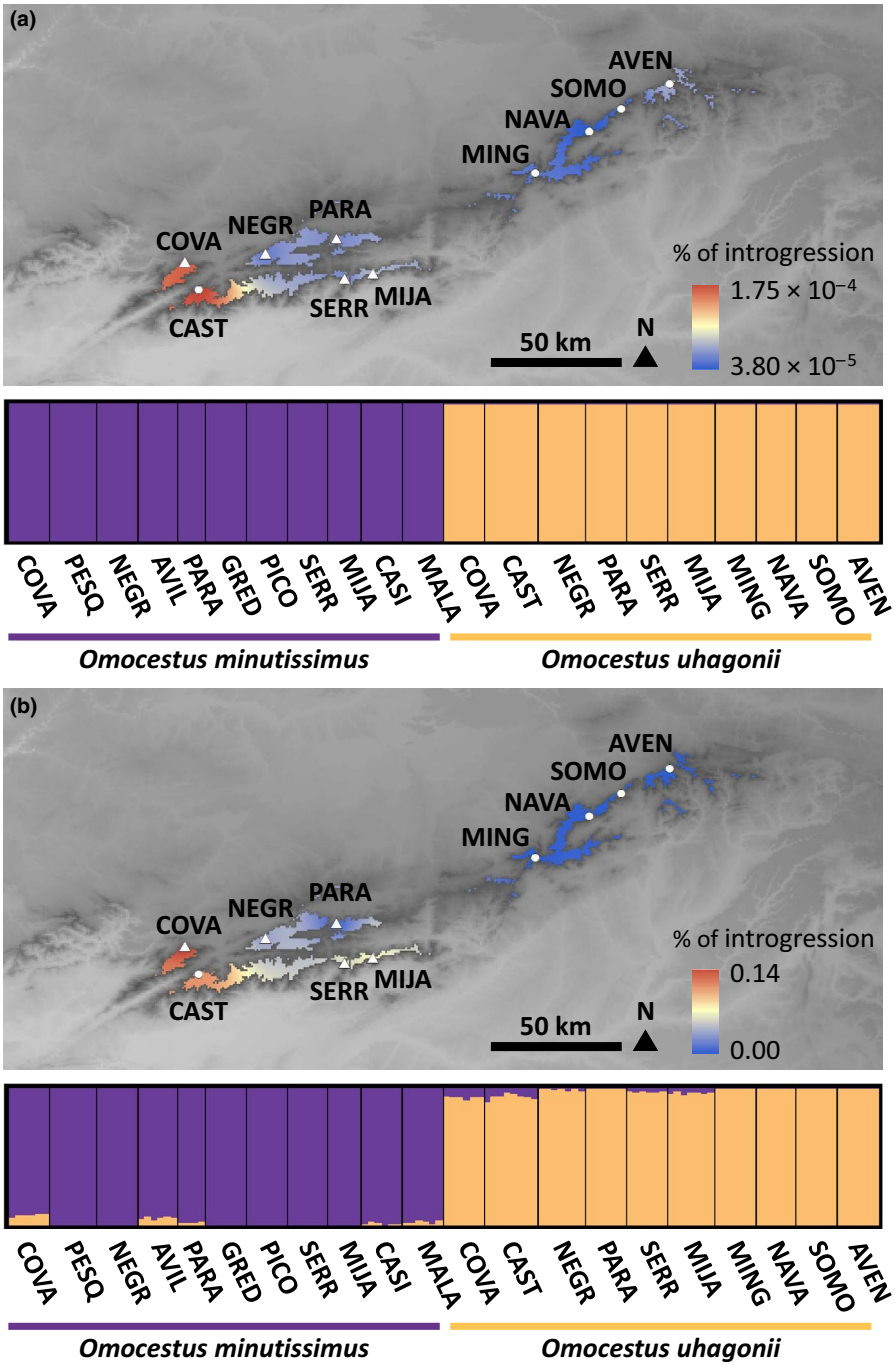

FIGURE 3 Genetic assignment of *Omocestus minutissimus* and *O. uhagonii* from the Central System based on the results of (a) FASTSTRUCTURE and (b) STRUCTURE. Individuals are partitioned into *K* coloured segments representing the probability of belonging to the cluster with that colour and thin vertical black lines separate individuals from different populations. Maps display the distribution of *O. uhagonii* and the probability of assignment of the populations of this species (spatial interpolation) to the genetic cluster of *O. minutissimus* (i.e., the degree of introgression from *O. minutissimus* to *O. uhagonii*). Population codes as in Table S1 [Colour figure can be viewed at wileyonlinelibrary.com]

of *O. minutissimus* into *O. uhagonii*: $F_{9,54}$ = 48.86, p < .001; introgression of *O. uhagonii* into *O. minutissimus*: $F_{10,53}$ = 96.68, p < .001; Figure 3b). Although visually imperceptible in the FASTSTRUCTURE bar plot (Figure 3a), probabilities of population membership inferred by this software also revealed that populations of both *O. uhagonii* and *O. minutissimus* present significant differences in the degree of introgression from the other species (one-way ANOVAs, introgression of *O. minutissimus* into *O. uhagonii*: $F_{9,54}$ = 14.01, p < .001; introgression of *O. uhagonii* into *O. minutissimus*: $F_{10,53}$ = 3.19, p = .003; Figure 3a). The degree of introgression from *O. minutissimus* into *O. uhagonii* estimated by either FASTSTRUCTURE or STRUCTURE decreased significantly with longitude (FASTSTRUCTURE: $F_{1,8}$ = 6.99, p = .030; STRUCTURE: $F_{1,8}$ = 16.22, p = .004; see maps in Figure 3), but did not differ significantly between currently sympatric and allopatric populations (FASTSTRUCTURE: $F_{1,8}$ = 0.06, p = .81; STRUCTURE: $F_{1,8}$ = 1.64, p = .236). In contrast, the degree of introgression from *O. uhagonii* into *O. minutissimus* estimated by either FASTSTRUCTURE or STRUCTURE did not decrease significantly with longitude (FASTSTRUCTURE: $F_{1,9}$ = 1.12, p = .317; STRUCTURE: $F_{1,9}$ = 0.41, p = .538) or differ between currently sympatric and allopatric populations (FASTSTRUCTURE: $F_{1,9}$ = 0.39, p = .550; STRUCTURE: $F_{1,9}$ = 0.03, p = .863). For illustrative purposes, we displayed on a map the probabilities of assignment of the populations of *O. uhagonii* to the genetic cluster of *O. minutissimus* (i.e., the degree of introgression from *O. minutissimus* into *O. uhagonii*) by conducting a spatial

interpolation using the Inverse Distance Weight (IDW) function available in ARCGIS version 10.5 (ESRI; Figure 3).

3.3 | Phylogenomic analyses and inference of historical hybridization

The test of introgression based on the *D*-statistic supported post-divergence gene flow between sympatric populations of *O. minutissimus* and *O. uhagonii* (BABA = 220.59; ABBA = 162.38; *D*-statistic = −0.152; Z = 3.39; $p < .001$). Accordingly, TREEMIX analyses supported a single migration event (Figure S3) corresponding to admixture between sympatric populations of *O. minutissimus* and *O. uhagonii* (Figure 4).

3.4 | Testing alternative models of gene flow

FASTSIMCOAL2 analyses showed that the best supported model (Model 5; ΔAIC = 0; Table 1) was the one considering symmetric interspecific gene flow between sympatric populations of *O. uhagonii* and *O. minutissimus* during a given period of time (T_{ADM1} and T_{ADM2}) and symmetric gene flow between sympatric (central) and allopatric (eastern) populations of *O. minutissimus*. Remarkably, models only considering interspecific gene flow during a given period of time tended to be slightly more well supported (ΔAIC > 0.6) than models only considering intraspecific gene flow between sympatric and allopatric populations of *O. minutissimus* (Table 1). Point estimates of demographic parameters under the best fitting model are presented in Table 2. Considering that the studied taxa are univoltine (i.e., 1-year generation time), FASTSIMCOAL2 analyses inferred that the separation of the two species (T_{DIV2}) and the split of eastern and central populations of *O. minutissimus* (T_{DIV1}) took place during the Early Pleistocene (Calabrian age; Table 2). Gene flow between sympatric populations of *O. minutissimus* and *O. uhagonii* was estimated to have occurred during a period of ~15 ka in the Late Pleistocene (Tarantian age; Table 2). Note, however, that although confidence

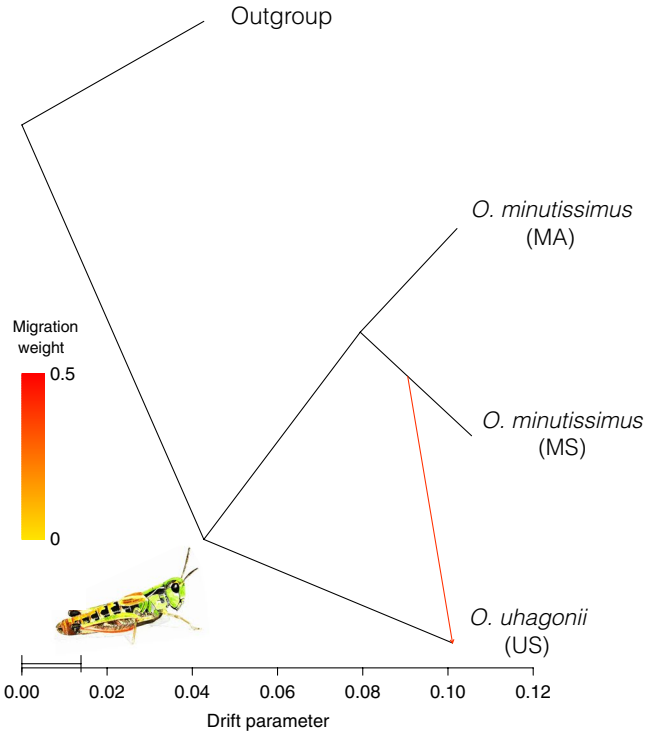

FIGURE 4 Maximum-likelihood tree inferred with TREEMIX for *Omocestus uhagonii* (US) and sympatric (MS) and allopatric (MA) populations of *O. minutissimus*. The direction of gene flow (from MS to US) for the most likely migration event ($m = 1$) inferred is represented with an arrow coloured according to the percentage of alleles (weight) originating from the source [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Comparison of alternative migration models (detailed in Figure 2) tested using FASTSIMCOAL2

Model	*k*	$\log_{10}$L	AIC	ΔAIC	ω_i
Model 0	6	−3,539.32	7,090.64	90.01	0.00
Model 1	7	−3,518.85	7,051.71	51.08	0.00
Model 2	8	−3,518.42	7,052.84	52.21	0.00
Model 3	9	−3,515.49	7,048.98	48.34	0.00
Model 4	10	−3,515.54	7,051.09	50.45	0.00
Model 5	**10**	**−3,490.32**	**7,000.63**	**0.00**	**0.86**
Model 6	12	−3,490.09	7,004.19	3.56	0.14

For each model, the table shows the maximum likelihood estimate of the model ($\log_{10}$L), the number of parameters (*k*), Akaike's information criterion score (AIC), the difference in AIC value of each model from that of the strongest model (ΔAIC) and AIC weight (ω_i). The best-supported model (ΔAIC < 2) is indicated in bold.

TABLE 2 Parameters inferred from coalescent simulations with FASTSIMCOAL2 under the best-supported demographic model (Model 5)

Parameter	Point estimate	Lower bound	Upper bound
θ_{ANC}	702,036	376,562	832,461
θ_{MS}	813,263	686,748	890,000
θ_{MA}	1,556,235	1,293,951	1,675,243
T_{DIV1}	1,199,191	922,762	1,380,088
T_{DIV2}	1,382,958	1,278,828	1,670,203
T_{ADM1}	14,090	1,632	23,626
T_{ADM2}	29,040	50,260	750,959
m_1	1.28×10^{-7}	9.02×10^{-8}	1.82×10^{-7}
m_2	2.41×10^{-7}	2.14×10^{-8}	1.37×10^{-7}

The table shows point estimates and lower and upper 95% confidence intervals. Note that the effective population size of *Omocestus uhagonii* is not presented because it was fixed in FASTSIMCOAL2 analyses to enable the estimation of other parameters (see the Materials and Methods section for further details). θ, mutation-scaled effective population sizes; T_{DIV} and T_{ADM}, timing of population divergence and admixture, respectively (given in number of generations); *m*, migration rates per generation. Each specific parameter is illustrated in Figure 2.

intervals around point estimates of most parameters were reasonably tight, there was considerable uncertainty around estimates for the two time parameters delimiting the period of interspecific gene flow (i.e., T_{ADM1} and T_{ADM2}; Table 2). Finally, migration rates between sympatric populations of *O. uhagonii* and *O. minutissimus* (m_2) did not differ significantly from those estimated between sympatric (western) and allopatric (eastern) populations of *O. minutissimus* (m_1) (i.e., 95% confidence intervals [CIs] of m_1 and m_2 overlapped; Table 2). This indicates that historical interspecific gene flow was of the same order of magnitude as intraspecific gene flow between the two allopatric genetic clusters of *O. minutissimus*.

4 | DISCUSSION

Hybridization has been extensively documented in contact zones where the distributions of closely related species with weak reproductive barriers meet (e.g., Folk, Soltis, Soltis, & Guralnick, 2018; Gugger & Cavender-Bares, 2013; Nadeau et al., 2013; Ortego, Gugger, Riordan, & Sork, 2014). Inferring events of past interspecific gene flow has important implications to understand the evolutionary history of organisms (e.g., humans; Prüfer et al., 2014; Wall et al., 2013), yet detecting the footprints of such processes is challenging (Payseur & Rieseberg, 2016; e.g., Eaton, Hipp, González-Rodríguez, & Cavender-Bares, 2015; Ortego et al., 2018). Here, by combining a suite of phylogenomic and population genetic tools and extensive population sampling across the entire distribution of our two focal species, including currently sympatric and allopatric populations, we found no evidence for contemporary hybridization. However, we did detect signals of past introgression in the geographical region where the distribution range of the two taxa currently overlap.

4.1 | Absence of contemporary interspecific gene flow

Bayesian clustering analyses showed a clear genotypic differentiation of the two species and further revealed the presence of two well-defined genetic clusters within *O. minutissimus*, corresponding to the populations of this taxon located in eastern (allopatric) and central (sympatric) Iberia (Figure 1). Detailed analyses across 21 populations from central Iberia where the distribution of the two species partially overlap and, thus, where they currently have the opportunity to hybridize, showed no evidence of ongoing interspecific gene flow (i.e., F_1 or first generation backcrosses). However, these analyses also revealed footprints of reciprocal introgression in the westernmost portion of the Central System, the area where the two species present overlapping distributions and some populations even co-occur. Although the degree of introgression did not differ statistically between currently sympatric and allopatric populations of the two species in the Central System, it was not spatially homogeneous. On the one hand, the proportion of genetic introgression differed significantly across populations of the two species. On the other hand, the degree of introgression from *O. minutissimus* into *O. uhagonii* increased westwards and the populations from the easternmost portion of the distribution range of this species, where *O. minutissimus* is not currently present, showed negligible signals of past hybridization (Figure 3). These results indicate spatial heterogeneity in the levels of introgression, suggesting that the magnitude and/or timing of historical hybridization differed among populations of the two species in the Central System (e.g., de Manuel et al., 2016; Ortego et al., 2018; Wall et al., 2013). The degree of introgression was consistently small in all populations of both species (STRUCTURE: <9%; FASTRUCTURE: <0.02%) and similar across individuals within populations. Thus, although our sample sizes are modest (128 individuals) and we cannot categorically discard that the two species sporadically hybridize, the observed patterns of introgression indicate that contemporary populations are at genotypic equilibrium (i.e., backgrounds of introgression are similar across all individuals within a given population) and suggest that hypothetical contemporary hybridization, if it even happens, is unlikely to have transcended F_1 hybrids at least in the last generations. This result is also supported by the fact that the five populations where the two species currently co-occur do not show higher levels of introgression than nearby allopatric populations, which points to the fact that the observed patterns of genetic introgression reflect historical rather than contemporary interspecific gene flow.

4.2 | Inferring historical hybridization

Both the phylogenomic analyses in TREEMIX and the *D*-statistic test yielded results compatible with those inferred by Bayesian clustering analyses and supported the hypothesis of historical hybridization between *O. uhagonii* and geographically overlapping populations of *O. minutissimus*. TREEMIX identifies gene flow in the context of competing hypotheses, taking into account the full sampled phylogeny when inferring admixture and introgression events (Pickrell & Pritchard, 2012). Even when we allowed several admixture edges for the two species, TREEMIX only inferred one event of introgression involving the sympatric populations of the two species. In agreement with the TREEMIX results, the *D*-statistic analysis detected the same pattern of interspecific gene flow. Bringing the TREEMIX and the *D*-statistic results together supports a scenario of historical hybridization and enhances the interpretation of the low levels of genetic introgression revealed by clustering analyses in currently coexisting populations.

Coalescent-based analyses in FASTSIMCOAL2, which provides detailed estimation of demographic parameters, further supported a scenario of past interspecific gene flow over a strictly bifurcating evolutionary history. Specifically, the most well-supported model was the one considering interspecific gene flow between sympatric populations of *O. uhagonii* and *O. minutissimus* during a given period of time and gene flow between sympatric (central) and allopatric (eastern) populations of *O. minutissimus*. The preferred model traced back the divergence of *O. uhagonii* and *O. minutissimus* to the

Early Pleistocene (~1.4 million years ago [Ma]) and this event was followed shortly after by the split of eastern and central lineages of *O. minutissimus* (~1.2 Ma). These divergence times agree with the Pleistocene diversification observed across most clades within the highly speciose acridid subfamily Gomphocerinae (Song at al., 2015). Additionally, the split time estimates are congruent with the crown age (<3 Ma) inferred for the recent radiation of the subgenus *Dreixius* based on mitochondrial DNA (García-Navas et al., 2017). The results of coalescent analyses also indicate that gene flow between *O. uhagonii* and *O. minutissimus* in the area of geographical overlap happened during a limited amount of time (~15,000 years) around the last glacial maximum (14–29 thousand years ago). During this period the two species engaged in gene flow at the same rate as the one estimated between the two lineages of *O. minutissimus*, indicating that interspecific gene flow, albeit low, was comparatively remarkable. The estimated timing of interspecific gene flow is compatible with the probable expansion of *O. uhagonii* to lower elevations during glacial periods. The shift to lower elevations might have put this species into extensive geographical contact with the more ubiquitous *O. minutissimus*, which presents many populations in foothills and valley bottoms where *O. uhagonii* is not present today. It must be noted, however, that confidence intervals around point estimates for the time of interspecific gene flow are wide (Table 2), particularly for the onset of this period (T_{ADM2}), and thus these results must be interpreted with extreme caution. Uncertainty in these estimates could in part be driven by heterogeneity in the timing and extent of hybridization among the different populations in the sympatric area, as suggested by the significant differences in the signal of genetic introgression observed among populations (see previous section).

4.3 | Inferred evolutionary scenario

Our genomic analyses point to a scenario in which *O. uhagonii* and ancestral populations of *O. minutissimus* probably diverged in allopatry followed by the split of *O. minutissimus* into two lineages, one of which came into secondary contact and hybridized with *O. uhagonii* during a limited period of time (Figure 5). Despite the fact that today the two species have ample opportunity to hybridize (i.e., a large proportion of their respective ranges currently overlap and several populations even co-occur in the Central System), our analyses did not find any evidence of contemporary hybridization,

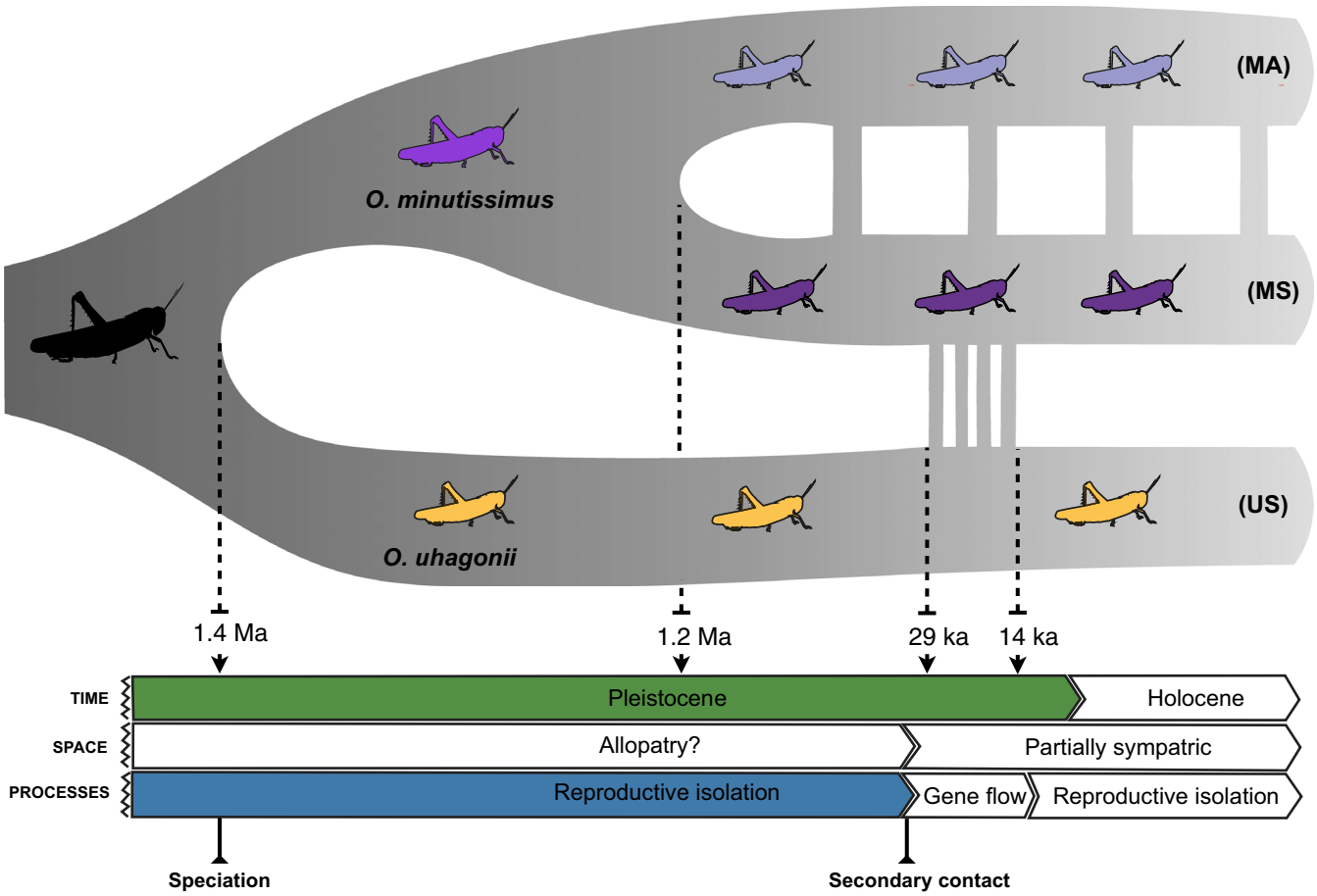

FIGURE 5 Schematic representation of the events documented in this study and the inferred biological processes. These correspond to the best-fitting demographic model (Model 5) for *Omocestus uhagonii* (US) and sympatric (MS) and allopatric (MA) populations of *O. minutissimus*. Vertical bars connecting MS and US represent historical gene flow. Note that geological reference time is not scaled and only point estimates inferred by FASTSIMCOAL2 are presented to simplify visualization [Colour figure can be viewed at wileyonlinelibrary.com]

which suggests that reproductive isolation probably evolved after secondary contact and historical gene flow (Figure 5). Speciation of Gomphocerinae and other grasshoppers has been generally linked to allopatric divergence (Mayer et al., 2010), a process that in the specific case of montane/alpine species of Pleistocene origin was probably caused by the extensive fragmentation of ancestral populations driven by Quaternary climatic oscillations (e.g., Huang, Hill, Ortego, & Knowles, 2020; Knowles, 2000; Scattolini, Confalonieri, Lira-Noriega, Pietrokovsky, & Cigliano, 2018). Different lines of evidence also point to allopatric divergence as the most plausible mode of speciation for *O. uhagonii* and *O. minutissimus*. First, the allopatric lineage of *O. minutissimus* from eastern Iberia presents a much larger distribution (Figure 1) and significantly higher levels of genomic diversity (one-way ANOVA: $F_{1,13}$ = 12.05, p = .004; Table S1) and effective population sizes (nonoverlapping 95% CIs for N_e estimates in FASTSIMCOAL2 analyses; Table 2) than the sympatric lineage of *O. minutissimus* from the Central System. This supports the view that *O. minutissimus* probably originated in eastern Iberia and subsequently colonized the Central System, where it came into secondary contact with *O. uhagonii*. Second, the ecological niches of *O. minutissimus* and *O. uhagonii* are very similar and the two species present graminivorous feeding habits and the same microhabitat preferences (Clemente et al., 1991; J. Ortego, personal observation). This points to considerable niche conservatism, typical of allopatric speciation, and rejects sympatric speciation via disruptive ecological selection (e.g., Grace, Wisely, Brown, Dowell, & Joern, 2010). Finally, our analyses showed no evidence of gene flow between *O. uhagonii* and ancestral populations of *O. minutissimus* (Figure 4), indicating that the two species probably evolved in allopatry and only exchanged gene flow after secondary contact in the region where their distribution ranges overlap (see also Sankararaman et al., 2014; Sankararaman, Patterson, Li, Paabo, & Reich, 2012).

The footprints of historical introgression among currently sympatric populations of *O. uhagonii* and *O. minutissimus* and the lack of evidence for contemporary gene flow lead us to hypothesize an evolutionary scenario in which reproductive isolation evolved after historical hybridization in the area where the distribution ranges of the two taxa currently overlap. The observed differences in the levels of introgression among sympatric populations suggest that barriers to gene flow might have evolved multiple times or, alternatively, could reflect heterogeneity in the proportion of the genome of the other species retained after the interruption of interspecific gene flow due to differences among the studied populations in their demographic histories (e.g., bottlenecks; Amorim et al., 2017; Lawson, Van Dorp, & Falush, 2018; Quilodran, Nussberger, Montoya-Burgos, & Currat, 2019) or spatial variation in the strength of hypothetical purifying selection acting against introgressed alleles (Juric, Aeschbacher, & Coop, 2016; Petr, Paabo, Kelso, & Vernot, 2019). Alternative processes might have led to the evolution of reproductive isolation after secondary contact and hybridization. It has been frequently documented that interspecific gene flow can increase phenotypic and genomic divergence via the evolution of reproductive isolation and character displacement (Garner, Goulet, Farnitano, Molina-Henao, & Hopkins, 2018; Hopkins, Levin, & Rausher, 2012; Pfennig & Pfennig, 2009). One of the many potential costs of hybridization is the ecological and genetic dysfunctions of hybrid offspring, which can reduce their fitness and drive reinforcement (Ortiz-Barrientos, Counterman, & Noor, 2004). In the reinforcement process, enhanced prezygotic isolation is favoured in sympatry in response to postzygotic isolation due to a strong selection against hybrids (Butlin, 1995; Coyne & Orr, 2004; Servedio & Noor, 2003). Selection for prezygotic isolation leads, in turn, to more divergent phenotypes and reproductive behaviours between species in sympatry than in allopatry (Moran, Zhou, Catchen, & Fuller, 2018). Thus, one possibility is that secondary contact and hybridization promoted the evolution of reproductive isolation via reinforcement, probably after an initial balance between dispersal and selection against hybrids in historical tension zones during which the two species experienced genetic exchange and introgression (Barton & Hewitt, 1985). An alternative explanation is that reproductive isolation evolved in geographical isolation as a consequence of genetic drift or as a fortuitous by-product of divergent selection on other traits (Coyne & Orr, 1989; Fitzpatrick, 2002; Sasa, Chippindale, & Johnson, 1998). The mosaic distribution of the two species in the Central System, with the presence of several sympatric populations but also large areas where the two species do not occur (e.g., eastern Central System and foothills; Figure 1), might have also provided ample opportunity for the evolution of reproductive isolation in geographically separated populations (Fitzpatrick, 2002).

5 | CONCLUSIONS AND FUTURE DIRECTIONS

The results of this study add to the growing body of evidence supporting that speciation-with-gene-flow is more prevalent in nature than formerly acknowledged (Nosil, 2008; Pinho & Hey, 2010; Roux et al., 2016). Our study system is very well suited to study the proximate mechanisms (e.g., reinforcement vs. genetic drift) that might have led to the evolution of reproductive isolation. Future research should focus on analysing mating preferences, phenotypic differentiation and reproductive character displacement (song, courtship behaviour, genitalic structures, etc.) between currently sympatric and allopatric populations of the two species (e.g., Butlin et al., 1991; Hollander, Smadja, Butlin, & Reid, 2013), determining mating success and the viability of offspring through experimental hybridization attempts in the laboratory (e.g., Coyne & Orr, 1989; Hoskin et al., 2005; Saldamando et al., 2005), and applying whole genome or transcriptome sequencing data to detect potential genomic signatures of reinforcement and/or identify loci that might be involved in reproductive isolation (Garner et al., 2018; Hopkins et al., 2012; Roda, Mendes, Hahn, & Hopkins, 2017).

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AUTHOR CONTRIBUTIONS

V.T., A.P. and J.O. conceived and designed the study and analyses. J.O. collected the samples. V.T. performed the laboratory work and analysed the data guided by J.O. V.T. wrote the manuscript with help of J.O., and with inputs from A.P.

DATA AVAILABILITY STATEMENT

Raw Illumina reads have been deposited at the NCBI Sequence Read Archive (SRA) under BioProject PRJNA543714. Input files for all analyses are available for download on Figshare (https://doi.org/10.6084/m9.figshare.12251600).

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