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The Strait of Gibraltar as a major biogeographic barrier in Mediterranean conifers: a comparative phylogeographic survey

J. P. JARAMILLO-CORREA,*† D. GRIVET,* A. TERRAB,‡§ Y. KURT,¶ A. I. DE-LUCAS,**†† N. WAHID,‡‡ G. G. VENDRAMIN§§ and S. C. GONZÁLEZ-MARTÍNEZ*††

*Departamento de Ecología y Genética, Centro de Investigación Forestal, CIFOR-INIA, Carretera de La Coruña, Km. 7.5, E28040 Madrid, Spain, †Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Apdo Postal 70-275, México D.F., México, ‡Department of Systematics and Evolutionary Botany, Institute of Botany, University of Vienna, Rennweg 14, A1030 Vienna, Austria, §Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Apdo. 1095, E41080 Sevilla, Spain, ¶Akdeniz University, Faculty of Arts and Sciences, Biology Department, 07058 Antalya, Turkey, **Laboratorio de Diagnóstico Genético, Departamento de Biotecnología, ITAGRA.CT, Campus Universitario ''La Yutera'', Avenida de Madrid 44, E34004 Palencia, Spain, ††Sustainable Forest Management Research Institute, UVa-INIA, Spain, ‡‡Laboratoire d'analyse et de valorisation des ressources environnementales, Département des Sciences de la Vie, Université Cadi Ayyad, Faculté des Sciences et Technique, BP 523, Béni-Mellal, Morocco, §§Istituto di Genetica Vegetale, Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, I50019 Sesto Fiorentino, Firenze, Italy*

Abstract

The Strait of Gibraltar (SG) is reputed for being both a bridge and a geographic barrier to biological exchanges between Europe and Africa. Major genetic breaks associated with this strait have been identified in various taxa, but it is unknown whether these disjunctions have been produced simultaneously or by independent biogeographic processes. Here, the genetic structure of five conifers distributed on both sides of the SG was investigated using mitochondrial (*nad*1 b/c, *nad*5-1, *nad*5-4 and *nad*7-1) and chloroplast (*Pt*1254, *Pt*15169, *Pt*30204, *Pt*36480, *Pt*71936 and *Pt*87268) DNA markers. The distribution of genetic variation was partially congruent between types of markers within the same species. Across taxa, there was a significant overlapping between the SG and the genetic breaks detected, especially for the four Tertiary species surveyed (*Abies pinsapo* complex, *Pinus nigra*, *Pinus pinaster* and *Taxus baccata*). For most of these taxa, the divergence of populations across the SG could date back to long before the Pleistocene glaciations. However, their strongly different cpDNA G_{ST} and R_{ST} values point out that they have had dissimilar population histories, which might include contrasting amounts of pollen-driven gene flow since their initial establishment in the region. The fifth species, *Pinus halepensis*, was genetically depauperated and homogenous on both sides of the SG. A further analysis of nuclear DNA sequences with coalescent-based isolation with migration models suggests a Pleistocene divergence of *P. halepensis* populations across the SG, which is in sharp contrast with the pre-Pleistocene divergence dates obtained for *P. pinaster*. Altogether, these results indicate that the genetic breaks observed across this putative biogeographical barrier have been produced by independent evolutionary processes related to the biological history of each individual species instead of a common vicariant phenomenon.

Keywords: *Abies*, cytoplasmic DNA, isolation with migration model, phylogeography, *Pinus*, Strait of Gibraltar, *Taxus*

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Correspondence: Santiago C. González-Martínez, Fax: (+34) 913572293;
E-mails: santiago@inia.es or santiago.c.gonzalez.martinez@gmail.com

Introduction

The most important aims of biogeography and phylogeography are the description of how biological and genetic diversity are geographically distributed, and the inference of how such observed patterns have come to occur (Beheregaray 2008). At the community or habitat scales, such goals are traditionally attained through paleobotanical and fossil reconstructions (e.g. Mai 1989; Carrión *et al.* 2003; Rodríguez-Sánchez & Arroyo 2008), while at the within-species or genus levels, they additionally rely on the analysis of molecular data (e.g. Burban & Petit 2003; Jaramillo-Correa *et al.* 2004; Magri *et al.* 2007; Liepelt *et al.* 2010). However, although the description of the current distribution patterns is important, the inference of the processes that may explain them has an even higher interest, especially when comparing data from co-distributed taxa. This interest is based on the dynamic nature of species ranges and on the rather fast microevolution of genetic lineages. Comparative inferences are of particular importance in areas such as islands, plate boundaries, mountain chains, isthmuses or straits, which have the potential to limit the historical movements of species, particularly those of sessile taxa like plants (e.g. Carstens *et al.* 2005; Cook *et al.* 2008; Papadupoulou *et al.* 2009).

The Strait of Gibraltar (SG) is one of such areas. Currently formed by two facing peninsulas, it has intermittently separated the African and Iberian plates since their collision in the late Miocene (Duggen *et al.* 2003). After this impact, and following the closing of the Mediterranean basin and the decrease in the sea level during the Messinian salinity crisis, the SG zone became a land bridge that allowed the exchange of species between Europe and Africa (e.g. Hsü 1972; Agustí *et al.* 2006). The strait re-opened at the beginning of the Pliocene, and since then, it has conserved a relatively mild climate because of the buffering influence of the Atlantic Ocean, even during the Pleistocene glaciations (e.g. Haywood *et al.* 2000; Jalut *et al.* 2000). This and other particular geological and edaphic characteristics have enhanced the biogeographical significance of the SG, making it a sanctuary of relict Tertiary taxa and an important Pleistocene glacial refugium (Mai 1989; Hewitt 2000; Rodríguez-Sánchez *et al.* 2008). Besides, during the different Pleistocene glacial maxima, the subsequent drop in the sea level and emergence of small islands would have narrowed the SG (Collina-Girard 2001; Lambeck & Chappell 2001) and alleviated its putative barrier effects.

An apparent botanical continuity across the SG has been reported in different comparative surveys (e.g. Valdés 1991; Ojeda *et al.* 1996; Marañón *et al.* 1999). However, the ranges of many plant taxa appear to be abruptly interrupted by the SG (Valdés 1991; Marañón *et al.* 1999). About 17% of the vascular plants registered in the Iberian flank are absent from the African side, while 27% of the southern species have not been found in the northern part of the strait (Rodríguez-Sánchez *et al.* 2008). Moreover, the presence of particular species on both shores of the SG does not imply a historical free movement of individuals between populations in the two sides. In fact, different phylogeographic studies on individual species have discovered genetic discontinuities associated with this strait (e.g. Castella *et al.* 2000; Burban & Petit 2003; Hampe *et al.* 2003; Terrab *et al.* 2008). However, it is still unknown whether these genetic breaks were originated by independent or simultaneous evolutionary processes. Thus, the study of co-distributed species seems necessary to gain a better understanding of the biogeographical events that have shaped the population genetic structure of this area.

Conifers, and in particular the Pinaceae, represent an ideal system for exploring the role of such processes. They form a monophyletic group and are among the most typical components of the Mediterranean forests; all of them sharing rather similar ecological requirements and life history traits (Barbero *et al.* 1998). There are at least eight conifer species found on both sides of the SG, and they seem to have been present in this region during the last 5–15 Myr (Mai 1989). Such a long time implies that they have faced most of the putative vicariant processes of this region; for instance the end of the Messinian salinity crisis, and the Pleistocene and Holocene interglacials. Moreover, given that most of these taxa (i.e. the Pinaceae) are pollinated and dispersed by the wind and have cytoplasmic genomes with contrasted modes of inheritance (paternal for the chloroplast and maternal for the mitochondrial genome), their study should allow the inference of events associated with differential levels of pollen and seeds gene flow (e.g. Burban & Petit 2003; Liepelt *et al.* 2010).

In the present study, we have used previously published (Burban & Petit 2003; Terrab *et al.* 2007; Soto *et al.* 2010) and newly collected cytoplasmic DNA data, or from the nuclear genome when cytoplasmic markers were monomorphic (González-Martínez *et al.* 2010), to search for the co-occurrence of genetic discontinuities across the SG in five Mediterranean conifers. A further coalescent approach was used on a separate set of nuclear DNA sequences to test for specific phylogeographic hypotheses related to the putative barrier effects of the SG, especially during the major disruptive geological events (Rosebaum *et al.* 2002). More specifically, we aim to answer the following questions: (i) Are both types of cytoplasmic DNA markers reflecting the same genetic structure within and among species? (ii) Is there a coincidence between the genetic discontinuities

detected in the different species and the Strait of Gibraltar? and (iii) Do the estimated times of divergence between populations on both sides of the SG correspond with a major disruptive historical event in this zone?

Materials and methods

Species selection and sampling

A total of five Mediterranean conifer taxa with distribution on both sides of the Strait of Gibraltar were selected and sampled in both southern Spain and Morocco. The remaining three taxa were excluded because of the scarcity of natural populations in one of the SG sides (i.e. *Tetraclinis articulata* in Spain), low amounts of genetic diversity and concerns about the natural origin of populations (i.e. *Pinus pinea*; see Vendramin *et al.* 2008), or unavailability of non-anonymous DNA markers (*Juniperus* spp.; see Terrab *et al.* 2008). For the five selected taxa, either needles or seed-lots were collected from between 8 and 36 individuals from at least five populations per species, with the total sample size ranging between 79 and 423 individuals per taxon (See Table S1 for population locations and exact figures on the number of individuals and populations sampled for each species). The included species were:

1 *Abies pinsapo* complex: this group of taxa includes *A. pinsapo* Boiss., *Abies marocana* Trab. and *Abies tazaotana* Villar. *A. pinsapo* is limited to three populations on the Betic Mountains in southern Spain, while the other two taxa are endemic to northwest Morocco. *A. marocana* is restricted to a few stands in the Rif Mountains, while *A. tazaotana* has been reported in only one population in the Tazaot Mountains (also in the Rif). Taxonomically, some authors (e.g. Farjon 1990) recognize only one species with three varieties, while others (e.g. Arista & Talavera 1994) recognize up to three different species (*A. pinsapo*, *Abies maroccana* and *A. tazaotana*).
2 *Pinus halepensis* Mill.: this species has a circum-Mediterranean range with highly fragmented and scattered populations distributed along the coast. It has relatively low levels of genetic diversity and low population structure (e.g. Morgante *et al.* 1998; Agúndez *et al.* 1999), with eastern Mediterranean populations showing higher genetic variation than western ones (Grivet *et al.* 2009).
3 *Pinus nigra* J. F. Arnold: it is mainly distributed in the northern shore of the Mediterranean, with only a few isolated stands reported in northern Africa, including the Rif Mountains of northern Morocco (Farjon 1984). This taxon has up to four recognized varieties (Christensen 1993) and has exhibited a rather strong population genetic structure with chloroplast DNA markers in western Europe (Afzal-Rafii & Dodd 2007).
4 *Pinus pinaster* Ait.: this species is limited to the western part of the Mediterranean and the Atlantic coast of Portugal, Spain and France. In its Mediterranean range, it has a fragmented distribution occupying a great variety of soils and altitudinal levels and has shown geographical population structure with different types of genetic markers (e.g. Burban & Petit 2003; Bucci *et al.* 2007), some authors distinguishing up to five landraces or varieties within this species (Resch 1974).
5 *Taxus baccata* Linn.: this is one of the conifers with the largest distribution in the world; ranging from the British Isles to central Iran, and from the High Atlas in Morocco to Norway, with a recent report in the Azores (Schirone *et al.* 2010). *T. baccata* is mostly a montane species that in the Mediterranean grows in small and discontinuous stands. Different local studies have revealed that it has high to moderate genetic diversity, with low to considerable genetic differentiation among populations (e.g. Myking *et al.* 2009; Dubreuil *et al.* 2010; González-Martínez *et al.* 2010).

DNA extraction and collection of genetic data

Total DNA was extracted with the Invisorb DNA Plant HTS 96 kit or the Dellaporta *et al.* (1983) protocol from either fresh or silica-gel-dried needles, or seedlings, following manufacturer instructions and standard procedures. Chloroplast DNA diversity was explored using six regions containing microsatellites (*Pt*1254, *Pt*15169, *Pt*30204, *Pt*36480, *Pt*71936 and *Pt*87268) that were amplified following previously described conditions (Vendramin *et al.* 1996; Soto *et al.* 2010). PCR products were electrophoresed through denaturing polyacrylamide gels in a Li-Cor 4300 sequencer and scored with the GeneIR program using the SequaMark DNA size marker and control samples of known size as standards. Chloroplast microsatellite data for the *A. pinsapo* complex were directly retrieved from Terrab *et al.* (2007), while partial data for *P. pinaster* (nine of 17 populations) were taken from Soto *et al.* (2010).

Mitochondrial DNA diversity was surveyed by direct sequencing of specific regions that have been previously reported as polymorphic in other conifers (e.g. Liepelt *et al.* 2002; Burban & Petit 2003; Jaramillo-Correa *et al.* 2004). More specifically, the fourth intron of the gene *nad*5 was sequenced in the *Abies* taxa, while the second intron of the gene *nad*1 was examined in *P. halepensis*,

P. nigra and *T. baccata*. The first intron of the genes *nad*5 and *nad*7 were additionally surveyed in *P. nigra*. Mitochondrial DNA data for *P. pinaster* were directly taken from Burban & Petit (2003).

For *T. baccata*, additional data had to be retrieved from seven nuclear microsatellite markers, as reported in González-Martínez *et al.* (2010), because of its almost complete lack of cytoplasmic DNA variation. Preliminary screens only revealed a rare cpDNA type in Iranian populations, while the remaining samples were all monomorphic (own unpublished results).

Genetic data analysis

To calculate standard cytoplasmic diversity estimates, such as the number of cpDNA and mtDNA types (n_{cp} and n_{mt}) and their respective diversity values ($H_{E\text{-}cp}$ and $H_{E\text{-}mt}$), all cpDNA and mtDNA polymorphisms were combined in chlorotypes and mitotypes for each individual species. For the particular case of *T. baccata*, standard nuclear diversity estimates, such as the number of alleles per locus (A), the observed (H_0) and expected (H_E) heterozygosities and the fixation index (F_{IS}) were calculated for each individual loci and population using the GDA program (Lewis & Zaykin 2001). Evolutionary relationships among mitotypes and chlorotypes of the same species were depicted with the software TCS (Clement *et al.* 2000), by fixing a connection limit at five steps, which allowed us to connect all haplotypes and recover all potentially missing intermediate forms (Templeton *et al.* 1992).

Population structure was analysed independently for each species by considering mtDNA and cpDNA (nuclear for *T. baccata*) data separately. For each data set, overall estimates of population differentiation were compared using the programs PERMUT and CPSSR (Pons & Petit 1996). Briefly, for mtDNA data, population differentiation was determined using parameters that take (N_{ST}) and do not take (G_{ST}) the relatedness among haplotypes into account. For nuclear and chloroplast microsatellite data, the parameter R_{ST}, which takes into account the mode of evolution of microsatellites to determine the distance between haplotypes, was used instead of N_{ST}. Then, a phylogeographic structure was assumed when the N_{ST} (R_{ST} for SSRs) estimates were significantly higher than the G_{ST} values.

The spatial distribution of genetic diversity was statistically surveyed only on the cpDNA or nuclear (for *T. baccata*) data sets. This was because of the low amounts of mtDNA variation observed among and within taxa (see Results). Populations of the same species were then clustered with a spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.* 2002), which maximizes the proportion of the total (cpDNA or nuclear) genetic variance that is due to differences among groups of stands (F_{CT}). The optimal configuration of K groups, which is the one that exhibited the largest F_{CT} value, was obtained with the simulated annealing process available on the software SAMOVA (Dupanloup *et al.* 2002) by considering 1000 initial conditions. Genetic discontinuities within species were inferred by loading 100 re-sampled Slatkin's (1995) linearized F_{ST} distance matrices in the software BARRIER ver. 2.2 (Manni *et al.* 2004). The Montmonier's maximum difference algorithm implemented in this software allowed us to retrieve the genetic discontinuities associated with the maximum genetic distance values and to calculate how many times each tessellation segment was included in one of the barriers inferred for each loaded matrix. These genetic breaks were then compared across species by importing the barriers into arcGIS and by searching for high density break zones with the lines density tool (see also Soltis *et al.* 2006; Thiel-Egenter *et al.* 2009). The resulting figures were visualized by colour density. The Spearman's *rho* correlation coefficient was finally used to test for break coincidence among species.

Estimation of divergence times

Nuclear DNA sequences were preferred to cytoplasmic DNA markers to infer the divergence time of populations on both sides of the SG. This was because of the lack of convergence across preliminary analyses performed on organelle DNA data sets to obtain estimates of demographic parameters (results not shown). To reduce computing time, only the two species exhibiting the most contrasting phylogeographic patterns, *P. halepensis* and *P. pinaster*, were retained for these analyses (see Results). Up to twelve individuals from populations sampled on both sides of the SG were sequenced in a previous study covering the full range of the species (see Grivet *et al.* 2010). From the 10 or 11 (depending on the species) gene regions surveyed (in most cases, homologous across taxa), eight appeared to be selectively neutral and were used for phylogeographic inferences herein. One of these gene fragments (*dhn5-Ps*) showed some correlations with climate at the range-wide scale in *P. pinaster* (Grivet *et al.* 2010); however, no substantial biases due to natural selection at the regional scale studied here are expected. Standard summary statistics, such as π_n (Tajima 1983), average number of nucleotide differences (K), Φ_{SC} and Φ_{CT}, were calculated for each gene region to test the assumptions of the isolation with migration (IM) model (see Hey & Nielsen 2007; Eckert *et al.* 2008 for more details).

Mutation rate-scaled demographic parameters, such as the effective population sizes ($\theta = 4N_e\mu$) of the

modern stands from southern Spain and Morocco (θ_{SS}, θ_M) and the ancestral population (θ_A), the rates of gene flow across regions ($M = m/\mu$), and the time of population divergence ($\tau = t\mu$), were estimated using the IM model implemented in the program IMa (Hey & Nielsen 2007). The upper bounds of the truncated uniform priors of each parameter (i.e. $\theta_{SS} = \theta_M = 5$, $\theta_A = 15$, $M_{SS \rightarrow M} = M_{M \rightarrow SS} = 15$, $\tau = 10$) were identified after multiple test runs of the program. Then, three final runs were performed for each species with the following conditions: a burn-in of 1.0×10^9 samples, followed by 1.0×10^6 simulations performed under the geometric heating scheme. Parameters were sampled every 1000 generations and graphed into a histogram that was used to determine the distribution of their respective marginal posterior probabilities. The peak of each distribution was assumed to be the estimate of each parameter. Convergence of the algorithm within the same run and across runs was evaluated for each species following the IMa documentation and Eckert *et al.* (2008).

Un-scaled mutation rates (μ) were estimated for each gene region using the average number of nucleotide differences (K) calculated with the Jukes-Cantor correction (Jukes & Cantor 1969) available in the program DNASP (Rozas *et al.* 2003). Estimates of μ were derived from the equation $\mu = K/2\tau$, where τ represents the divergence times between *P. halepensis* or *P. pinaster* and three different outgroups (*Pinus taeda*, *P. nigra* and *Pinus sylvestris*) as reported in recent molecular clock studies (Willyard *et al.* 2007; Gernandt *et al.* 2008). These mutation rates were then used to transform the divergence times estimated with the IM model into un-scaled values, which were finally compared with reported dates of the main geological and climatic events that have occurred in the SG region (e.g. Rosebaum *et al.* 2002; Carrión *et al.* 2003; Duggen *et al.* 2003).

Results

Amounts of genetic diversity

Overall, the different cytoplasmic genetic diversity estimates were lower in *Pinus halepensis* and the *Abies pinsapo* complex than in *Pinus nigra* and *Pinus pinaster* (Table 1). The total and mean (per population) number of chloroplast DNA haplotypes (chlorotypes) and mean cpDNA diversities ($H_{E\text{-}cp}$) ranged between 12, 2.8 and 0.217 in *P. halepensis*, to 124, 17 and 0.951 in *P. nigra*, respectively. At least one mtDNA region was polymorphic for all species except *P. halepensis* and *Taxus baccata*, which were monomorphic for all markers surveyed. As observed for the cpSSRs, *P. nigra* was the most diverse species with four mtDNA haplotypes and a mean $H_{E\text{-}mt}$ value of 0.12, followed by *Pinus pinaster* and the *A. pinsapo* complex with two mitotypes each. Nevertheless, all populations of these taxa were fixed for a particular mitotype, which translated into null mtDNA within-population diversities. In most species, populations of southern Spain were more diverse than the Moroccan stands, especially for the cpDNA markers (Table 1). The only exception to this trend was the *A. pinsapo* complex, which exhibited similar amounts of cytoplasmic DNA diversity on both sides of the SG.

In *T. baccata*, all the nuclear SSR's analysed were polymorphic and exhibited a mean of 5.4 alleles (A) and a mean expected heterozygosity (H_E) of 0.621 per population. Interestingly, the Moroccan stands had a higher mean A value and a lower H_E estimate than the Spanish populations, while there was a significant excess of homozygous in most stands, especially in Morocco (Table 1). Indeed, most loci were not in Hardy–Weinberg (H–W) equilibrium in all populations, with the exception of the southernmost Spanish stand (Sierra Tejeda), which exhibited a mean fixation index (F_{IS}) of −0.182 and was in H–W equilibrium for all loci (data not shown). All the original mtDNA sequences gathered during this study are available on GenBank (accessions nos HQ185286 – HQ185293).

Genetic and geographic population structures

All species surveyed except *P. halepensis* showed a significant genetic structure with at least one type of marker (cpDNA or mtDNA, or ncDNA for *T. baccata*). In all these cases, the observed structure was related to geography and it was generally associated with the presence of the Strait of Gibraltar (Figs 1 and 2).

Differentiation between populations of the *A. pinsapo* complex was maximal for mtDNA (i.e. $G_{ST} = N_{ST} = 1$), with each taxa fixed for a different mitotype. These two mitotypes differed by a single substitution (data not shown). Interestingly, the mtDNA variant found in Moroccan populations (*Abies maroccana*) was identical to the one previously reported for *Abies numidica* (Algerian fir) and was more distantly related to those previously reported for *Abies alba* (a European species; Liepelt *et al.* 2002) than the mitotype observed in Spanish stands (*A. pinsapo*; Fig. 1). The distribution of cpDNA variation in this species complex followed a similar trend, showing a significant phylogeographic structure ($R_{ST} = 0.78 > G_{ST} = 0.31$; $P < 0.0001$) that was strongly correlated with the continental location of each taxa. Both the SAMOVA and BARRIER analyses revealed that the maximal genetic (i.e. cpDNA) differentiation was obtained when populations were partitioned in two groups, corresponding to Morocco and southern Spain, respectively (Fig. 2a; see Terrab *et al.* 2007 for more details on cpDNA for this species complex).

Table 1 Sample size and mean cytoplasmic (nuclear for *Taxus baccata*) genetic diversity parameters estimated in populations of five conifer taxa distributed on both sides of the Strait of Gibraltar. Standard deviations are shown in parentheses

	mtDNA						cpDNA						
Species/Region	N Pop	Mean N	Total n_{mt}	Mean n_{mt}	Mean $H_{E\text{-}mt}$	G_{ST}/N_{ST}	N Pop	Mean N	Total n_{cp}	Mean n_{cp}	Mean $H_{E\text{-}cp}$	G_{ST}/R_{ST}	Ref
Abies pinsapo complex													
southern Spain (SS)	1	10	1	1	0	–	3	30	20	9.7	0.839	0.056/0.53*	PS; 1
Morocco (M)	1	10	1	1	0	–	5	27	21	9.4	0.843	0.051/0.256*	
Total/Mean (Standard deviation)	2	10	2	1 (0)	0 (0)	1/1(–/–)	8	28.13 (4.96)	34	9.5 (2.6)	0.842 (0.076)	0.312/0.779* (0.030/0.078)	
Pinus halepensis													
SS	2	5	1	1	0	–	9	23.23	12	3	0.221	0.088/0.034	PS
M	1	5	1	1	0	–	7	24.14	5	2.7	0.212	0.038/0.004	
Total/Mean (Standard deviation)	3	5	1	1 (0)	0 (0)	– (–)	16	23.63 (1.73)	12	2.8 (1.5)	0.217 (0.171)	0.065/0.018 (0.009/0.005)	
Pinus nigra													
SS	6	10	3	1.34	0.14	0.881/0.883	6	25	118	17.7	0.959	0.034/0.038	PS
M	1	10	1	1	0	–	1	30	13	13	0.903	–	
Total/Mean (Standard deviation)	7	10	4	1.29 (0.45)	0.12 (0.189)	0.828/0.843 (0.124/0.132)	7	25.71 (2.71)	124	17 (3.2)	0.951 (0.049)	0.036/0.057 (0.018/0.027)	
Pinus pinaster													
SS	5	10	1	1	0	–	10	24.6	106	13.5	0.912	0.069/0.355*	PS; 2; 3
M	4	10	2	1.25	0	1/1	7	25.29	53	8.1	0.704	0.120/0.139	
Total/Mean (Standard deviation)	9	10	2	1 (0)	0 (0)	1/1 (0/0)	17	24.88 (1.99)	153	11.3 (4.2)	0.936 (0.201)	0.113/0.261* (0.025/0.117)	

	nuDNA						
	N Pop	Mean N	A	H_E	F_{IS}	G_{ST}/R_{ST}	Ref
Taxus baccata†							
southern Spain	3	11.67	4.81	0.658	0.235	0.137/0.032	4
Morocco	2	22	5.94	0.575	0.518	0.236/0.071	
Total/Mean (Standard deviation)	5	15.80 (10.40)	5.4 (1.88)	0.621 (0.075)	0.377 (0.183)	0.187/0.112 (0.048/0.098)	

Abbreviations: N Pop, number of sampled populations; Mean N, mean number of sampled individuals per population; Total n_{mt}, total number of mitochondrial haplotypes (mitotypes); Mean n_{mt}, mean number of mitotypes per population; Mean $H_{E\text{-}mt}$, mean mitotype diversity per population; Total n_{cp}, total number of chloroplast haplotypes (chlorotypes); Mean n_{cp}, mean number of chlorotypes per population; Mean $H_{E\text{-}cp}$, mean chlorotype diversity per population; G_{ST}/N_{ST} and G_{ST}/R_{ST}, genetic differentiation among populations by considering un-ordered (G_{ST}) and ordered alleles (N_{ST} and R_{ST}); Ref., references; PS, present study; 1, Terrab *et al.* (2007); 2, Burban & Petit (2003); 3, Soto *et al.* (2010); 4, González-Martínez *et al.* (2010).

†Diversity values for *Taxus baccata* correspond to seven nuclear microsatellites. A = mean number of alleles per locus, H_E = mean expected heterozygosity, F_{IS} = mean fixation index, and G_{ST}/R_{ST} = genetic differentiation among populations by considering un-ordered and ordered alleles, respectively.

*Estimates of G_{ST} and R_{ST} significantly different at $P = 0.01$.

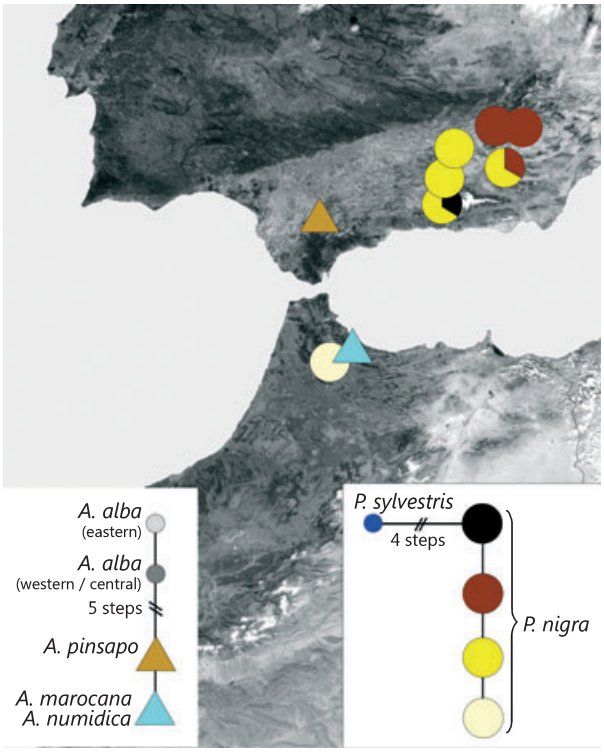

Fig. 1 Geographic location of the mitochondrial DNA haplotypes (mitotypes) found in populations of *Abies pinsapo* complex (triangles; based on variation in *nad*5 intron 4) and *Pinus nigra* (circles; based on variation in *nad*7 intron 1) distributed on both sides of the Strait of Gibraltar. The evolutionary relationships among mitotypes of the same species are presented within the two inserts. The outgroups used to 'root' the networks (*Abies alba* and *Pinus sylvestris*) are only shown for reference.

All mtDNA regions surveyed in *P. halepensis* were monomorphic. Chloroplast data revealed low and geographically unstructured differentiation between populations (R_{ST} = 0.02, G_{ST} = 0.07; $P > 0.1$), which is probably the result of such low genetic diversity. The only genetic breaks disclosed for this species involved marginal populations from the High Atlas and the Betic Mountains, in southern Morocco and southeastern Spain, respectively (breaks 'a' and 'b' on Fig. 2b).

The two other pine species surveyed (*P. nigra* and *P. pinaster*) exhibited strong population genetic structures, especially with the mtDNA markers. In *P. nigra*, mitochondrial DNA variation was high (N_{ST} = 0.843, G_{ST} = 0.828), but not phylogeographically significant ($N_{ST} \approx G_{ST}$; P = 0.89). Interestingly, from the four mtDNA types detected in this taxon, the mitotype fixed in and exclusive to the Moroccan population was the most recently originated, as indicated by its peripheral position, relative to the outgroup, in the mtDNA network (light yellow mitotype on Fig. 1). The distribution of the chloroplast DNA variation exhibited a similar pattern than the mtDNA one, although its among-population differentiation values were much lower (R_{ST} = 0.057, G_{ST} = 0.036; Fig. 2c). Indeed, both types of markers showed that the population of northern Morocco was significantly differentiated from the Iberian ones (barrier 'a', and purple group on Fig. 2c), which formed a far more homogenous group with only a few marginally diverging stands (barriers 'b' and 'c').

In *P. pinaster*, the distribution of both cytoplasmic DNA variants clearly separated the Moroccan populations, except the northernmost stand of Punta Cires, from the Spanish populations (Fig. 2d; see Burban & Petit 2003 for more details on mtDNA for this species). Variation in the cpSSRs further revealed that some populations from southeastern Iberia and northern Morocco could be differentiated from these two main clusters. However, the level of statistical support for these genetic discontinuities was not high (barriers 'b' and 'd' on Fig. 2d). As observed for the other species, the overall population differentiation in *P. pinaster* was higher for mtDNA ($G_{ST} = N_{ST} = 1$; Burban & Petit 2003) than for cpDNA markers (R_{ST} = 0.261, G_{ST} = 0.113; $R_{ST} > G_{ST}$; $P < 0.001$).

Cytoplasmic DNA variation in *T. baccata* was inexistent for all the genomic regions sequenced in the study area, and nuclear SSRs had to be used to infer genetic structure. The mean population differentiation value for the seven microsatellites surveyed was high for a conifer (G_{ST} = 0.187), but not phylogeographically significant (R_{ST} = 0.112). Both the SAMOVA and the BARRIER analyses showed that the Spanish and Moroccan populations were differentiated from each other. However, a genetic break found between the southernmost Spanish stand and the other populations surveyed had more statistical support than the discontinuity between the Moroccan and the Iberian stands (Fig. 2e).

Altogether, the genetic barriers detected across species showed two zones of high breaks density. The first zone approximately coincides with the Betic Mountains in southeastern Spain, and the second one with the Strait of Gibraltar (Fig. 3). Such pattern diverges significantly from the null hypothesis of random distribution of genetic barriers across the study region ($P < 0.01$) and further points that these two geographic features might have structured the genetic diversity of the surveyed taxa in similar ways.

Parameter estimation of the IM model and divergence time across the SG

Patterns of nucleotide diversity for the nuclear gene regions surveyed showed a similar trend than the

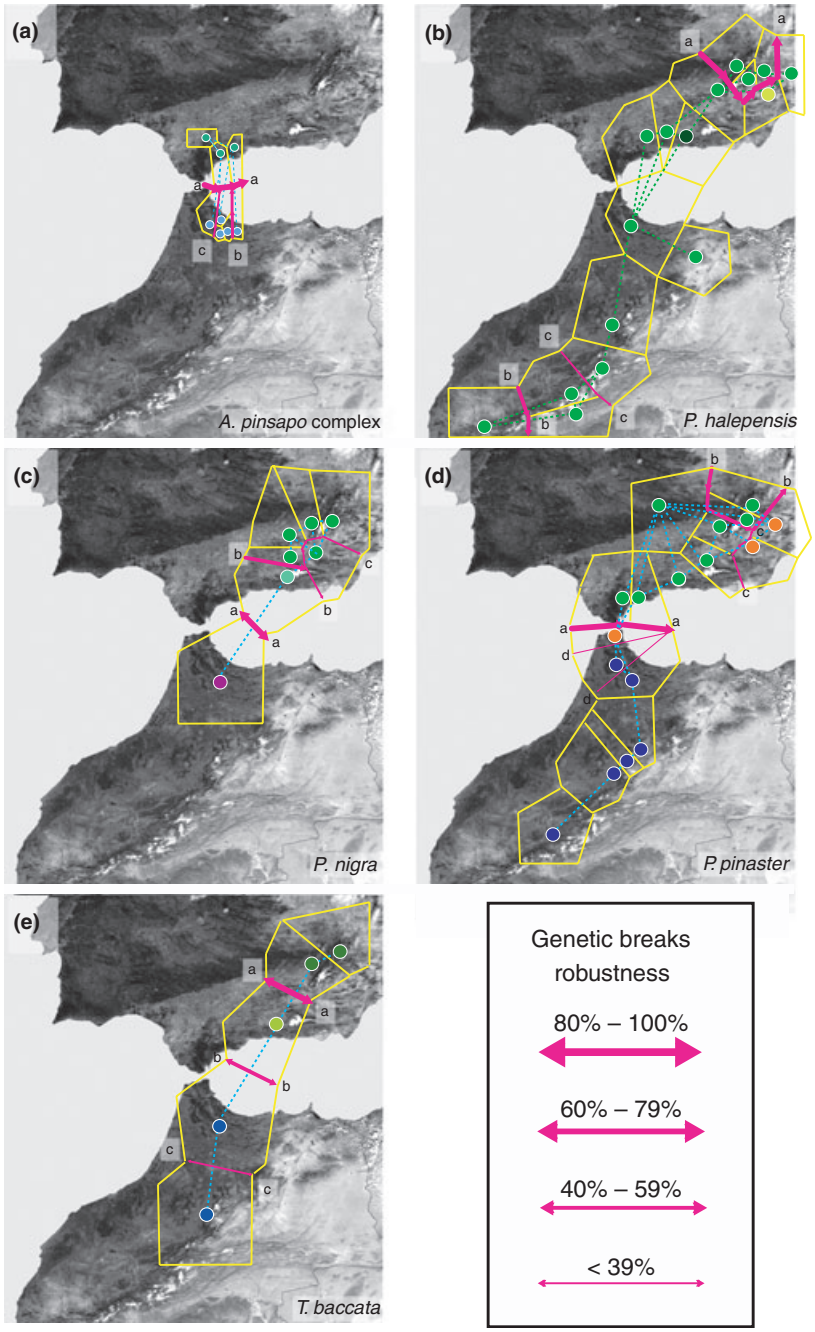

Fig. 2 Genetic boundaries (bold lines) obtained with the Montmonier's maximum difference algorithm on genetic distances derived from chlorotype frequencies, and clustering of populations (coloured dots) made with spatial analyses of molecular variance (SAMOVA) in five conifer species distributed on both sides of the Strait of Gibraltar. (a) *Abies pinsapo* complex (reanalysed data from Terrab *et al.* 2007) (b) *Pinus halepensis*; (c) *Pinus nigra*; and (d) *Pinus pinaster*. Boundaries and clusters for *Taxus baccata* (e) were derived from variation on seven nuclear microsatellite markers (reanalysed data from González-Martínez *et al.* 2010). The thickness of the genetic boundaries represents their statistical robustness, ranging from 1% to 100%, as indicated in the bottom right insert.

cytoplasmic DNA markers, with *P. halepensis* having less diversity than *P. pinaster* (Table 2). In the first species, five of eight gene regions (*4cl-Pt-a*, *4cl-Pt-b*, *dhn2-Ps-b*, *lp31-Pt-a* and *lp31-Pt-b*) showed moderate levels of polymorphism and exhibited similar nucleotide diversity (π_n) values in the two stands surveyed. The remaining three genes were monomorphic (*dhn2-Pp-b* and *lp33-Pp*) or almost monomorphic (*dhn2-Pp-a*) in both populations. On the other hand, all genes were polymorphic in all stands of *P. pinaster*. Overall, the Spanish

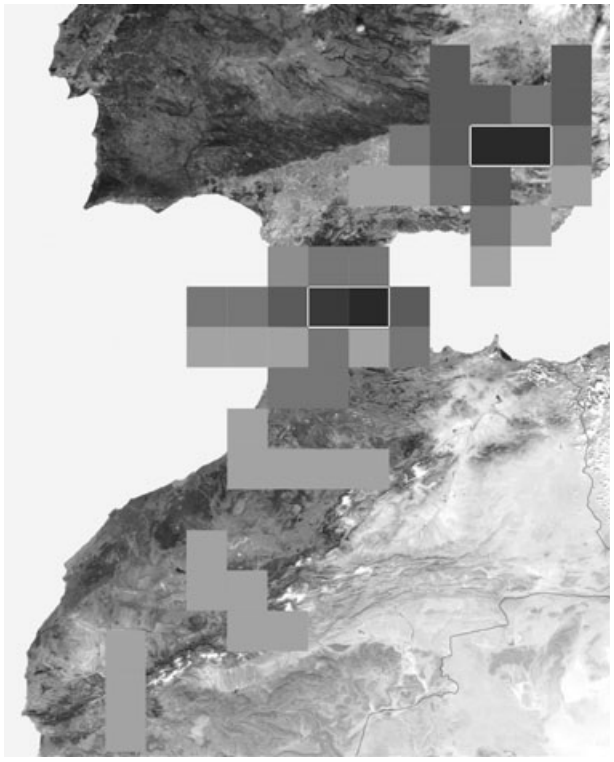

Fig. 3 Density of the genetic breaks identified for populations of five conifer species distributed on both sides of the Strait of Gibraltar (see Fig. 2) as calculated with the 'Lines Densities' tool in ArcView. Cells surrounded by white lines have a significantly higher density of breaks than expected under the null hypothesis that these are randomly distributed across the study area.

populations exhibited significantly higher π_n values than their Moroccan counterparts (Kruskall–Wallis test, $P < 0.05$).

Population differentiation (Φ_{ST}) varied notably among DNA regions in *P. halepensis*, with two genes showing significantly high Φ_{ST} estimates (*dhn2-Ps-b* and *lp31-Pt-b*), and the remaining polymorphic regions exhibiting values close to zero. In *P. pinaster*, differentiation within groups of stands (Φ_{SC}) was nonsignificant for all genes, while differentiation across geographic regions (Φ_{CT}) was consistently and significantly high for all DNA regions (Table 2).

For both species (*P. halepensis* and *P. pinaster*), the posterior probability distributions generated by the IM model were fully contained within the prior bounds of each estimated demographic parameter, and most of them showed single unambiguous peaks. The only exception was the gene flow from Morocco to southern Spain ($M_{M \rightarrow SS}$) in *P. halepensis*. Independent runs of this model appeared to converge on similar distributions and median values (Kolmogorov–Smirnov tests; $Q > 0.05$), while the rates of autocorrelation of each parameter were low, and the ESS estimates were all above 100 (data not shown). In *P. halepensis*, the values of the population-splitting parameter (s) estimated with the IM model indicated that a large proportion (about 66%) of the ancestral population gave origin to the lineages currently observed in Morocco, while only 34% of this ancestral stand founded the lineages from southern Spain (Table 3). Scaling the estimated ancestral population size with this s value pointed out that both populations have been rather stable since their initial separation (i.e. θ_{SS} and $\theta_M \approx \theta_A \times s$). In *P. pinaster*, the estimated s parameter suggested the inverse pattern, with most of the ancestral population originating the lineages from southern Spain (72%). Furthermore, a population decrease following the separation of these populations from the Moroccan stands was inferred on both sides of the SG when the estimated ancestral population size was scaled with the splitting parameter (i.e. θ_{SS} and $\theta_M < \theta_A \times s$).

Mutation rates varied between four and seven-fold across genes within the same species (Table 2). When using these rates and the more recent calibration dates (τ_1) for the divergence of *P. halepensis* and two outgroups from their common ancestor [see Table 2 and Willyard *et al.* (2007) and Gernandt *et al.* (2008) for more details], the separation of the populations on both sides of the SG was estimated in about 0.28 Ma (95% credible interval: 0.15–1.67; Table 3, Fig. 4). The most conservative divergence times (τ_2) approximately doubled this estimate to 0.58 (95% credible interval: 0.31–3.40), which situates the divergence of the Spanish and Moroccan populations of *P. halepensis* on the late Pleistocene, before the beginning of the last major glacial cycle, 0.11 Ma. In *P. pinaster*, the estimated mutation rates combined with the earlier calibration dates (τ_1; see Table 2) produced a divergence time of 1.90 Ma (95% credible interval: 1.41–2.76), while the most conservative dates increased this estimate almost twofold (3.70 Ma; 95% credible interval: 2.73–5.32). These divergence dates could be placed during the Pliocene, after the end of the Messinian salinity crisis, 5 Ma (Fig. 4).

Discussion

The Strait of Gibraltar as a vicariant factor

Disjunctions in the distribution of plant genetic diversity are often consequence of past environmental changes, which are usually associated with geographical features that have limited the historical flow of genetic material between populations (e.g. Carstens *et al.* 2005; Rodríguez-Sánchez *et al.* 2008; Papadupoulou *et al.* 2009). In this study, we show that there is a

Table 2 Summary statistics and estimates of mutation rates for eight gene regions sequenced in *Pinus halepensis* and *Pinus pinaster* populations located on both sides of the Strait of Gibraltar

			π_n ($\times 10^{-4}$)					Mut rate ($\mu \times 10^{-7}$)‡	
Species/Gene region	Length (bp)	*h*	SS	M	Φ_{SC}	Φ_{CT}†	*K*	$\tau 1$	$\tau 2$
Pinus halepensis									
4cl-Pt-a	461	13	45.3	57.6	–	0.016	6.86[a]	4.57	2.21
4cl-Pt-b	708	13	127.3	112.3	–	0.012	6.02[a]	4.01	1.94
dhn2-Pp-a	403	5	0	0	–	0	16.03[a]	10.69	5.17
dhn2-Pp-b	450	3	0	4.2	–	0	17.03[a]	11.35	5.49
dhn2-Ps-b	486	6	4.3	2.2	–	0.387*	16.79[b]	15.26	8.39
lp31-Pt-a	488	4	32.8	39.3	–	0.271**	14.28[a]	9.52	4.61
lp31-Pt-b	366	4	24.1	22.7	–	0.019	8.46[a]	5.64	2.73
lp33-Pp	375	2	0	0	–	0	23.27[a]	15.51	7.51
Total/Mean	3737	6.25	29.23	29.79	–	0.088	13.59	8.54	4.20
P. pinaster									
4cl-Pt-a	554	4	22.2	9.2	0.001	0.461*	9.03[a]	6.02	2.91
dhn2-Pp-a	494	11	59.3	45.2	0.008	0.152*	15.11[a]	13.74	7.56
dhn2-Ps-a	526	14	50.7	68.6	0.001	0.091***	45.45[c]	41.32	22.73
dhn2-Ps-b	752	14	79.9	34.5	0.011	0.212*	35.74[b]	32.49	17.87
dhn5-Ps	468	5	36.7	33.4	0.002	0.510*	12.92[c]	11.75	6.46
lp31-Pt-a	576	3	53.1	21.4	0.028	0.092**	13.07[a]	8.71	4.22
lp31-Pt-b	463	7	71.5	44.4	0.046	0.194*	11.49[a]	7.66	3.71
lp33-Pp	449	9	33.4	54.4	0.027	0.089*	24.23[a]	16.15	7.82
Total/Mean	4282	8.37	50.85	38.89	0.016	0.218	20.88	13.92	7.18

Abbreviations: *h* = number of haplotypes (including only those defined by unambiguous indels); π_n = nucleotide diversity (Tajima 1983) within groups of populations: SS = southern Spain, M = Morocco; Φ_{SC} = genetic differentiation of populations within regions; Φ_{CT} = genetic differentiation between regions (†These values correspond to Φ_{ST} for *P. halepensis*); *K* = average number of pairwise differences between homologous sequences of each species and an outgroup: [a] = *Pinus taeda*, [b] = *Pinus nigra*, [c] = *Pinus sylvestris*.
‡ Mutation rates per year were estimated from the equation $\mu = K/2\tau$ (See Materials and Methods), using the estimated *K* values and assuming the following divergence times (τ) between each species and the corresponding outgroup: *P. halepensis – P. taeda*: $\tau 1$ = 15 Ma, $\tau 2$ = 31 Ma; *P. halepensis – P nigra*: $\tau 1$ = 11 Ma, $\tau 2$ = 20 Ma; *P. pinaster – P. taeda*: $\tau 1$ = 15 Ma, $\tau 2$ = 31 Ma; *P. pinaster – P. nigra*: $\tau 1$ = 11 Ma, $\tau 2$ = 20 Ma; *P. pinaster – Pinus sylvestris*: $\tau 1$ = 11 Ma, $\tau 2$ = 20 Ma; see Willyard *et al.* (2007) and Gernandt *et al.* (2008) for more details.
Estimates of Φ_{CT} significant at *P = 0.001; **P = 0.01; and ***P = 0.05.

significant co-location of such genetic breaks in five Mediterranean conifers distributed on both sides of the Strait of Gibraltar (Figs 1-3). Among the species surveyed, three (*Abies pinsapo* complex, *Pinus nigra* and *Pinus pinaster*) were polymorphic for the mtDNA regions sequenced, and these polymorphisms were differentially distributed across the SG. Similar genetic breaks were observed for these three species with cpSSR markers. Indeed, among the taxa studied, only *Pinus halepensis* seemed to have a relatively homogeneous distribution of its rather low genetic diversity between the Spanish and Moroccan populations. The fifth species surveyed, *Taxus baccata*, exhibited no cytoplasmic genetic diversity in the study region, but the analyses of seven nuclear SSRs also revealed, among others, a significant differentiation between stands on both sides of the SG. Altogether, these results suggest that in spite of the putative homogenising effects of the gene flow driven by wind-dispersed pollen, the SG has represented a significant biogeographic barrier for these conifers, thus preserving an important differentiation between the Spanish and African populations.

The effect of this strait as a significant biogeographical barrier has been reviewed for several plant and animal species (e.g. Rodríguez-Sánchez *et al.* 2008; García-Mudarra *et al.* 2009). For instance, genetic breaks have been observed in populations on both sides of the SG for *Frangula alnus* (Hampe *et al.* 2003), *Juniperus thurifera* (Terrab *et al.* 2008), *Hypochaeris glabra* (Ortiz *et al.* 2009), *Myotis myotis* (Castella *et al.* 2000) and *Otis tarda* (Broderick *et al.* 2003). Similarly, divergence between Atlantic and Mediterranean populations of marine organisms because of limited gene flow through the SG has been reported for *Stenella coeruleoalba* (García-Martínez *et al.* 1999), *Thunnus alalunga* (Viñas *et al.* 2004) and *Cymodocea nodosa* (Alberto *et al.* 2008), among others.

Table 3 Estimates of mutation-scaled and un-scaled parameters for the isolation with migration model on eight nuclear gene regions of *Pinus halepensis* and *Pinus pinaster*

	P. halepensis		*P. pinaster*	
Parameter	Estimate	95% CI	Estimate	95% CI
Mutation-scaled				
θ_{SS}	0.61	0.01–1.27	1.51	0.81–2.58
θ_{M}	0.99	0.51–2.19	0.39	0.19–1.40
θ_{A}	1.82	0.91–2.76	3.48	2.48–12.98
$M_{SS \rightarrow M}$	3.38	1.75–5.98	7.18	1.45–9.81
$M_{M \rightarrow SS}$	–	–	1.91	0.21–5.30
s	0.34	0.21–0.44	0.72	0.41–0.89
τ	0.24	0.13–1.43	2.65	1.96–3.82
Un-scaled				
τ_1				
$N_{SS(g25)}$*	7200	120–14900	10900	5800–18600
$N_{SS\ (g100)}$*	1800	60–7600	2700	1500–4600
$N_{M\ (g25)}$*	11600	5600–25700	2800	1400–10100
$N_{M\ (g100)}$*	2900	1500–6500	700	340–2500
$N_{A\ (g25)}$*	21300	10700–32400	25000	17800–93300
$N_{A\ (g100)}$*	5300	2700–8100	6300	4500–23400
t (Ma)**	0.28	0.15–1.67	1.90	1.41–2.76
τ_2				
$N_{SS\ (g25)}$*	14500	240–30300	21000	11300–36000
$N_{SS\ (g100)}$*	3600	60–7600	5300	2800–9000
$N_{M\ (g25)}$*	23600	12200–52200	5500	2700–19500
$N_{M\ (g100)}$*	5900	3000–13000	1400	660–4900
$N_{A\ (g25)}$*	43400	21700–65700	48500	34600–180800
$N_{A\ (g100)}$*	10800	5400–16400	12100	8600–45200
t (Ma)**	0.58	0.31–3.40	3.70	2.73–5.32

Abbreviations: θ_{SS} = southern Spain scaled population size; θ_{M} = Morocco scaled population size; θ_{A} = ancestral scaled population size; $M_{SS \rightarrow M}$ = gene flow from SS into M; $M_{M \rightarrow SS}$ = gene flow from M into SS; s = population-split parameter; τ = mutation-scaled divergence time between SS and M; N_{SS} = effective size of the SS population; N_{M} = effective size of the M population; N_{A} = effective size of the ancestral population; t = un-scaled divergence time between SS and M.

*Effective population sizes were calculated from the equation $N_e = \theta/4g\mu$ using the appropriate θ value (e.g. θ_{SS} for the SS population), the μ estimates from Table 2, and by assuming generation times (g) of 25 (as in Brown *et al.* 2004; Eckert *et al.* 2008) and 100 years (as in Provan *et al.* 1999; Navascués & Emerson 2005).

**Un-scaled divergence times were determined from the equation $t = \tau/\mu$.

However, in sharp contrast with the examples above, and similar to the patterns found herein for *P. halepensis*, no genetic breaks associated with the SG were found in *Narcisus papyraceus* (Pérez-Barrales *et al.* 2009), some Mediterranean oaks (Magri *et al.* 2007; Lumaret & Jabbour-Zahab 2009), *Psammodromus algirus* (Carranza *et al.* 2006) and *Galerida cristata* (Guillaumet *et al.* 2008), among others. Altogether, the different patterns observed across species might be accounted for by particular historical or biological features inherent to each taxon, such as habitat preferences, dispersal and competition capabilities, establishment requirements (Rodríguez-Sánchez *et al.* 2008), specific anthropogenic impacts (e.g. Badal 1998), or the location and degree of isolation of ancestral populations (Aguinagalde *et al.* 2005). In addition, for the particular case of forest trees, and given their long generation times, the stability of the ecosystem where they evolve seems to be one of the key factors to explain such differences (Petit & Hampe 2006).

Different species, different stories

Although there is a general correspondence in the geographic location of genetic disjunctions across conifers in the SG area (Fig. 3), most of the genetic breaks observed seem to reflect, at least in part, the individual biological history of each species, instead of a common vicariant phenomenon that shaped simultaneously the distribution of genetic diversity of all taxa. For instance, there is a strong similarity in the distribution of mitotypes of *P. nigra*, *P. pinaster* and the *A. pinsapo* complex, which points to an early divergence of populations on opposite sides of the SG (given the

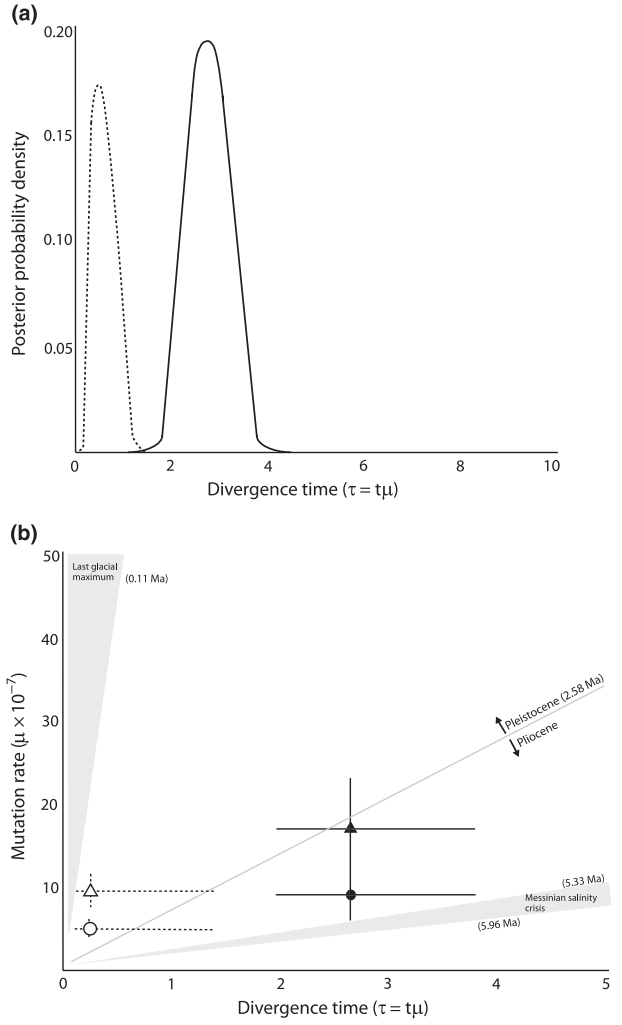

Fig. 4 Estimated divergence times between populations of *Pinus halepensis* (empty symbols and dotted lines) and *Pinus pinaster* (filled symbols and solid lines) located on both sides of the Strait of Gibraltar (SG). (a) Distribution of marginal posterior probabilities for the population divergence time. (b) Representation of the two-dimensional parameter space used for estimating the un-scaled divergence times. Shaded areas illustrate the portions of this space that are consistent with major geological and climatic events in Europe and the Mediterranean. Circles and triangles represent estimates performed with two different calibration times (τ_1 and τ_2; see Table 2) for each species. Error bars along the x and y axis represent the 95% credible intervals of the divergence time and one standard deviation of the mutation rate, respectively.

normally slow rates of evolution of mitochondrial genomes). However, the strong variation observed in the chloroplast G_{ST} and R_{ST} values across species suggests that they have had dissimilar population histories since their establishment in the region. Such different histories might include contrasting amounts of pollen-driven gene flow or stochastic forces acting at different levels and with different strength across taxa.

In recent years, evidence suggesting that northern Africa was colonized by vegetation from two independent fronts expanding simultaneously through the SG and the Strait of Sicily (i.e. between southern Italy and northern Algeria) has been accumulating (e.g. Petit *et al*. 2005a,b; Lumaret & Jabbour-Zahab 2009). The mitotype networks obtained for *P. nigra*, the *A. pinsapo* complex (Fig. 1) and, especially, for *P. pinaster* (Burban & Petit 2003) would support this hypothesis. For all these taxa, the mtDNA variants found in the Moroccan stands were the most recently derived, while the Iberian mitotypes were intermediate between these Moroccan types and the outgroups. Such an observation implies that for these species, the Moroccan populations were all founded by stands expanding through the SG. Moreover, for the particular case of the *Abies* complex, the fact that *Abies maroccana* and *A. numidica* shared a common mitotype additionally implies that they had a common ancestor with a rather wide distribution, which probably covered most of the coastal mountains between Morocco and Algeria (i.e. the Rif and the Kabylies). This possibility further suggests a putative secondary contact between *A. numidica* and *Abies alba*, via pollen exchange through the Strait of Sicily, such as recently deduced from cpDNA data (Liepelt *et al.* 2010).

On the other hand, previous paleoecological and biogeographic surveys have shown that many of the modern Mediterranean tree species are actually 'relicts' that have been present in the region since the Tertiary (Mai 1989). Such taxa include *Abies*, *P. pinaster*, *P. nigra* and *T. baccata*, whose fossils have been found in deposits from the early Miocene to the late Pleistocene in the Pyrenees, Sicily, the Balearic Islands, and southern Spain (e.g. Depape 1928; Menéndez & Florschültz 1964; Mai 1989; Carrión *et al*. 2003; González-Martínez *et al*. 2010 and references therein). A recent study (Magri *et al.* 2007) associated the distribution of chlorotypes in another Mediterranean paleoendemic, *Quercus suber*, with the Neogene tectonic events that occurred between 25 and 15 Ma. Such an association implies that these oaks survived in the rifting microterranes and blocks during the shaping of the west Mediterranean basin without any further population genetic modifications. This observation further points that the long-lasting ecological stability of this region should be reflected not only in extended periods without speciation but also in enduring patterns of repartition of within-species genetic diversity (Magri *et al*. 2007).

The striking similarities in the distribution of cpDNA types of *Q. suber* and mitotypes of *P. pinaster* (Burban & Petit 2003) were interpreted by Magri *et al*. (2007) as evidence of a common vicariant history for Mediterranean Tertiary trees, something that seems to be

complemented by the additional convergent patterns observed herein for *P. nigra* and the *A. pinsapo* complex (Fig. 1). Altogether, these results, those from previous studies (Gerber *et al.* 1995; Burban & Petit 2003; Afzal-Rafii & Dodd 2007; Bucci *et al.* 2007; Terrab *et al.* 2007), and the low mutation rates estimated for the conifer mitochondrial genome (e.g. Wolfe *et al.* 1987; Drouin *et al.* 2008), point out that the divergence of mtDNA lineages located on opposite sides of the SG may be dated back to long before the Pleistocene glaciations. Indeed, if we follow Magri *et al.*'s (2007) interpretation, this date could be as early as the split of the Betic-Rif block during the mid-Miocene (Rosebaum *et al.* 2002). However, our simulations on nuclear DNA data suggest that, at least for the particular case of *P. pinaster*, this divergence should have occurred later, after the end of the Messinian salinity crisis (see below).

The fourth Tertiary species analysed herein, *T. baccata*, showed no cytoplasmic variability in the study region and exhibited intermediate values of nuclear diversity with a significant excess of homozygous individuals in most stands. Furthermore, this species had high levels of nuclear genetic differentiation when compared to other conifers (e.g. Nybom 2004; Petit *et al.* 2005b and references therein), especially between populations on both sides of the SG and between the southernmost Spanish stand and the remaining populations surveyed. These results suggest that the SG and other geographic barriers such as the Betic Mountains (see Fig. 2e) were, and probably still are, hampering the exchange of genetic material among stands of *T. baccata*. Unfortunately, the low number of polymorphic markers available for this species (i.e. seven) limited the reliability of the Bayesian analyses performed (data not shown), which restrained us from determining whether the differentiation among the main genetic clusters of this species preceded or not the Pleistocene glaciations, as suggested for the other Tertiary conifers. However, the long-lasting presence of *T. baccata* in the fossil records (e.g. Mai 1989; Hageneder 2007) and its putative capability to adapt to extinction-recolonization dynamics (Dubreuil *et al.* 2010) suggest that its presence in the region might be indeed very old (see also González-Martínez *et al.* 2010). On the other hand, the excess of homozygous individuals observed herein and in other studies (e.g. Myking *et al.* 2009), together with the significant divergence detected among and within stands (e.g. Dubreuil *et al.* 2010), highlights the importance of stochastic forces in exacerbating the vicariant effects of the different geographic barriers. These forces, along with deforestation, could be the direct result of the ecological decline that *T. baccata* has suffered during the last 4000 years in Europe (Paule *et al.* 1993).

Contrary to the previous four conifers, the low amounts of genetic diversity and the homogeneous distribution of cytoplasmic and nuclear DNA diversity in *P. halepensis* suggest a rather fast and recent southward expansion of this taxon followed by strong bottlenecks (see Grivet *et al.* 2009). Indeed, there is a lack of population differentiation between stands on opposite sides of the SG for both nuclear and chloroplast DNA markers (Tables 1, 2 and S1, Supporting information). These results are consistent with those from previous studies performed at the species-range scale with cpDNA-SSRs (Bucci *et al.* 1998; Morgante *et al.* 1998) and other markers (Schiller *et al.* 1986; Gómez *et al.* 2001). However, our IM simulations suggested an older divergence date (i.e. before the last glacial maximum) than the one proposed in these surveys, and even than the one inferred from palynological and fossil records (e.g. Nahal 1962; Schiller *et al.* 1986), which suggested that *P. halepensis* colonized the western Mediterranean region during the current interglacial.

Divergence time of populations across the SG

The inferences made with the IM model on *P. halepensis* and *P. pinaster* confirmed the opposite phylogeographic patterns observed with the cytoplasmic DNA markers and illustrated how the vicariant effects of the SG have differed across taxa. For the particular case of *P. halepensis*, the divergence time between populations on both sides of the SG was estimated between 0.28 and 0.58 Ma, while for *P. pinaster* it ranged between 1.9 and 3.7 Ma.

The late divergence of *P. halepensis* stands is backed up by paleoecological evidence, as previously discussed, and also from additional demographic models fitted on the variation of ten nuclear candidate genes (Grivet *et al.* 2009). Such models revealed that *P. halepensis* went through a strong bottleneck in the western Mediterranean just after the last glacial maximum, 25 000–18 000 yr BP, which might explain the low levels of genetic diversity currently observed in this species. However, the estimates obtained herein for the divergence time across the Strait of Gibraltar are far older than those previously inferred and probably represent more ancient colonization events. Indeed, coalescent simulation approaches, such as the one developed in Grivet *et al.* (2009), are not normally able to distinguish between patterns of polymorphism produced by a single or successive bottlenecks. Alternatively, because the accuracy of our values depends on the assumption that the IM model adequately explains the patterns observed, and in spite that no major violations were determined for *P. halepensis*, its low amounts of diversity could have led to imprecise or even overestimated parameters (Hey & Nielsen 2007). This is reflected in the large credible intervals obtained with the different simulations. However, even if these estimated dates seem inflated, they are still more

than one order of magnitude lower than those from *P. pinaster*, which suggests that, in agreement with the paleoecological records, we still can reject the hypothesis of a pre-Pleistocene disjunction for *P. halepensis*.

The estimated dates of divergence for *P. pinaster* are situated after the end of the Messinian salinity crisis, 5.5 Ma (Krijgsman *et al.* 1999; Duggen *et al.* 2003). During this period, which extended over more than 0.6 Ma (Krijgsman *et al.* 1999), the passage between the Atlantic Ocean and the Mediterranean was closed, and the SG became a land bridge that allowed free biological exchanges between Europe and Africa (e.g. Hsü 1972; Agustí *et al.* 2006). The re-opening of the strait should have thus led to the interruption of this exchange and to the gradual divergence of populations on both shores. The ecological significance of this event has been thoroughly discussed and invoked to explain the genetic breaks observed in the distribution of different Tertiary taxa in this region (Agustí *et al.* 2006; Rodríguez-Sánchez *et al.* 2008), while limited amounts of gene flow between both sides of the SG would have helped maintaining the observed genetic structure, as deduced from our simulations (Table 3).

The probable existence of a pre-Pleistocene disjunction across the SG for *P. pinaster* is further reinforced by the strong congruence observed between the nuclear DNA and cytoplasmic DNA markers (see Fig. 2 and Burban & Petit 2003) and points out to an ancient divergence of genetic lineages in this and other Tertiary species of the region as suggested by Magri *et al.* (2007). Nevertheless, additional simulations should be performed in other Mediterranean Tertiary trees to test whether the Messinian salinity crisis, or other ancient biogeographical events, can be held responsible for the divergence of populations of different taxa across the Strait of Gibraltar.

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JPJ-C is a researcher interested in the molecular ecology and evolution of forest trees, and their relationships with historical and environmental factors. DG is interested in population genetics and genomics to study the evolution, adaptation and conservation of temperate forest trees. AT is interested in the phylogeographical patterns of North-African and South-European flora. YK develops research on forest genetics and breeding of eastern Mediterranean forest trees. AIde-L's and NW's studies focus on population and conservation genetics of forest trees. GGV and SCG-M have broad interests in population genetics and genomics, phylogeography, molecular ecology and conservation genetics of Mediterranean plants.

Supporting information

Additional supporting information can be found in the online version of this article.

Table S1 Geographic location and estimates of chloroplast (nuclear for *Taxus baccata*) DNA diversity in populations of five conifer taxa distributed on both sides of the Strait of Gibraltar

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