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Word count, main text: 7345

Running title: **Genomic footprints of domestication in the carob**

Title: **Genome-wide footprints in the carob tree (*Ceratonia siliqua*) unveil a new domestication pattern of a fruit tree in the Mediterranean**

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mec.16563

Abstract

Intense research efforts over the last two decades renewed our understanding of plant phylogeography and domestication in the Mediterranean basin. We aim to investigate the evolutionary history and the origin of domestication of the carob tree (*Ceratonia siliqua*), which has been cultivated for millennia for food and fodder. We used >1000 microsatellite genotypes to delimit seven carob evolutionary units (CEUs). We investigated genome-wide diversity and evolutionary patterns of the CEUs with 3557 SNPs generated by restriction-site associated DNA sequencing (RADseq). To address the complex wild vs. cultivated status of sampled trees, we classified 56 sampled populations across the Mediterranean basin as wild, semi-natural or cultivated. Nuclear and cytoplasmic loci were identified from RADseq data and separated for analyses. Phylogenetic analyses of these genomic-wide data allowed us to resolve west-to-east expansions from a single long-term refugium likely located in the foothills of the High Atlas Mountains near the Atlantic coast. Our findings support multiple origins of domestication with a low impact on the genetic diversity at range wide level. The carob was mostly domesticated from locally selected wild genotypes and scattered long-distance westward dispersals of domesticated varieties by humans, concomitant with major historical migrations by Romans, Greeks and Arabs. Ex-situ efforts to preserve carob genetic resources should prioritize accessions from both western and eastern populations, with emphasis on the most differentiated CEUs situated in Southwest Morocco, South Spain and Eastern Mediterranean. Our study highlights the relevance of wild and seminatural habitats in the conservation of genetic resources for cultivated trees.

Introduction

Fruit trees played a major role in the development of Mediterranean civilizations during the last millennia (Zohary and Hopf, 2012). Several species survived in refugia during the Pleistocene climatic changes and suffered repeated range expansions and contractions, which shaped their genetic diversity and structure. Their evolutionary histories represent examples of plant evolution under three important drivers, geological, climatic and human, which have been defined as the Mediterranean triptych (Thompson, 2020). Albeit human activities represent the most recent driver of the Mediterranean triptych on shaping global biodiversity (Boivin et al., 2016). Indeed, humans have profoundly modified Mediterranean ecosystems for thousands of years, resulting in a continuum between forest and agrosystems (Quézel and Médail, 2003). As a result, it is difficult to document the evolutionary history of fruit trees, which may have cultivated, feral or wild populations in the same region (Besnard et al., 2018). In exceptional cases, intense dispersal by humans across the Mediterranean has led to the lack of robust genetic structure (e.g., in the chestnut (Fineschi et al., 2000) or the stone pine (Vendramin et al., 2008). By contrast, recent phylogeographic studies have revealed that imprints of ancestral populations preceding agriculture are still present in the genetic diversity structure of some Mediterranean cultivated tree species such as the olive tree and the wild date palm (Gros-Balthazard et al., 2017; Besnard et al., 2018). Identifying the ancestral genetic legacy is essential to conserve properly genetic resources in the Mediterranean region, and to improve our understanding of the domestication process.

As a general pattern, the domestication of plants in the Mediterranean started in the East, in the Fertile Crescent, and was followed by human-mediated westward dispersals of crops across the Mediterranean basin (Zeder, 2008; Zohary and Hopf, 2012). However, recent studies suggest that the cultivation of useful plants was not a rare phenomenon throughout the Mediterranean and may have involved local resources

from several diversity centers in a protracted process during which genetic admixture, within or between species, played a crucial role (Fuller et al., 2011; Purugganan, 2019; Thompson, 2020).

The carob (*Ceratonia siliqua*, Fabaceae) is a common tree in traditional Mediterranean orchards which has traditionally been valued and still is, for its ability to produce food and fodder in marginal lands, notably during unfavourable years. Thus, the domestication of the carob tree aimed at increasing the pulp in the fruit (Zohary, 2002). New uses have recently emerged, such as producing bioethanol or obtaining galactomannan from seeds as a food stabilizer. A recent review outlined the potential of carob for developing health-beneficial food products (Brassesco et al., 2021). The carob tree has also been the subject of trials for the ecological restoration and afforestation of degraded lands (Domínguez et al., 2010). Cultivars are propagated clonally by grafting, branches (scions) from selected productive trees are grafted on carob trees (rootstocks) often obtained from seedlings. Then it is assumed that the spread of its cultivation and domestication was linked to the development of grafting methods ca. 3,000 years ago (Zohary, 2002; Meyer et al., 2012). As for several crops, the Near East and the Eastern Mediterranean regions were initially proposed as the center of domestication for the carob tree (de Candolle, 1883; Zohary, 2002; Ramon-Laca and Mabberley, 2004). Due to the lack of international breeding programs, each country possesses its own carob varieties and few international exchanges of selected varieties have been reported so far. The countries with the highest numbers of cultivated varieties are Spain, Portugal and Italy (Tous et al., 2013), although a lack of geographic pattern explaining the genetic structure was initially found for Spanish and Italian cultivars (Caruso et al., 2008; La Malfa et al., 2014). A recent study of the world's largest germplasm collection detected genetic structure between cultivars from South Spain and Morocco, and separated from cultivars of Northeast Spain, using microsatellite and plastid markers (Di Guardo et al., 2019). This finding was congruent with studies including wild carob populations (Viruel et al., 2018, 2020), which recognized four main genetic groups across the Mediterranean and a strong West-East structure, as documented for several Mediterranean plants (Désamoré et al., 2011; Nieto Feliner,

2014; Chen et al., 2014; Migliore et al., 2018; Garcia-Verdugo et al., 2021). Integrating the results from these studies supports a regional use, cultivation and domestication of wild carob trees in several parts of the Mediterranean. The mixed ancestry found in current cultivars was likely the result of the diffusion of selected productive, female or hermaphrodite genotypes via grafting (Di Guardo et al., 2019). Nevertheless, the effects of domestication were not homogeneous across the Mediterranean basin. For example, carob cultivation in orchards was less intensive in Andalusia and Morocco than in the rest of the distribution range (Di Guardo et al., 2019). In these two regions, the carob pods from cultivars have a low pulp content, similar to the wild type. By contrast, in the eastern and central Mediterranean areas, especially in Sicily, Crete and Cyprus, carob cultivation is more intensive and supports several traditional uses, suggesting an ancient history of selection and domestication. This geographical variation of domestication efforts is congruent with the habitats and plant communities where carob trees occur in the Mediterranean, being more heterogeneous in the Western Mediterranean (Baumel et al., 2018).

In this study, we aim to resolve the complex evolutionary scenario of the carob tree in the Mediterranean and to investigate the footprints of domestication on the overall genetic diversity of this tree. We hypothesize i) a stronger impact of domestication on the genetic diversity is expected in Central and Eastern Mediterranean populations, and ii) human-mediated gene flow occurred from east to west of domesticated carob trees mainly influenced by the spread of cultivars by Arabs during the Middle Ages (Ramon-Laca and Mabberley, 2004).

Our first objective was to define evolutionary units based on geographic distribution and genetic structure data obtained from SSR polymorphisms and SNPs obtained by Viruel et al. (2018, 2020). We aimed at delimiting geographically homogenous genetic groups of carob populations (hereafter called CEUs for Carob Evolutionary Units). Then, using these CEUs, we investigated carob genome-wide diversity and differentiation with data developed using a reduced-representation genomic approach, restriction

associated DNA sequencing (RADseq), which has been successfully used to decipher evolutionary history in several tree species (Hodel et al., 2017; Borrell et al., 2018; Warschefsky and von Wettberg, 2019; Hipp et al., 2020). Our second objective was to assess the potential impact of domestication on the genome-wide diversity of the carob tree. For this, we performed a comparative diversity analysis and search of candidate loci for carob populations in different conditions (wild versus cultivated). Our third objective was to reconstruct the evolutionary history of the CEUs, including population splits and gene flow estimations.

Materials and Methods

Plant material

We used material collected from populations of *Ceratonia siliqua* across the Mediterranean basin as described in Table S1 (Supplementary Material). Along with the field sampling, it was challenging distinguishing between cultivated and wild trees because carob is currently observed following a gradient of ecological conditions from natural habitats to cultivated lands. Since leaves for DNA extraction were collected from the canopy, they could belong to the grafted part of the tree. Indeed, clones were reported in Viruel et al. (2020, see also Table S1), but they were very scarce. We classified populations as wild when trees showed several trunks and small curved fruits (wild type). By contrast, trees cultivated in orchards with large and non-curved fruits were identified as grafted cultivars. However, in many cases, we could not find any evidence of cultivation, grafted scars or no fruits. Moreover, we often faced abandoned cultivated areas, some of which were burnt, and rootstocks probably replaced the cultivars. Therefore, to address uncertainty regarding wild or cultivated status in our analyses, we assigned each sampled carob tree to one of three types of habitats. Based on field observations, the habitat of each population was recorded as 'wild', 'semi-natural' or 'cultivated'. Examples of these three types of habitats found in our

sampling are provided in Supplementary Material (Fig. S1). Habitats where human impact is low and there is no evidence of recent land use were recorded as 'wild'. These wild habitats correspond mainly to cliffs, rocky slopes or riverbanks. In wild habitats, carob trees are probably of wild or feral origin, but they have an almost null probability of having been planted or grafted. 'Semi-natural' habitats lack evidence of current cultivation but show the clear presence of past human activities such as pasture or old farming where carobs, often consociated with almond or olive trees, are occasionally harvested. In these semi-natural habitats, the carob trees may be of wild origin, originating from close thermophilous vegetation that recolonises the fields and terraces (Baumel et al., 2018), or they can be the descendants of cultivated carob trees (feral trees) or even abandoned cultivated trees. In this case, single plants could be grafted with cultivars and clones were observed (Table S1). Habitats containing carob trees in specialized or consociated orchards (with cereals or other fruit trees), were recorded as 'cultivated'. In these cultivated habitats carob trees have a higher probability of having been grafted as verified in some cases (Table S1). When microsatellite makers revealed clones, only one representative genotype was kept.

Preliminary delimitation of *Ceratonia siliqua* evolutionary units using microsatellite data

We selected localities with at least ten carob genotypes per population. We used 17 microsatellite markers for genotyping 1019 individuals collected in 56 localities across the Mediterranean basin as described in Table S1. We also scored 15 SNPs present in the flanking regions of the 17 SSR loci following Viruel et al. (2018). We calculated genetic differentiation between localities using the D index (Winter, 2012; Mmod R package) and converted it into Euclidean genetic distances based on the coordinates of an NMDS (Non-metric Multidimensional Scaling; vegan R package). The Euclidean genetic distances and Euclidean geographic distances were then processed by ClustGeo (Chavent et al., 2018; clustgeo R package), which relies on Ward's method to minimize intra-group variance for both geographical and genetic distances by calculating K and α parameters: K is the number of clusters and α is a mixing parameter determining the

weight of the spatial constraint. Several values of α were tested to optimize the spatial contiguity of populations without deteriorating genetic differentiation structure. Several values of K were also tested. This clustering method provided genetically homogeneous groups of spatially adjacent carob populations, named "CEUs" (Carob Evolutionary Units), which were used in subsequent analyses. A neighbor-joining tree based on pairwise genetic differentiation (Gst, Mmod package) among the seven CEUs was built to display the overall differentiation structure.

RADseq methodology and sequencing

CEUs were used to select 376 samples representative of the genetic diversity and structure of *Ceratonia siliqua* for RADseq analysis. We sampled eight trees of the only other species in the genus, *C. oreothauma* native in the Arabian Peninsula, kindly sent by the Oman Botanical Garden, which was used as an outgroup in our analysis. Genomic library preparation and sequencing were conducted by Microsynth ecogenics GmbH (Blagach, Switzerland). DNA samples (200-400 ng input) were digested with the restriction enzymes *Eco*RI/*MSe*I following heat inactivation according to the manufacturer's protocol (New England Biolabs, NEB). Fragments between 500 and 600 bp were selected by automated gel cut, Illumina Y-shaped adaptors were ligated, and ligation products were bead purified. Each library was then individually barcoded by PCR using a dual-indexing strategy. Individually barcoded libraries were pooled and subsequently purified before sequencing on an Illumina NextSeq platform (300 million of 75 bp reads per run).

Bioinformatic pipeline to extract and filter SNPs from RADseq data

The bioinformatic approach used in this study is summarized in Figure 1. FASTQC reports (multiQC, Ewels et al., 2016) were used to exclude 26 *C. siliqua* and 4 *C. oreothauma* samples due to low sequencing coverage (i.e., below 0.5 million reads). The remaining 354 samples (350 *C. siliqua* and 4 *C. oreothauma*) had an average of 3 million raw reads, ranging between 0.7 and 15 million. Assembly (Fig. 1) was performed using ipyrad (Eaton and Overcast 2020) in a high-performance computing cluster (HPC

pytheas). Thereafter, a "locus" is a RADseq marker of 65 bp, that resulted from the ipyrad workflow, and a "SNP" is a polymorphic position of a specific locus, considering that a locus can contain several SNPs. We conducted an assembly limited to *C. siliqua* following two steps. First, we selected 36 samples representative of the diversity in *C. siliqua* with a sequencing coverage ranging from 1.3 to 5.3 million reads to conduct four *de novo* assemblies with varying thresholds of clustering reads (*clust_threshold*) between 0.9 and 0.96, the minimum number of samples per locus (*min_sample_locus*) fixed to 30, the minimum depth (*mindepth_statistical*) for base calling fixed to 8, the maximum depth fixed to 1000, the minimum size of reads fixed to 50 and the first 5 bp of both ends of locus trimmed. The maximum number of indels and the maximum percentage of SNPs per locus were set to 1 and 10%, respectively. Default values were used for all other parameters. Considering the number of loci, heterozygosity, and error rates, we estimated an optimal clustering threshold of 0.94 (see Tab. S1 supplementary material). A reference file was generated by extracting the first sequence of each locus with pyrad2fasta script (available on https://github.com/pimbongaerts). Second, this file was used for a subsequent reference assembly for the 354 samples with the same parameters as previously except for the minimum number of samples per loci, which was fixed to 45. The dataset constructed using this reference assembly contained more than 64% missing data. The vcf file obtained from ipyrad was then processed to run a principal components analysis (PCA) using the glPca function of adegenet R package, and a neighbor-joining tree, based on pairwise genetic differentiation (Gst, Mmod R package) among the seven CEUs, to check that the structure from RAD markers was congruent with previous analysis based on microsatellite markers.

We used *Matrix condenser* software (de Medeiros and Farrell, 2018; de Medeiros, 2019) to visualize missing data across samples aiming at maintaining a balance between reducing missing data and not significantly discarding SNPs. We filtered samples to optimize locus coverage and to keep the sampling equilibrium among CEUs and reconducted the reference assembly with ipyrad on a new set of 190 *C.*

siliqua samples. The data was then reduced to one SNP per locus, based on two criteria: SNPs having the maximal minimum allelic frequency per locus and only keeping SNPs with an allelic frequency above 1.05 % (i.e., rarest allele present in at least two individuals).

To examine the carob genetic diversity based on neutral processes, such as genetic drift and gene flow, we reduced the effect of outlier loci (i.e., having unexpectedly high differentiation among populations, which could have a great effect on the variance among populations). We used Outflank software (Whitlock & Lotterhos, 2015), which produces a lower false-positive rate compared to other methods (e.g. Silliman 2019), considering CEUs as populations. The false discovery rate (qvalue) was fixed to 0.05. BLAST searches using the nucleotide NCBI database limited to flowering plant sequences were conducted for outliers, which were mostly assigned to plastid (pDNA) or mitochondrial (mtDNA) genomes. These outlier loci were removed to create a final data set of 3557 SNPs (one SNP/locus) for 190 *C. siliqua* and 4 *C. oreothauma*. Since a subsequent Outflank scan failed to detect further outliers (see results), we considered this set of SNPs as a genome-wide data set of neutral markers.

Recovery of plastid and mitochondrial data

To obtain plastid and mitochondrial markers we performed ipyrad reference assembly with default parameters, and changed the maximum alleles per site to one. We used NCBI reference sequence NC-047061.1, a plastid genome of *C. siliqua* produced by Zhang et al. (2020), as a reference for plastid assembly, and for mitochondrial assembly, we used the Genbank sequence MW448447, the mitochondrial genome of *C. siliqua* produced by Choi et al. (2021). In both cases, we produced data sets with and without missing data by using trimAl (Capella-Gutierrez et al., 2009).

Analysis of genetic differentiation and admixture

The final data set of 3557 SNPs and 190 *C. siliqua* was converted from genind to genlight, vcf and treemix data formats using dartR (Gruber et al., 2018) and radiator (Gosselin, 2020) R packages. Population structure analysis was performed using snmf (R LEA package, Frichot and François, 2015), which estimates admixture coefficients from the genotypic matrix assuming K ancestral populations. Snmf runs fast even on large data sets and without loss of accuracy compared to other Bayesian modelling software such as ADMIXTURE (Alexander et al., 2009; Frichot et al., 2014). Snmf was run for K = 2 to 15, 100 repetitions, regularization parameter set to 250 and 25% of the genotypes masked to compute the cross-entropy criterion. Barplots showing ancestry coefficients were obtained with the compoplot function in adegenet R package (Jombart and Ahmed, 2011), with genotypes sorted according to CEUs. To visualize the genetic diversity structure, we performed a PCA using the glPca function of adegenet R package. Pairwise differentiation among CEUs (Gst) was also computed with the Mmod package.

Phylogenetic relationships between CEUs

Plastid and mitochondrial DNA alignments were used to reconstruct maximum likelihood phylogenetic trees with and without missing data as implemented in IQ-TREE (Minh et al. 2020). All parameters were set to default and node robustness was estimated by ultrafast bootstrap analysis (1,000 iterations). The cophylo function from phytools (Revell, 2012) was used to build a figure comparing pDNA versus mtDNA phylogenetic trees.

The nuclear data set, which includes four *C. oreothauma* samples as outgroup, was used to estimate a coalescent-based tree with the SVDquartets method (Chifman and Kubatko, 2014) using PAUP*4.0a (Swofford, 2018). Comparative studies using coalescent inferences have demonstrated that the SVDquartets approach is statistically consistent for different data types (Wascher and Kubatko, 2021). Ten million randomly selected quartets were analyzed and node support was assessed by 1,000 bootstrap replicates. We used the 'distribute' option for heterozygous sites. To infer population splits

and mixtures across the evolutionary history of the carob tree, we performed a Treemix analysis (Pickrell and Pritchard, 2012). A pilot analysis including *C. oreothauma* genotypes was first run to check for the position of the root followed by a second one without *C. oreothauma* to obtain more accurate branch lengths within *C. siliqua*. Treemix builds a backbone tree based on population allelic frequency without gene flow and then adds reticulate branches, representing gene flow, aiming at improving the fit of the data. For this analysis, we used CEUs as populations. In the final analysis, one of the CEUs was fixed as an outgroup in agreement with the results obtained in previous analyses.

Screening for footprints of domestication in RADseq data

Footprints of domestication were investigated by estimating genetic diversity and/or the presence of candidate loci under selection (outliers) for the CEUs. A stronger effect of domestication is expected in cultivated habitats compared to wild and seminatural habitats, and in the eastern CEUs compared to the western ones (Zohary, 2002). Therefore, the analyses were organized according to combinations of these two factors. Observed heterozygosity (H_{obs}), Nei genetic diversity (H_{exp}), inbreeding coefficient (f) and the standardized association index ($\bar{r}_d$), which indicates a lack of genetic mixing when it increases, were estimated using *diveRsity* and *poppr* R packages (Keenan et al., 2013; Kamvar et al., 2014). Outliers were searched with the Outflank method with an FDR threshold of 0.05 (as described previously) considering combinations of habitats and CEUs as populations.

Results

Delimitation of Carob Evolutionary Units (CEUs) using microsatellite data

Based on genotypes from 17 microsatellite loci (including SSR and SNPs) and geographical coordinates, we grouped 1,019 carob trees into CEUs. The ClustGeo method, which considers geographic and genetic

distances, found seven genetic groups of genotypes which are geographically non-overlapping (Fig. 2). Four of these CEUs are in the Western Mediterranean (W1 to W4), C1 in Central Mediterranean, and E1 and E2 in Eastern Mediterranean. These CEUs are grouped in two clusters by ClustGeo. W1, W3 and W4 formed the first cluster whereas W2 grouped in the second cluster with C1, E1 and E2. Based on microsatellite SSR markers, the overall genetic differentiation among these seven CEUs is 16% (Gst). Pairwise Gst values visualized by a neighbor-joining tree (Fig. 2) confirmed the ClustGeo clustering scheme but placed W1 and E2 in intermediate positions along the NJ tree instead of grouping these two CEUs. The sampling and data composition of the seven CEUs in shown in Table 1.

Assembly of RADseq loci and SNPs filtering

The *de novo* assembly conducted on 36 samples, with a clustering threshold of 0.94 and a minimum number of samples by loci of 30, produced a mean number of 48,828 loci by sample (Tab. S1), which were reduced to 13,371 loci retained after ipyrad filtering. The sequences of these loci were used as a reference for the assembly of 354 genotypes for 10,012 loci which revealed a pattern of genetic structure roughly congruent with microsatellite pattern of differentiation (Fig. S2). However, this data presented an overall missing data rate of 64%.

To overcome the limitations of that 64% of missing data could have in our results, we searched for genotypes and loci having the highest missing rate as implemented in Matrix condenser, and their subsequent removal produced a matrix with 190 samples and 12,767 loci, with 14% missing data rate. After filtering to keep one SNP per locus, the data set included 190 samples for 3,613 loci (or SNPs), with 9.5% overall missing data and a 3.5% median missing rate by genotype. The Outflank method detected 56 outlier markers within the data set of 190 samples by 3,613 SNPs (Fig. S3 in supplementary material). The global differentiation computed for these outliers was six times higher than for other SNPs (Gst 0.74 vs. 0.12). Nucleotide BLAST searches revealed that 27 outliers were assigned to the plastid genome, 14

to the mitochondrial genome, 11 to nuclear genomes and four were not assigned. Excluding the outliers detected by Outflank, the new data set included 3,557 unlinked SNPs. For SVDquartets and Treemix analyses, we filtered out samples with missing data rates above 20%, which generated a matrix containing 171 *C. siliqua* and 4 *C. oreothauma* genotypes for 3,284 unlinked SNPs and 8% missing data rate.

The plastid and mitochondrial data sets, assembled for 190 *C. siliqua* and four *C. oreothauma* samples, held 135,525 and 191,840 bp with 47 and 52% of missing data, respectively. After missing data removal, the pDNA alignment had 13,931 bp with 61 parsimony informative sites whereas the mtDNA had 10,306 bp with 19 parsimony informative sites.

Phylogenetic and genetic structure of carob trees across the Mediterranean based on RADseq data

The maximum likelihood phylogenetic trees reconstructed with plastid and mitochondrial DNA data obtained without (Fig. S5) and with (Fig. 3) missing data had similar topology, but the bootstrap values are higher when missing data is included (Fig. S4). Using the alignments without missing data, IQ-Tree selected the F81+F+I substitution model for both pDNA and mtDNA. Haplotypes from the west CEUs are almost all grouped in a clade whereas central and eastern haplotypes (the red dotted square in Fig. 3) were nested in one (pDNA) or two (mtDNA) clades. The overall phylogenetic topology shown by cytoplasmic lineages is supporting a west-east split except for haplotypes from W1 (green), which appear in both west and central-east ones. All these haplotypes are from the northern part of W1, situated north of Agadir in the Imouzzer Ida Ou Tanane area. Therefore, the cytoplasmic phylogenetic diversity is almost entirely present in Southwest Morocco (W1).

By reducing the rate of missing data and filtering to obtain unlinked SNPs, the genetic structure was more evident as shown in the PCA plot (Fig. 4 compared to Fig. S2). The phylogenetic tree reconstructed with nuclear data using SVDquartets, which included 171 samples of *C. siliqua* and four samples of *C.*

oreothauma as outgroup, confirmed the monophyly of *C. siliqua* and resolved a strongly supported first split of the W1 clade from the remaining *C. siliqua* lineages (Fig. 4A). A subsequent split separated the remaining western CEUs (W2, W3 and W4) from Central and Eastern CEUs (C1, E1, E2). This phylogenetic topology based on 3,557 SNPs is congruent with plastid and mitochondrial data in supporting Southwest Morocco (W1) as sister to all other lineages in the carob evolutionary history. SVDquartets phylogenetic topology is also highly congruent with the K=4 genetic structure obtained with SSR data (Fig. 4A), and these four genetic groups match the following clades: W1, W3 + W4, E1 + E2, C1. The North Moroccan W2 clade is sister to the other three western CEUs in the SVDquartets tree, which is congruent with its mixed assignation to different genetic clusters for RADseq data (Fig. 4A,C). The genetic clustering estimated with snmf based on RADseq data was repeated from K=2 to K=7 ancestral populations. The cross-entropy criterion suggested two optimal solutions for 5 and 7 groups (Fig. S6A in supplementary material), of which K=7 had the highest probability. The most likely genetic clustering, K=7 solution (Fig. 4A, C), resolved the differentiation of four ancestral populations mostly present in Southwest Morocco (W1), South Spain (W3and W4) and the eastern CEUs (E1 and E2). However, departures from a good match between microsatellite based CEUs and RADseq genetic groups are substantial too. C1 includes individuals with admixture from three ancestral groups, two of them (in orange and yellow, Fig. 4) almost restricted to this CEU and the third one, coloured in turquoise (Fig. 4), present in all CEUs although with higher proportions in W2, W3 and C1. W2 was resolved as admixed with the contribution of several ancestral populations. Although admixture of W2 was already seen with microsatellite data, it was closer to central and eastern CEUs than with RADseq. The PCA results are consistent with the main genetic groups found with snmf, showing a clear delimitation of three groups, W1, W3 + W4 and E1+E2, but partial overlap between W2 and C1 (Figure 4B, C). RADseq genetic differentiation among the seven CEUs (Tab. S2. in supplementary material) was moderate with an overall Gst of 11%; all values above the

mean involved a west-east differentiation, the highest differentiation being between W4 and E2 (Gst = 19%).

Impact of dispersals on the genetic diversity and structure of *Ceratonia siliqua*

For the Treemix analysis, we rooted *C. siliqua* phylogenetic tree with W1 following SVDquartets reconstructions with *C. oreothauma*. Tree topology reconstructed by Treemix (Fig. 5) is similar to that of SVDquartets, showing the same west-east differentiation. The main differences concerned the position of W2. SVDquartets phylogenetic tree reconstructed W2 sister to other western CEUs, in congruence with cytoplasmic lineages, whereas Treemix resolved W2 as intermediate between western (W1, W3, W4) and central and eastern CEUs (C1, E1, E2). Treemix agrees with the highly admixed pattern in W2 inferred by snmf (Fig. 2 A) and with its low to moderate differentiation with respect to other CEUs (Tab. S2 in supplementary material). The Treemix model without gene flow explained 96% of the covariance (Fig. 3A), and the addition of four dispersals resulted in 99.8% of the total covariance explained (Fig. 3B). Three of the four dispersals identified E2 as the source of introgression, into W1, W3 and W2, whereas the fourth dispersal connected the central-eastern CEUs to W4, again indicating a westward dispersal.

In summary, the phylogeographic history of the carob tree is mainly derived from west-to-east expansions. Southwest Morocco (W1) was reconstructed sister to all other lineages of *C. siliqua*, and the populations northern part of this area are phylogenetically related to the eastern lineages. On the one hand, genomic information from cytoplasmic lineages, as well as from multi-species coalescent analyses from nuclear data (SVDquartets), support two dispersal routes, one originating the western CEUs and the second originating the eastern CEUs. On the other hand, Treemix, admixture and pairwise Fst analyses, all based on allelic frequencies, support a simplified west-to-east differentiation through northern Morocco (W2). The secondary east-west dispersals detected by the Treemix were in agreement with

microsatellite data, for example, the shared co-ancestry between W1 and E2 genotypes (Fig. 4 A SSR) or the high mixing rate of W2.

Impact of carob cultivation effort on carob genetic diversity

Overall, for microsatellite markers, the admixture plot (Fig. 4A) revealed the same pattern within each of the CEUs, that is, a similar gradient of ancestries from non-admixed to highly admixed regardless of the type of habitat. A slightly higher admixture can be observed in semi-natural populations. However, considering the RADseq markers, a striking difference is observed for C1 where 12 genotypes collected in cultivated habitats constitute a genetically pure (yellow, Fig. 4) group. These genotypes correspond to grafted branches of monumental carob trees living near Raguza in Sicily. Although not forming clones, they are genetically very similar, a pattern that was not detected with microsatellites. This genetic cluster is rarely found outside Sicily, although it scarcely occurs in France (C1) and North Morocco. In C1, the other genotypes sampled in cultivated habitats, coming from other monumental trees or known cultivars, are more genetically mixed and are closer to the genotypes found in semi-natural habitats, which are mainly composed of a cluster that is almost exclusive to this area (orange, Fig. 4). Higher admixture was detected in Western Spain (W3) with North Morocco (W2) than in Eastern Spain (W2). In Eastern Mediterranean, a genetic group (light red, mostly in E2) was mainly detected in semi-natural populations, and it constituted the predominant genetic group in Lebanon (Fig. 4A).

Genetic diversity values are reported in Table 2. For microsatellite data, Nei diversity (Hexp) values decrease from western CEUs and wild or seminatural habitats to eastern and/or cultivated habitats, regardless of the markers. This is less pronounced for RADseq data. Interestingly, the association index ($\bar{r}_d$), which increases as mixing decreases, shows a very clear effect of decreased genetic mixing in cultivated habitats for microsatellite data. The reduced sampling for RADseq does not allow such comparison but support that central (C1) and eastern CEUs experienced less mixing than western ones.

The inbreeding index (f) suggests both deficit and excess of heterozygosity. The deficit is significant for both RADseq and SSR data for W1, W2, E1 and E2 whereas an overall excess is observed for C1 for RADseq and a few other cases. The highest values of genetic diversity are observed in the western CEUs, whereas in the east, but also with its cultivation, the genetic diversity of carob decreases as does the genetic mixture (increased $\bar{r}_d$, Table 2).

Based on the differentiation between genetic groups formed by the combination of CEUs and habitats (Table 2), OUTFLANK did not detect candidate loci with a false discovery rate (qvalue) below 0.05, which indicates that the hypothesis of neutrality cannot be rejected (Fig. S7 in supplementary material).

Discussion

Intense research on plant phylogeny and phylogeography over the last two decades have allowed the discovery of several major biogeographical trends in the Mediterranean basin (Comes, 2004; Nieto Feliner, 2014; Garcia-Verdugo et al., 2021) and renewed our understanding of plant domestication (Purugganan, 2019). Following an initial focus on biogeographic refugia, recent studies have revealed the genetic imprints of past environmental changes and dispersal processes, some involving the entire Mediterranean basin (see reviews in Médail and Diadema, 2009; Fady & Conord, 2010; Nieto Feliner, 2011; Nieto Feliner, 2014; Migliore et al., 2018; Vargas et al., 2018; Thompson, 2020; Garcia-Verdugo et al., 2021). Our study untangles a new phylogeographic scenario for a Mediterranean tree species: the ancestral gene pools of carob (i.e., CEUs) originated from a biogeographic refugium probably located in Southwest Morocco, and a subsequent west-to-east expansion. Our results also highlight that carob domestication has mainly relied on the use of locally selected varieties, albeit punctuated by long-distance westward dispersal events by humans, which match major cultural migration waves by Greeks, Romans and Arabs.

Evolutionary history of the carob tree

Our phylogeographic reconstruction conclusively rejects a long-standing hypothesis that proposes an introduced origin of the carob tree in most of the Mediterranean. An Eastern Mediterranean or Southern Arabian origin, followed by human-mediated expansions, were proposed by several authors partly based on linguistic evidence from vernacular names, *Ceratonia siliqua* and *C. oreothauma* occurrences in western Asia and carob agricultural practices (reviewed in Ramon-Laca & Mabberley, 2004). However, genetic data from SSR and plastid markers based on a thorough population sampling across the Mediterranean (Viruel et al., 2020) revealed a strong west vs. central-east pattern suggesting a low human influence on the main current patterns of genetic diversity and structure of the carob tree across the Mediterranean. The comprehensive review of carob fossil data presented in Viruel et al. (2020) did not provide support for an eastern origin of *C. siliqua*. Instead, fossil records show a mostly continuous presence of *Ceratonia* around the palaeo-Mediterranean Sea since the Oligocene with a progressive decline starting c. 20 Ma.

Compared to SSR data, the increased resolution of the RADseq results presented here has allowed bridging phylogenetic and population genetic inferences (Parchman et al., 2018). In addition, the inclusion of *Ceratonia oreothauma*, the sister species of *C. siliqua* —diverged around 6.4 Ma (Viruel et al., 2020)— allows rooting the carob tree history, thus providing a relative timeframe for the successive splits that occurred along with the evolutionary history of this species. Coalescent-based models based on SSR data could not discard the existence of smaller refugia in the eastern Mediterranean, and two disjunct refugia after LIG was suggested as the most likely evolutionary scenario for the current genetic structure of *C. siliqua* (Viruel et al., 2020). The RADseq data generated in our study provides a substantially higher resolution for the evolutionary history of the carob tree. The fact that Southwest Morocco CEU (W1) exhibits a high genetic diversity and is sister to all other Mediterranean carob

lineages in the phylogenies (Table 2, Figures 3 and 4) supports the existence of a long-term refugium in the foothills of the High-Atlas Mountains near the Atlantic coast, from where the ancestral population of *C. siliqua* has possibly emerged. From this ancestral population the carob tree likely followed two dispersal routes: one northward that reached Northwest Africa and South Spain (W2, W3 and W4 CEUs), and another towards the east that gave rise to the central-eastern CEUs. Mitochondrial and plastid evidence extracted from RADseq data also support the existence of an ancestral pool in Southwest Morocco. Specifically, mtDNA and pDNA haplotypes present in the northern part of Southwest Morocco (Imouzzer Ida Ou Tanane area) are closely related to eastern haplotypes thus indicating this latter area as close to the source of the eastern populations. This pattern was already apparent in 1472 bp-long plastid markers exhibiting only three substitutions (Viruel et al., 2020). This new evolutionary scenario explains the west-east split in the carob genetic diversity by dispersal from an ancestral population. Footprints of this ancestral population were found in the genetic profiles of the populations located in Southern Morocco. However, the number of dispersal routes estimated from this ancestral population varied in different analyses. Two dispersal routes were less supported by analyses based on allelic frequencies (pairwise Fst, admixture plots or Treemix). Indeed, they positioned north Morocco (W2) at the crossroad of west, central and east CEUs suggesting that more than one genepool from the west contributed to central and eastern populations. This is represented by the "turquoise" genetic cluster (Fig. 4A) inferred with RADseq data, which is mostly present in north Morocco (W2), Southwest Spain (W3), and Central Mediterranean (C1), with a slight presence in the east (E1). However, it is noteworthy that all cytoplasmic haplotypes from north Morocco belong to the western group and are not related to eastern haplotypes, both when using RADseq or Sanger sequencing (Viruel et al. 2020). This strong nuclear-cytoplasmic incongruence supports that north Morocco mostly played a receiver rather than a donor role (Currat et al., 2008) during carob history, thus supporting two dispersal routes as the most likely scenario.

Viruel et al. (2020) estimated that the divergence between the west and east part of the carob range could be as old as 1815 generations ago (95% CI: 400-4640 generations). This age estimate is therefore in favor of a west-to-east expansion before the Last Glacial (ca. 21 Ka ago). Species distribution modelling (SDM) indicates that both the Last Interglacial (ca. 116 Ka ago) and the Last Glacial Maximum were periods of range contraction for the carob tree during the Pleistocene (Viruel et al., 2020). Moreover, SDM predicted that some areas in the North African and South European Atlantic coasts could have been continuously suitable over the last 130 ka. Southwest Morocco has been identified as a biogeographic refugium and even as a diversification cradle for several taxonomic groups, e.g., *Dracaena draco* and several elements of the Macaronesian flora (Médail and Quézel, 1999), *Olea europaea* L. ssp. *maroccana* (Médail et al. 2001); *Hypochaeris* sp. (Ortiz et al., 2009), *Daboiea* sp. vipers (Martínez-Freiría et al., 2017), *Astragalus edulis* (Bobo-Pinilla et al., 2018), *Lavatera maritima* (Villa-Machío et al., 2018), and *Buthus* sp. scorpions (Klesser et al., 2021). Although Mediterranean phylogeographic studies focused mostly on glacial refugia, five recent studies have highlighted South and West Morocco as a long term stable refugium for plant populations during the LIG and LGM (Garcia-Castaño et al. 2014; Villa-Machío et al., 2018; Bobo-Pinilla et al., 2018; Migliore et al., 2018; Viruel et al., 2020). This area is characterized by a highly complex topography with the foothills of the Atlas Mountains near the Atlantic Ocean that may have buffered environmental conditions (i.e., climatic stability, frequent sea fogs) favoring higher species persistence during unfavorable periods of pronounced climate continentality.

Footprints of domestication in the current genetic structure of the carob tree across the Mediterranean

Although disentangling the history of cultivated plants is complex, our phylogeographic investigation in the carob tree sheds light on its domestication history. Our previous study based on SSR data (Viruel et al., 2020) suggested that local domestication events from wild populations were the most likely scenario. Consistent with this, the RADSeq data, based on a more intense genome-wide sampling, cannot explain

domestication solely based on historical translocations. Agricultural practices in the carob tree are based on propagation by grafting (Zohary, 2002), although seeds could have also been transported for propagation or their use as a unit of weight for gemstones (Turnbull et al., 2006). In either case, if domestication was based only on westward propagations of cultivars from the east, maternal (eastern) haplotypes would remain today in the western Mediterranean. Instead, our results are conclusive in supporting that although the dispersal of selected varieties (vegetatively propagated) between remote geographical areas may have contributed to the local gene pools, this was not the main force of domestication in carob tree.

The use of genomic data at the intraspecific level has permitted identifying footprints of domestication in crop models where PCR-based molecular markers had previously failed. In the case of the date palm, genomic data revealed that human-mediated dispersal imprints were superimposed on a previous phylogeographical structure (Gros-Balthazar et al., 2017; Flowers et al., 2019). In the carob tree, translocation of eastern domesticated varieties into differentiated western populations does fit with the patterns found in geographically intermediate groups (W2, C1, and E1). These are less differentiated, which is explained by high rates of admixture (Fig. 2). To untangle the role of human-based dispersals in these strong genetic admixtures, we used allelic frequency-based models aiming at estimating the intensity and origin of dispersal events throughout the carob evolutionary tree (Fig. 5): results of Treemix recover westwards dispersals that were mostly originated from E2 (Lebanon and Crete), or central-eastern CEUs (E2, E1, and C1). These translocations match with the beginning of carob agriculture in the East, its dispersal by Greeks, Romans, and after by Arabs in historical times (Ramon Laca and Mabberley, 2001; Viruel et al., 2020). They may have contributed to the genetic admixed pool used locally for cultivation as observed in North Morocco (W2).

The second footprint of domestication was observed in genetic diversity. Our analyses revealed an association between lower genetic diversity (and lower genetic admixture) and a higher carob cultivation intensity. This pattern was well observed in the central CEU (C1), where cultivated varieties are most diffused compared to other CEUs (Di Guardo et al., 2019). In C1, we detected a genetic group of individuals lacking admixture, corresponding to the monumental carobs of the Ragusa district (Sicily, Italy). These monumental carobs have been harvested without interruption for centuries, and they are genetically very close to each other, although not clones. Other cultivars collected near Raguza, either from monumental trees or recently grafted, were closely related to genotypes found in seminatural habitats of C1 and differentiated from other CEUs (orange Fig. 4A). These genetic patterns of cultivated C1 individuals constitute additional support for diffusion of selected genotypes at the local scale, rather than long-distance dispersal. This local process played a major role in the domestication of carob. Despite this pattern, we did not detect any candidate loci under selection due to domestication pressures, which could be explained by the limitations of our method and sampling or by a low effect of domestication on the carob genome, or even by the suitability of carob wild traits for human needs, which might have been good enough for their use as fodder.

Compared to other perennial crop species for which candidate and adaptive loci have been found by whole genome sequencing as well as RADseq (Cornejo et al., 2018; Alves-Pereira et al., 2020; Groppi et al., 2021), a relatively lower impact of selection is likely in carob. Moreover, domestication leading to fine-tuning of gene expression patterns rather than genome-wide evolution, as observed in olive (Gros-Balthazard et al., 2019), may be almost undetectable by a reduced-representation genomics approach such as RADseq. In North Morocco, forest-agrosystems continuity coupled with close wild-domesticated relationships characterizes the local domestication of olive and fig trees (e.g. Achtak et al., 2010; Aumeeruddy-Thomas et al., 2017). However, a low impact of domestication on genetic diversity at the range-wide level, as shown here for the carob tree, is unique by comparison to other Mediterranean

fruit trees. This is probably the reason why the footprints of demographic history are still present and detectable.

Conservation of genetic diversity within Carob Evolutionary Units (CEUs)

Defining the structure of genome-wide diversity is essential for preserving the genetic resources of cultivated species and for future breeding practices (Purugganan, 2019). We used an integrative approach that combines geographic and genetic differentiation to characterize evolutionary units for *Ceratonia siliqua* across the Mediterranean. In a survey including 1019 samples, seven non-overlapping CEUs were identified as the best solution to minimize intra-group variance and obtain homogenous groups without geographic overlap. A thorough sampling across the Mediterranean using nuclear SSR and SNP data allows grouping the seven CEUs into four genetic clusters (Fig. 4): Southwest Morocco (W1), Iberian Peninsula (W3, W4), Central Mediterranean, and north Morocco (C1, W2) and eastern Mediterranean (E1, E2). RADseq data further resolved these genetic structuring across the Mediterranean by identifying seven genetic clusters (Fig. 4 A, C), which in some cases fully matched with a CEU (e.g. W1) or two CEUs (W3, W4), whereas, in other cases, a mixture of more than one genetic cluster was observed (e.g. C1). These data permit a better interpretation of the genetic diversity patterns between CEUs and are thus important for future designs of *ex situ* conservation actions. Our results suggest that a moderate genetic diversity is uniformly distributed across CEUs (Table 2). Only a slightly higher genetic diversity was estimated in western CEUs mostly based on SSR loci. Although W2 and C1 CEUs are highly admixed, these factors did not entail an increase in genetic diversity compared to non-admixed clusters. Conservation of genetic resources for the carob tree should recover genetic diversity found across the Mediterranean by preserving populations from western and eastern CEUs prioritizing the most differentiated ones (Table S3 in supplementary material). C1, which contains three genetic

groups and for which carob cultivars have been well characterized, specifically in Italy, should benefit from more investigations on carob evolution under domestication.

Acknowledgments

This study is part of the DYNAMIC project supported by the French national agency of research (ANR-14-CE02-0016) and benefited from equipment and services from the molecular biology facility (SCBM) at IMBE (Marseille, France). All bioinformatics and simulations were done on the High-Performance Computing Cluster from the Pytheas informatic facility (OSU Institut Pytheas Aix Marseille Univ, INSU-CNRS UMS 3470). J.V. benefited from a Postdoc Fellowship funded by DYNAMIC and a Marie Skłodowska-Curie Individual Fellowship (704464 - YAMNOMICS - MSCA-IF-EF-ST). The authors thank for their help to complete our sampling: Annette Patzelt (Oman Botanic Garden), Minas Papadopulos (Department of Forests of the Republic of Cyprus), Zahra Djabeur (Oran University), Nabil Benghanem (Tizi-Ouzou University), Gianluigi Bacchetta (Cagliari University), Sonja Yakovlev (Paris-Sud University), Errol Vela (Montpellier University), Maria Panitsa (Patras University), and the services of Junta de Andalucia. We thank the three anonymous reviewers for their helpful comments.

Author contributions

H. S., A.B., F.M., S.L.M., M.B.K., L.O., G.N.F. and J.V. conceived, planed the study and collected samples. F.L.M. performed the DNA extraction and quality assessment. J.V, A.B. and V.F. performed curation and analysis of microsatellite data. A.B. performed RADseq data curation and SNPs filtering. A.B. and J.V provided the analysis, tables and figures. A.B., J.V., G.N.F. and F.M. interpreted the results. A.B. and J.V. drafted the manuscript. J.V., G.N.F., S.L.M. and M.D.G. edited the manuscript. J.V., G.N.F. and A.B. wrote the final manuscript. H.S. was in charge with funding acquisition and project administration. All authors read and approved the final version.

Data Availability Statement

Full information on population sampling and microsatellite data are available in Viruel et al. (2020) and deposited in DRYAD (https://doi.org/10.5061/dryad.k7m020r).

Raw RADseq reads are deposited at NCBI SRA database under Bioproject accession PRJNA793764.

Assemblies from ipyRAD (vcf files), filtered R data files and R scripts of analyses are available at https://osf.io/t5m8y/.

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Supporting information

Tab. S1: Excel file describing each locality of sampling, with SSR diversity statistics.

Tab. S2: Statistics from four assemblies conducted on RADseq data (36 samples) elaborated with ipyrad with varying the clustering threshold from 0.9 to 0.96 % of similarity.

Tab. S3: pairwise Gst differentiation among CEUs based on RADseq data. Values above the overall Gst (11%) in bold.

Fig. S1: *Ceratonia siliqua* in three types of habitats considered in this study. (a) to (c) Wild habitats in Southwest Morocco, south Spain and Cyprus, respectively. (d) to (i) Seminatural habitats in France, Greece, north Morocco, Lebanon, Lebanon, and southwest Morocco, respectively. (j) to (l) Cultivated habitats in Cyprus, Sicily and Sicily, respectively.

Fig. S2: Genome-wide diversity structure of 350 carob trees based on 10,012 RADseq loci with an overall missing data rate of 64%. (A) PCA scatter plot of 350 carob RADseq genotypes. (B) Neighbor joining tree of pairwise Gst differentiations among seven Carob Evolutionary Units (CEUs).

Fig. S3: FST per loci distribution (1 SNP by locus) with 56 loci identified as outliers by OUTFLANK due to their unexpectedly high Fst differentiation (FDR <0.05). The blue line is the inferred neutral distribution.

Fig. S4: IQ-Tree maximum likelihood trees constructed with (A) plastid and (B) mitochondrial alignments obtained from RADseq data for the carob tree. PDNA and mtDNA have 135,525 and 191,840 bp for 47 and 52 % of missing data, respectively. See color codes in Fig. S2. *Ceratonia oreothauma*, the outgroup, is in black. Bootstrap values are indicated near the nodes.

Fig. S5: Plastid (left) versus mitochondrial (right) IQ-Tree maximum likelihood trees obtained for 190 C. siliqua and 4 C. oreothauma samples. Bootstrap support are shown for the main nodes. The dotted red square corresponds to central and eastern CEUs (in yellow and red) except for haplotypes from the northern area of W1 (in green). PDNA and mtDNA alignments accounted for 13,931 and 10,306 bp respectively with no missing data.

Fig. S6: Population genetic structure of the carob tree according to RADseq. A) Genetic admixture plots for 190 carob trees from k=2 to K=7 ancestral populations obtained with the snmf method (LEA package) performed on 3,557 unlinked SNPs. The West and East lineages refer to organellar haplogroups (Fig. S4). B) Cross-entropy criterion suggesting two optimal solutions with K= 5 or 7.

Fig. S7: FST per loci distribution (1 SNP by locus). OUTFLANK method did not detect any outlier (FDR <0.05). The blue line is the inferred neutral distribution.

Figures and tables captions

Figure 1: Bioinformatic pipeline used to call and filter SNPs from RADseq data for the carob tree. Stars (* and **) are corresponding to the two data sets of Table 1. Raw data and filtered vcf files (*,**) are available (see data availability statement).

Figure 2: Identification of Carob Evolutionary Units (CEUs) using ClustGeo method, which considers Euclidean genetic and geographic distances. The analysis used 1,019 genotypes from 56 localities based on 17 SSRs and 15 SNP markers from microsatellite loci. The Ward dendrogram of 56 carob populations with a partition in K=7 clusters (A) was obtained with a normalized proportion α of explained inertia of 0.2 for the geographic distance and 0.8 for the genetic distance. The seven clusters, or CEUs, are mapped (B) and a Neighbor Joining tree (C) shows pairwise genetic differentiation (Gst SSR markers) among the seven clusters. W1 = Southwest Morocco, W2 = mainly North Morocco but also West Algeria and Portugal, W3 = West Andalusia, W4 = East part of Andalusia, C1 = France, Italy and Algeria, E1 = Greece, Turkey and Cyprus, E2 = Lebanon and Crete (Greece), see details in Table 1.

Figure 3: Plastid (left) versus mitochondrial (right) IQ-Tree maximum likelihood trees obtained for 190 *C. siliqua* and 4 *C. oreothauma* samples. Black dots are indicating bootstrap support above 95% for the main nodes. The dotted red square corresponds to central and eastern CEUs (in yellow and red) except for haplotypes from the northern area of W1 (in green). PDNA and mtDNA have 135,525 and 191,840 bp for 47 and 52 % of missing data respectively. Color codes are corresponding to Carob Evolutionary Units (CEUs) and *Ceratonia oreothauma*, the outgroup, is indicated in black.

Figure 4: Population genetic structure of the carob tree. A) SVDquartets tree of seven genetically and geographically homogeneous groups (CEUs) based on RADseq markers. Genetic admixture plots are based on four ancestral populations for SSR markers (1019 genotypes, 17 loci) and on 7 ancestral populations for RADseq markers (190 genotypes, 3557 neutral unlinked SNPs). Within CEUs, genotypes were organized by habitats and according to their admixture coefficient (c= cultivated, s= seminatural and w= wild). B)) PCA scatterplots of RADseq genotypes (accumulated variance of the first four components = 15.2%). C) Map of genetic admixture based on RADseq markers.

Figure 5: Evolutionary history of the carob tree reconstructed with Treemix. Maximum likelihood trees obtained without (A) and with gene flow (B) events explaining 96% and 99% of the variance, respectively. The color of the arrows indicates the gene flow weight which is the fraction of ancestry derived from the gene flow edge.

Table 1: Sampling and data summarized for seven Carob Evolutionary Units (CEUs) identified by ClustGeo analysis based on genetic differentiation and geographic isolation of 1,019 genotypes sampled from 56 locations.

Table 2: Estimates of genetic diversity based on microsatellites (17 SSR loci) and RADseq markers (3557 SNPs) for seven *Ceratonia siliqua* units (CEUs). Within each CEU, samples were split into groups according to three habitat types (cultivated, seminatural or wild).

Table 1

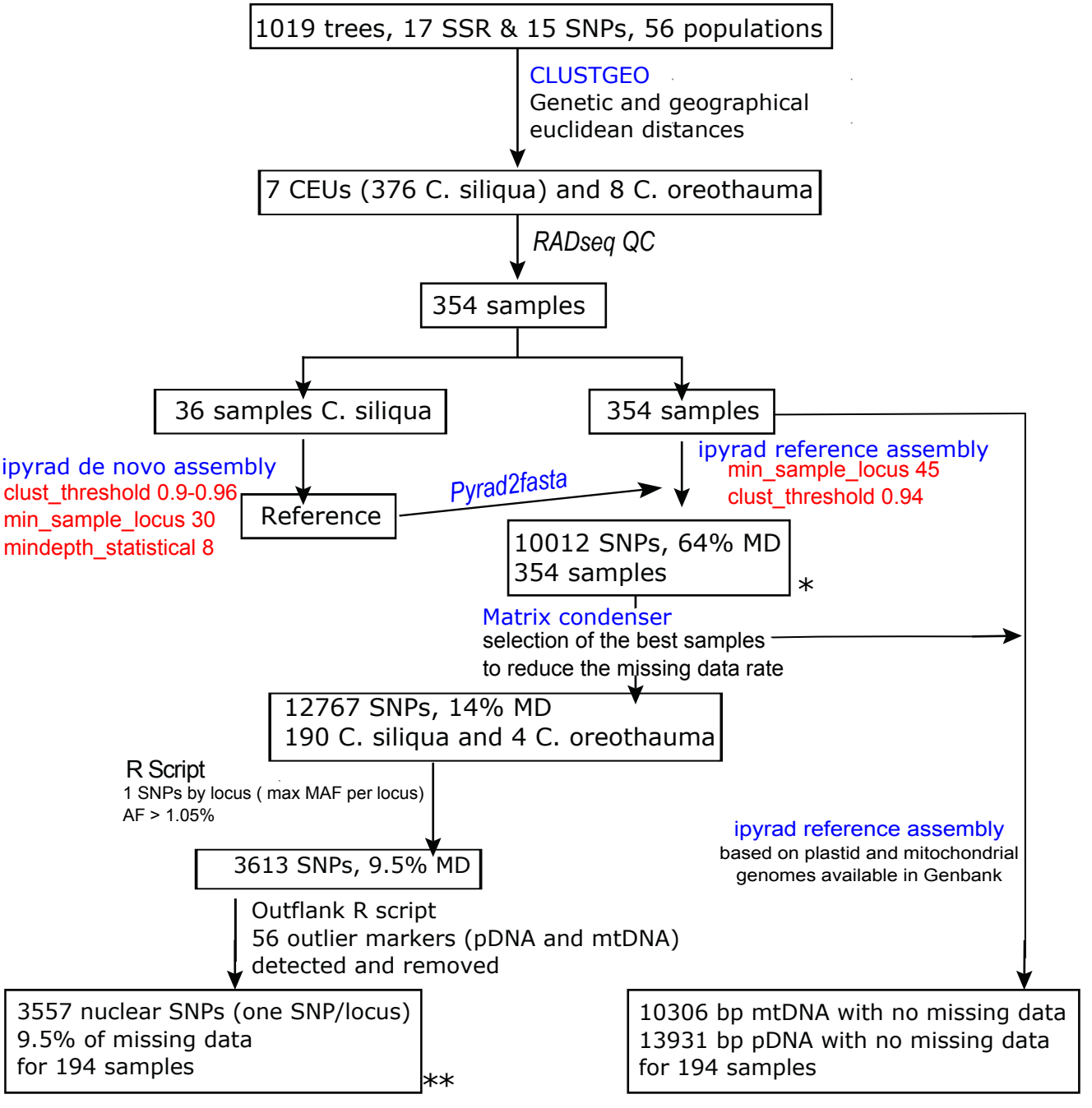
CEU	Observations	Sampling for SSR	Sampling for RAD first selection *	Sampling for RAD second selection **
W1	Southwest Morocco, carob trees often exploited, mainly in seminatural habitats but also in wild riparian vegetation and rocky slopes.	9 sites, 169 genotypes	7 sites, 62 genotypes	7 sites, 30 genotypes
W2	Mainly North Morocco but also West Algeria and Portugal, carob trees, almost all exploited, are found in cultivated and seminatural habitats, no carob in wild habitats	11 sites, 173 genotypes	6 sites, 30 genotypes	6sites, 23 genotypes
W3	West Andalusia, carob trees rarely exploited, carob trees frequent in wild habitats mostly on rock outcrops and cliffs.	5 sites, 104 genotypes	5 sites, 35genotypes	5 sites, 18 genotypes
W4	East part of Andalusia, carob trees more exploited than in the rest of the west but still frequently found in wild habitats	6 sites, 101 genotypes	5 sites, 35 genotypes	5 sites, 21 genotypes
C1	France, Italy and Algeria, high carob crop intensity in Sicily, mostly in seminatural habitats otherwise	10 sites, 193 genotypes	7 sites, 71 genotypes	7 sites, 42 genotypes
E1	Greece, Turkey and Cyprus, high frequency of carob trees in landscape, in seminatural or cultivated habitats and almost all exploited. Only carobs found in riparian vegetation are expected to be wild.	10 sites, 173 genotypes	8 sites, 53 genotypes	7 sites, 23 genotypes
E2	Lebanon and Crete (Greece), high frequency of carob trees in the landscape but mostly in abandoned field or orchards in Lebanon but cultivated and exploited in Crete.	5 sites, 106 genotypes	5 sites, 64 genotypes	5 sites, 33 genotypes

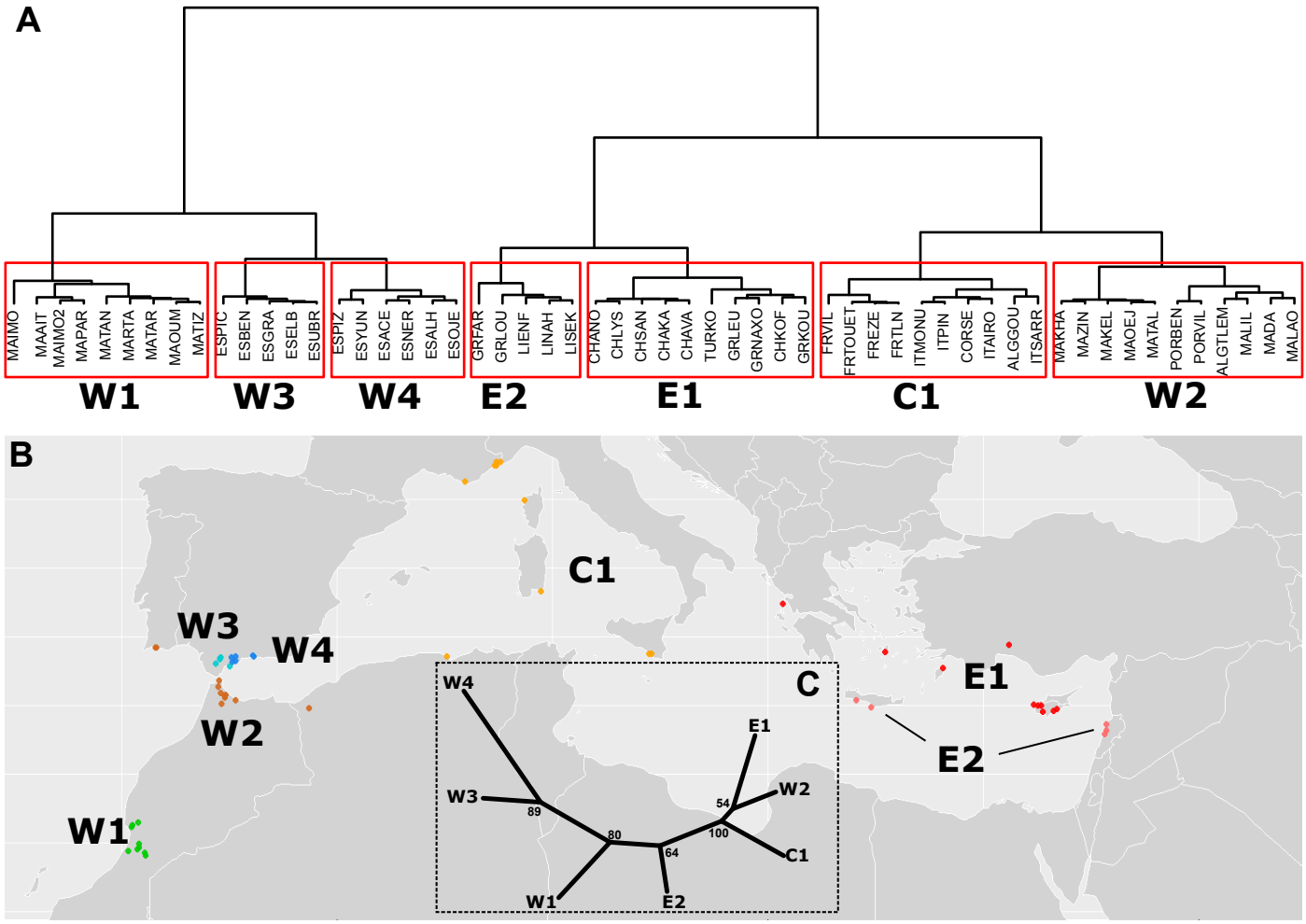
*,**: See M&M and Fig. 1 to see how these data sets were obtained

Table 2

	SSR					RAD				
	N	**Hobs**	**Hexp**	**f**	$\bar{r}_d$	**N**	**Hobs**	**Hexp**	**f**	$\bar{r}_d$
W1_cultivated	30	0.496	0.564	0.088*	22*	4	-	-	-	-
W1_seminatural	119	0.508	0.578	0.106*	18*	21	0.223	0.257	0.088*	6*
W1_wild	20	0.532	0.576	0.052 ns	5 ns	5	-	-	-	-
W1	169	0.509	0.579	0.109*	17*	30	0.222	0.260	0.109*	6*
W3_cultivated	20	0.513	0.5	-0.062 ns	68*	3	-	-	-	-
W3_seminatural	48	0.533	0.559	0.04 ns	8 ns	0	-	-	-	-
W3_wild	36	0.549	0.532	-0.038 ns	8 ns	15	0.220	0.241	0.037 ns	3*
W3	104	0.535	0.544	0.016 ns	17*	18	0.221	0.239	0.037_ns	3*
W4_cultivated	50	0.505	0.516	0.018 ns	42*	8	0.222	0.207	-0.135*	28*
W4_seminatural	31	0.502	0.56	0.084 ns	15*	9	0.227	0.237	-0.026 ns	4*
W4_wild	20	0.594	0.543	-0.128*	5 ns	4	-	-	-	-
W4	101	0.522	0.545	0.040 ns	24*	21	0.224	0.229	-0.010 ns	9*
W2_cultivated	62	0.456	0.48	0.011 ns	36*	10	0.208	0.228	-0.075*	19*
W2_seminatural	111	0.477	0.524	0.069*	16*	13	0.183	0.233	0.085 ns	195 ns
W2	173	0.47	0.513	0.06*	25*	23	0.194	0.236	0.114*	167_ns
C1_cultivated	22	0.412	0.473	0.074 ns	25*	19	0.217	0.189	-0.059*	188*
C1_seminatural	171	0.417	0.459	0.071*	18*	23	0.243	0.238	-0.029*	23*
C1	193	0.416	0.461	0.076*	17*	42	0.230	0.221	-0.016*	54*
E1_cultivated	33	0.473	0.471	-0.019 ns	122*	1	-	-	-	-
E1_seminatural	113	0.448	0.506	0.096*	18*	18	0.219	0.237	0.032 ns	60*
E1_wild	27	0.459	0.479	0.013 ns	20*	5	-	-	-	-
E1	173	0.454	0.501	0.076*	20*	24	0.217	0.240	0.060*	43*
E2_cultivated	39	0.425	0.477	0.106 ns	34*	10	0.224	0.241	0.010 ns	11*
E2_seminatural	67	0.475	0.517	0.067 *	16*	22	0.200	0.223	0.067 ns	11*
E2	106	0.456	0.517	0.106*	23*	32	0.208	0.235	0.091*	14*

N= number of genotypes analysed, Hobs= observed heterozygosity, Hexp= expected heterozygosity or Nei genetic diversity, f=Wright's inbreeding coefficient, $\bar{r}_d$ = standardized form of the index of association accounting for multilocus linkage (x1000). * indicates f or $\bar{r}_d$ significantly different from zero based upon 500 and 100 bootstrap iterations respectively.

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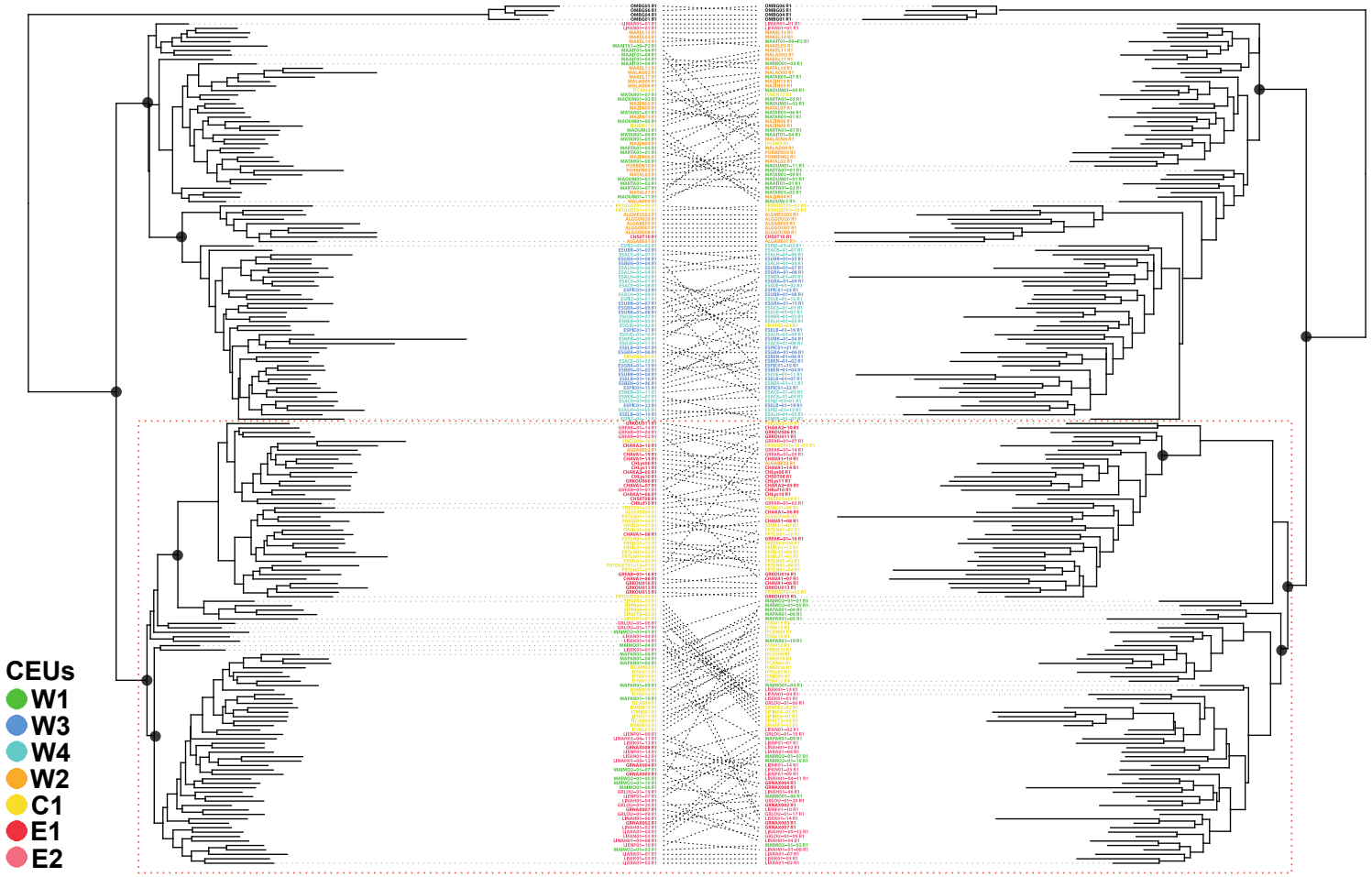
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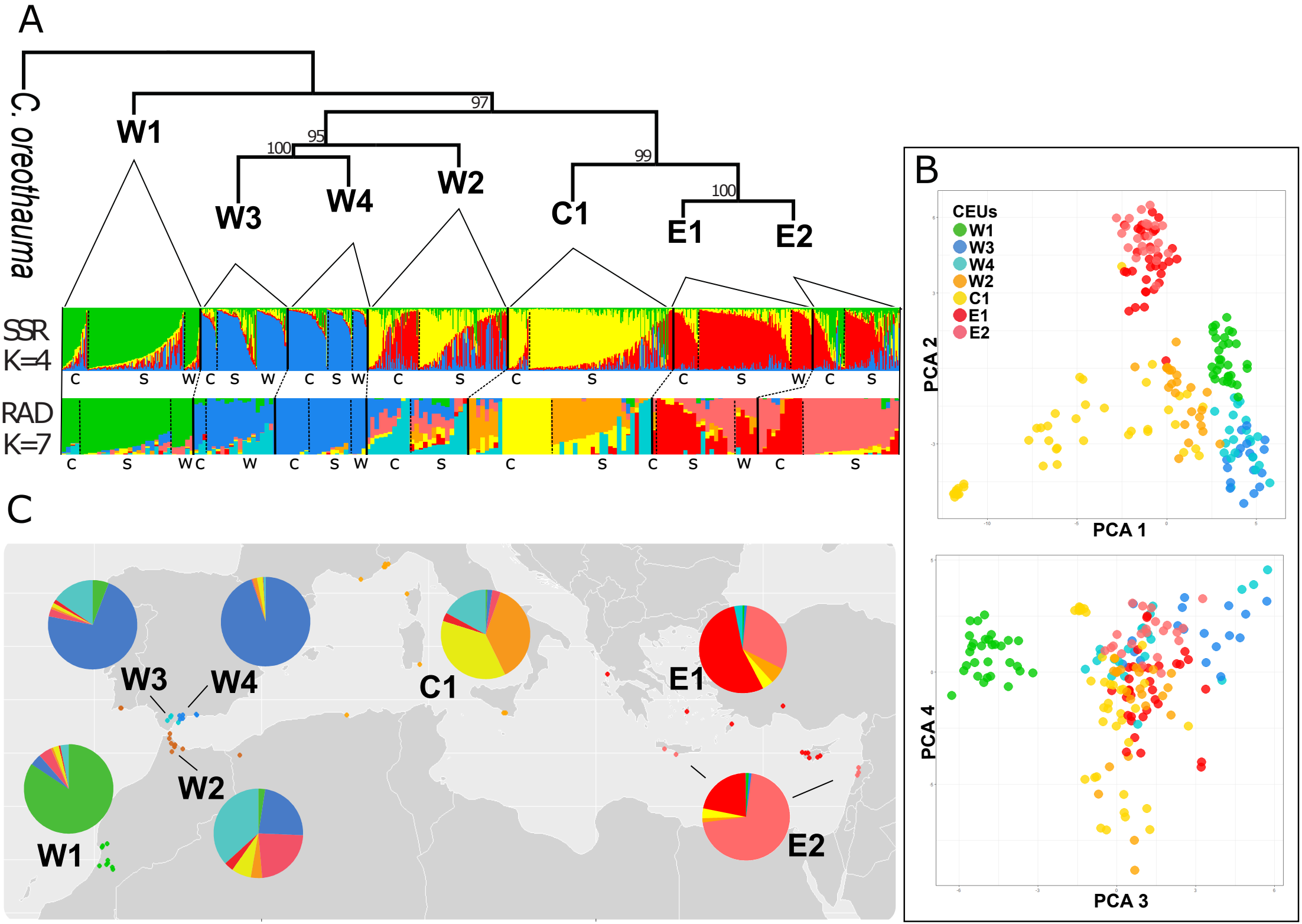
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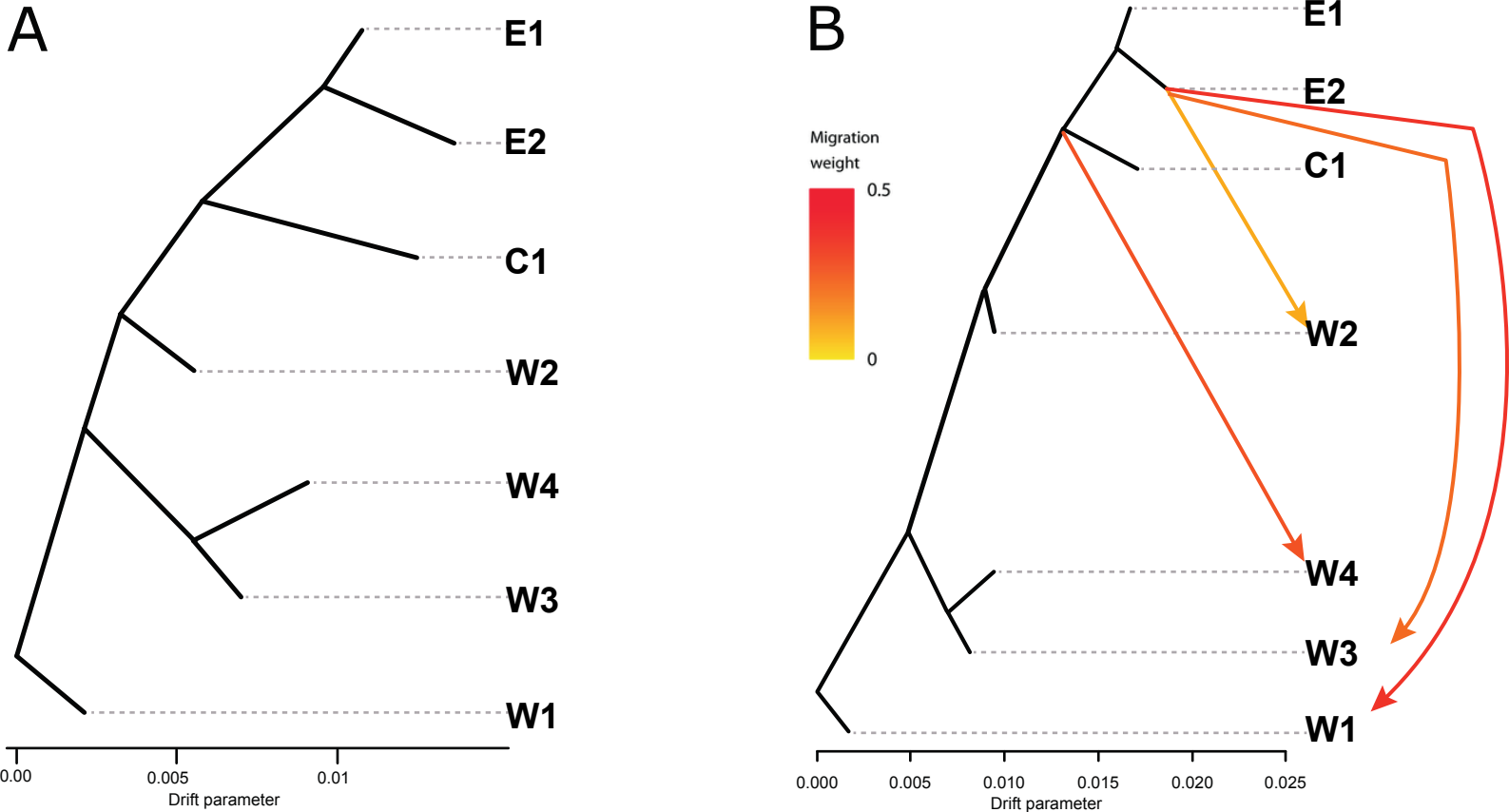
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