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► To cite this version:

A. I. De Lucas, S. C. González Martínez, E. Hidalgo, F. Bravo, Myriam Heuertz. Admixture, one-source colonization or long-term persistence of maritime pine in the Castilian Plateau? [Spain]. Insights from nuclear microsatellite markers. *FOREST SYSTEMS*, 2009, 18 (1), pp.3-12. 10.5424/fs/2009181-01045 . hal-02657751

HAL Id: hal-02657751

<https://hal.inrae.fr/hal-02657751>

Submitted on 30 May 2020

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Admixture, one-source colonization or long-term persistence of maritime pine in the Castilian Plateau? Insights from nuclear microsatellite markers

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Abstract

Atlantic and Mediterranean populations of maritime pine are very different in quantitative and adaptative traits, even to be considered different subspecies. Different authors have suggested the existence of glacial refugia for this species both in Portugal and eastern Spain based on molecular markers and supported by pollen and fossil records. In addition, based on the existence of high levels of haplotypic variation in this region, the Castilian Plateau is suggested as a contact zone between Atlantic and Mediterranean gene pools of the species. This hypothesis is tested using Bayesian clustering methods and exclusion tests based on multilocus genotypes obtained by genotyping 13 populations with three highly polymorphic nuclear microsatellites. The results show that Castilian Plateau populations constitute a different gene pool by itself that can not be fully originated from the Mediterranean or the Atlantic gene pools, or from a mixture of both. Therefore, three different hypotheses have been considered as plausible causes to give rise to Castilian Plateau maritime pine populations: i) fast colonization of the region from one or more Mediterranean refugial areas, ii) existence of cryptic refugia for the species in the zone, and iii) introduction of exotic material by humans.

Key words: Glacial refugia, population structure, nuclear microsatellites, Castilian Plateau, Iberian Peninsula, maritime pine.

Resumen

¿Mezcla de orígenes, colonización desde un solo refugio glacial o persistencia del pino negral en la Meseta Castellana? Perspectivas utilizando marcadores microsatélites nucleares

Las poblaciones atlánticas y mediterráneas de *Pinus pinaster* en la Península Ibérica son muy diferentes en cuanto a caracteres cuantitativos se refiere, llegando a considerarse dos subespecies diferentes. Diversos autores, basándose en marcadores moleculares y en registros polínicos y fósiles, sugieren la existencia de refugios glaciares para la especie en Portugal y en el este de España. Por otro lado, y debido a la elevada variación haplotípica encontrada en las poblaciones de pino negral de la Meseta Castellana, se ha sugerido que esta región pueda ser una zona de contacto entre los acervos genéticos Atlántico y Mediterráneo. Para comprobar esta hipótesis se han genotipado 13 poblaciones de la especie con tres marcadores microsatélites nucleares muy polimórficos, y se han utilizando métodos de agrupamiento Bayesianos y análisis de exclusión. Los resultados obtenidos muestran que las poblaciones de la Meseta Castellana constituyen un acervo genético singular y que su origen no proviene de los acervos Atlántico o Mediterráneo, ni de una mezcla entre ambos. Por tanto, se han considerado tres hipótesis diferentes como posibles causas que han dado origen a las poblaciones de pino negral de la Meseta Castellana: i) rápida colonización de la región a partir de uno o varios refugios glaciares del este peninsular, ii) existencia de refugios crípticos para la especie en la zona, e iii) introducción antrópica de material foráneo.

Palabras clave: Refugios glaciares, estructura de poblaciones, microsatélites nucleares, Meseta Castellana, Península Ibérica, pino negral.

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Received: 09-11-08. Accepted: 22-02-09.

Introduction

During the last glacial maximum (LGM) (25,000 to 17,000 years ago), large areas of northern, central and western Europe were covered in permafrost and by ice-sheets, but the southern European peninsulas remained relatively ice-free (CGMW-ANDRA, 1999). As stated by the 'southerly refugial model', the Iberian, Italian and Balkan peninsulas in the northern part of the Mediterranean basin are considered to have been the major refugia for many animal and plant species during this cold stage (Bennet *et al.*, 1991; Hewitt, 1999). However, recent findings, both fossil and genetic, have indicated that northerly regions may also have supported cryptic refugia consisting of small and scattered populations of plants and animals that would have persisted in mild environments such as protected valleys (Bhagwat and Willis, 2008; Provan and Bennet, 2008 and references therein).

In the Iberian peninsula, genetic evidence, often supported also by fossil records, has identified glacial refugia for several forest tree species, most of them also supported by fossil records: *Alnus glutinosa* (King and Ferris, 1998; Carrión *et al.*, 2000), *Quercus* spp. (Petit *et al.*, 2002; Carrión *et al.*, 2000), *Pinus sylvestris* (Sinclair *et al.*, 1999; Willis *et al.*, 1998), *Populus nigra* (Cottrell *et al.*, 2005) or *Fraxinus excelsior* (Heuertz *et al.*, 2004; Carrión *et al.*, 2000). In particular, several studies based on different biochemical and DNA markers have been carried out in maritime or cluster pine (*Pinus pinaster* Aiton), both at wide-range (e.g. Bucci *et al.*, 2007) and local (e.g. Salvador *et al.*, 2000) scales. For the Iberian Peninsula, Baradat and Marpeau (1988), based on biochemical markers, hypothesized the presence of a maritime pine refugium in the southwest of Portugal with two different pathways of recolonization after the Würm glaciation: i) Towards the North of Portugal, Spain and France and ii) Towards the South of Spain, France and Italy. However, more recent studies of genetic variability based on chloroplast microsatellites (Vendramin *et al.*, 1998; Bucci *et al.*, 2007) and isozymes (Salvador *et al.*, 2000) rejected this hypothesis since they found low levels of genetic diversity in western Iberia. Salvador *et al.* (2000) considered the existence of glacial refugia in both southern and eastern Iberian as starting point of the recolonization process towards inland and the Atlantic region. These authors also suggested the disappearance of *P. pinaster* from Portugal during the Holocene. However, palynological evidence indicated the presence of maritime pine in central Portugal (south of Lisbon) dur-

ing the Atlantic period (>7,000 yr BP, García-Amorena *et al.*, 2007). In addition, results based on chloroplast microsatellites did not find clear population structure in Portuguese populations of *P. pinaster* (probably due to recent human activity and extensive gene flow among populations), but confirmed the existence of a differentiated gene pool in Portugal, related to a putative glacial refugium in this range (Ribeiro *et al.*, 2001 and references therein). More recently, Bucci *et al.* (2007), in the most comprehensive study published to date in maritime pine (1339 trees from 48 populations), supported the existence of glacial refugia in southeastern Spain and the Atlantic coast of Portugal, from which northward recolonization of central Spain and the northwestern range of the species might have taken place, backing up the hypotheses of Salvador *et al.* (2000) and Ribeiro *et al.* (2001).

Despite these controversial interpretations about glacial refugia and colonization pathways based on molecular markers (probably caused by the use of different markers assayed and the uneven distribution of populations sampled), the existence of glacial refugia of *Pinus pinaster* both in Portugal and eastern Spain are well documented based on pollen and fossil (charcoal) records. In Portugal, the earliest evidences of maritime pine date back to the Middle Würm 55,000-25,000 yr BP and ¹⁴C 33,000 yr BP based on palynological records and charcoal analysis, respectively, and these findings come from the lowlands of Portuguese Estremadura (north of the river Tejo) (Figueiral, 1995; Figueiral and Carcaillet, 2005 and references therein). These authors conclude that the species was able to survive the latest glaciation in sheltered areas at low altitude and close to the Atlantic coast. In the case of Spain, palaeological analysis indicates that *Pinus pinaster* survived the last glacial in eastern and southeastern locations, the oldest records stemming from Navarrés peatbog (in the province of Valencia) and from Siles lake (in the province of Jaén), dating back to ~30,000 yr BP and ~20,000 yr BP, respectively (Carrión *et al.*, 2000; Carrión, 2002). In both cases, the species seems to have retreated considerably during the LGM and the Younger Dryas event, recovering its importance during the Atlantic period (~7,000-8,000 yr BP).

Apart from the molecular marker and palaeoecological evidence reported above, the existence of two differentiated gene pools in maritime pine associated with Atlantic and Mediterranean refugial areas is evidenced by notable differences in quantitative and adaptive traits, as shown by numerous common garden experi-

ments (Alía and Moro, 1996; Alía *et al.*, 1997). These remarkable differences have conducted some authors (e.g. Barbéro *et al.*, 1998) to distinguish two different subspecies: *Pinus pinaster* subsp. *atlantica* (maritime pine) and *Pinus pinaster* subsp. *mesogeensis* (cluster pine or Mediterranean maritime pine), which have served as basis for numerous silvicultural studies (e.g. Barrio *et al.*, 2006; Rodríguez-García *et al.*, 2007 or Bravo-Oviedo *et al.*, 2008) that have a great influence on operational forestry. From a geographical point of view, these two gene pools or subspecies would have entered into contact in the Castilian Plateau, an elevated plain land in central Spain, where maritime pine forms nowadays large forests, but that was occupied by tundra-like vegetation, including cold-tolerant conifers such as Scots pine, during glacial times (Franco-Múgica *et al.*, 2001).

The objective of this study was to test whether maritime pine populations from the Castilian Plateau (i) have an admixture origin (with contributions from both Atlantic and Mediterranean gene pools), (ii) were formed by colonization from a single source (either Atlantic or Mediterranean) or (iii) have an ancient origin (through persistence in cryptic refugia). To achieve this objective, Bayesian clustering methods and exclu-

sion tests based on multilocus genotypes obtained by genotyping 13 populations (603 individuals) with three highly-informative nuclear microsatellites were used.

Materials and methods

Plant material and molecular markers

In this study, we screened 13 maritime pine populations (603 individuals) with three highly polymorphic nuclear microsatellite markers (*frpp94*, *itph4516* and *frpp91*, see Derory *et al.*, 2002). Details on DNA extraction and lab protocols can be found in Mariette *et al.* (2001) and de-Lucas (2009). Populations were classified into five groups (see Table 1): Atlantic (comprising coastal and inland Portuguese populations), Castilian Plateau (represented by two populations sampled in Cuéllar in the province of Segovia), Castilian peripheral (including populations found in mountain ranges surrounding the Castilian Plateau), Mediterranean (those from mountain ranges relatively close to the Mediterranean Sea) and marginal (populations that present notable levels of isolation or are found at the edges of the maritime pine range).

Table 1. Pairwise genetic differentiation, as estimated by F_{ST} (above diagonal), and Nei's genetic distance (below diagonal) for 13 populations of maritime pine located across a transect from the Atlantic to the Mediterranean coast; in bold are values that were higher than the average

Region/Type	Population	ID	Pairwise genetic differentiation (above diagonal) and genetic distance (below diagonal)												
			J	E	B	L	P1	P5	AV	SO2	CU2	T	LE	Z	V
Atlantic	Leiria	J		0.0305	0.0217	0.0130	0.0887	0.0697	0.0220	0.0168	0.0522	0.0592	0.0664	0.0441	0.0689
	Figueira da foz	E	0.1139		0.0377	0.0182	0.0873	0.0698	0.0434	0.0485	0.0434	0.0698	0.1173	0.0447	0.0476
	Oleiros	B	0.0827	0.1614		0.0027	0.0860	0.0497	0.0116	0.0180	0.0479	0.0410	0.0331	0.0257	0.0330
	Manteigas	L	0.0358	0.0703	0.0089		0.0487	0.0287	0.0005	0.0008	0.0211	0.0250	0.0442	0.0132	0.0440
Castilian Plateau	Cuéllar 1	P1	0.5246	0.5586	0.6156	0.3500		0.0240	0.0561	0.0592	0.0578	0.0773	0.1082	0.0418	0.1170
	Cuéllar 2	P5	0.3747	0.4091	0.2960	0.1926	0.1559		0.0380	0.0299	0.0520	0.0544	0.0662	0.0349	0.0797
Castilian peripheral	Arenas de S. P.	AV	0.0756	0.2026	0.0509	0.0051	0.4132	0.2589		0.0025	0.0328	0.0270	0.0356	0.0114	0.0523
	San Leonardo	SO2	0.0592	0.2316	0.0863	0.0070	0.4146	0.1859	0.0133		0.0298	0.0284	0.0449	0.0198	0.0474
Mediterranean	Boniches	CU2	0.2398	0.2082	0.2739	0.1357	0.4526	0.3911	0.2152	0.1803		0.0085	0.0834	0.0311	0.0290
	Olba	T	0.2954	0.3951	0.2306	0.1611	0.6113	0.3736	0.1720	0.1700	0.0571		0.0437	0.0376	0.0363
Marginal	S. del Teleno	LE	0.2728	0.6185	0.1376	0.2029	0.7537	0.3627	0.1530	0.2042	0.4859	0.2115		0.0580	0.0851
	Fuentelapeña	Z	0.2152	0.2446	0.1492	0.1028	0.3117	0.2468	0.0882	0.1305	0.2365	0.2532	0.3179		0.0554
	Quatretonda	V	0.3067	0.2092	0.1494	0.2228	0.9330	0.4949	0.2732	0.2368	0.1332	0.1780	0.4146	0.3138	

Genetic analyses of population genetic structure

Here we will focus on population genetic structure, as genetic diversity analyses have been presented elsewhere (see Derory *et al.*, 2002; González-Martínez *et al.*, 2004; de-Lucas, 2009). First, pairwise genetic differentiation (as estimated by F -statistics, Weir and Cockerham, 1984) and Nei's genetic distance (Nei, 1978) were computed using SPAGeDi software (Hardy and Vekemans, 2002). Then two different approaches were used for spatial genetic analysis, as follows: The first approach consisted in a Bayesian clustering method that does not rely on previous definition of populations but on classification of individuals in a predefined number of gene pools, K . This method, implemented in the STRUCTURE software (Pritchard *et al.*, 2000), has been extensively used to determine population genetic structure patterns in forest trees (e.g. Heuertz *et al.*, 2004; Born *et al.*, 2008). Genetic drift can substantially modify allele frequencies in marginal populations blurring general genetic structure patterns. Thus, these analyses were based only on the ten core populations (three marginal populations removed). For the STRUCTURE method, we used the admixture model (allowing for individuals with mixed gene pool composition) and correlated allele frequencies, performing ten independent runs for each K value ranging from 1 to 5 with a burn-in period of 50,000 steps followed by 500,000 MCMC replicates. The optimal number of gene pools, K , was selected using the guidelines provided by the authors and the method described in Evanno *et al.* (2005). Finally, the ten independent runs from each simulation were averaged using algorithms found in CLUMP (Cluster Matching and Permutation Program, Jakobsson and Rosenberg, 2007) and represented in bar graphs using DISTRUCT (Rosenberg, 2004). As bar graph representation does not take into account spatial position of populations, we also constructed interpolated maps for each gene pool using R code from Olivier François (available at http://www-timc.imag.fr/Olivier.Francois/admix_display.html).

Because of founder effects during colonization and genetic drift, sister populations can share most of their alleles while harbouring very different allele frequencies, thus showing significant genetic differentiation. Different origins of recently expanded populations would only be proved, then, if not only differences in allele frequencies are found but also different genetic variants. To test whether Castilian Plateau populations originated from Mediterranean or Atlantic gene pools, we used exclusion tests to identify Castilian Plateau individuals with multi-

locus genotypes that have low probability of assignment to the Mediterranean or Atlantic gene pool of the species, given current allele frequencies. Three different statistical criteria were used, as implemented in GENECLASS2 (Piry *et al.*, 2004): Cavalli-Sforza and Edwards' (1967) Chord distance, the likelihood frequencies-based method of Paetkau *et al.*, (1995) extended by Piry *et al.* (2004), and a Bayesian method derived by Baudouin and Lebrun (2000) from Rannala and Mountain (1997). Monte Carlo resampling of 10,000 individuals was used to compute the probability of the multilocus genotype of each individual from the Castilian Plateau to be found in a given reference population, Mediterranean or Atlantic, following Paetkau *et al.*, (2004).

Finally, admixture scores for single trees (obtained from STRUCTURE analyses described above) were used to compute the distribution of individuals from each geographical region (Atlantic, Mediterranean and Castilian Plateau) with a significant component of the Atlantic or Mediterranean gene pools.

Results

Pairwise genetic differentiation (F_{ST}) and genetic distance estimates showed that the two coastal Portuguese maritime pine populations (Leiria and Figueira da Foz) were well differentiated from Mediterranean populations (average F_{ST} of 0.059) and more similar to inland Portuguese populations (average F_{ST} of 0.023, Table 1). Surprisingly, populations from the Castilian Plateau also showed high differentiation from most other populations (average F_{ST} and Nei's genetic distance of 0.064 and 0.433, respectively), notably from Mediterranean and from Atlantic ones. In addition, the marginal populations of Sierra del Teleno (northwestern Spain) and Quatretonda (coastal Mediterranean), while relatively similar to nearby populations, showed remarkable genetic singularity. This might be a consequence of recurrent bottlenecks caused by frequent forest fires and/or isolation, both enhancing genetic drift.

Both non-spatial (Figure 1, top) and spatial (Figure 1, bottom) representation of Bayesian clustering results showed clearly the existence of three gene pools, centred around the Portuguese Atlantic coast, the Castilian Plateau and the Mediterranean mountains, and the existence of substantial gene flow across them. Contrarily to what is currently believed, the Castilian Plateau gene pool (represented by populations P1 and P5, both in Cuéllar, Segovia) did not fully originate from the Mediterranean

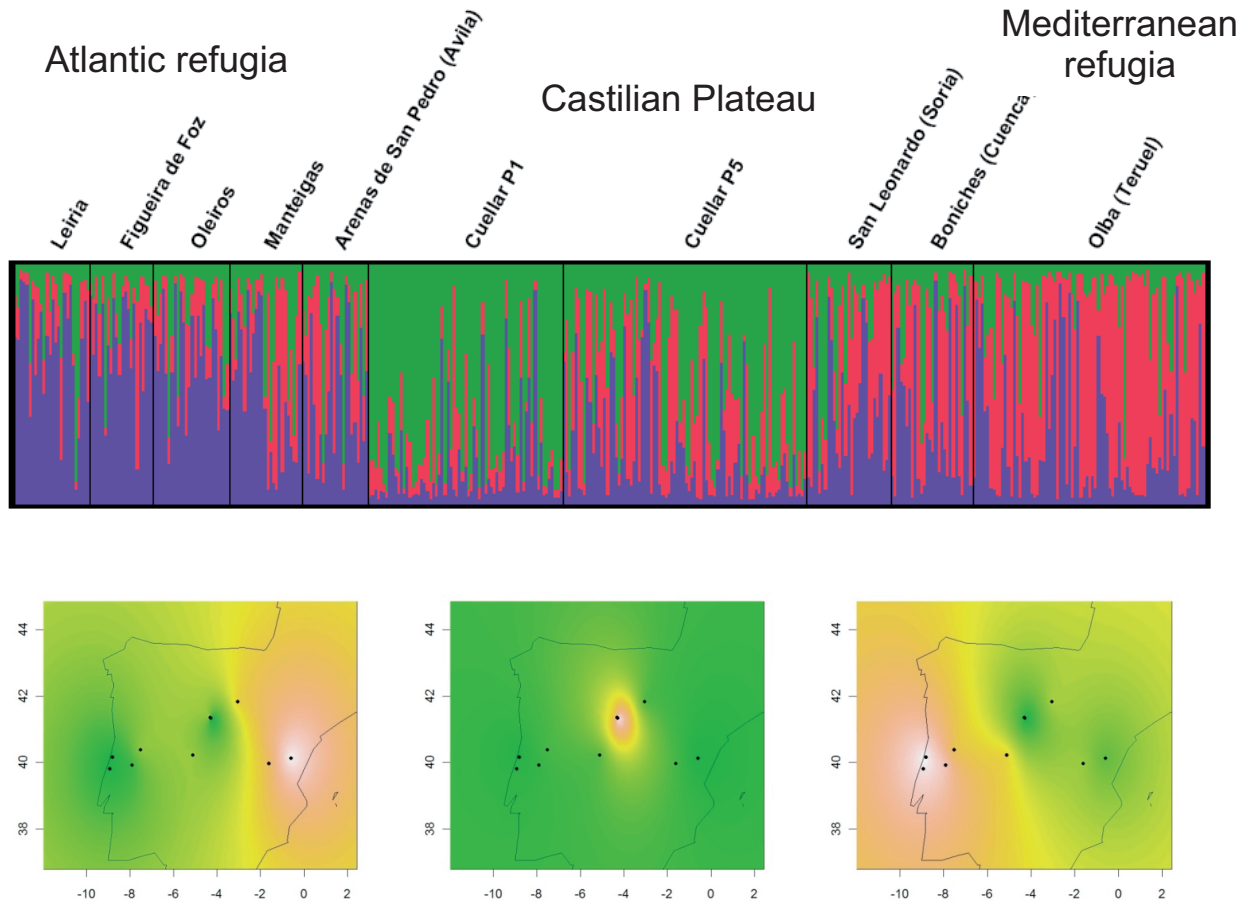


Figure 1. Bar plot (non-spatial; top) and spatial representation (bottom) of Bayesian clustering for $K=3$, the optimal number of gene pools found in maritime pine studied populations. Axes on the maps represent latitude and longitude degrees.

or the Atlantic gene pools, or even a mixture of both, but constitutes a different gene pool by itself. This conclusion was supported also by assignment tests, as there were ~5-39% of individuals from this range (depending on the method used) that could not possibly be generated from allele frequencies of current Atlantic or Mediterranean gene pools (Table 2). Finally, detailed analyses of individual admixture coefficients showed that contributions of the Mediterranean refugia to the Castilian Plateau gene pool were higher than those potentially coming from the Atlantic range (Figure 2), supporting the view of closer genetic relationship between Castilian Plateau and Mediterranean populations.

Discussion

Little palaeobotanical information is available from the Castilian Plateau due to the lack of paleopollen

deposits in the Iberian plain lands and to the high degree of human disturbance in these areas (Franco-Múgica *et al.*, 2005). Although the oldest record for *Pinus pinaster* (based on charcoal macrofossils) dated to the end of the Holocene (1,400 14^C yr BP, Alcalde *et al.*, 2004), the stability of pine forest (albeit probably belonging to cold-tolerant Scots or Black pines) in this region through the whole Holocene period (9,000-10,000 yr BP) has been confirmed by pollen and macrofossils (García-Antón *et al.*, 1995; Franco-Múgica *et al.*, 2001; 2005).

The results obtained from both the Bayesian clustering method and the exclusion tests performed in this study allowed us to reject (at least partially, see below) the hypothesis (suggested by Bucci *et al.*, 2007, among others) of population admixture from Atlantic and Mediterranean gene pools as unique explanation to give rise to Castilian plateau populations. ‘Transient’ or ‘contact zones’ of genetic diversity must entirely be

Table 2. Percentage of individuals from the Castilian Plateau that cannot be generated (at probability 0.01 and 0.05) from allele frequencies found in Atlantic and Mediterranean gene pools of maritime pine or a mixture of both. See details on assignment methods in the text

Assignment method	Atlantic gene pool		Mediterranean gene pool		Mixture of Atlantic and Mediterranean gene pools	
	0.01	0.05	0.01	0.05	0.01	0.05
Distance	25.17	49.67	15.23	35.76	7.95	21.85
Frequency	56.95	76.82	23.84	49.01	13.91	39.07
Bayesian	19.87	46.36	6.62	23.84	0.00	4.64

composed of the genetic contributions from the source refugial populations or their descendents (Provan and Bennet, 2008) but, in our study, an important proportion of individuals from the populations in Cuéllar could not

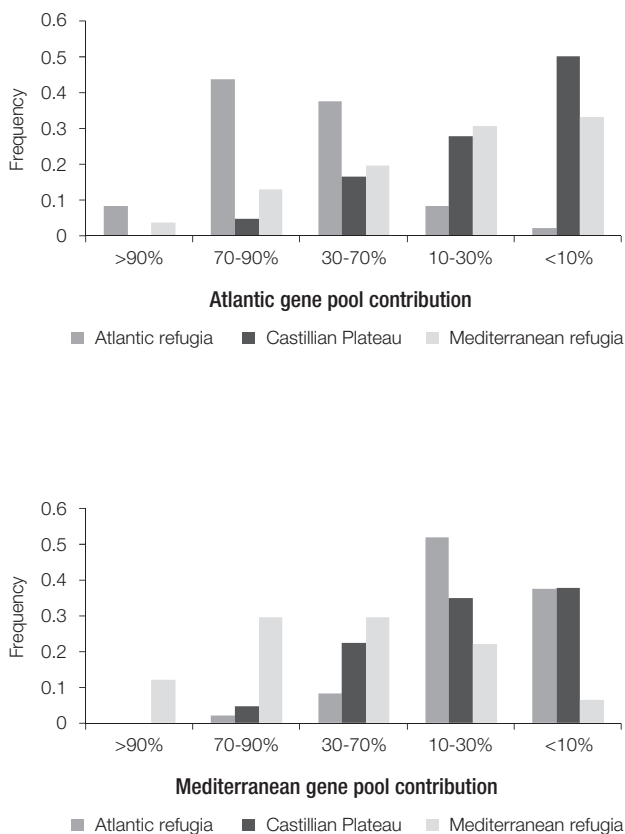


Figure 2. Contributions of Atlantic (top) and Mediterranean (bottom) gene pools to Atlantic, Castilian Plateau and Mediterranean populations based on admixture coefficients of individual trees. The x-axis indicates the percentage of each individual that is assigned to the corresponding gene pool and the y-axis the relative frequency in the different population groups.

be generated from current Atlantic or Mediterranean gene pools. This finding based on nuclear microsatellite allelic frequencies is consistent with inferences drawn from chloroplast data, since a considerable number of private haplotypes has been found in this range (see Table S2 in Bucci *et al.*, 2007 Supplementary Material). Then, three different hypotheses (that do not exclude each other) can be considered to explain the genetic singularity of Castilian Plateau populations:

Hypothesis I: Fast colonization from one or more Mediterranean refugial areas.

Castilian Plateau populations could have diverged quite fast from their source populations, due to colonization bottlenecks (de-Lucas, 2009) and subsequent population expansion. Under this scenario, novel microsatellite alleles generated in the expanding population (because of the high microsatellite mutation rates) would have a higher probability of persistence (Nordborg, 2001). Considering the close genetic relationships between Castilian Plateau and Mediterranean populations and the absence of clear signatures of admixture with Portuguese populations, it is more probable that Castilian Plateau maritime pine populations were originated in eastern Spain. Genetic diversity of Portuguese populations of maritime pine are very low (see Salvador *et al.*, 2000 for isozymes; Bucci *et al.*, 2007 for cpSSRs) whereas high levels of genetic diversity have been found in eastern Spain and the Castilian Plateau, indicating a plausible expansion in the East-West direction for this species. These views are supported by the fact that nowadays Mediterranean Iberia shows higher tree species richness and bigger refugium sizes for numerous tree species than other zones of Europe (Petit *et al.*, 2005).

Hypothesis II: Cryptic refugia in the Castilian Plateau

Alternatively, despite the lack of fossil data confirming the presence of the species earlier than the end of the

Holocene, cryptic refugia for maritime pine might have existed in the Castilian Plateau. Cryptic refugia are suggested to have been located in small micro-environmentally favourable sites where generalist species (i.e. adapted to a wide range of habitat types) may have been able to persist in isolated pockets (Willis and van Andel, 2004). The ability of maritime pine to grow in a great variety of different edaphic and climatic conditions could have allowed its survival during the LGM in inland sheltered areas as well as in refugia near to the Atlantic and Mediterranean coasts. Besides, Bhagwat and Willis (2008) suggest that during the LGM, many species in southern areas were sheltered on mountains slopes and in valley bottoms which may have been corridors for species spread. The valley of the Cega River, where Cuéllar is located, could have acted as a corridor and a refugium for a vicariant gene pool of maritime pine and possibly other species. Nowadays, this valley shelters relict populations of Eurosiberian trees such as *Pinus sylvestris*, *Pinus nigra* and *Populus tremula* that are thought to have been widespread in the high plains during glacial times (see Robledo-Arnuncio *et al.*, 2005) and are now mainly found in the mountains surrounding the Castilian Plateau. Interestingly, Cuéllar populations of *P. pinaster* showed notable genetic differentiation from nearby mountain populations such as Arenas de San Pedro (Ávila) and San Leonardo (Soria), highlighting the importance of mountain chains as barriers to gene flow.

Hypothesis III: Introduction of exotic material by humans

Finally, another possible explanation to the genetic singularity of the Castilian Plateau populations of maritime pine is the introduction by humans of exotic material, as this region has been subjected to forest exploitation since long. However, as far as we know, the silviculture applied in these maritime pine populations (at least since the first regulated management plans, dating back late XIX century) has been based on natural regeneration following a shelterwood system adapted to resin tapping. Moreover, this region has traditionally been a source of reproductive material for plantations in other ranges, given the facility of seed collection and management in the relatively low density and accessible forests of the Castilian Plateau (as an example, about 100 000 kg of seed from this region were used annually in plantations in the 1950s-60s, one of the periods of higher effort in plantation establishment in Spain, Gil *et al.*, 1990). Hence, the importation of foreign material for seedlings or plantations in this area in modern times

seems highly improbable. Nevertheless, the first historic reference to this species in the Castilian Plateau come from a document from King Alfonso VIII dated in 1210 (Gil, 2008) and the first silvicultural regulations dated back the XVI century (Corral, 1988), thus earlier introductions of exotic material, although improbable, cannot be fully ruled out.

As a conclusion, in addition to clarifying historical questions related to the distribution and use of forest tree species, the identification of glacial refugia and the knowledge of how genetic diversity changes over time and space can help us understand and predict the future distributions of species in a changing climate. Moreover, this information is useful to determine conservation units and adaptive management strategies to cope with future environmental and management conditions.

Acknowledgements

The authors wish to thank C. Ordóñez and J.C. Santana, for field and lab assistance, respectively and to M.M. Ribeiro for providing DNA from Portuguese populations of maritime pine. A.I. de Lucas was supported by a PhD scholarship from the ‘Universidad de Valladolid’. The work was funded by CICYT AGL2007-65795-C02-01 and REPROFOR AGL2005-07440-C02-01/FOR projects. M. Heuertz is a postdoctoral researcher of the National Fund for Scientific Research of Belgium (FRS-FNRS) and acknowledges an FNRS-funded scientific visit to CIFOR-INIA.

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