

Original Article

Hybridization and introgression are prevalent in Southern European *Erysimum* (Brassicaceae) species

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Short title: Hybridization and introgression in *Erysimum* species

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ABSTRACT

Background and Aims: Hybridization is a common and important force in plant evolution. One of its outcomes is introgression - the transfer of small genomic regions from one taxon to another by hybridization and repeated backcrossing. This process is believed to be common in glacial refugia, where range expansions and contractions can lead to cycles of sympatry and isolation, creating conditions for extensive hybridization and introgression. Polyploidization is another genome-wide process with a major influence on plant evolution. Both hybridization and polyploidization can have complex effects on plant evolution. However, these effects are often difficult to understand in recently evolved species complexes.

Methods: We combined flow cytometry, transcriptomic and genomic analyses, and pollen-tube growth assays to investigate the consequences of polyploidization, hybridization, and introgression on the recent evolution of several *Erysimum* (Brassicaceae) species from the South of the Iberian Peninsula, a well-known glacial refugium. This species complex differentiated in the last 2Myr, and its evolution has been hypothesized to be determined mainly by polyploidization, interspecific hybridization, and introgression.

Key Results: Our results support a scenario of widespread hybridization involving both extant and “ghost” taxa. Several taxa studied here, most notably those with purple corollas, are polyploids, likely of allopolyploid origin. Moreover, hybridization in this group might be an ongoing phenomenon, as prezygotic barriers appeared weak in many cases.

Conclusions: The evolution of *Erysimum* spp. has been determined by hybridization to a large extent. The adaptive value of such genomic exchanges remains unclear, but our results indicate the importance of hybridization for plant diversification across evolutionary scales.

Keywords: Hybridization, Introgression, Polyploidy, Allopolyploidy, Glacial refugium, Brassicaceae, *Erysimum* spp.

INTRODUCTION

Hybridization is widespread across the tree of life, determining the branching and diversification patterns of many taxonomic groups (Rieseberg and Carney, 1998; Coyne and Orr, 2004; Abbott et al., 2013; Arnold, 2016). Because of its pervasiveness, hybridization has been a subject of research for a long time (Stebbins, 1959; Anderson, 1953; Arnold et al., 1999). However, it is only recently, with the advent of next-generation sequencing, that scientists have started to analyze the dynamics of hybridization at the scale of whole genomes, thus rekindling interest in the evolutionary relevance of this phenomenon. Although the patterns of hybridization remain unexplored for many groups, the renewed research efforts have undoubtedly increased our understanding of the role of hybridization in nature (Payseur and Rieseberg, 2016; Goulet et al., 2017; Taylor and Larson, 2019).

Hybridization is particularly relevant for plant evolution, with many plant species showing hybrid origins (Mallet, 2005; Soltis and Soltis, 2009). The evolutionary outcomes of hybridization may vary widely. Interspecific hybridization can hinder speciation and therefore diversification (Mayr, 1992; Schemske, 2000; Mallet, 2005; Saari and Faeth, 2012; Gómez et al. 2015a), but in other cases, hybridization can actually foster the formation of new species (Rieseberg et al., 2003; Stelkens and Seehausen, 2009) or the introgression of novel genetic variation (by hybridization and repeated backcrossing; Anderson and Hubricht, 1938; Anderson, 1953; Rieseberg and Wendel, 1993). In addition, the fusion of genomes between two hybridizing species can lead to changes in ploidy levels (i.e., allopolyploidization; Soltis et al., 2014). There is evidence that introgression might even span ploidy levels (e.g., gene flow between diploid and tetraploid species of *Senecio*; Chapman and Abbott, 2010), which opens intriguing questions about the interplay of introgression and polyploidization. However, the specifics of how hybridization, introgression, and polyploidization interact to affect the evolution of particular plant groups remain poorly understood. Advancements in genomic sequencing technology and analyses are now making the challenges of characterizing these processes far more feasible, even in recently diverged lineages and taxa.

Erysimum L. is one of the largest genera of the Brassicaceae, comprising more than 200 species (Polatschek, 1986), and has been described as a taxonomically complex genus with a reticulated evolutionary history in which polyploidization may have affected the evolution of some clades (Marhold & Lihová, 2006; Turner, 2006; Abdelaziz, 2013; Muñoz-Pajares, 2013). This genus is distributed mainly in Eurasia, with some species in North America and North Africa (Warwick et al., 2006). Notably, more than a hundred species have been described in the Mediterranean region (Greuter et al., 1986) with particular abundance in the Iberian Peninsula, where twenty-one (Polatschek, 1979; Polatschek, 2014) or twenty-three (Nieto-Feliner, 1993; Mateo et al., 1998) species have been described. Most Iberian *Erysimum* species have yellow flowers, but six have purple corollas (Nieto-Feliner, 1993; Gómez et al., 2015b). Interestingly, previous studies suggested that some purple species may have a recent, hybrid, and allopolyploid origin (Nieto-Feliner, 1992, Nieto-Feliner, 1993; Abdelaziz et al., 2014; Gómez et al. 2014). A history of hybridization could further suggest the possibility that the purple flower color has been transferred across the Iberian clade through hybridization and then maintained by natural selection. This scenario would indicate that introgression and polyploidization are intertwined in this group and might have contributed to the adaptive evolution of *Erysimum* spp.

Here we studied signals of hybridization across six species of *Erysimum* (*E. mediohispanicum*, *E. nevadense*, *E. fitzii*, *E. popovii*, *E. baeticum*, *E. bastetanum*) that inhabit the Baetic Mountains, an important and dynamic glacial refugium (Médail and Diadema, 2009). The evolution of several plant species has been hypothesized to have been affected by speciation and secondary contacts in this region (Médail and Diadema, 2009; Nieto-Feliner, 2011). The repeated expansion and contraction of ranges and the subsequent cycles of sympatry and isolation might have created conditions for extensive hybridization, introgression, and allopolyploid formation. This species group appears to have differentiated relatively rapidly within the last 2Myr (Osuna-Mascaró et al., 2021). Previous authors have hypothesized that this rapid evolution has been strongly affected by polyploidization and

hybridization, as this group spans several ploidy levels, and some species pairs have been reported to produce fertile hybrids (Abdelaziz et al., 2014; Abdelaziz et al., 2021). Species of this group show characteristics that may facilitate ongoing introgression, such as growing in sympatry in some locations and having a generalist pollination system that renders gene flow among different species possible.

The main goal of this study is to disentangle the history of hybridization for the *Erysimum* species complex in the Baetic Mountains. Specifically, we considered both whole-genome effects of hybridization (i.e., the interplay between hybridization and polyploidization) and local, potentially important, introgression of specific genomic regions. Moreover, we also quantified prezygotic barriers among extant taxa to estimate the likelihood of gene flow among them. We test the hypotheses that a) Genomes of this species complex must exhibit signals of multiple hybridization events; b) Some taxa might be allopolyploid, and c) If purple corollas are the product of introgression, hybridization and gene-flow should be detectable, and prezygotic barriers may be weak between (at least some) yellow and purple taxa.

MATERIAL AND METHODS

Plant samples

We studied six species in the genus *Erysimum* collected in the Baetic Mountains, South of Spain (Table 1; Figure 1). Specifically, we sampled three different populations for *E. mediohispanicum* (yellow corollas; Em21, Em39, Em71), *E. nevadense* (yellow corollas; En05, En10, En12), *E. popovii* (purple corollas; Ep16, Ep20, Ep27), *E. bastetanum* (purple corollas; Ebt01, Ebt12, Ebt13), and *E. baeticum* (purple corollas; Ebb07, Ebb10, Ebb12), and one population for *E. fitzii* (yellow corollas; Ef01). Some of these species appear in sympatry in some of the sampled localities (e.g., *E. popovii*, Ep20, and *E. mediohispanicum*, Em39; Table 1). Additionally, we sampled one population of *E. lagascae* (Ela07), an allopatric diploid species with purple corollas inhabiting Central Spain, posited as one potential parental species of the Baetic Mountain species studied here (Nieto-Feliner, 1993). We collected fully

developed flower buds for transcriptomic analyses (five buds from an individual per population) and leaves for flow cytometry (6-10 individuals per population).

Flow cytometry analyses

We used flow cytometry to assess genome size and estimate DNA ploidy levels. Nuclei were isolated from fresh leaf tissues by simultaneously chopping with a razor blade 0.5 cm² of leaf and 0.5 cm² of an internal reference standard (Galbraith et al., 1983). We used *Solanum lycopersicum* L. 'Stupické' with 2C = 1.96 pg or *Raphanus sativus* L. with 2C = 1.11 pg as internal reference standards (Doležal et al., 1992). The nuclei extraction was made on a Petri dish containing 1 ml of WPB buffer (Loureiro et al., 2007). Then, the nuclear suspension was filtered using a 50 µm nylon mesh, and DNA was stained with 50 µg ml⁻¹ of propidium iodide (PI, Fluka, Buchs, Switzerland). Additionally, 50 µg ml⁻¹ of RNase (Fluka, Buchs, Switzerland) was added to degrade dsRNA. After a 5 min incubation, the samples were analyzed in a Sysmex CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW). Results were acquired using FloMax software v2.4d (Partec GmbH, Münster, Germany) in the form of four graphics: histogram of fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. The FL histogram was gated using a polygonal region defined in the FL vs. SS histogram to avoid debris signals. At least 5,000 particles were analyzed per sample. Only CV values of 2C peak of each sample below 5% were accepted; otherwise, a new sample was prepared and analyzed until quality standards were achieved (Greilhuber et al., 2007). In a few cases, samples produced histograms of poorer quality even after repetition due to the presence of cytosolic compounds. Thus, it was impossible to estimate ploidy level and/or genome size for some samples (Table 2).

We obtained the genome size in mass units (2C in pg; sensu Greilhuber et al., 2005) using the formula: sample 2C nuclear DNA content (pg) = (sample G1 peak mean/reference standard G1 peak mean)* genome size of the reference. The ploidy levels were inferred for each sample based on chromosome counts and genome size estimates available for the genus and species.

RNA extraction and sequencing

Details of the sampling, RNA extraction, and sequencing appear in Osuna-Mascaró et al. (2021). In summary, we stored collected flower buds of each individual in liquid nitrogen until RNA extraction. Floral buds were ground with a mortar and a pestle in liquid nitrogen. We used the Qiagen RNeasy Plant Mini Kit following the manufacturer's protocol to isolate total RNA from 17 samples (one individual per population; three populations of *E. baeticum*, *E. bastetanum*, *E. mediohispanicum*, *E. nevadense*, and *E. popovii*, and one population of *E. fitzii* and *E. lagascae*). Then, we checked the quality and quantity of the RNA using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, United States) and agarose gel electrophoresis. Library preparation and RNA sequencing were conducted at Macrogen Inc. (Seoul, Korea). Before sequencing, the quality of the RNA was analyzed again with the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc., Santa Clara, California, United States), and an rRNA-depletion procedure with Ribo-Zero (Illumina, San Diego, California, United States) was used to enrich mRNA content and to avoid the sequencing of rRNA. Library preparation was performed using the TruSeq Stranded Total RNA LT Sample Preparation Kit (Plant). Sequencing of the 17 libraries (one per individual) was carried out using the HiSeq 3000-4000 sequencing protocol and TruSeq 3000-4000 SBS Kit v 3 reagent, following a paired-end 150 bp strategy on the Illumina HiSeq 4000 platform. A summary of sequencing statistics is shown in Table S1 (Supporting Information).

Transcriptome assembly and annotation

Details of the read quality control, trimming, and de novo transcriptome assembly and annotation can be found in Osuna-Mascaró et al. (2021). Briefly, we used FastQC v0.11.5 (Andrews, 2010) to analyze the quality of each library's raw reads. Then, we trimmed the adapters in the raw reads using cutadapt v1.15 (Martin, 2011), and we quality-filtered the reads using Sickle v1.33 (Joshi and Fass, 2011). After trimming, we used FastQC v0.11.5 (Andrews, 2010) again to verify the trimming efficiency. To assemble the resulting high-quality, cleaned reads into contigs, we followed a de novo approach using

Trinity v 2.8.4 (Grabherr et al. 2011). Before assembly, each library was normalized in silico to validate and reduce the number of reads using the "insilico_read_normalization.pl" function in Trinity (Haas et al., 2013). Then we used the parameter 'min_kmer_cov 2' to eliminate single occurrence k-mers heavily enriched in sequencing errors, following Haas et al. (2013). Candidate open reading frames (ORF) within transcript sequences were predicted and translated using TransDecoder v 5.2.0 (Haas et al., 2013). We performed functional annotation of Trinity transcripts with ORFs using Trinotate v 3.0.1 (Haas, 2015). Sequences were searched against UniProt (UniProt Consortium, 2014), using SwissProt databases (Bairoch and Apweiler, 2000) (with BLASTX and BLASTP searching and an e-value cutoff of 10⁻⁵). We also used the Pfam database (Bateman et al., 2004) to annotate protein domains for each predicted protein sequence. Transcripts were filtered through the egglog (Jensen et al., 2007), GO (Gene Ontology Consortium, 2004), and Kegg (Kanehisa and Goto, 2000) annotation databases.

Orthology inference

To reduce redundancy, we clustered the translated sequences using cd-hit v 4.6 (Li and Godzik, 2006), following the steps of the pipeline described in Yang and Smith (2014). For the inference of orthologs, we excluded UTRs and non-coding transcripts, using only coding DNA sequences (CDS) in order to avoid the inclusion of sequencing errors (Yang and Smith, 2014). We identified ortholog genes using the OrthoFinder v 2.3.3 pipeline (Emms and Kelly, 2015). In brief, this pipeline first made a BLASTP analysis with the protein sequences as input for searching the orthogroups (a set of potentially orthologs protein-coding genes derived from a single gene in the last common ancestor of all the species sampled), then clustered and aligned the orthologous sequences using MAFFT v 7.450 (Katoh and Standley, 2013) with default parameters. Finally, we obtained the maximum-likelihood phylogenetic gene trees for all orthogroups using IQ-Tree v 1.6.1 (Nguyen et al., 2014). Then, each orthogroup that contained sequenced from all sampled species was used to infer a species tree using STAG v 1.0.0 (Emms and Kelly, 2019). Then, we used DLCpar v 1.1 (Wu et al., 2014) to reconcile the

species tree with the gene trees, considering gene duplication, losses, and incomplete lineage sorting (ILS) as potential causes of discordance among trees.

Phylogenetic reconstruction

We obtained a coalescent species tree using ASTRAL v 5.6.3 (Mirarab et al., 2014) with default parameters. This method reconstructs a species tree from unrooted gene tree topologies. We used the gene trees previously obtained by maximum likelihood by using IQ-Tree v 1.6.1 as input. We used FigTree v 1.4.0 (Rambaut and Drummond, 2012) to visualize and edit the species tree. Then, we compared the alternative tree topologies with the phylogeny obtained from whole chloroplast genome analyses for the same species (presented in Osuna-Mascaró et al., 2021) using the Shimodaira-Hasegawa Test (SH-Test; Shimodaira and Hasegawa, 1999) from the R package phangorn v 2.5.5 (Schliep, 2011). Both phylogenies were also compared visually, plotting them as mirror images with the function cophyloplot, using the R package ape v 5.4 (Paradis et al. 2004).

Variant calling

We first ran a variant calling analysis, using the *E. lagascae* transcriptome as a reference. We indexed the *E. lagascae* transcriptome using BWA v 0.7.17 (Li and Durbin, 2009) to create a reference and then mapped all the trimmed raw reads to it using the BWA v 0.7.17 "mem" option. We used SAMtools v 1.7 (Li et al., 2009) to convert and sort the alignment files. We then called SNPs using the SAMtools v 1.7 "mpileup" command. Lastly, we used bcftools v 1.9 to filter the SNPs (Narasimhan et al., 2016), running the SAMtools v 1.7 Perl script "vcfutils.pl VarFilter" with default parameters to filter down the candidate variants and to eliminate false positives.

Discriminant Analysis of Principal Components (DAPC)

We conducted a Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010) of the SNP data to group the different genotypes avoiding any prior subjective bias using the R package adegenet v 2.1.3 (Jombart and Ahmed, 2011). DAPC is a multivariate method that identifies and describes clusters of genetically related individuals from large datasets, providing a measure of the

optimal number of genetic clusters (K) across a range of K values by using the Bayesian Information Criterion (BIC). We set a range of K values from two to seven since K=7 is the number of different species in our dataset. The existence of significant hybridization and introgression would result in K < 7. To identify the optimal number of K, we selected the model with the lowest BIC.

Phylogenetic inference of introgression

As a first step to detect introgression events between species pairs, we computed phylogenetic species networks. This approach provides a graphical extension of the phylogenetic tree model, representing the gene flow by edges connecting the OTUS that are likely to be linked by introgression. We used the software PhyloNet v 3.6.9 (Than et al., 2008; Wen et al., 2018), which implements a phylogenetic network method based on the frequencies of rooted trees accounting for incomplete lineage sorting (ILS). To generate the input for PhyloNet, we first ultrametricized the trees obtained previously with IQ-Tree v 1.6.1, using the "nnls" method in the "force.ultrametric" function within the R package phytools v 0.6-99 (Revell, 2012). Due to computational limitations, we inferred the species networks using a maximum pseudo-likelihood method (MPL) (Yu and Nakhleh, 2015). We performed the search five times to avoid getting stuck at local optima. We estimated optimal networks among an optimal computational range of 0 to 15 introgression events, determining the most likely network based on Akaike's Information Criterion (AIC; Bozdogan, 1987) with the generic function for AIC in R package stats v 3.6.1. As AIC may not provide precise values when using pseudo-likelihood phylogenetic networks (Cao et al., 2019), we also estimated the more optimal network by slope heuristic of log-likelihood values. The optimal network was then visualized with Dendroscope v 3.5.10 (Huson and Scornavacca, 2019).

ABBA-BABA statistic

To assess gene flow between species, we calculated D-statistics, also known as the ABBA-BABA statistic (Durand et al., 2011). To evaluate introgression among the seven species, we used the software Dsuite v 0.1 (Malinsky, 2019), which allows the assessment of gene flow across large datasets and

directly from a variant call format (VCF) file. This algorithm computes the D statistic by considering multiple groups of four populations: P1, P2, P3, and O, grouped in asymmetric trees of the form (((P1, P2), P3), O). The site patterns are ordered such that the pattern BBAA refers to P1 and P2 sharing the derived allele (B-derived allele, A-ancestral allele), ABBA to P2 and P3 sharing the derived allele, and BABA to P1 and P3 sharing the derived allele. The ABBA and BABA patterns are expected to occur with equal frequencies, assuming no gene flow (null hypothesis), while a significant deviation from that suggests possible introgression. To assess whether D is significantly different from zero, Dsuite uses a standard block-jackknife procedure (Green et al., 2010; Durand et al., 2011), obtaining approximately normally distributed standard errors. As recommended by Malinsky (2019), we used a conservative approach estimating the statistic Dmin, which gives the lowest D-statistic value in a given trio. We used the ruby script "plot_d.rb" to plot into a heatmap the introgression among all the pairs of samples. To complement these analyses, we computed the Fbranch statistic implemented in Dsuite v 0.1 (Mallinsky et al., 2018, Mallinsky et al., 2019). The statistic allows the identification of gene flow events within specific internal branches of a phylogeny. Thus, evaluating the excess sharing of alleles between one species and the descendant or ancestral species, helping to understand when the gene flow happened. We used the whole chloroplast genomes phylogeny from Osuna-Mascaró et al. (2021) in Newick format to establish a reference phylogeny and specify which species could be more accurately treated as sister species (i.e., as P1 and P2) while always using *E. lagascae* as an outgroup.

Pollen tube growth

The existence of prezygotic barriers can fully impede interspecific hybridization. Therefore, the existence of such barriers may indicate that gene flow across a given set of species is highly unlikely, while the lack of such barriers may indicate plausible hybridization and introgression. To explore the existence of prezygotic barriers, we carried out a preliminary experiment on the growth of pollen tubes on a reduced set of co-occurring species (Table 1). We collected 20 individual plants of each *E. mediohispanicum*, *E. bastetanum*, and *E. popovii* from natural populations. We grew the plants in a

common garden (University of Granada facilities) and moved them into a greenhouse before flowering to exclude pollinators. When the flowers opened, we performed hand-pollination experiments by tipping the anther with a small stick to remove the pollen and placing it on the stigma of a flower from different species previously emasculated (hybrid crosses) or of a flower from the same species but different populations previously emasculated (intra-specific crosses). Moreover, we emasculated some flowers and hand-pollinated them with their own pollen (forced selfing crosses), and some flowers were not manipulated and left for spontaneous self-pollination (spontaneous selfing crosses).

We collected the pistils after 72 hours and preserved them in ethanol at 4°C until staining of pollen tubes, following the Mori et al. (2006) protocol with minor modifications. In brief, each pistil was cleaned in 70% EtOH for ten minutes and then moved to 50% EtOH, 30% EtOH, and finally distilled water. We softened the samples by placing them into a small petri dish of 8 M NaOH for one hour at room temperature (as recommended by Kearns and Inouye, 1993). Then, we transferred the pistils to distilled water for ten minutes, and afterward, the stigmas were incubated with 0.1 % aniline blue in phosphate buffer (pH 8.3) for two hours. The final slide preparations were examined under a fluorescence microscope with blue light (410 nm) to observe and measure pollen tube development.

RESULTS

Ploidy levels

Flow cytometry revealed a wide variation in genome size and, therefore, in DNA ploidy levels across but also within species (Table 2). We found that all samples of *E. fitzii* and *E. nevadense* were diploid. The other species with yellow corollas, *E. mediohispanicum*, also appeared to be predominantly diploid, although the Em71 population deviated from this pattern being tetraploid. The genome size of *E. lasgacae* also corresponded to that of a diploid, while the other purple corolla species showed ploidy levels higher than diploidy (Table 2). Moreover, ploidy levels differed across populations in two of these species. Populations of *E. bastetanus* varied between 4x and 8x, while in *E. popovii*, the range

was even greater, from 4x to 10x. In three cases (Ebb12, En05, and En10; Table 2), it was not possible to establish the ploidy level of the samples, and we used those reported in Blanca et al. (1992).

Transcriptome assembly and orthology inference

The sequencing results and the corresponding summary statistics of the assembled transcriptomes can be found in Osuna-Mascaró et al. (2021). In summary, we obtained between 104K and 382K different Trinity transcripts, producing between 66K and 235K Trinity isogenes. The total assembled bases ranged from 92 Mbp (in the Em21 population of *E. mediohispanicum*) to 319 Mbp (in En10 population of *E. nevadense*). The number of annotated unigenes ranged between 71,606 (*E. nevadense*, En12) and 197,069 (*E. baeticum*, Ebb10); mean value 146,314.35. The highest proportion of annotated unigenes was obtained using BLASTX to search against the SwissProt reference database. Details of the annotated unigenes using different protein databases can be found in Osuna-Mascaró et al. (2021). OrthoFinder assigned 1,519,064 protein gene sequences (96.4% of total) to 92,984 gene families (orthogroups) (Table S2). Among them, 16,941 orthogroups were shared by all species, and their corresponding gene trees were used for further analyses.

Phylogenetic trees and population clustering

We inferred a coalescence tree using the 16,941 maximum likelihood gene trees obtained with IQ-Tree as input for ASTRAL (Figures 2 and S1). This species tree was nearly fully resolved with high support, having only four nodes with low quartet scores results (posterior probabilities for these nodes: 0.78, 0.77, 0.70, and 0.53; see Figure S1). In this tree, rooted with *E. lagascae*, the 4× population of *E. mediohispanicum* (yellow corollas; Em71) appeared as the first branching OTU. Three clades, although with low supports, were evident. A clade formed by *E. bastetanum* and *E. baeticum*, both species were having purple corollas; another clade including *E. fitzii* (yellow corollas) and the three populations of *E. popovii* (purple corollas); and the last clade including the populations of *E. nevadense* and the 2x populations of *E. mediohispanicum*, both species with yellow corolla. Although there is some species clustering, not all species appear to be monophyletic, supporting a history of hybridization. Moreover,

when comparing the species tree with the whole chloroplast genomes phylogeny (Figure 2), we find clear cytonuclear discordances resulting in a significant SH test result (Diff -ln L= 345426.4, p-value < 0.01). This lack of congruence among both phylogenies also supports the hybridization hypothesis.

The discriminant analysis revealed K=4 and K=5 as the most likely number of genetic clusters (Figure 3), both with very similar BIC values (K=4, BIC= 189.99; K=5, BIC= 188.99). The clusters corresponding to K=4 produced the same clusters that appeared in the coalescence tree (Figure 2). However, the clusters corresponding to K=5 included three monospecific groups (for *E. lagascae* -purple corollas-, *E. fitzii* -yellow corollas-, and *E. popovii* -purple corollas-), one for the diploid species with yellow corollas (the three populations of *E. nevadense* and the diploid populations of *E. mediohispanicum*), and the last including all the populations of *E. baeticum* (purple corollas), *E. bastetanum* (purple corollas), *E. popovii* (purple corollas) and Em71, the 4x population of *E. mediohispanicum* (yellow corollas).

Analysis of introgression

The network with 13 reticulation instances appeared as the most reliable based on the AIC values for the log-likelihood of the networks (Table S3). The estimates of slope heuristic of log-likelihood values also supported the network with 13 reticulation instances as the most reliable network estimated. This network shows frequent hybridization events in the genealogy of these populations involving yellow and purple species (Figure 4), as indicated by the edges connecting tree branches between different populations and species. Notably, this network includes edges connecting non-terminal branches (see Figure 4), which indicates reticulations with past extinct taxa (i.e., “ghost species”) or incomplete sampled taxa.

The ABBA-BABA analyses support this scenario of frequent hybridization, even using a conservative approach (D-min). We summarized the tested topologies and the inferred D-statistics with corrected p-values for all triplet combinations in Table S4 and Figure S2. The highest signal of introgression occurred between *E. fitzii* (yellow corollas) and *E. baeticum* (purple corollas; populations

Ebb12 and Ebb10) and *E. popovii* (purple corollas; Ep16); and between *E. popovii* (purple corollas; Ep16) and *E. bastetatum* (purple corollas; Ebt12) and *E. baeticum* (purple corollas; Ebb07, Ebb10, Ebb12). There was also evidence of interspecific gene flow as manifested by the fbranch statistic (Figure S3) that identifies gene flow events into specific internal branches of the phylogeny while accounting for potential false-positive results due to correlated introgression signatures among closely-related species. Specifically, we found the highest signal of gene flow between *E. bastetatum* (purple corollas; Ebt12) and *E. mediohispanicum* (yellow corollas; Em71, Em21), *E. nevadense* (yellow corollas; En12, En05), and *E. baeticum* (purple corollas; Ebb07, Ebb10); between *E. bastetatum* (purple corollas; Ebt13) and *E. mediohispanicum* (yellow corollas; Em71) and *E. popovii* (purple corollas; Ep20); between *E. bastetatum* (purple corollas; Ebt01) and *E. mediohispanicum* (yellow corollas; Em21, Em39) and *E. nevadense* (yellow corollas; En12). In addition, we have detected other gene flow events with ancestral or non-sampled taxa (Figure S3).

Pollen tube growth

A total of 103 preparations of *Erysimum* pistils were examined: 52 from hybrid crosses, 24 from forced selfing crosses, 16 from spontaneous selfing crosses, and 11 from intra-specific crosses (Table S5). Our results showed full growth of pollen tubes (i.e., reaching the ovary) in 63.33 % of intra-specific crosses, 51.92 % of hybrid crosses ($\chi^2 = 0.50$, p-value = 0.513 compared with intra-specific crosses), and only in 29.16 % of forced selfing crosses ($\chi^2 = 3.73$, p-value = 0.074), and in 25.16 % of spontaneous selfing crosses ($\chi^2 = 4.03$, p-value = 0.057). Although these last values were not significant, when selfing classes were pooled, it showed a significant reduction in the growth of pollen tubes ($\chi^2 = 4.93$, p-value = 0.039) (Figure S4). Cases in which pollen tubes grew but did not reach the ovary were treated as non-growing. In these cases, we could not estimate whether tube growth had completely stopped or if it was ongoing but developed too slowly to reach the ovary during the duration of the experiment.

DISCUSSION

Our results suggest that the *Erysimum* species studied here have a strong signature of hybridization and introgression in their genomes. This result is supported by the pollen tube growth experiments that showed that pollen tubes could grow all the way to the ovary in some hybrid crosses, indicating very weak or non-existent prezygotic barriers. Moreover, we found that species with purple flowers are polyploid and have a strong signature of introgression, suggesting an allopolyploid origin. We also found a hybridization signature in the (mostly diploid) yellow species, indicating that hybridization occurred across both colors and ploidy levels.

Several phylogenetic reconstructions have been performed for western Mediterranean *Erysimum* species (e.g., Abdelaziz et al. 2014; Gómez et al. (2014, 2015); Züst et al. (2020)) that have used different strategies (several populations per species or only one representative per species; several nuclear and cytoplasmic sequences or NGS transcriptomic data). Abdelaziz et al. (2014) found that populations of *E. mediohispanicum* and *E. bastetanum*, species analyzed here, did not appear as monophyletic (with some populations placed within other branches of the phylogeny), which was indicative that introgression probably produced important reticulation at the population level. Our analyses support this hypothesis. The reticulate nature of these phylogenies imposes some caution in interpreting phylogenies based on only a few nuclear or cytoplasmic sequences, as suggested by Chan & Levin (2005). In these cases, major divisions may reflect the reality of some old phylogenetic splits. However, it will be challenging for more recent speciation events to obtain a clear picture of the phylogeny without interrogating complete genomes or transcriptomes.

Overall, our results support a hybrid origin for the purple polyploid *Eysimum* Iberian species, as suggested in previous studies (Nieto-Feliner, 1993; Abdelaziz et al., 2014; Gómez et al., 2015b; Osuna-Mascaró, 2020). In particular, we found support for *E. popovii* (purple corollas and polyploid) and *E. fitzii* (yellow corollas and diploid) as sister species. Also, the genome of *E. popovii* exhibited signatures of a hybridization process in which *E. fitzii* may have been involved. The possible hybrid origin of *E.*

popovii with *E. fitzii* as a potential parental taxon was previously proposed by Nieto-Feliner (1993) based on morphology. Similarly, a hybrid origin of *E. baeticum* (purple corollas and polyploid) had been previously suggested, in this case with *E. nevadense* (yellow corollas and diploid) implicated as a potential parent (Nieto-Feliner, 1992). Our results showed that these two species appear closely related, and *E. baeticum* may have had an introgression signature of *E. nevadense*. Moreover, our results also suggested a complex scenario for *E. bastetanum* (purple corollas and polyploid), which appears closely related to *E. baeticum* (purple corollas and polyploid). In fact, *E. bastetanum* has been considered a subspecies of *E. baeticum* until recently (Lorite et al., 2014). Therefore, the general pattern that emerged from our results is that these purple species are polyploids of hybrid origin, descending from crosses between an unidentified parent and some diploid, often yellow taxon.

However, our results also suggested a complex evolutionary history for the mostly diploid yellow species. The contributing lineages also often involve unidentified taxa. This might be attributable to insufficient sampling, as we did not include some *Erysimum* species (*E. rondae*, *E. myriophyllum*, -yellow corollas-, and *E. cazorlense* -purple) that also inhabit the Baetic Mountains, although with a limited distribution (Nieto-Feliner, 1993). At this point, it is impossible to establish whether these taxa may have acted as a source of introgression. In any case, our results show that hybridization and introgression are major contributors to the evolutionary history of this species complex, deserving further research.

Interestingly, we did not find a consistent, predictable pattern of hybridization for most species. Populations of the same species showed differences in their hybridization history, as shown by the ABBA-BABA test (which detected multiple and diverse introgression events) and the PhyloNet reconstructions (which yielded a tree with 13 reticulations as the most optimal network). In the same vein, the DAPC results did not support a scenario with populations clustered by species. Our results are similar to those of previous studies describing asymmetric hybridization patterns as a consequence of differences in ecological pressures across populations and geographical areas (Payton et al., 2019; Sujii

et al., 2019; Wang et al., 2019). At this stage, we cannot unambiguously identify any ecological factor behind the asymmetries we detected. However, we did observe variation in pollinators' preferences and flowering time across populations, which might lead to local differences in gene-flow patterns. Thus, to fully understand this asymmetry in hybridization and why some populations have more introgression signatures than others, future studies considering different ecological pressures for these species and including pollinator censuses of wild populations are required.

Evidence of hybridization between at least some of these species has been reported previously (Abdelaziz 2014). Thus, *E. mediodispanicum* and *E. nevadense* show a hybrid zone in a sector of the Spanish Sierra Nevada (Abdelaziz et al., 2021). Pollinators do not appear to constitute strong pre-pollinating barriers since all of these species are extreme generalists and share most pollinators (Gómez et al. 2015b). Moreover, we have found that prezygotic, post-pollination barriers may not be effective since pollen tubes are often growing in hybrid crosses. Contemporary gene flow between different cytotypes of *E. mediodispanicum* seems negligible, as evidenced by an almost complete absence of triploids and other minority cytotypes in the contact zone between tetraploid and diploid populations of this species (Muñoz-Pajares et al. 2018). Historical dynamics of genetic isolation and sympatry might have also played a role (Albaladejo and Aparicio, 2007; Rifkin et al., 2019; Zielinski et al., 2019). These *Erysimum* species are located in a well-known glacial refugium (Médail and Diadema, 2009; Hughes and Woodward, 2017), and thus, the isolation and then re-establishment of gene flow (i.e., secondary contact zones) among populations of different species may have favored locally specific hybridization patterns (Coyne, 2004; Harrison and Larson, 2014; Arnold, 2015). A better knowledge of the historical dynamics of species and populations and past ranges overlap is required to fully understand the genomic pattern of divergence between closely related species. For instance, combining macroecological methods with niche models and phylogenetic approaches could clarify the opportunity for hybridization through evolutionary time (Folk et al., 2018; Aguirre-Liguori et al., 2021).

Furthermore, we detected signatures of ghost introgression, implying that ancestral species have influenced the hybridization history of these *Erysimum* species. This result was first evidenced by cytonuclear discordance, which might be due to past organellar introgression from extinct species (Huang et al., 2014; Folk et al., 2017; Lee-Yaw et al., 2019). We also found a clear signature of ancestral introgression in the phylogenetic species network, in which some of the reticulations appeared from introgression involving "ghost" taxa. Similarly, the fbranch statistic identified gene flow events in internal branches that concurred with introgression with ghost species. Specifically, we observed that some ancestral form of *E. popovii* (purple corollas and polyploid) could have been related to *E. fitzii* (yellow corollas and polyploid). Also, we detected evidence of gene flow between an ancestor of *E. mediohispanicum* (yellow corollas and diploid; Em21), *E. bastetanum* (purple corollas and polyploid), and *E. baeticum* (purple corollas and polyploid). Moreover, the results showed that many past gene flow events could have occurred between *E. baeticum* (purple corollas and polyploid), *E. nevadense* (yellow corollas and diploid), and *E. bastetanum* (purple corollas and polyploid). In light of these results, it seems that some unidentified ancestral species played a role as introgression sources for both the purple and yellow species. However, as previously noticed, we include only a subset of the Iberian *Erysimum* species in this study; accordingly, we may be mistaking the signal of the unsampled species for that of ancestral taxa. Further research about the ghost introgression's influence on *Erysimum* evolution, including all the Iberian species and high-quality genome assemblies, would be required to understand the hybridization history thoroughly.

CONCLUSIONS

Our results indicate that complex evolutionary dynamics have shaped present-day Iberian *Erysimum* diversity. The genomes of extant taxa are the product of multiple polyploidizations, hybridization, and introgression events. Understanding these multi-faceted processes and their interplay is crucial to characterize the evolution of *Erysimum* spp. and probably, of angiosperms in general. Although the evolution of the Iberian *Erysimum* might have been particularly dynamic, this group could be

representative of the evolutionary response of multi-species complexes to drastic environmental fluctuations. Further research that incorporates a wider taxonomic sample, whole-genome sequences, and complex demographic and evolutionary statistical methods is needed to precisely characterize the patterns described here.

Author contributions

COM, RR, JMG, and FP conceived and designed the study. COM analyzed the data with the help of FP, JL, and RH. JL and SC performed the flow cytometry analyses. COM wrote the first draft. The final version of the M.S. was redacted with the contribution of all the authors.

Acknowledgments

The authors thank Modesto Berbel Cascales, Tatiana López Pérez, Mercedes Sánchez Cabrera, Raquel Sánchez Fernández, Javier Valverde, and Mohamed Abdelaziz, for their help in the lab and fieldwork. Thanks to Pamela Soltis and Douglas Soltis for their help during the first steps of this work. The authors thank the Sierra Nevada National Park headquarters for providing the permits to work in the National Park.

Funding information

This research is supported by grants from FEDER/Junta de Andalucía-Consejería de Economía y Conocimiento A-RNM-505-UGR18 and P18-FR-3641. This research was also funded by the Spanish Ministry of Science and Innovation (CGL2016-79950-R, CGL2017-86626-C2-2-P), including EU FEDER funds. COM was supported by the Ministry of Economy and Competitiveness (BES-2014-069022).

LITERATURE CITED

Abbott R, Albach D, Ansell S, et al. 2013. Hybridization and speciation. *Journal of Evolutionary Biology* 26: 229-246.

501 Abdelaziz M. 2013. How species are evolutionarily maintained? Pollinator-mediated divergence and
502 hybridization in *Erysimum mediohispanicum* and *Erysimum nevadense*. PhD Thesis,
503 Universidad de Granada, Spain.

504 Abdelaziz M, Muñoz-Pajares AJ, Lorite J, Herrador MB, Perfectti F, Gómez Reyes JM. 2014.
505 Phylogenetic relationships of *Erysimum* (Brassicaceae) from the Baetic Mountains (se Iberian
506 peninsula). *Anales del Jardín Botánico de Madrid* 71: e005.

507 Abdelaziz M, Muñoz-Pajares AJ, Berbel M, García-Muñoz A, Gómez JM, Perfectti F. 2021.
508 Asymmetric reproductive barriers and gene flow promote the rise of a stable hybrid zone in the
509 Mediterranean high mountain. *Frontiers in Plant Science* 12.

510 Aguirre-Liguori JA, Ramírez-Barahona S, Gaut BS. 2021. The evolutionary genomics of species'
511 responses to climate change. *Nature Ecology & Evolution* 5: 1-11.

512 Ai H, Fang X, Yang B, et al. 2015. Adaptation and possible ancient interspecies introgression in pigs
513 identified by whole-genome sequencing. *Nature Genetics* 47: 217-225.

514 Albaladejo RG, Aparicio A. 2007. Population genetic structure and hybridization patterns in the
515 Mediterranean endemics *Phlomis lychnitis* and *P. crinita* (Lamiaceae). *Annals of Botany* 100:
516 735-746.

517 Anderson E. 1953. Introgressive hybridization. *Biological Reviews* 28: 280-307.

518 Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data.

519 Arnold ML, Bulger MR, Burke JM, Hempel AL, Williams JH. 1999. Natural hybridization: how low
520 can you go and still be important?. *Ecology* 80: 371-381.

521 Arnold ML. 2015. Divergence with genetic exchange. OUP Oxford.

522 Arnold BJ, Lahner B, Da Costa JM, et al. 2016. Borrowed alleles and convergence in serpentine
523 adaptation. *Proceedings of the National Academy of Sciences* 113: 8320-8325.

524 Bairoch A, Apweiler R. 2000. The SWISS-PROT protein sequence database and its supplement
525 TrEMBL in 2000. *Nucleic Acids Research* 28: 45-48.

526 Barlow A, Cahill JA, Hartmann S, et al. 2018. Partial genomic survival of cave bears in living brown
527 bears. *Nature Ecology & Evolution* 2: 1563-1570.

528 Bateman A, Coin L, Durbin R, et al. 2004. The Pfam protein families database. *Nucleic Acids Research*
529 32: D138-D141.

530 Bozdogan H. 1987. Model selection and Akaike's information criterion (AIC): The general theory and
531 its analytical extensions. *Psychometrika* 52: 345-370.

532 Cao Z, Liu X, Ogilvie HA, Yan Z, Nakhleh L. 2019. Practical aspects of phylogenetic network analysis
533 using phylonet. *BioRxiv* doi: <https://doi.org/10.1101/746362>.

534 Chan KM, Levin SA. 2005. Leaky prezygotic isolation and porous genomes: rapid introgression of
535 maternally inherited DNA. *Evolution* 59: 720-729.

536 Chapman MA, Abbott RJ. 2010. Introgression of fitness genes across a ploidy barrier. *New Phytologist*
537 186: 63-71.

538 Coyne JA, Orr HA. 2004. Speciation Sinauer Associates. *Sunderland MA* 276: 281.

539 Doležal J, Sgorbati S, Lucretti S. 1992. Comparison of three DNA fluorochromes for flow cytometric
540 estimation of nuclear DNA content in plants. *Physiologia Plantarum* 85: 625-631.

541 Doležal J, Greilhuber J, Suda J. 2007. Flow cytometry with plant cells: analysis of genes, chromosomes
542 and genomes. John Wiley & Sons.

543 Durand EY, Patterson N, Reich D, Slatkin M. 2011. Testing for ancient admixture between closely
544 related populations. *Molecular Biology and Evolution* 28: 2239-2252.

Emms, D. M., & Kelly, S. (2015). OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. *Genome Biology* 16: 157.

Emms DM, Kelly S. 2019. OrthoFinder: phylogenetic orthology inference for comparative genomics. *Genome Biology* 20: 1-14.

Folk RA, Mandel JR, Freudenstein JV. 2017. Ancestral gene flow and parallel organellar genome capture result in extreme phylogenomic discord in a lineage of angiosperms. *Systematic Biology* 66: 320-337.

Folk RA, Visger CJ, Soltis PS, Soltis DE, Guralnick RP. 2018. Geographic range dynamics drove ancient hybridization in a lineage of angiosperms. *The American Naturalist* 192: 171-187.

Galbraith DW, Harkins KR, Maddox JM, et al. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220: 1049-1051.

Gene Ontology Consortium. 2004. The Gene Ontology (GO) database an informatics resource. *Nucleic Acids Research* 32: D258-D261.

Gokhman D, Mishol N, de Manuel M, et al. 2019. Reconstructing denisovan anatomy using DNA methylation maps. *Cell*, 179: 180-192.

Gómez JM, González-Mejías A, Lorite J, Abdelaziz M, Perfectti F. 2015a. The silent extinction: Climate change and the potential for hybridization-mediated extinction of endemic high-mountain plants. *Biodiversity and Conservation* 24: 1843–1857.

Gómez JM, Perfectti F, Lorite J. 2015b. The role of pollinators in floral diversification in a clade of generalist flowers. *Evolution* 69: 863-878.

Gómez JM, Perfectti F, Klingenberg CP. 2014. The role of pollinator diversity in the evolution of corolla-shape integration in a pollination-generalist plant clade. *Philosophical Transactions B* 369: 1–11

568 Goulet BE, Roda F, Hopkins R. 2017. Hybridization in plants: old ideas, new techniques. *Plant*
569 *Physiology*, 173: 65-78.

570 Grabherr MG, Haas BJ, Yassour M, et al. 2011. Full-length transcriptome assembly from RNA-Seq
571 data without a reference genome. *Nature Biotechnology* 29: 644.

572 Green RE, Krause J, Briggs AW, et al. 2010. A draft sequence of the Neandertal genome. *Science* 328:
573 710-722.

574 Greilhuber J, Doležel J, Lysak MA, Bennett MD. 2005. The origin, evolution and proposed
575 stabilization of the terms ‘genome size’ and ‘C-value’ to describe nuclear DNA contents. *Annals*
576 *of Botany* 95: 255-260.

577 Greuter W, Burdet HM, Long G. 1986. Dicotyledones (Convolvulaceae-Labiatae). *Med-Checklist* 3:
578 106-116.

579 Haas BJ, Papanicolaou A, Yassour D, et al. 2013. *De novo* transcript sequence reconstruction from
580 RNA-Seq using the Trinity platform for reference generation and analysis. *Nature Protocols* 8:
581 1494.

582 Haas BJ. 2015. Trinotate: transcriptome functional annotation and analysis.

583 Hanušová K, Ekrt L, Vit P, Kolář F, Urfus T. 2014. Continuous morphological variation correlated with
584 genome size indicates frequent introgressive hybridization among *Diphasiastrum* species
585 (Lycopodiaceae) in Central Europe. *PLoS One* 9: e99552.

586 Harrison RG, Larson EL. 2014. Hybridization, introgression, and the nature of species boundaries.
587 *Journal of Heredity* 105: 795-809.

588 Huang DI, Hefer CA, Kolosova N, Douglas CJ, Cronk QC. 2014. Whole plastome sequencing reveals
589 deep plastid divergence and cytonuclear discordance between closely related balsam poplars,
590 *Populus balsamifera* and *P. trichocarpa* (Salicaceae). *New Phytologist* 204: 693-703.

591 Hughes PD, Woodward JC. 2017. Quaternary glaciation in the Mediterranean mountains: a new
592 synthesis. *Geological Society London Special Publications* 433: 1-23.

593 Huson DH, Scornavacca C. 2019. User Manual for Dendroscope V 3.6.2.

594 Jensen LJ, Julien P, Kuhn M, et al. 2007. eggNOG: automated construction and annotation of
595 orthologous groups of genes. *Nucleic Acids Research* 36: D250-D254.

596 Jombart T, Devillard S, Balloux F. 2010. Discriminant analysis of principal components: a new method
597 for the analysis of genetically structured populations. *BMC Genetics* 11: 94.

598 Jombart T, Ahmed I. 2011. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data.
599 *Bioinformatics* 27: 3070-3071.

600 Joshi NA, Fass JN. 2011. Sickel: A sliding-window, adaptive, quality-based trimming tool for FastQ
601 files (Version 1.33) [Software].

602 Kanehisa M, Goto S. 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*
603 28: 27-30.

604 Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements
605 in performance and usability. *Molecular Biology and Evolution* 30: 772-780.

606 Kuhlwilm M, Han S, Sousa VC, Excoffier L, Marques-Bonet T. 2019. Ancient admixture from an
607 extinct ape lineage into bonobos. *Nature Ecology & Evolution* 3: 957-965.

608 Lee-Yaw JA, Grassa CJ, Joly S, Andrew RL, Rieseberg LH. 2019. An evaluation of alternative
609 explanations for widespread cytonuclear discordance in annual sunflowers (*Helianthus*). *New*
610 *Phytologist* 221: 515-526.

611 Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or
612 nucleotide sequences. *Bioinformatics* 22: 1658-1659.

613 Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows–Wheeler transform.
614 *Bioinformatics* 25: 1754-1760.

615 Li H, Handsaker B, Wysoker A, et al. 2009. The sequence alignment/map format and SAMtools.
616 *Bioinformatics* 25: 2078-2079.

617 Liu L, Bosse M, Megens HJ, et al. 2019. Genomic analysis on pygmy hog reveals extensive
618 interbreeding during wild boar expansion. *Nature Communications* 10: 1-9.

619 Loureiro J, Rodriguez E, Doležal J, Santos C. 2007. Two new nuclear isolation buffers for plant
620 DNA flow cytometry: a test with 37 species. *Annals of Botany* 100: 875-888.

621 Malinsky M, Matschiner M, Svardal H. 2021. Dsuite-Fast D-statistics and related admixture evidence
622 from VCF files. *Molecular Ecology Resources* 21: 584-595.

623 Mallet J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology & Evolution* 20: 229-
624 237.

625 Mateo G, Villalba MBC, Udias SL. 1998. Acerca del orófito minusvalorado de la Sierra de Javalambre
626 (Teruel). *Flora Montiberica* 9: 41-45.

627 Marhold K, Lihová J. 2006. Polyploidy, hybridization and reticulate evolution: lessons from the
628 Brassicaceae. *Plant Systematics and Evolution* 259: 143-174.

629 Martin M. 2011. Cutadapt removes adapter sequences from highthroughput sequencing reads. *EMBnet*
630 *J* 17: 10–12.

631 Mayr E. 1992. A local flora and the biological species concept. *American Journal of Botany* 79: 222-
632 238.

633 Médail F, Diadema K. 2009. Glacial refugia influence plant diversity patterns in the Mediterranean
634 Basin. *Journal of Biogeography* 36: 1333-1345.

635 Meyer M, Kircher M, Gansauge MT, et al. 2012. A high-coverage genome sequence from an archaic
636 Denisovan individual. *Science* 338: 222-226.

637 Mirarab S, Reaz R, Bayzid MS, Zimmermann T, Swenson MS, Warnow T. 2014. ASTRAL:
638 genome-scale coalescent-based species tree estimation. *Bioinformatics* 30: i541-i548.

- Mori T, Kuroiwa H, Higashiyama T, Kuroiwa T. 2006. Generative Cell Specific 1 is essential for angiosperm fertilization. *Nature Cell Biology* 8: 64-71.
- Narasimhan V, Danecek P, Scally A, Xue Y, Tyler-Smith C, Durbin R. 2016. BCFtools/RoH: a hidden Markov model approach for detecting autozygosity from next-generation sequencing data. *Bioinformatics* 32: 1749-1751.
- Nieto-Feliner G. 1992. Los " *Erysimum*" orófilos nevadenses de flor amarilla y purpúreo-violácea: ¿son coespecíficos?. *Anales del Jardín Botánico de Madrid* 50: 272-274.
- Nieto-Feliner G. 1993. *Erysimum* L. In: Flora iberica. Vol. IV. Cruciferae-Monotropaceae 48–76.
- Nieto-Feliner G. 2011. Southern European glacial refugia: a tale of tales. *Taxon* 60: 365-372.
- Muñoz-Pajares AJ. 2013. *Erysimum mediohispanicum* at the evolutionary crossroad: phylogeography, phenotype, and pollinators. Phd Thesis, Universidad de Granada, Spain.
- Muñoz-Pajares AJ, Perfectti F, Loureiro J, et al. 2018. Niche differences may explain the geographic distribution of cytotypes in *Erysimum mediohispanicum*. *Plant Biology* 20: 139-147.
- Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. 2014. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution* 32: 268-274.
- Paradis E, Claude J, Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* 20: 289-290.
- Payseur BA, Rieseberg LH. 2016. A genomic perspective on hybridization and speciation. *Molecular Ecology* 25: 2337-2360.
- Payton AC, Naranjo AA, Judd W, Gitzendanner M, Soltis PS, Soltis DE. 2019. Population genetics, speciation, and hybridization in *Dicerandra* (Lamiaceae), a North American Coastal Plain endemic, and implications for conservation. *Conservation Genetics* 20: 531-543.

- Polatschek A. 1978. Die arten der gattung *Erysimum* auf der Iberischen Halbinsel. *Annalen des Naturhistorischen Museums in Wien* 325-362.
- Polatschek A. 1986. *Erysimum*. In Strid A. [ed.], Mountain flora of Greece, 1, 239–247. Cambridge University Press, Cambridge, UK.
- Polatschek A. 2014. Revision der gattung *Erysimum* (Cruciferae): Nachträge zu den bearbeitungen der Iberischen. Halbinsel und Makaronesiens. *Annalen des Naturhistorischen Museums in Wien. Serie B für Botanik und Zoologie* 87-105.
- Rambaut A, Drummond AJ. 2012. FigTree version 1. 4. 0.
- Rendón-Anaya M, Montero-Vargas JM, Saburido-Álvarez S, et al. 2017. Genomic history of the origin and domestication of common bean unveils its closest sister species. *Genome Biology* 18: 60.
- Revell LJ. 2012. phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution* 3: 217-223.
- Rieseberg LH, Wendel JF. 1993. Introgression and its consequences in plants. *Hybrid Zones and the Evolutionary Process* 70: 109.
- Rieseberg LH, Carney SE. 1998. Plant hybridization. *The New Phytologist* 140: 599-624.
- Rieseberg LH, Raymond O, Rosenthal DM, et al. 2003. Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* 301: 1211-1216.
- Rifkin JL, Castillo AS, Liao IT, Rausher MD. 2019. Gene flow, divergent selection and resistance to introgression in two species of morning glories (Ipomoea). *Molecular Ecology* 28: 1709-1729.
- Saari S, Faeth SH. 2012. Hybridization of Neotyphodium endophytes enhances competitive ability of the host grass. *New Phytologist* 195: 231-236.
- Schemske DW. 2000. Understanding the Origin of Species 1. *Evolution* 54: 1069-1073.
- Schliep KP. 2011. phangorn: phylogenetic analysis in R. *Bioinformatics* 27: 592-593.

- Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* 16: 1114-1114.
- Soltis PS, Soltis DE. 2009. The role of hybridization in plant speciation. *Annual Review of Plant Biology* 60: 561-588.
- Soltis DE, Visger CJ, Soltis PS. 2014. The polyploidy revolution then and now: Stebbins revisited. *American Journal of Botany* 101: 1057-1078.
- Stebbins GL. 1959. The role of hybridization in evolution. *Proceedings of the American Philosophical Society* 103: 231-251.
- Stelkens R, Seehausen O. 2009. Genetic distance between species predicts novel trait expression in their hybrids. *Evolution: International Journal of Organic Evolution* 63: 884-897.
- Suarez-Gonzalez A, Hefer CA, Christe C, Corea O, Lexer C, Cronk QC, Douglas CJ. 2016. Genomic and functional approaches reveal a case of adaptive introgression from *Populus balsamifera* (balsam poplar) in *P. átrichocarpa* (black cottonwood). *Molecular Ecology* 25: 2427-2442.
- Suarez-Gonzalez A. 2017. Adaptive introgression from *Populus balsamifera* (balsam poplar) in *P. trichocarpa* (black cottonwood), PhD Thesis, University of British Columbia, Canada.
- Suarez-Gonzalez A, Lexer C, Cronk QC. 2018. Adaptive introgression: a plant perspective. *Biology Letters* 14: 20170688.
- Sujii PS, Cozzolino S, Pinheiro F. 2019. Hybridization and geographic distribution shapes the spatial genetic structure of two co-occurring orchid species. *Heredity* 123: 458-469.
- Sušnik S, Weiss S, Odak T, Delling B, Treer T, Snoj A. 2007. Reticulate evolution: ancient introgression of the Adriatic brown trout mtDNA in softmouth trout *Salmo obtusirostris* (Teleostei: Salmonidae). *Biological Journal of the Linnean Society* 90: 139-152.
- Taylor SA, Larson EL. 2019. Insights from genomes into the evolutionary importance and prevalence of hybridization in nature. *Nature Ecology & Evolution* 3: 170-177.
- Than C, Ruths D, Nakhleh L. 2008. PhyloNet: a software package for analyzing and reconstructing reticulate evolutionary relationships. *BMC Bioinformatics* 9: 322.

- Turner BL. (2006). Taxonomy and nomenclature of the *Erysimum asperum*- *E.capitatum* complex (Brassicaceae). *Phytologia* 88: 279-287.
- UniProt Consortium. 2014. UniProt: a hub for protein information. *Nucleic Acids Research* 43: D204-D212.
- Wang, D., Wang, Z., Kang, X., & Zhang, J. (2019). Genetic analysis of admixture and hybrid patterns of *Populus hopeiensis* and *P. tomentosa*. *Scientific Reports* 9: 1-13.
- Warwick SI, Francis A, Al-Shehba IA. 2006. Brassicaceae: species checklist and database on CD-Rom. *Plant Systematics and Evolution* 259: 249-258.
- Wen D, Yu Y, Zhu J, Nakhleh L. 2018. Inferring phylogenetic networks using PhyloNet. *Systematic Biology* 67: 735-740.
- Wu YC, Rasmussen MD, Bansal MS, Kellis M. 2014. Most parsimonious reconciliation in the presence of gene duplication, loss, and deep coalescence using labeled coalescent trees. *Genome Research* 24: 475-486.
- Yang Y, Smith SA. 2014. Orthology inference in nonmodel organisms using transcriptomes and low-coverage genomes: improving accuracy and matrix occupancy for phylogenomics. *Molecular Biology and Evolution* 31: 3081-3092.
- Yu Y, Nakhleh L. 2015. A maximum pseudo-likelihood approach for phylogenetic networks. *BMC Genomics* 16: S10.
- Zieliński P, Dudek K, Arntzen JW, et al. 2019. Differential introgression across newt hybrid zones: Evidence from replicated transects. *Molecular Ecology* 28: 4811-4824.

735

736

List of tables

Table 1. Population code, location, and details of sympatry status for all of the populations sampled.

Table 2. Genome size estimates and ploidy levels of the *Erysimum* species samples included in this study. Genome sizes were estimated with flow cytometry analyses.

Species	Population	Location	Elevation	Geographical coordinates	Flower color	Sympatry with
<i>E. baeticum</i>	Ebb07	Sierra Nevada, Almería, Spain	2128	37°05'46"N, 3°01'01"W	purple	
	Ebb10	Sierra Nevada, Almería, Spain	2140	37°05'32"N, 3°00'40"W	purple	En12
	Ebb12	Sierra Nevada, Almería, Spain	2264	37°05'51"N, 2°58'06"W	purple	
<i>E. bastetatum</i>	Ebt01	Sierra de Baza, Granada, Spain	1990	37°22'52"N, 2°51'49"W	purple	
	Ebt12	Sierra de María, Almería, Spain	1528	37°41'03"N, 2°10'51"W	purple	
	Ebt13	Sierra Jureña, Granada, Spain	1352	37°57'10"N, 2°29'24"W	purple	Em71
<i>E. fitzii</i>	Ef01	Sierra de la Pandera, Jaén, Spain	1804	37°37'56"N, 3°46'46"W	yellow	
<i>E. lagascae</i>	Ela07	Sierra de San Vicente, Toledo, Spain	516	44°05'49"N, 4°40'40"W	purple	
<i>E. mediohispanicum</i>	Em21	Sierra Nevada, Granada, Spain	1723	37°08'04"N, 3°25'43"W	yellow	
	Em39	Sierra de Huétor, Granada, Spain	1272	37°19'08"N, 3°33'11"W	yellow	Ep20
	Em71	Sierra Jureña, Granada, Spain	1352	37°57'10"N, 2°29'24"W	yellow	Ebt13
<i>E. nevadense</i>	En05	Sierra Nevada, Granada, Spain	2074	37°06'35"N, 3°01'32"W	yellow	
	En10	Sierra Nevada, Granada, Spain	2321	37°06'37"N, 3°24'18"W	yellow	
	En12	Sierra Nevada, Granada, Spain	2255	37°05'37"N, 2°56'19"W	yellow	Ebb10
<i>E. popovii</i>	Ep16	Jabalcul, Jaén, Spain	796	37°45'26"N, 3°51'02"W	purple	
	Ep20	Sierra de Huétor, Granada, Spain	1272	37°19'08"N, 3°33'11"W	purple	Em39
	Ep27	Llanos del Purche, Granada, Spain	1470	37°07'46"N, 3°28'48"W	purple	

760 Table 1. Population code, location, and details of sympatry status for all of the populations sampled.

Species	Population	DNA Ploidy level		Genome size (2C, pg)					
		2n	N	Mean	SD	CV	Min	Max	N
<i>E. baeticum</i>	Ebb07	8x	5	2.08	0.08	3.85	1.93	2.17	2
	Ebb10	8x	6	2.07	0.09	4.35	1.93	2.17	5
	Ebb12	8x	-	-	-	-	-	-	-
<i>E. bastetanum</i>	Ebt01	4x	4	1.06	0.06	5.66	0.97	1.10	4
	Ebt12	4x	2	1.06	0.12	11.32	0.97	1.15	2
	Ebt13	8x	64	1.96	0.06	3.06	1.87	2.17	60
<i>E. fitzii</i>	Ef01	2x	3	0.44	0.004	0.91	0.44	0.45	3
<i>E. lagascae</i>	Ela07	2x	10	0.46	0.02	4.35	0.44	0.50	10
<i>E. mediohispanicum</i>	Em21	2x	2	0.44	0.01	2.27	0.43	0.44	2
	Em39	2x	21	0.46	0.02	4.35	0.43	0.49	19
	Em71	4x	59	0.98	0.04	4.08	0.93	1.13	59
<i>E. nevadense</i>	En05	2x	-	-	-	-	-	-	-
	En10	2x	-	-	-	-	-	-	-
	En12	2x	3	0.45	0.03	6.67	0.42	0.47	3
<i>E. popovii</i>	Ep16	4x	3	0.98	0.02	2.041.86	0.95	1.00	3
	Ep20	10x	15	2.49	0.06	2.416	2.40	2.60	10
	Ep27	4x	39	0.96	0.04	4.17	0.92	1.05	9

Table 2. Genome size estimates and DNA ploidy levels obtained in populations of *Erysimum*. The following data are given for each population and ploidy level: mean, the standard deviation of the mean (SD), coefficient of variation (CV, %), minimum (Min) and maximum values (Max) of the holoploid genome size (2C, pg) followed by sample size for genome size estimates (N); DNA ploidy level (2n) and respective sample size (N) for ploidy estimates. DNA ploidy levels: 2x, diploid; 4x, tetraploid; 8x, octoploid; 10x, decaploid. For Ebb12, En05, and En10 samples were not possible to estimate the ploidy levels, and we have used the described in Blanca et al. (1992).

List of figures

Figure 1. Map of the Iberian Peninsula showing the location of the sampled populations.

Figure 2. Cyto-nuclear discordance in *Erysimum* spp.. The phylogeny on the left was obtained using whole plastid genomes in Osuna-Mascaró et al. (2021). The phylogeny on the right is a representation of nuclear genome evolution and was generated from the 16,941 maximum likelihood gene trees computed using the SNP data described in the present paper (see text for details).

Figure 3. Membership probability plot showing the DAPC results representing the populations grouped into predetermined different clusters.

Figure 4. Optimal species network. The graph represents a maximum pseudo-likelihood (MPL) tree with 13 reticulations computed using PhyloNet. These events are represented by edges connecting the tree branches between different individuals and indicate likely hybridization between different taxa. Note that in some instances, introgression appears to involved ancestral or extinct taxa (i.e., ghost species, dotted lines).



Figure 1. Map of the Iberian Peninsula showing the location of the sampled populations. The insert shows a more detailed map of the Baetic mountains. Purple species are represented with purple symbols and yellow species are represented with yellow symbols. The populations Ebt13 – Em71, Ebb10 – En12, and Em39 – Ep27 represent population pairs located in sympatry.

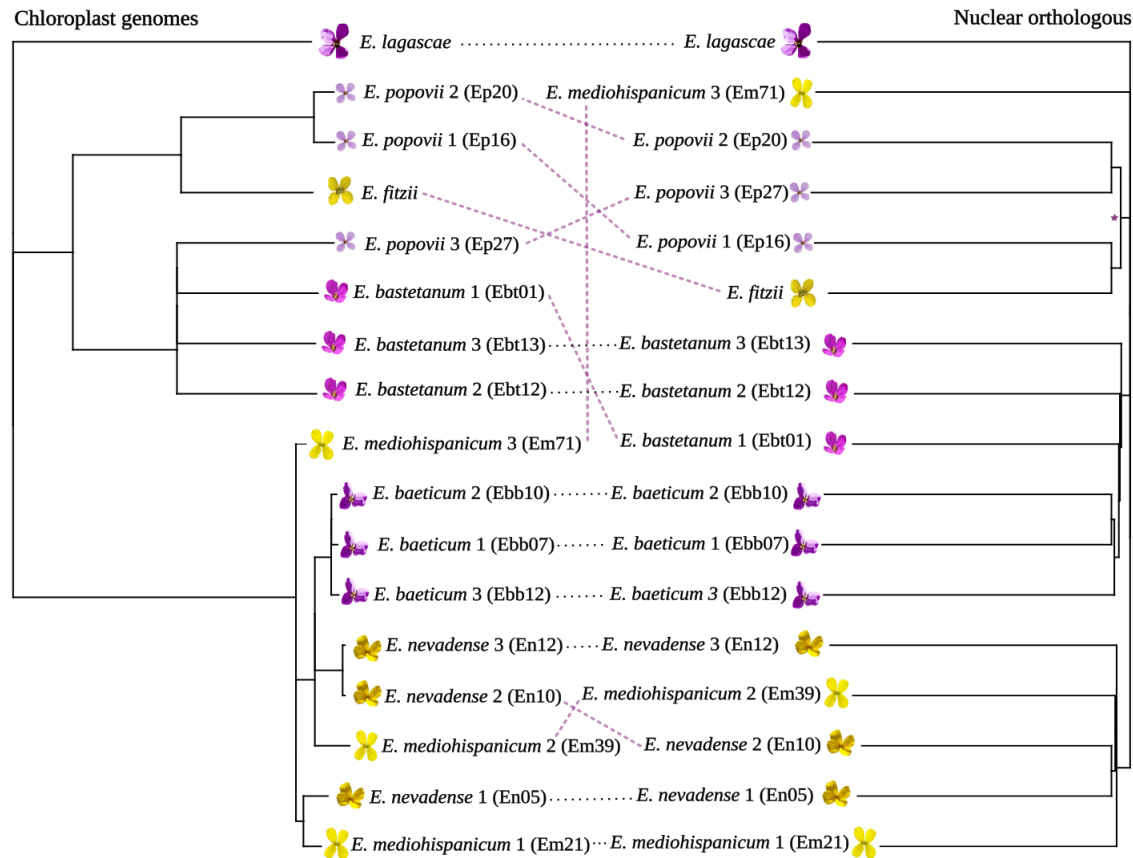


Figure 2. Cyto-nuclear discordance in *Erysimum* spp.. The phylogeny on the left was obtained using whole plastid genomes in Osuna-Mascaró et al. (2021). The phylogeny on the right is a representation of nuclear genome evolution and was generated from the 16,941 maximum likelihood gene trees computed using the SNP data described in the present paper (see text for details).

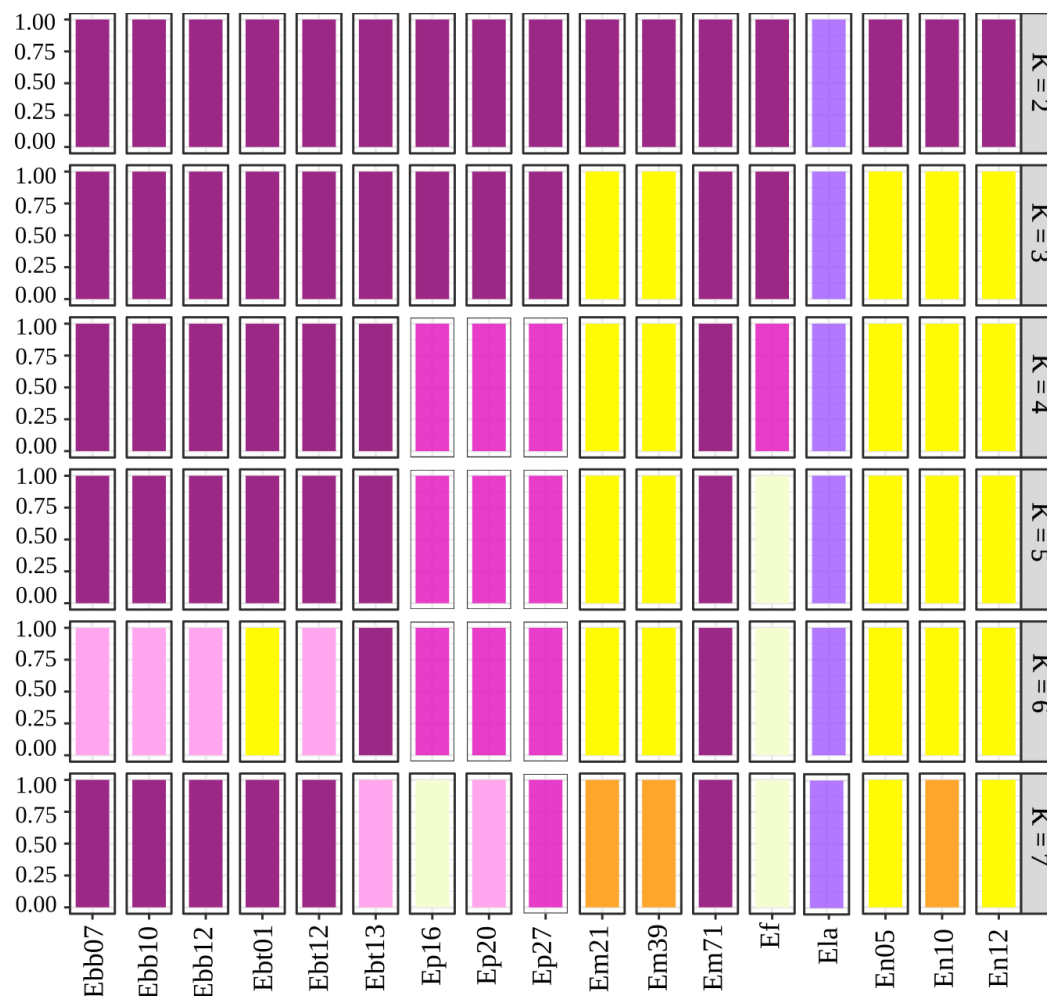
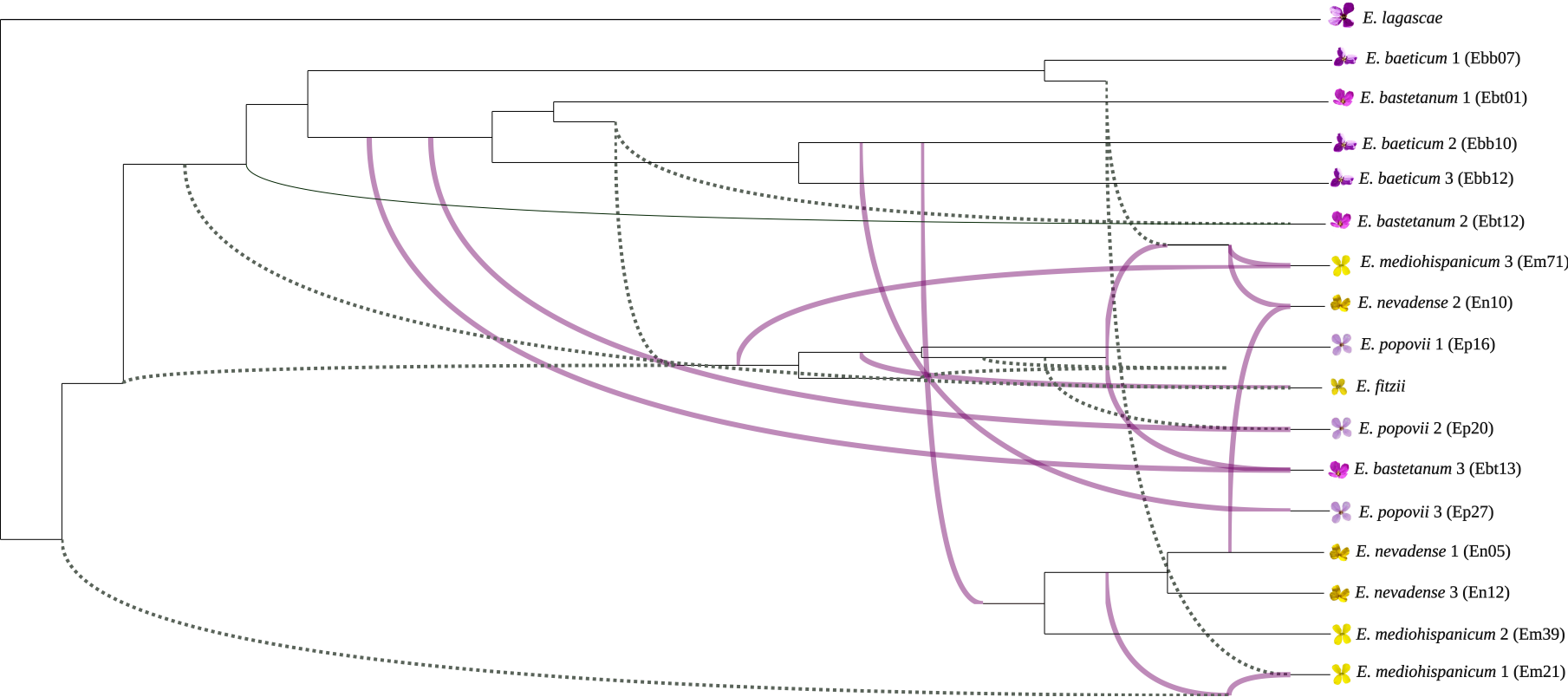


Figure 3. Membership probability plot showing the DAPC results representing the populations grouped into predetermined different clusters (ranging from K=2 to K=7, where each color represents a cluster). The Bayesian analyses (BIC) revealed K = 5 as the most likely number of genetic clusters.



848 Figure 4. Optimal species network. The graph represents a maximum pseudo-likelihood (MPL) tree with 13 reticulations computed using
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