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A complex genetic structure of *Tetraclinis articulata* (Cupressaceae) in the western Mediterranean

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Some tree species have distributions on both sides of the Strait of Gibraltar and the Strait of Sicily. It is a challenge to determine whether such distributions result from the Tertiary or Pleistocene or from more recent dispersal related to human activities. *Tetraclinis articulata* (Cupressaceae) is a gymnosperm that offers an ideal model to deal with this problem because it has a limited area of distribution and has been used only moderately by humans. Three hundred and twenty-three individuals from 30 populations covering the entire distribution of the species were analysed. A multiple approach was developed: (1) by assessing the genetic structure through two molecular techniques, AFLP and nSSR markers, which were used to evaluate the genetic diversity of these populations and the relationships among them and (2) by estimating past distributions. Four lineages of populations that are geographically intermixed to a certain extent are documented here. Results obtained are discussed in the context of palaeontological records and climatic models. There is evidence of an ancient widespread distribution, including Europe, and the subsequent appearance of four isolated lineages that, nowadays, are partially intermixed. Nevertheless, the origin of the current populations could not be fully ascertained through this work, although logical deductions are discussed that consider human activities or, much less probably, wind dispersal of seeds out of refugia in northern Africa-southern Europe during the Quaternary, including shore connections.

ADDITIONAL KEYWORDS: AFLP – dispersal – fossils – glacial refugia – gymnosperm – microsatellites – phylogeography – Tertiary.

INTRODUCTION

Migration routes, refugia and speciation have led the Mediterranean Basin to contain large numbers of endemic species in relatively small areas. This, with significant threats of habitat loss, led Myers *et al.* (2000) to consider it among the 25 most important biodiversity hotspots. Its geological history in the last 30 Myr has been dynamic. Land bridges between the northern and southern shores of the Mediterranean Sea existed during the Oligocene–Miocene, especially during the Messinian salinity crisis, which was followed by opening of the Strait of Gibraltar (Bocquet, Widler & Kiefer, 1978; Krijgsman *et al.*, 1999; Broderick *et al.*, 2003; Thompson, 2005). During the Miocene and Pliocene, microplate tectonics in the south-western Mediterranean (Rosenbaum, Lister & Duboz, 2002) modified contacts between palaeoshores, making long-term vicariance events possible when microplates separated (Pfenninger *et al.*, 2010). More recently, glaciations in the Quaternary shaped current distributions of many tree species, including *Abies alba*

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Mill. or *Fagus sylvatica* L. (Hewitt, 1999). The number of phylogeographical studies on plant species that live on both shores of the Mediterranean Sea reached a substantial level in the last dozen years (Ortiz *et al.*, 2009; Fernández-Mazuecos & Vargas, 2010; González-Martínez *et al.*, 2010; Sánchez-Gómez *et al.*, 2018; Sękiewicz *et al.*, 2018). A review of older studies was presented by Rodríguez-Sánchez *et al.* (2008). In these types of phylogeographical investigation, a wide assortment of molecular approaches, especially AFLP (amplified fragment length polymorphisms; García-Castaño *et al.*, 2014; Sánchez-Gómez *et al.*, 2018) and nSSRs (nuclear simple sequence repeats, Sánchez-Robles *et al.*, 2014a), have been used. The number of studies focusing on the Mediterranean southern shore, however, is low, despite the sensitivity of this area to climate change (Houghton *et al.*, 2001). Furthermore, the scarcity of fossil records in this region makes phylogeographical reconstructions difficult (Elenga *et al.*, 2000; Magri & Parra, 2002; García-Castaño *et al.*, 2014).

Tetraclinis articulata (Vahl) Masters, commonly known as the sandarac gum tree, Barbary thuja, araar tree or Mediterranean alerce (among other names), is an evergreen, medium-sized coniferous tree in a currently monospecific genus in the cypress family (Cupressaceae). It is 6(–12) m tall, with a reddish-brown scented trunk, flattened branches, scale-like leaves and female cones with four characteristic scales. It is endemic to the mountain ranges of northern Africa (Morocco, Algeria and Tunisia, up to 1800 m a.s.l.), with isolated populations mainly occurring in east Spain (near Cartagena, Murcia province) and Malta (Tutin, 1964; do Amaral Franco, 1986; Charco, 2001; see Fig. 1). There are also scattered populations that are considered naturalized or of uncertain origin in Cyprus (Hand, 2010), the Canary Islands (Santos Guerra & Reyes-Betancort, 2014) and the southern Spanish provinces of Málaga (Monte San Antón), Huelva (Doñana), Granada (Barranco de Lanjarón) and Almería (Bajo Almanzora, Sierra de Gádor and Sierra de Lúcar), among others (Rosúa *et al.*, 2001; Farjon, 2005; Casimiro-Soriguer & Pérez Latorre, 2008; Baonza Díaz, 2010; Pérez Latorre & Cabezudo, 2011; Casimiro-Soriguer, Pérez Latorre & Cabezudo, 2014; Raab-Straube, 2014). Reports from Libya were not substantiated by Farjon (2005).

The hardness, veneer, durability and fragrance of *Tetraclinis articulata* wood have encouraged traditional human use in construction, handcrafting, wood carving, cabinet making and even improving the flavour of drink [Plinius Secundus (better known as Pliny the Elder), 1st century AD; Smith, 1857; Khabbach *et al.*, 2012; Azémard, Ménager & Vieillescazes, 2017]. Its impermeability to water and resistance to fungal decay (Fidah *et al.*, 2015) offer important

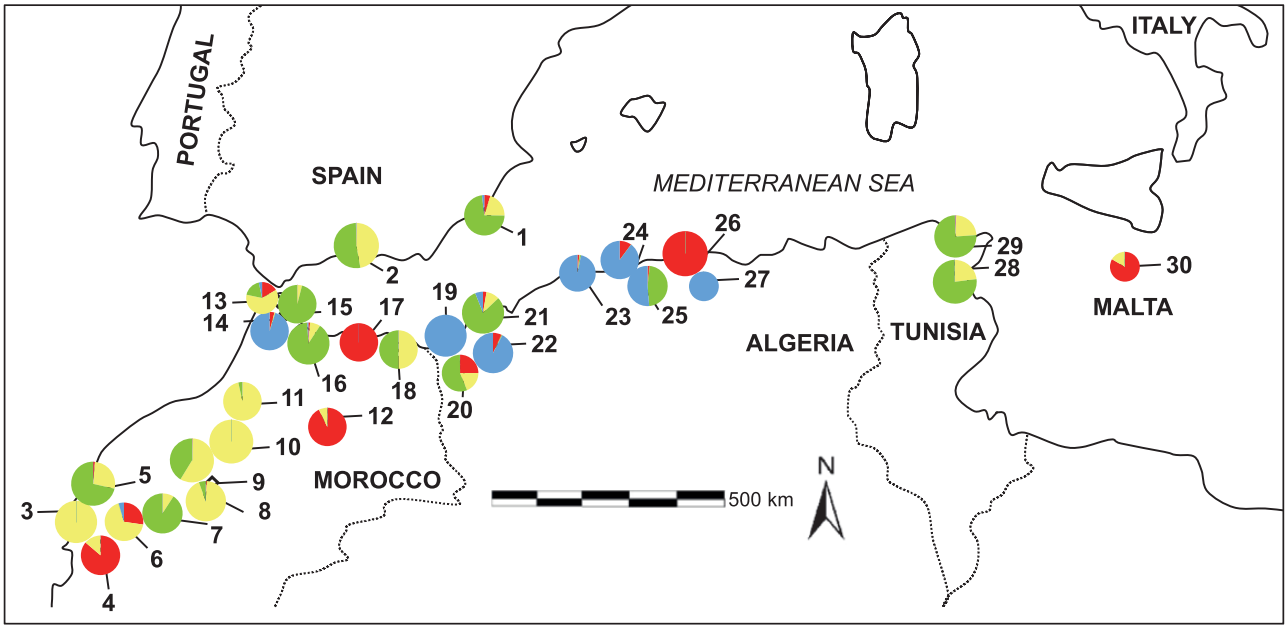

Figure 1. Geographical distribution of the 30 sampled populations of *Tetraclinis articulata* and BAPS results based on AFLP data. Four groups were distinguished [log (marginal likelihood) of the optimal partition = –45 269.95]: the WC-Moroccan group of populations (yellow), the Algerian group of populations (blue) and two groups [widespread (green) and Moroccan–Algerian (red)] that appeared to some extent in different countries. Circle size proportional to the number of individuals genotyped (for population codes and details, see Table 1).

characteristics for shipbuilding. Nevertheless, the intensity of these uses is far from that of species like *Olea europaea* L. (Besnard & Rubio de Casas, 2016). According to Charco (1999), traditional practices, as well as grazing pressure and uncontrolled fires, have led to degradation of many forests (Hadjadj-Aoul, Chouïeb & Loisel, 2009; López-Hernández, 2000; Baonza Díaz, 2010). Although resistance to fire and ability to resprout have helped avoid extinction of this species (López-Hernández *et al.*, 1995), large trees are scarce and density is low (DREF, 2002). Listed as a 'least concern' species in the IUCN Red List of Threatened Species (v.2020–3) with a 'decreasing' population trend, the populations in Spain and Malta are regionally 'endangered' (Sánchez-Gómez *et al.*, 2011; Thomas, 2017).

Despite the ecological and economic importance of this species (Charco, 1999), little information exists on the genetic structure and variability of its natural populations. However, Sánchez-Gómez *et al.* (2013) studied genetic diversity and differentiation of 14 populations based on inter-simple sequence repeats (ISSR markers). Their study showed that 83% of the total genetic variation was within populations, grouping Spanish populations near Cartagena with those from Malta and Tunisia. Nevertheless, partly due to the use of only one type of genetic marker, many important questions, such as the origin of the non-African populations, remained unsolved.

We propose an ancient widespread distribution followed by a reduction that led to the appearance of isolated lineages. Populations were probably spread further by human activities (trade and deliberate introductions for its wood) or naturally by wind-mediated pollen or seed dispersal, leading to a connection between the shores of the Mediterranean Basin. The aims of this study were: (1) to clarify the genetic diversity and population structure of *T. articulata*; and (2) to investigate the evolutionary history of this species in its area of distribution. These objectives were addressed by (1) assessing the genetic structure through two molecular markers, AFLPs and nSSRs, and (2) estimating past distributions based on niche modelling. Results obtained are discussed in the context of palaeontological records, climatic models, reproductive biology of the species and the influence of human culture.

MATERIAL AND METHODS

Material collection and DNA extraction

Young leafy branches were collected from six to 14 individuals in 30 populations throughout the entire area of distribution in Spain (populations 1–2), Morocco (populations 3–18), Algeria (populations 19–27), Tunisia (populations 28–29) and Malta (population 30), resulting in a total of 323 samples (Table 1; Fig. 1). All the populations analysed here are considered as wild, except population 2 (from Monte San Antón, Málaga, Spain), which is recognized as non-native. In most cases, the collected specimens were sufficiently far apart to avoid collecting the same individual. Moreover, the number of individuals per population was based on collector observations in the field and, in some cases, on bibliographic sources. Material was preserved in silica gel, and DNA was extracted using an Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany). A related species, *Platycladus orientalis* (L.) Franco (Yang, Ran & Wang, 2012), also Cupressaceae, was used to root the tree including the sampled populations of *T. articulata*. Vouchers from all sampled populations were deposited at the Herbarium of the University of Seville (SEV, Spain; see Table 1).

AFLP analyses

AFLP was performed following the protocols of Vos *et al.* (1995). A previous screening of selective primers was performed on eight individuals from eight populations, with 72 two- and three-selective nucleotide primer combinations. The final six selected primer combinations, based on polymorphism and success, were (fluorescent dye in brackets): *Eco*RI(FAM)-ACC/*Mse*I-CAG, *Eco*RI(VIC)-ACG/*Mse*I-CTA, *Eco*RI(NED)-AGC/*Mse*I-CAG, *Eco*RI(FAM)-ACC/*Mse*I-CGA, *Eco*RI(VIC)-ACG/*Mse*I-CTG and *Eco*RI(NED)-AGC/*Mse*I-CGA. Fifty-eight individuals (18%), with one to two individuals per population, were replicated to calculate the error rate according to Bonin *et al.* (2004), starting from DNA extracts. Assuming that genotyping and scoring errors were randomly distributed and no bias at the population level would occur, a 0.1-limit error was followed as recommended in Skrede *et al.* (2006) and Bonin, Ehrich & Manel (2007).

The fluorescence-labelled selective amplification products were separated by capillary gel electrophoresis on an automated sequencer (3730 DNA Analyzer, PE Applied Biosystems, Foster City, CA, USA) with an internal size standard (GeneScan - 500 LIZ, Applied Biosystems) at the Genomics Unit of the Madrid Science Park (Complutense University, Madrid, Spain). Raw data were exported to GeneMarker v.1.8 (SoftGenetics, LLC, PA, USA) for scoring of fragments. The scoring was normalized after automatic runs. Amplified fragments from 75 to 503 bp were scored. Scoring results were exported as an absence/presence (0/1) matrix.

Genetic diversity was assessed for each population using the total number of AFLP fragments present

Table 1. Population samples of *Tetraclinis articulata*. Origin (country, population, locality/region, coordinates, associated voucher number), sample size (n), population size (N)*. (I) AFLP. n_{ph}: number of phenotypes, $Frag_{tot}$: number of fragments, $Frag_{poly}$: number of polymorphic fragments, $Frag_{priv}$: number of private fragments, H_D: average gene diversity, F_{IS}: inbreeding coefficient. (II) nSSR. MLGs: number of multilocus genotypes, *A_NA*: average number of alleles per locus, *P*: number of private alleles per population, *RA*: allelic richness after rarefaction, H_o: observed heterozygosity, H_e: expected heterozygosity, r_d: standardized index of association (significant values in bold), F_{IS}: inbreeding coefficient. SD: standard deviation, SE: standard error, CI: confidence interval

							AFLP						nSSR							
Country	Population code	Locality/Region	Coordinates	Associated voucher number	n	N*	n_{ph}	$Frag_{tot}$	$Frag_{poly}$	$Frag_{priv}$	H_D (SD)	F_{IS} (95% CI)	MLGs	*A_NA* (SE)	*P* (SE)	*RA* (SE)	H_o	H_e	r_d	F_{IS} (95% CI)
Spain																				
	1	Sierra Minera de Cartagena-La Unión (Murcia)	37 °35′N 0 °50′W	SEV223187	11	5000	11	228	189	2	0.13 (0.07)	0.78 (0.61–0.93)	10	4.00 (0.49)	0.01 (0.01)	3.12 (0.34)	0.34	0.51	0.10	0.24 (0.02–0.42)
	2	Monte San Antón (Málaga)	36 °44′N 4 °21′W	SEV286925	14	35	14	216	178	1	0.12 (0.06)	0.78 (0.61–0.92)	14	2.43 (0.20)	0.00 (0.00)	2.35 (0.19)	0.47	0.49	0.01	0.00
Morocco																				
	3	Igouzouln	31 °08′N 9 °42′W	SEV223079	12	> 1000	12	275	247	2	0.09 (0.05)	0.93 (0.77–1.00)	11	5.43 (1.36)	0.07 (0.04)	4.25 (0.90)	0.61	0.60	0.03	0.00
	4	Ait Assaiu	30 °36′N 9 °21′W	SEV223075	11	> 5000	11	220	175	3	0.13 (0.07)	0.92 (0.80–1.00)	11	4.29 (1.15)	0.01 (0.01)	3.54 (0.80)	0.67	0.65	0.08	0.00
	5	Tawbalt	31 °30′N 9 °42′W	SEV223081	13	> 5000	13	226	183	2	0.12 (0.06)	0.83 (0.65–0.96)	13	5.71 (1.41)	0.04 (0.03)	4.23 (0.90)	0.36	0.64	0.02	0.42 (0.27–0.59)
	6	Imi-n-Tanannat	31 °07′N 8 °57′W	SEV223074	10	500–1000	10	194	151	3	0.10 (0.05)	0.91 (0.75–1.00)	10	5.43 (1.34)	0.18 (0.11)	4.38 (0.94)	0.68	0.69	0.01	0.00
	7	El Tnium	31 °20′N 7 °45′W	SEV223070	11	1000–5000	11	232	187	3	0.14 (0.07)	0.77 (0.57–0.92)	11	5.57 (1.43)	0.13 (0.09)	4.40 (0.99)	0.44	0.59	0.03	0.00
	8	Tanannat	31 °53′N 6 °49′W	SEV223069	11	> 1000	11	188	154	3	0.11 (0.06)	0.91 (0.74–0.99)	11	4.57 (0.81)	0.10 (0.08)	3.78 (0.58)	0.36	0.55	0.04	0.27 (0.09–0.46)
	9	Ait-Attab	32 °09′N 6 °47′W	SEV223067	13	> 1000	12	226	183	5	0.13 (0.07)	0.84 (0.67–0.98)	13	6.43 (1.17)	0.19 (0.11)	4.63 (0.71)	0.67	0.68	-0.04	0.00
	10	Oued Zem	32 °49′N 6 °34′W	SEV223063	13	100–500	7	269	239	1	0.09 (0.05)	0.92 (0.77–1.00)	13	5.57 (1.41)	0.12 (0.09)	4.28 (0.88)	0.43	0.68	-0.03	0.17 (0.16–0.45)
	11	Maaziz	33 °39′N 6 °18′W	SEV223062	10	100–500	10	239	208	3	0.10 (0.05)	0.86 (0.64–0.98)	10	4.43 (0.75)	0.01 (0.01)	3.74 (0.62)	0.54	0.58	0.02	0.00
	12	Jbel Bou-Iblan	33 °38′N 4 °14′W	SEV227744	10	> 1000	10	219	180	0	0.14 (0.08)	0.92 (0.77–1.00)	10	5.00 (0.69)	0.01 (0.01)	4.42 (0.59)	0.49	0.63	0.05	0.24 (0.10–0.41)
	13	Bni-Karrich	35 °29′N 5 °26′W	SEV218743	7	> 1000	7	248	212	2	0.14 (0.08)	0.76 (0.52–0.95)	7	4.29 (0.47)	0.30 (0.18)	4.16 (0.42)	0.20	0.58	0.03	0.63 (0.44–0.79)
	14	Chefchaouen	35 °15′N 5 °16′W	SEV218764	10	100–500	6	309	292	0	0.05 (0.03)	0.77 (0.46–0.99)	10	4.57 (0.53)	0.01 (0.01)	4.03 (0.44)	0.67	0.68	0.00	0.00
	15	Between Chefchaouen and Oued-Laou	35 °18′N 5 °13′W	SEV218772	10	> 1000	9	241	192	2	0.16 (0.09)	0.76 (0.56–0.95)	9	5.00 (0.69)	0.04 (0.04)	4.31 (0.52)	0.33	0.66	**0.12**	0.50 (0.35–0.64)
	16	Between El Khemis and Assifane	35 °10′N 4 °59′W	SEV218800	12	> 1000	12	226	188	0	0.13 (0.07)	0.83 (0.64–0.97)	12	5.00 (0.53)	0.01 (0.01)	4.19 (0.48)	0.38	0.63	-0.02	0.38 (0.24–0.52)
	17	Imoussatene	35 °11′N 3 °25′W	SEV218855	10	> 1000	10	227	188	1	0.15 (0.08)	0.88 (0.70–1.00)	10	4.57 (0.69)	0.05 (0.04)	4.04 (0.54)	0.27	0.60	-0.05	0.55 (0.39–0.69)
	18	Kebdana Mountains	34 °58′N 2 °45′W	SEV218914	10	> 1000	9	201	161	1	0.10 (0.05)	0.73 (0.50–0.91)	8	4.71 (0.87)	0.13 (0.08)	4.00 (0.65)	0.31	0.58	**0.18**	0.43 (0.26–0.60)
Algeria																				
	19	Honaine	35 °12′N 1 °36′W	SEV286926	12	> 10000	11	299	268	1	0.07 (0.04)	0.87 (0.68–1.00)	12	5.71 (0.84)	0.13 (0.08)	4.56 (0.52)	0.43	0.69	0.05	0.38 (0.25–0.51)
	20	Tlemcen	34 °45′N 1 °11′W	SEV286927	9	> 1000	9	264	232	2	0.13 (0.07)	0.70 (0.47–0.89)	9	5.43 (0.84)	0.04 (0.02)	4.77 (0.70)	0.43	0.61	0.11	0.28 (0.11–0.45)
	21	Misserghin	35 °39′N 0 °44′W	SEV286928	12	> 10000	11	237	212	7	0.14 (0.07)	0.80 (0.61–0.94)	12	4.57 (0.75)	0.11 (0.11)	3.89 (0.53)	0.34	0.59	-0.02	0.43 (0.28–0.57)
	22	Foret de Messer (near Hassi Dahou)	35 °08′N 0 °36′W	SEV286929	11	> 1000	10	213	175	3	0.09 (0.05)	0.75 (0.52–0.94)	11	5.00 (0.79)	0.01 (0.01)	4.06 (0.51)	0.41	0.61	-0.04	0.29 (0.15–0.48)
	23	Tenes	36 °32′N 1 °20′E	SEV286930	9	> 10000	8	263	226	0	0.10 (0.05)	0.74 (0.52–0.91)	9	4.43 (0.53)	0.10 (0.10)	4.03 (0.47)	0.38	0.63	0.06	0.40 (0.25–0.57)
	24	Tipasa	36 °34′N 2 °33′E	SEV286931	10	> 1000	8	251	214	4	0.08 (0.04)	0.84 (0.63–0.99)	10	5.00 (0.58)	0.02 (0.01)	4.30 (0.44)	0.40	0.62	-0.02	0.32 (0.14–0.49)
	25	Chiffa	36 °25′N 2 °45′E	SEV286932	11	> 1000	10	246	210	6	0.17 (0.09)	0.82 (0.63–0.95)	11	4.57 (0.72)	0.00 (0.00)	3.84 (0.57)	0.36	0.60	0.04	0.37 (0.20–0.51)
	26	Bouira	36 °39′N 3 °56′E	SEV286933	14	> 1000	13	235	193	7	0.14 (0.07)	0.81 (0.64–0.94)	14	4.71 (0.57)	0.02 (0.02)	3.81 (0.44)	0.65	0.65	-0.01	0.00
	27	Mchedallah	36 °22′N 4 °16′E	SEV286934	6	> 10	6	165	115	0	0.09 (0.05)	0.65 (0.36–0.89)	3	3.00 (0.31)	0.01 (0.01)	3.00 (0.31)	0.57	0.52	**0.55**	0.00
Tunisia																				
	28	Zaghouan	36 °23′N 10 °08′E	SEV286935	13	> 1000	13	208	174	0	0.11 (0.06)	0.87 (0.69–0.99)	13	5.00 (0.62)	0.00 (0.00)	4.28 (0.48)	0.62	0.65	-0.01	0.08 (0.00–0.21)
	29	Hammam Lif	36 °43′N 10 °20′E	SEV286936	12	> 10000	11	220	184	0	0.12 (0.06)	0.87 (0.70–0.99)	12	4.71 (0.84)	0.02 (0.02)	3.85 (0.60)	0.43	0.66	-0.01	0.00
Malta																				
	30	Mellieha	35 °57′N 14 °22′E	SEV286937	6	350	4	193	138	0	0.14 (0.08)	0.75 (0.48–0.94)	6	2.43 (0.20)	0.00 (0.00)	2.43 (0.20)	0.29	0.47	**0.32**	0.35 (0.07–0.61)

*Number estimated according to the observation of the different collectors in field and/or according to the bibliography.

($Frag_{tot}$), the number of polymorphic fragments ($Frag_{poly}$) and the number of private fragments ($Frag_{priv}$). We also calculated the average Nei's gene diversity (1987) H_D with Arlequin v.3.11 (Schneider *et al.*, 1997; Excoffier, Laval & Schneider, 2005): $H_D = 1 - \Sigma x_i^2$; where x_i is the population frequency of each phenotype 'allele' (0 or 1) at locus i. The average gene diversity was the resulting mean across all loci. AFLPDAT (Ehrich, 2006) was used to calculate the number of AFLP phenotypes (n_{ph}) after scoring-error correction (i.e. considering the average number of loci subject to error per individual). Population F_{IS} values were calculated using the Bayesian method of estimation of the within-population inbreeding coefficient (F_{IS} analogue) with the software I4A (I4A, Inbreeding For AFLP), with the default priors (Chybicki, Oleksa & Burczyk, 2011). This method assumes independency of allele frequencies among sub-populations and linkage equilibrium, which was tested by means of the exact test for haplotypic data implemented in Arlequin v.3.11. Moreover, ascertainment bias in AFLP markers might introduce bias to F_{IS} estimates and there is also risk of bias when assessing extreme values. Nevertheless, it works reasonably well if the actual inbreeding coefficient shows high variance, which, as long as some variation due to, for example, mixed mating system exists, provides robust estimates.

The population structure was examined by an approach based on statistical inference with Bayesian clustering methods, applying BAPS v.5.3 (Corander, Waldmann & Sillanpää, 2003; Corander *et al.*, 2004; see also Supporting Information, Fig. S1 for a parallel Structure approach). BAPS was run on diploid data with the maximal number of groups (K) set to 30 (i.e. a number equal to the sampled populations), which was replicated 30 times, and the results were averaged according to the obtained likelihood scores. Subsequently, admixture analysis (Corander & Marttinen, 2006) was conducted with the following settings: 50 iterations to estimate the admixture coefficients for the individuals, and a minimal size of clusters of one individual and 20 iterations to estimate the admixture coefficients for 50 reference individuals. Migration rates, based on the estimated population sizes, were indirectly calculated to assess the confidence of the optimal K value obtained (Cullingham *et al.*, 2020).

Cavalli-Sforza and Edwards' chord population distances (Cavalli-Sforza & Edwards, 1967; Takezaki & Nei, 1996) were calculated with FAMD v.1.30 (Schlüter & Harris, 2006) by applying the Bayesian method to calculate null allele frequencies and with the among-population non-uniform prior assumption (Zhivotovsky, 1999). The matrix was exported to SplitsTree v.4.5 (Huson & Bryant, 2006) where a Neighbor-Net (Bryant & Moulton, 2004) was constructed to establish the different relationships between sampled populations. The support for each node was calculated with FAMD v.1.30, using bootstrap replicates based on the mentioned chord distances for 1000 neighbor-joining trees. To investigate genetic distances and relationships between individuals and populations, we applied two principal coordinate analyses (PCoAs) with the software FAMD v.1.30, based on (1) squared Euclidean distances, averaged per population and (2) chord distances.

Analyses of molecular variance (AMOVA) were conducted with Arlequin v.3.11, in haplotypic format, based on 20 022 permutations. Two approaches were carried out: (1) for all the populations and (2) only for the populations of the main area of distribution, i.e. continental northern Africa, in four groups delimited by the three countries sampled and splitting Morocco into two regions (closer to the Atlas Mountains or closer to the Rif): i.e. south and centre of Morocco, north Morocco, Algeria and Tunisia (four geographically delimited groups from west to east). To test for isolation by distance, the population chord distances were compared with their geographical distance using Mantel bilateral tests based on Spearman's correlations (on 10 000 random permutations; XLSTAT v.2016.03.30882, Addinsoft), for all the populations and only for the northern African ones. Finally, we tested the existence of a diversity (based on H_D) or an inbreeding pattern along the continental northern African west–east axis, which was highly correlated to the south–north axis (Spearman's $\rho = 0.885$, $P < 0.0001$; $n = 27$) by a Spearman's correlation, as well as the relationship between genetic diversity and population size, which was not correlated to the west–east axis (Spearman's $\rho = 0.093$, $P < 0.645$; $n = 27$) (JMP v.6.0.0; SAS Institute Inc., 2005).

For the BAPS-inferred groups of populations, considering those individuals completely assigned to one of them, the $Frag_{tot}$, $Frag_{poly}$ and $Frag_{priv}$ were calculated, as well as H_D and n_{ph} after scoring-error correction. A Neighbor-Net was constructed based on $\rho_{ST(2)}$ values, used here to compensate for the high degree of inequality in the sample sizes (El Mousadik & Petit, 1996).

NUCLEAR SSR ANALYSES

A total of seven nuclear microsatellite (nSSR) markers previously developed for *T. articulata* were used for plant genotyping: *Tetra1*, *Tetra2*, *Tetra4*, *Tetra15*, *Tetra19*, *Tetra29* and *Tetra49*. Details about microsatellite characteristics, PCR cycle profiles and amplification conditions can be found in Lorenzo *et al.* (2014). Amplification and labelling of amplified products were performed at the University of Seville

Research, Technology and Innovation Centre (CITIUS, University of Seville, Seville, Spain) and analysed on a 3730 DNA Analyzer sequencer (Applied Biosystems) at the Genomics Unit of the Madrid Science Park (Complutense University, Madrid, Spain). nSSR markers were scored using GeneMarker v.1.8.0 (SoftGenetics, State College, Pennsylvania, USA) and they were further manually checked. Genotyping reproducibility was checked in 60 independent repeats (19% of total), starting from DNA extracts.

First, the number of multilocus genotypes (MLGs) was calculated for each population. When individuals with the same MLGs resulted, an estimate of the probability for a genotype to be obtained by chance (P_{gen}; Arnaud-Haond *et al.*, 2007) was taken into account. Investigations as to whether the populations were clonal (where significant disequilibrium is expected due to linkage among loci) were carried out, estimating the standardized index of association (r_d; Agapow & Burt, 2001). Additionally, to test whether MLG replicates were the result of a clonal spread rather than a distinct recombination event, P_{sex} was estimated (Arnaud-Haond *et al.*, 2007). These tests were performed with the R package *poppr* v.2.3.0 (Kamvar, Tabima & Grünwald, 2014; Kamvar, Brooks & Grünwald, 2015; Kamvar *et al.*, 2016). To assess the genetic diversity of each population, the average number of alleles per locus (*A_NA*), and the observed and the expected heterozygosity (H_o and H_e, respectively) were computed with the R package *adegenet* v.2.0.1 (Jombart, 2008). In addition, allelic richness (*RA*) and rarity (private alleles per locus, *P*) were estimated following a generalized rarefaction approach using ADZE v.1.0 (Szpiech, Jakobsson & Rosenberg, 2008).

As presence of null alleles may affect estimates of inbreeding, INEST v.2.2 (Chybicki & Burczyk, 2009; Chybicki *et al.*, 2011) was used to estimate, at the same time, the number of null alleles, the genotyping errors and the inbreeding coefficients of the populations. INEST was run for different models for each population (500 000 generations plus 5000 burn-in steps): 'nfb' (accounting for null alleles, inbreeding and genotyping errors), 'nb' (null alleles and genotyping errors), 'nf' (null alleles and inbreeding), 'b' (just genotyping errors), 'f' (just inbreeding), 'n' (just null alleles) and 'null' (null model). To select which model performed better, the deviance information criterion was used (DIC; Spiegelhalter *et al.*, 2002).

Bayesian analyses were developed with Structure v.2.3.4 (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2007) from $K = 1$ to $K = 8$ (20 runs per K, burn-in lengths of 100 000 and runs of 500 000 steps). The optimal K value was selected according to Evanno, Regnaut & Goudet, (2005) and migration rates were indirectly calculated, based on the estimated population sizes, to assess the confidence of the optimal K value obtained (Cullingham *et al.*, 2020). Furthermore, among-population genetic distances (Reynolds, Weir & Cockerham, 1983) were calculated from nSSR data and a neighbor-joining tree was built with *poppr* v.2.3.0 (10 000 replicates). The partitioning (within and among populations) of genetic variation was determined with an AMOVA (Schneider *et al.*, 1997; Excoffier, Laval & Schneider, 2005) performed in *poppr* v.2.3.0 over all the studied samples. As for AFLP, two approaches were used: (1) for all the populations; and (2) only for the northern African ones, grouped for southern and central Morocco, northern Morocco, Algeria and Tunisia. To test for isolation by distance, comparison of the population Reynolds' distances with their geographical distance using Mantel bilateral tests based on Spearman's correlations (on 10 000 random permutations; XLSTAT v.2016.03.30882, Addinsoft) was carried out for all the populations and for the northern African ones only. Finally, we sought for a diversity (based on H_e) or an inbreeding pattern along the west–east axis by a Spearman's correlation and a relationship between genetic diversity and population size (JMP v.6.0.0; SAS Institute Inc., 2005).

AFLP-nuclear SSR comparison

To assess the congruence between the molecular approaches, three analyses were done. The first one consisted of an AMOVA on the AFLP and nSSR data, considering as populations those groups of individuals completely assigned to one of the BAPS-inferred groups for the AFLP data. The second one estimated the correlation between diversity indexes and F_{IS} values obtained for both markers (JMP v.6.0.0; SAS Institute Inc., 2005). Finally, a Mantel bilateral test was applied to detect a potential correlation between AFLP-based chord and nSSR-based Reynolds' population distances (on 10 000 random permutations; XLSTAT v.2016.03.30882, Addinsoft).

Niche modelling

To investigate the historical range of *T. articulata*, niche-modelling analyses based on presence/pseudo-absence data were conducted for three temporal climatic frames: the last interglacial period (*c.* 140 000–120 000 years BP; Otto-Bliesner *et al.*, 2006), the last glacial maximum (*c.* 21 000 years BP; Braconnot *et al.*, 2007) and the present (*c.* 1950–2000; Hijmans *et al.*, 2005). The last glacial maximum scenario was assessed by means of two models: CCSM and MIROC. CCSM is based on a framework that divides the climate system into four components connected by a coupler

in a completely asynchronous manner: atmosphere, sea ice, land and ocean, each exchanging data with the coupler only (Collins *et al.*, 2006). MIROC consists of five components: atmosphere, land, river, sea ice and ocean. The atmospheric component interacts with the land and the sea ice components. The air–sea exchange is realized exclusively between the atmosphere and the sea ice components, and the ocean component interacts only with the sea ice component. The river component receives ground runoff water from the land component and drains riverine runoff water into the sea ice component (Hasumi & Emori, 2004). Bioclimatic and soil data were used to develop predictive analyses by using the maximum entropy method with the software MaxEnt (Phillips, Anderson & Schapire, 2006), following methodology as in García-Castaño *et al.* (2014).

RESULTS

AFLP ANALYSES

The six AFLP primer combinations of the analysed individuals resulted in 435 fragments, which ranged from 100 to 503 bp, three of which were private for *Platycladus orientalis*, with an average scoring-error rate value of 8.9%. For *T. articulata*, 427 were polymorphic (Table 1) and the number of fragments per population ranged from 165 (population 27) to 309 (population 14), polymorphism from 69.7% (population 13) to 94.5% (population 14), the number of private fragments from zero (populations 16, 23, 27, 28, 29 and 30) to seven (populations 21 and 26) and gene diversity from 0.05 (population 14) to 0.17 (population 25), with European populations (Spain and Malta) showing relatively high values (0.12–0.14; Table 1). After scoring-error correction, the number of phenotypes (n_{ph}) per population was similar to the population sample size except for some populations (populations 10, 14, 24 and 30; see Table 1). Linkage disequilibrium was, generally, high (88.7% ± 19.3%, mean ± SD). These high values might have influence on the accuracy of the estimation of the F_{IS} values, although a correlation between linkage disequilibrium and F_{IS} values was not found (Spearman's $\rho = -0.071$, $P = 0.711$; $N = 30$).

BAPS analysis (see Supporting Information, Fig. S1 for the parallel Structure approach) distinguished four groups [log (marginal likelihood) of the optimal partition = –45 269.95; Fig. 1]. Two groups of populations showed geographical coherence, a western-central (WC, hereafter)-Moroccan group of populations and an Algerian group of populations, whereas the other two groups (widespread and Moroccan–Algerian) did not, as they were distributed to some extent in Morocco, Algeria, Tunisia and even in Spain; Malta contained mostly only one of these two widely spread groups. Nevertheless, the degree of intermixing was remarkably high, and the number of pure or nearly pure populations was low. The relationships of these populations at a genetic level are shown in a chord distance-based Neighbor-Net together with bootstrap values, which supported three out of the four BAPS population clusters (Fig. 2). The four BAPS groups can also be recognized in the PCoA analysis based on averaged per population individuals (Fig. 3). Mean PCo1 axis (9.5%) split two groups, the Moroccan–Algerian group of populations and the remaining ones (closer to each other), and Mean PCo2 axis (5.0%) separated the WC-Moroccan and the Algerian groups of populations; the widespread group of populations located at a diffuse intermediate position. When the analysis was developed directly at a population level, the percentages of variation explained by the axes were 31.7 and 14.4% for PCo1 and PCo2, respectively.

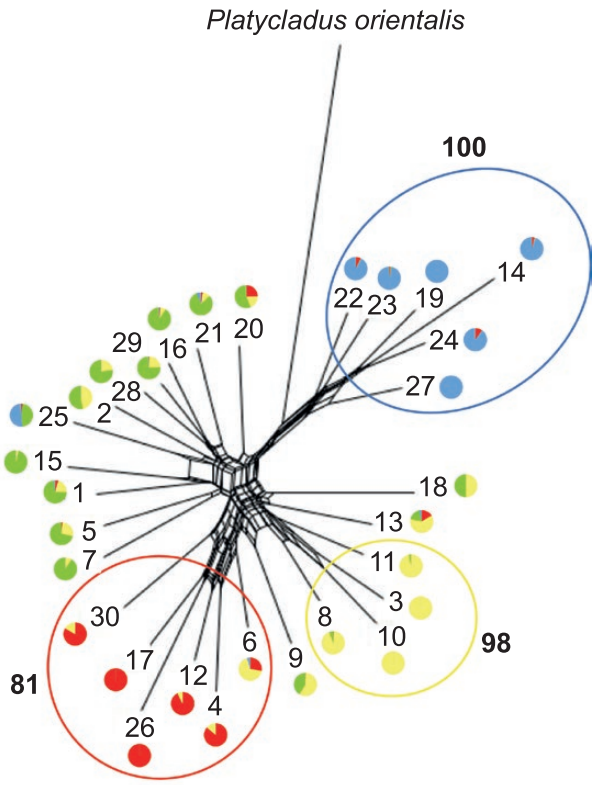

Figure 2. Neighbor-Net for the populations of *Tetraclinis articulata* sampled, including *Platycladus orientalis* as an outgroup representative, based on chord distances from AFLP data. Bootstrap values (> 80%) obtained on the chord distances for the neighbor-joining tree (see Table 1 for population codes). Colours: WC-Moroccan (yellow), Algerian (blue), widespread (green) and Moroccan–Algerian (red) group of populations.

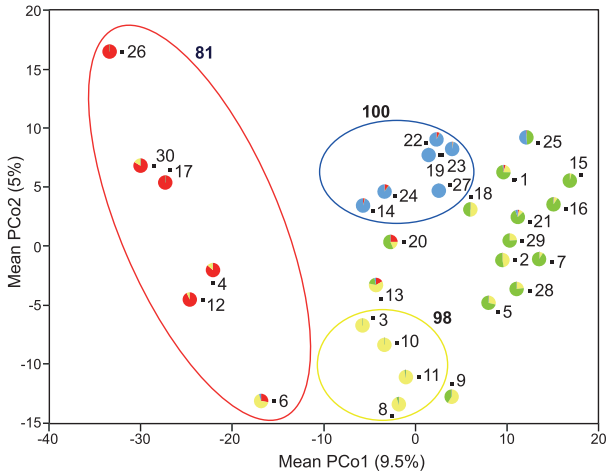

Figure 3. Mean PCoA for the first two axes, showing the different Bayesian-inferred groups of populations for population means from squared Euclidean distances between individuals of *Tetraclinis articulata*, from AFLP data (within brackets, amount of variation the axis accounts for). Bootstrap values (> 80%) obtained on the chord distances for the neighbor-joining tree; when the analysis was developed directly at a population level, PCo1 and PCo2 accounted for 31.7 and 14.4% of variation, respectively (see Table 1 for population codes). Colours: WC-Moroccan (yellow), Algerian (blue), widespread (green) and Moroccan–Algerian (red) group of populations.

The general AMOVA analysis showed high intrapopulational variation (88.43%; Table 2A), which resulted in low F_{ST} values (0.116, $P < 0.0001$; estimated migration rate for most populations resulted in $m = c.$ 0.005). If populations in North Africa were clumped into four geographically delimited groups (from west to east: southern and central Morocco, northern Morocco, Algeria and Tunisia), these absorbed more than a third of the variance among populations (4.14% out of 11.83%, $P < 0.0001$; Table 2B). A slight relationship was found between geographical and genetic population distance (Spearman's $\rho = 0.098$, $P = 0.042$), which increased when only those populations from North Africa were considered (Spearman's $\rho = 0.136$, $P < 0.011$). For these populations, there was no evident genetic diversity pattern along the west–east axis (Spearman's $\rho = -0.005$, $P = 0.981$; $N = 27$). Nevertheless, F_{IS} values increased in a westward direction (west–east Spearman's $\rho = -0.406$, $P = 0.036$; $N = 27$). AFLP diversity and population size were not correlated (Spearman's $\rho = 0.207$, $P = 0.272$; $N = 30$).

According to the Neighbor-Net built for the four BAPS groups, considering only those individuals fully assigned to one of them (Table 3), no conspicuous pattern could be stood out but a significant separation between them and *Platycladus orientalis* (Fig. 4).

Nuclear SSR analyses

Sign of clonality, in relation to the number of multilocus genotypes, was found only in a few populations (populations 1, 3, 15, 18 and 27; Table 1), the overall rate of clonality per population being low (0.03). Congruently, the standardized index of association values found in these few populations was, frequently, significantly different from zero. The probability of repeated MLGs evolved by chance was low (P_{gen} range: 1.2×10^{-15}–8.2×10^{-4}), which suggested a high power of MLG identification of the used loci. Similarly, the probability for the MLG replicates to originate from distinct sexual reproductive events was low ($P_{gen} < 0.05$, range 3.9×10^{-13}–2.3×10^{-1}) except for the replicated MLGs in population 1. Estimates of genetic diversity for the studied populations are presented in Table 1. On average, the microsatellite loci revealed 4.72 ± 0.16 (mean ± SE) different alleles per locus. The heterozygosity observed was almost always lower than expected (exceptions were populations 3, 4 and 27). Although diversity measures as the allelic richness or the average number of alleles per locus were similar throughout the range of the species, in general, European populations (Spain and Malta) showed lower values. More than half of the populations showed significant inbreeding after accounting for null alleles. The absolute values of the inbreeding coefficient (F_{IS}) varied from 0.00 to 0.63 (the latter in population 13; Morocco). Null alleles were detected by INEST in ten out of the 30 populations, which showed a population average over loci of 7.3%.

The optimal K obtained from the Structure results was $K = 3$, that distributed without a clear pattern but for population 2, which was homogeneous (for the parallel BAPS approach, slight peculiarities where additionally found in populations 1, 27 and 30 for the optimal, maximum set, $K = 30$; see Supporting Information, Fig. S2). nSSR-based neighbor-joining phylogram for *T. articulata* showed poorly resolved internal relationships (all bootstrap values < 60; phylogram not shown). For this reason, no significant inference could be drawn according to this source. AMOVA for nSSR data (Table 2A) revealed that most of the variation occurred within populations (88.49%; see similar m values estimated in the previous 'AFLP analyses' section). As for AFLP data, when considering the four North African groups of populations, more than a third of the variance among populations was contained above the population level (2.67% out of 6.33%, $P < 0.0001$; Table 2B). It was found a significant relationship between geographical and genetic population distance (Spearman's $\rho = 0.284$, $P < 0.001$), which was also present when only those populations from North Africa were considered (Spearman's $\rho = 0.241$, $P < 0.001$). Nevertheless, for these populations, neither a significant genetic

Table 2. Results of the AMOVAs applied to AFLP and nSSR data: A, considering all the populations and B, grouping the North African populations in southern and central Morocco, northern Morocco, Algeria and Tunisia. C, AMOVA based on the four Bayesian-inferred groups obtained on AFLP data, selecting those individuals fully assigned to a group. See Tables 1 and 3 and Figure 1 for details

A							
		AFLP			nSSR		
Source of variation	d.f.	SS	Variance component	%	SS	σ	%
Among populations	29	1777.92	3.33*	11.57	285.38	0.53*	11.51
Within populations	293	7461.33	25.47	88.43	1201.80	4.10	88.49
Total	322	9239.25	28.80		1487.18	4.64	
B							
		AFLP			nSSR		
Source of variation	d.f.	SS	Variance component	%	SS	σ	%
Among groups	3	390.61	1.18*	4.14	140.12	0.40*	2.67
Among populations within groups	23	1122.69	2.20*	7.69	451.85	0.54*	3.66
Within populations	265	6677.46	25.20*	88.16	3668.54	13.84*	93.67
Total	291	8190.76	28.58		4260.51	14.78	
C							
		AFLP			nSSR		
Source of variation	d.f.	SS	Variance component	%	SS	σ	%
Among BAPS groups	3	699.65	2.91*	9.88	27.06	0.06*	1.35
Within populations	287	7609.55	26.51	90.12	1310.01	4.56	98.65
Total	290	8309.20	29.42		1337.07	4.63	

*$P < 0.0001$.

Table 3. Bayesian-inferred, based on AFLP data, groups of populations. n: sample size, n_{ph}: number of phenotypes, $Frag_{tot}$: number of fragments, $Frag_{poly}$: number of polymorphic fragments, $Frag_{priv}$: number of private fragments, H_D: average gene diversity, F_{IS}: inbreeding coefficient. SD: standard deviation, CI: confidence interval

BAPS group	n	n_{ph}	$Frag_{tot}$	$Frag_{poly}$	$Frag_{priv}$	H_D (SD)	F_{IS} (95% CI)
WC-Moroccan	84	57	330	314	15	0.10 (0.05)	0.33 (0.21–0.47)
Algerian	58	41	334	320	21	0.09 (0.04)	0.41 (0.27–0.55)
Widespread	98	89	346	331	15	0.15 (0.07)	0.28 (0.18–0.41)
Moroccan–Algerian	51	48	319	293	16	0.15 (0.08)	0.13 (0.05–0.21)

diversity (Spearman's $\rho = -0.069$, $P = 0.732$; $N = 27$) nor a significant inbreeding coefficient pattern along the west–east axis (Spearman's $\rho = -0.130$, $P = 0.517$; $N = 27$) was detected. nSSR diversity and population size were not correlated (Spearman's $\rho = 0.189$, $P = 0.317$; $N = 30$).

AFLP-NUCLEAR SSR COMPARISON

The BAPS lineages (obtained from AFLP data) explained only 1.35% variation of the nSSR data whereas 9.88% was explained for the AFLP ones (a similar value to the populational AMOVA but for only four values instead of 30; Table 2C).

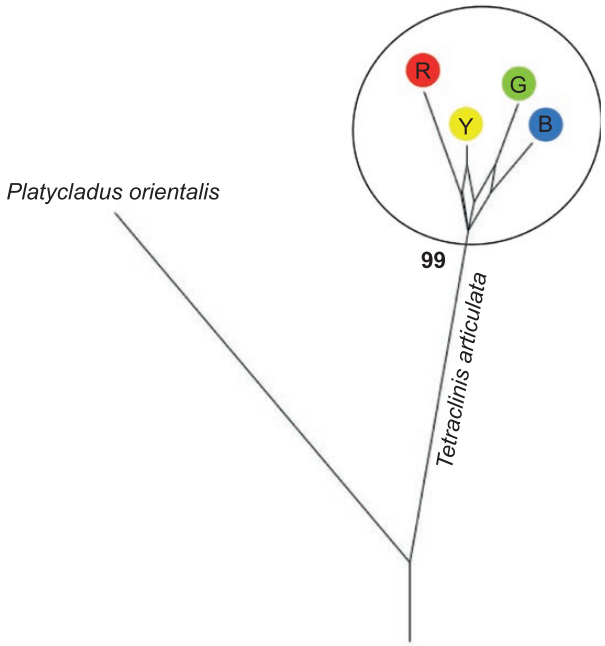

Figure 4. Rooted Neighbor-Net for the Bayesian-inferred groups of *Tetraclinis articulata* (yellow: WC-Moroccan; blue: Algerian; green: widespread; red: Moroccan–Algerian; see Table 3), including *Platycladus orientalis* as an outgroup representative, based on the $\rho_{ST(2)}$ distances from AFLP data. Bootstrap value for the four groups, obtained on the chord distances for the neighbor-joining tree.

Additionally, from the perspective of a mirrored Bayesian approach, it was not possible to assess for incongruence between both markers, as no pattern (except a clear distinctiveness in population 2 and slight peculiarities in populations 1, 27 and 30) was found from the nSSR data. There was also a lack of correlation between the diversity values obtained by both types of markers (Spearman's $\rho = -0.197$, $P = 0.296$; $N = 30$), the F_{IS} values ($\rho = -0.291$, $P = 0.119$; $N = 30$) and the chord and Reynolds' distances ($\rho = 0.029$, $P = 0.538$).

Niche modelling

Results from niche modelling suggested a narrow potential distribution of *T. articulata* during the last interglacial period (Fig. 5A). It was even narrower on the northern shore of the Mediterranean during the last glacial maximum, although the degree of this narrowing was highly dependent on the atmospheric model chosen (Fig. 5B, C). Nevertheless, on the southern shore, occurrence was probable for several groups. At least the two BAPS-defined linages with a current geographical entity (i.e. western/central Morocco and Algeria) showed some potential areas of permanence during this glacial period. The estimated present and interglacial suitable areas were similar and largely corresponded to the current distribution of *T. articulata* on the southern shore. However, the suitable areas in the Iberian Peninsula did not fit the nowadays-restricted distribution in Spain (Fig. 5D).

DISCUSSION

Complex genetic structure and patterns of diversity of *Tetraclinis articulata*

The molecular markers used (AFLPs and microsatellites), although non-redundant, are not incongruent, and show different aspects of the genetic structure of *T. articulata*. AFLP data point to the existence of four lineages, two of them located in separate geographical regions (western/central Morocco and Algeria), but the other two highly dispersed and intermixed. These results might indicate a complex scenario of expansion from four areas (after generation by isolation or colonization). The current area of distribution could be the result of a pattern of vicariance that differentiated two areas, one in Morocco and one in Algeria; additionally, two other sources, which are of uncertain geographical origin, appear to have spread throughout the present area of distribution. No centre of diversity was distinguished as a candidate for an unambiguous origin. There was no clear genetic difference between populations on the northern and southern Mediterranean shores, whereas other authors did detect differences for other Mediterranean species, such as *Aegilops geniculata* Roth (Arrigo *et al.*, 2010), *Erophaca baetica* (L.) Boiss. (Casimiro-Soriguer *et al.*, 2010) or *Juniperus thurifera* L. (Teixeira, Rodríguez-Echeverría & Nabais, 2014).

As also found by Mariette *et al.* (2002), diversity values for both markers were not correlated and the nSSR analyses, contrary to the AFLP ones, did not manage to group significantly different populations. This lack of redundancy might be due to the different mutation rates both types of marker experience: rapid for AFLP but even faster for SSR markers (Mariette *et al.*, 2002, Gaudeul *et al.*, 2004; Garoia *et al.*, 2007; Tang *et al.*, 2008; Grünwald & Goss, 2011). Therefore, where nSSR data could not suggest any grouping, AFLP data could draw four population groups due to less recent events. Moreover, the populational structure that was obtained did not fit the previous results from ISSR data (Sánchez-Gómez *et al.*, 2013). The low percentage of genetic diversity present among populations (< 12% for both markers) makes it difficult to assess any pattern.

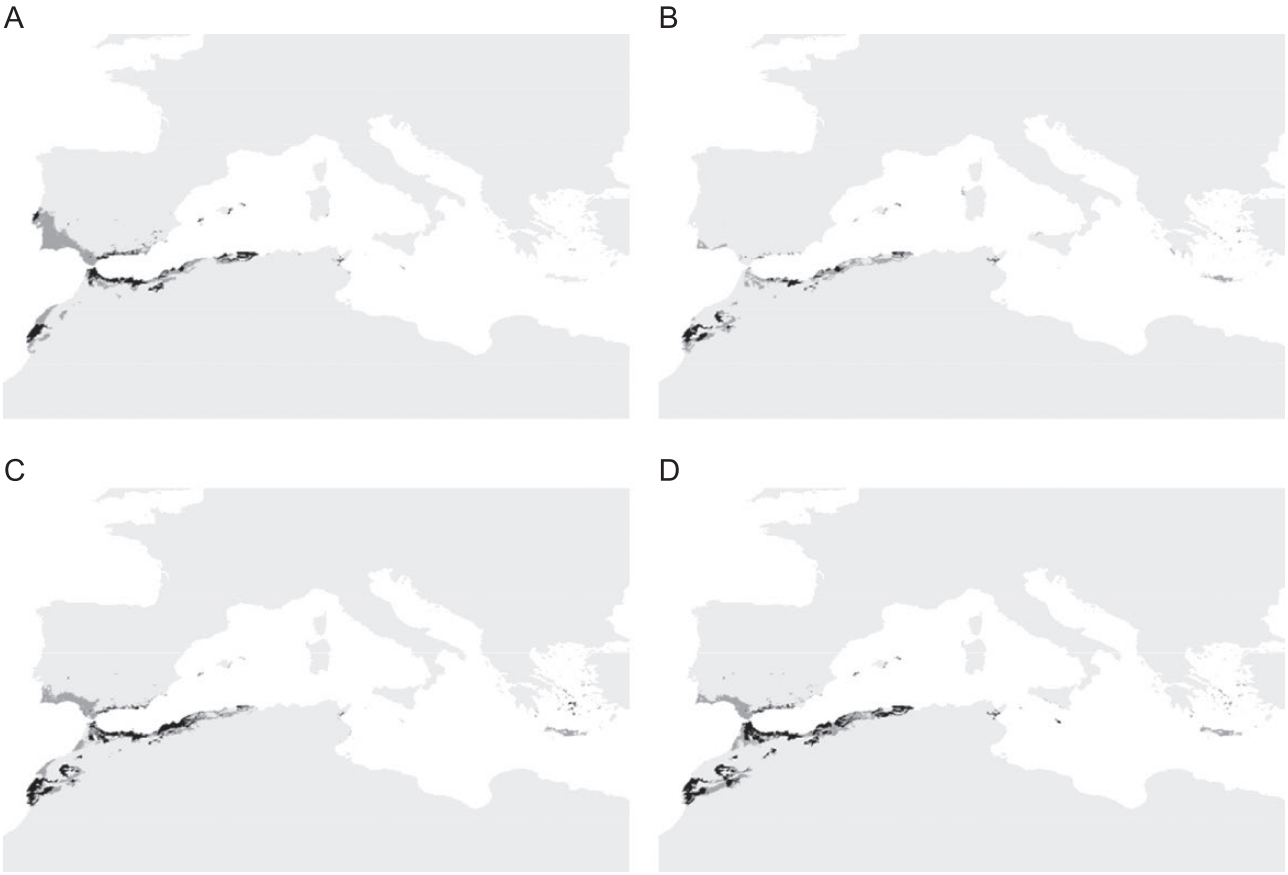

Figure 5. Suitable areas for *Tetraclinis articulata* estimated in three periods. A, Last interglacial (LIG, ~140 000–120 000 years BP; B, last glacial maximum (LGM, ~21 000 years BP) for the CCSM atmospheric model; C, for the MIROC atmospheric model and D, the present period (~1950–2000). Results shown for two thresholds: maximum training sensitivity plus specificity logistic threshold (in dark grey) and ten-percentile training presence logistic threshold (in black). LIG and LGM are represented with current coastlines.

In addition, the inbreeding coefficients (F_{IS}), both from AFLP and nSSR data, were frequently far from zero, pointing to high within-population inbreeding. Nevertheless, the high linkage disequilibrium, found for the AFLP data, might be behind these values and this could be due to other reasons (e.g. selection, genetic drift or clonality). A plausible explanation for this high inbreeding might relate to the reproductive characteristics of the species since anemophily might encourage selfing through huge pollen clouds near the male cones; the latter, i.e. a pollen influence concentrated in the vicinity of the father trees, has been documented specifically in other gymnosperms (Restoux *et al.*, 2008; Sánchez-Robles *et al.*, 2014b). In addition, its post-fire resprouting capacity (López-Hernández *et al.*, 1995) may help explain the high within-population diversity, allowing the persistence of the ancestral gene pool and increasing the generation time of this species (Premoli & Steinke, 2008).

Old origin and expansion: integrating molecular and fossil information

The weak spatial genetic pattern and the low number of fossil records on the southern shore of the Mediterranean are major impediments for phylogeographical reconstructions in *T. articulata* (but see the record in the Canary Islands for the Miocene–Pliocene; Anderson, Channing & Zamuner, 2009). Nevertheless, some attempts regarding the origin and evolutionary history of this species can be brought assisted by historic literature. Fossil evidence indicates that *Tetraclinis* Masters spread in the past from North America to Europe (Hably, Kvaček & Manchester, 2000). Tertiary macrofossils are known from Oregon (Meyer & Manchester, 1997), France and Austria (Kvaček, 2010), Germany (Mai, 1994), Poland (Szafer, 1961), Czech Republic (Kvaček & Walther, 1998), Hungary and Slovenia (Erdei *et al.*, 2012), northern Spain (Barrón, 1999, Barrón *et al.*, 2006), Sicily (Teodoridis

et al., 2015) and Crete (Zidianakis, Mohr & Fassoulas, 2007); Palamarev (1987) also reported *Tetraclinis* for Romania and Bulgaria. As *Tetraclinis* was known in Europe in the early Eocene–Palaeocene (Mai, 1994; Kvaček, Manchester & Schorn, 2000) and at this time Europe and most of the African continent were not in contact, a northern origin for *T. articulata* and a subsequent generation of the different lineages are supported. *Tetraclinis* could have become distributed on both sides of the Mediterranean via land bridges at the Strait of Sicily, which connected Italy to Tunisia during the Oligocene–Miocene, or through the Strait of Gibraltar during the Messinian salinity crisis (Reguant, 1986; García-Castellanos & Villaseñor, 2011; Troia, Raimondo & Geraci, 2012). A similar pattern was found in other gymnosperms, including *Juniperus thurifera* (Jiménez *et al.*, 2003) or Mediterranean firs (Balao *et al.*, 2020), and angiosperms such as *Chamaerops humilis* L. (García-Castaño *et al.*, 2014).

Pliocene and Quaternary ecological niche contraction

The past distribution of *Tetraclinis*, which was not necessarily strictly continuous, and the current AFLP genetic structure across the southern Mediterranean area indicate extinction and fragmentation after the Tertiary of the species range. Extinction could have started at the northern side in the late Miocene–Pliocene following a global cooling (Ravelo *et al.*, 2004) and generation by isolation among the four *Tetraclinis* lineages could have been subsequently encouraged by Quaternary glaciations. Results of the niche modelling indicate that the potential distribution during the last interglacial was not extensive, and they support the hypothesis of massive habitat loss and fragmentation during the last glacial maximum. According to the CCSM model, the range of *T. articulata* would have been extremely narrow and confined to a few regions in North Africa and some refugia in the Iberian Peninsula (cf. *Abies pinsapo* Boiss.; Sánchez-Robles *et al.*, 2014a). Nevertheless, the presence was probable, under both the CCSM and the MIROC models, for the four lineages of northern African populations considered. Additionally, and according only to the MIROC model, there was a low probability of occurrence in Malta. The effects of Pleistocene glaciations on the distribution of other Mediterranean species have been widely demonstrated both in plants and animals (Hampe *et al.*, 2003; Fady & Conord, 2010; Conord, Gurevitch & Fady, 2012; Sánchez-Robles *et al.*, 2014a; García-Castaño *et al.*, 2014, Balao *et al.*, 2017). Specifically, the importance of the south-eastern corner of the Iberian Peninsula has been considered in relation to the presence of different species of gymnosperms and angiosperms during the last glacial stage (see González-Sampériz *et al.*, 2010 for a review). From some of these potential refugia, estimated by the niche-modelling analyses, a slight expansion could have occurred.

Last, genetic diversity from AFLP data did not vary in North Africa from west to east, suggesting the existence of a genetically homogeneous distribution for a period of time. Nevertheless, there was an increase of inbreeding (F_{IS}) as we went westwards, which might have occurred due to ecological reasons (e.g. less open areas with less intense wind activity in the south in comparison to those in the north). The nSSR data, pointing to more recent events, showed the same lack of variation for the genetic diversity in North Africa from west to east and for the inbreeding estimated (F_{IS}) values that, for instance, might have risen in the north by the inclusion of more different nuclei in a recent expansion.

Assessing potential explanatory causes leading to high genetic intermixing

In accordance with AFLP and nSSR analyses, a high level of intermixing among the four *T. articulata* lineages occurred during post-glacial colonization. Moreover, low numbers of AFLP private fragments and nSSR private alleles (null in Malta) were found. These patterns suggest post-glacial migration to distant locations. High within-population diversity and relatively low among-population differentiation are consistent with this. Additionally, high inbreeding (F_{IS}) values point to a high degree of geitonogamy, as pollen would tend to concentrate near its source (Restoux *et al.*, 2008; Sánchez-Robles *et al.*, 2014b). Subsequently, populations connected more likely via sporophytes (e.g. seeds) than via gametophytes (pollen), which would have led to admixing.

Human-driven migration and seed dispersal by wind, potentially and not exclusively, might explain the intermixing found in the, not necessarily always continuous, distribution range for the groups obtained from the AFLP data, which sometimes appear in a very similar way despite the long distance between some populations (e.g. those in Spain and Tunisia). The first source of intermixing points to a long-distance expansion implemented by the moderate traditional use of this species by humans. The Moroccan–Algerian and the widespread lineages (and, partially, the WC-Moroccan and the Algerian ones) are related to known trade routes, as old as those practised, for example, by the Phoenicians (Benjamin & Wiesner-Hanks, 2015). Later peoples throughout the long history of humans in the western Mediterranean (Carthaginians, Romans or Arabians) might be pointed out as potential dispersing agents (Abulafia, 2011; Sánchez-Gómez *et al.*, 2013). Similar human-driven dispersal has been suggested for many other plant species as a result of centuries

of human occupation in the region (Blondel, 2006). Specifically, cultivation of *T. articulata* has been recorded by Mai (1994) and El-Hilaly, Hmammouchib & Lyoussi (2003). A second source of intermixing points to wind-dispersed seeds (do Amaral Franco, 1986). Nevertheless, because of their weight, they tend to concentrate near their mother trees (Esteve-Selma *et al*., 2017) making this improbable.

In Spain, because of the origin of the populations (natural vs. naturalized), two different situations would occur. On the one hand, populations from Sierra Minera de Cartagena-La Unión (Murcia), presumably natural (Esteve-Selma *et al*., 2017), showed a shared pattern with those in Tunisia. Tertiary *Tetraclinis* fossils in Spain (Barrón, 1999, Barrón *et al.*, 2006) and antracological records attributed to *T. articulata* for the Chalcolithic in an archaeological site only *c*. 200 km away (Rodríguez-Ariza & Vernet, 1991; García Martínez, 2009) make a foreign foundation unnecessary. Therefore, this similarity, which is based on two of the four detected AFLP groups, points to: either (1) intermixing between both sides, i.e. south-eastern Spain and Tunisia, or (2) the possibility for south-eastern Spain to act as the only source (Sánchez-Gómez *et al.*, 2013), in both cases due to human activities. Additionally, its low nSSR diversity and slight peculiarity (Supporting Information, Fig. S2) point to a relatively intense bottleneck. On the other hand, the naturalized population from Monte San Antón in Málaga (Pérez Latorre & Cabezudo, 2011), based on the detected AFLP groups, showed two lineages that did not necessarily reach the place directly from two different sources, i.e. no independent transplantation was needed; however, possibly because of a strong bottleneck in the foundation process, the nSSR diversity was low and distinctive (Supporting Information, Fig. S2).

In Malta, the indigenous origin of *T. articulata* has never been doubted (Baldacchino, 1985). However, its complete native origin cannot be taken for granted. This study showed that Malta has a high AFLP genetic diversity that can be interpreted as intermixing of genes coming from different sources. The hypothesis that *T. articulata* expanded naturally from Tunisia into the Maltese territory when these lands were possibly connected by a land bridge during the Messinian salinity crisis (Troia *et al.*, 2012) is not supported in this study. When Al-Himjari described Malta during the Ottoman Empire between 870 and 1054 stated that 'the island of Malta remained an uninhabited ruin (*hirba*), but it was visited by shipbuilders, because the wood in it is of the strongest kind by the fishermen' (Brincat, 1995). This matches important characteristics of *T. articulata*, the hard and durable wood of which is impermeable to water and resists fungal decay (Fidah *et al.*, 2015), offering important characteristics for shipbuilding; nevertheless, other timber alternatives, such as *Pinus* L. or *Quercus* L., are not excluded (Smith, 1857; Gambin *et al.*, 2016). Therefore, a plausible explanation for the population in Malta might combine a man-mediated dispersion, added or not to a natural occurrence. The wild populations declined gradually over the last five centuries and became depleted to a point of facing extinction (Borg, 1927; Baldacchino, 1985), which might explain diversity loss and slight peculiarity of the more recent nSSR markers (Supporting Information, Fig. S2).

Conservation remarks

Currently, *T. articulata* populations face different threats. Human activities, such as habitat conversion, logging for timber, overgrazing and forest fires, have led to intense reduction of the distribution of this species in recent times (López-Hernández, 2000; Hadjadj-Aoul *et al.*, 2009; Baonza Díaz, 2010; Sánchez-Gómez *et al.*, 2011). Other factors, such as competence with other species such as *Pinus halepensis* Mill., affect its occurrence (Esteve-Selma & Miñano Martínez, 2010; Esteve-Selma *et al*., 2017). Finally, studies in south-eastern Spain have shown that the impact of future climate change on the distribution of *T. articulata* is not completely clear (Esteve-Selma *et al*., 2010, 2012, 2017).

Nevertheless, our results reveal that populations represent < 12% of the total genetic variance in *T. articulata*. This value is similar to the 14–17% found in other studies on *T. articulata* (Sánchez-Gómez *et al.*, 2013; Makkaoui *et al.*, 2020), based on ISSR studies, and low among those found for other Cupressaceae in AFLP-based studies, which can range from 8% in *Cunninghamia konishii* Hayata (Li *et al.*, 2019) to 34% in *Calocedrus formosana* (Florin) Florin (Chien *et al.*, 2020) or 37% in *Juniperus phoenicea* L. (Sánchez-Gómez *et al.*, 2018); specifically, the value of 12% found in *Cunninghamia lanceolata* (Lamb.) Hook. was labelled as low (Chunga *et al.*, 2004). For this reason, conservation should be focused on each of the four groups detected by the AFLP analyses rather than on every population. Already proposed conservation strategies for these lineages, based on both *in situ* and *ex situ* measures, include preservation of natural areas and source-controlled reintroductions, and use of germplasm banks, respectively. All of them are potentially adequate to preserve the genetic structure when considering the source (at least, when the same AFLP group is controlled); other factors, such as economics or logistics, would determine which one to use.

On the one hand, these measures can be developed in North Africa (Makkaoui *et al.*, 2020), where its abundance leads to consider the species conservation status as 'least concern' with a 'decreasing' population trend (Sánchez-Gómez *et al.*, 2011). On the other, for

the European populations of south-eastern Spain and Malta, considered 'endangered' (Thomas, 2017), we only found slight nSSR peculiarities (Supporting Information, Fig. S2), presumably associated to recent bottlenecks. Nevertheless, in the context of their geographical and historical singularities, recently addressed protection plans are worthy (Moreno, 2011, and Esteve-Selma *et al*. 2017 for Spain; L.N. 12, 2001, and Baldacchino, 2015, for Malta).

CONCLUSIONS

Four lineages of populations, geographically intermixed to a certain extent, could be documented. Our work supports the hypothesis of an ancient wide distribution, including Europe, and the appearance of four isolated lineages. Nevertheless, we could not fully determine the recent origin of the current populations that may have been generated or aided by human activities or, much less probably, from seeds dispersed by wind out of refugia in northern Africa-southern Europe in the Quaternary, including shore connections.

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DATA ARCHIVING STATEMENT

AFLP, nSSR and presence data for this research are available at a GitHub repository doi: 10.5281/zenodo.84173074 (https://github.com/fbalao/tetraclinis/tree/v1.0). The separate files refer to information related to AFLP data and primers information, nSSR data and presence data from SEV Herbarium.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Parallel Bayesian analyses for AFLPs with Structure v.2.3.3 (Pritchard, Stephens & Donnelly, 2000; Falush *et al.*, 2007), from $K = 1$ to $K = 8$ (10 runs per K, burn-in lengths of 100 000 and runs of 500 000 steps). A, According to Evanno *et al.* (2005), the optimal K was $K = 2$, with a suboptimal value of $K = 4$ (the same K value selected by BAPS). STRUCTURE results for B, $K = 2$ and C, $K = 4$, together with D, the BAPS v.5.3 results for $K = 4$.
Figure S2. A, Structure v.2.3.4 results on nSSRs for $K = 3$, which, B, according to Evanno *et al.* (2005), was the optimal value. C, BAPS v.5.3 results on nSSRs for $K = 30$ (optimal value).