

# **Molecular and Quantitative Genetics of Stone Pine (*Pinus pinea* L.)**

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## **Abstract**

The Mediterranean stone pine is currently on its way to domestication. Its genuine Mediterranean pine nuts are among the most expensive nuts in the world because they are mainly wild-collected from pine forests and woodlands. Despite the wide current distribution of stone pine over the whole Mediterranean biome, old-growth forests are scarce, often associated locally to dynamics on loose sands, coastal dunes or former estuary marshes. The species has been found to be genetically depauperate, putatively due to a population bottleneck in a local refugium during the Last Glacial Maximum confirmed in southern Iberia, and a possibly anthropic range expansion during Holocene. Only recently, cone harvesting and processing mechanisation have allowed for profitable pine nut production from orchard plantations. In Spain and Portugal, first elite clones have been registered for their use as grafted orchard crop.

**Keywords:** Mediterranean stone pine, pine nuts, genetic depletion, domestication

## 1 Botany, origin and relevance of the species

Edible nuts from gymnosperms are usually harvested from wild forests rather than constituting an agronomic crop. Rather uniquely, the Mediterranean stone pine, *Pinus pinea* L., is currently on its way to domestication for the production of pine nuts, one of the most expensive nuts in the world, known since Antiquity as gourmet ingredient of traditional Mediterranean dishes (Thirgood 1981; Agrimi and Ciancio 1993; Prada et al. 1997; Gil 1999).

The genus *Pinus* comprises over 100 species, including some major forest trees of the northern hemisphere providing relevant ecosystem services, notably timber. Among about twenty pine species with large, nut-like edible seed kernels, Mediterranean stone pine is known as the first one to have nourished humans: its pine nuts were already consumed by Neanderthals in the Middle Palaeolithic, before the last glacial maximum in Europe (Weyrich et al. 2017). The small edible, ivory-white kernels are obtained by shelling pine nuts after extraction from cones. The seed coat is thick and woody, pine nuts yielding only 25% kernel in weight, *i.e.* in average 0.15 g out of 0.6 g per unit, respectively. Mean cone weight is 250-350 g when harvested in winter (relative humidity 50%), yielding 15-20% seeds and only 3.5-4% kernels in weight (Fig. 1). Cone picking has traditionally been done by tree climbers using long, hooked poles, although during the last decades, the use of specific mechanical tree shakers has been generalised where tractors can access tree stems (Mutke et al. 2012).



**Fig. 1** Mediterranean stone pine cone, green harvested and sundried opened, nuts in shell and kernels

Mediterranean stone pine is an evergreen conifer tree that can reach 25-35 m height, exceeding 1 m stem diameter in monumental specimens, though in its natural, often resource-limited forest habitats, even dominant trees are more likely to culminate at 12–20 m height and 40–50 cm diameters at the end of the silvicultural rotation (80–120 years). Open-grown stone pines present a characteristic polyarchic (candelabra-like) crown, due to the low apical dominance at each branching point. The resulting crown shape is quite singular for a conifer, first spherical, later characteristically umbrella-like when lower branches are dead or pruned (Fig. 2). In absence of pruning, stem form is poor and volume growth is slow, the timber not being appreciated. Most of the needle mass of the canopy is situated in an outward layer at the upper crown surface formed by many co-dominants, upwards-growing shoots, which bear also the female cones (Lanner 1989; Fady et al. 2004; Mutke et al. 2005c, 2012).



**Fig. 2** Young stone pines (left) and open-grown tree, overtopping oak woodlands (right)

Among the seven species of the taxonomic group of Mediterranean pines (subgenus and section *Pinus*, subsection *Pinaster*), stone pine is the only one with seeds larger than 15 mm, reaching up to 20 mm, and ripening only two and a half year after pollination, a year later than in most other pine species (Klaus 1989; Montero et al. 2004). Geographically, stone pine is native Mediterranean, sparsely spread from the Portuguese Atlantic coast to the shores of the Black Sea and the slopes of Mount Lebanon, from sea level up to 1,000 meters, occasionally to 1,600 m (Quézel and Médail 2003). Within the last hundred years, its forest area has more than doubled to 0.75 million hectares as results of forestation, for soil protection, ecosystem restoration and sustainable production of both timber and pine nuts. Additionally, during the last 30 years private land owners have invested more than 20 million euros in new stone pine plantations aimed for cone production, motivated by high prices paid for pine nuts. Portugal and Turkey sum more than 0.25 million hectares of new plantations, often on abandoned farmland that yield higher cone crops than forest land (Calado 2012; Mutke et al. 2012; Kilci et al. 2014).

The actual geographic origin of Mediterranean stone pine has long been discussed. During the Antiquity, the species was already present in most Mediterranean countries. Despite its ubiquity as an old cultural element of Mediterranean landscape, the ecological or phytosociological role of stone pine is only secondary in mixed Mediterranean woodlands. Rather than forming its own, closed canopy layer, it grows as scattered trees or small groves overtopping the dominant lower oak or bush layer (Fig. 2), quite similarly to *Pinus sabiniana* spreading over blue oak woodlands and chaparrals in California (Lanner 1999). Pure or dominant stone pine forests are locally limited, often associated to poor sites such as coastal dunes, loose or shifting sands, on sandpits or in former marshes or estuaries, or shallow soils over sandstone, gneiss or granite bedrocks, sometimes on limestone. In absence of forest management, on the long run they will often be replaced by succession of mixed stands (Blanco et al. 1997; Prada et al. 1997; Gil 1999; Soto et al. 2010; Ganatsas 2007; Mutke 2013).

In some parts of its range (e.g. the southern Iberian Peninsula), Mediterranean stone pine was an integral element of the open woodland and dry steppe habitat dynamics that prevailed before the Last Glacial Maximum (LGM, 27-18 kyr BP). The oldest archaeological evidences for human pine nut hoarding and consumption date from 49 kyr BP (Oakley 1958, cit. in Gil

1999), putatively even 150 kyr BP (Cortés-Sánchez et al. 2011), corresponding to Mousterian Neanderthals dwelling in caves and rock shelters along the Costa del Sol (Málaga, Spain). Pine nuts are ideal staple food, easy-to-store because the hard shell conserves dry-stored nuts for years. Kernels are highly nutritive, with 50% fats and 35% proteins, as much as raw soybeans. The northern shore of the Alboran Sea is actually considered to have been the refugium of the last ever Neanderthal population surviving until 28 kyr BP. Also in later periods, i.e. Upper Palaeolithic (Gravettian, 29-24 kyr BP), Epipaleolithic (Solutrean, 23-19 kyr BP; Magdalenian 17-12 kyr BP) and early Neolithic (7,500 yr BP), the continued presence of charred pine cone and seed fragments in caves and shelters such as Gorham's Cave (Gibraltar), Bajondillo and Nerja (Málaga, Spain) has confirmed that *P. pinea* was widely gathered by man along this littoral fringe (Badal 1998, 2001; Jordá Pardo et al. 2003; Finlayson et al. 2006, 2008; Córtez-Sánchez et al. 2008; Carrión et al. 2008; Stringer et al. 2008).

High-resolution palaeoclimate scenarios coincide to pinpoint the same coastal area as the most likely, if not the only, glacial refugium for Mediterranean stone pine on the Iberian Peninsula during the LGM (Benito-Garzón et al. 2007). Later, during the Neolithic, Copper, Bronze and Iron Ages, the presence of stone pine also continued in this coastal region, where Phoenicians would establish the settlements of *Malaka* (Málaga, Spain) and *Calpe* (Gibraltar). By that time, the bioclimatic niche suitable for stone pine (Benito-Garzón et al. 2007) would have already been expanded to all main areas in the Iberian Peninsula with presence of spontaneous stone pine forests today (Blanco et al. 1997; Prada et al. 1997). Nevertheless, the active role of men spreading useful tree species should not be neglected (Aranbarri et al. 2015; Levis et al. 2017). Protohistoric presence of stone pine has been confirmed by archaeological evidences in the sandy estuaries of rivers like Tagus and Sado near the old Phoenician colonies *Keition* and *Bevipo* (Alcácer do Sal, Portugal), or Odiel, Tinto, Guadalete and Guadalquivir in the Gulf of Cadiz, territories of the ancient local *Tartessian* culture. When the Phoenician founded *Onoba* (Huelva) and *Gadir* (Cádiz, Spain), Mediterranean stone pine constituted already the dominant forest type in the sandy coastal plains. In 8<sup>th</sup> century BCE, pine nuts were being object of trade when Mycenaean and Phoenician goods started to make their way into Iberia (Gil 1999; Martínez et al. 2003; Martínez and Montero 2004). But even in the early 3<sup>rd</sup> millennium BCE, long-distance trade had existed already in the area, confirmed for archaeological sites from megalithic SW Iberia by items such as amber, ostrich eggs, and even ivory not only from African, but also from Asian elephants (Fig. 3; García Sanjuan et al. 2013). In the same epoch, Maritime Bell-Beaker ceramics were spreading from Western Iberia all over Europe, following Atlantic coasts and river valleys (Cunliffe 2010).



**Fig. 3** Ivory vessel from Copper Age, carved in relief of four-sided pyramids, which might have been inspired in a pine cone (Photograph: M.A. Blanco de la Rubia. Reproduction: courtesy of Research Group ATLAS (HUM-649), University of Sevilla)

The presence of stone pine has been confirmed also in other, later Iberian archaeological sites (Fig. 4), such as the Catalonian littoral near the Hellenic *Emporion* (Ampurias, Spain) or in the central Duero basin inhabited during Iron Age by the Celtiberian *Vaccaeii*, where a region of several hundred square kilometres of inland sand deposits between Valladolid and Segovia has preserved its natural cover of extended pine forests with *Pinus pinaster*, *P. pinea*, and relicts of *P. silvestris* and *P. nigra*, called *Tierra de Pinares* (“Land of pinewoods”) (Uzquiano 1995; Gil 1999; Franco-Múgica et al. 2005; García-Amorena et al. 2007; García-Antón et al. 2011; Morales-Molino et al. 2011).

It remains unclear, however, if any stone pine population survived glaciations in other refugia than southernmost Iberia. Elsewhere in the Mediterranean basin, anthracological evidence for its presence during the LGM and before the Holocene is absent, except one report by one author for two sites in southern France dated in 22 and 18 kyr BCE, though in a dubious floristic context with *Pinus sylvestris* and *Sorbus domestica* (Bazile-Robert 1981). In the late Neolithic (2,500 BCE) and Bronze Age (2,500-1,200 BCE), stone pine might have been already present in the Crau, an open Mediterranean steppe southeast of Arles (Provence, France) shaped by agro-pastoral practices since the Neolithic. However, the corresponding charcoal remains, smaller than 2 mm, did not allow distinguishing between *Pinus pinea* and *P. halepensis*, the latter being definitively native (Henry et al. 2010).



**Fig. 4** Archaeological presence of *Pinus pinea* before the Roman Empire: 1 NW Portugal (Bronze Age to Roman, Figueiral 1993); 2 Ponta da Passadeira (Neolithic, Carrión Marco 2005); 3 Vila Nova de S. Pedro (Neolithic, Alfonso do Poço 1954); 4 Zambujal, (Eneolithic, Hopf 1981); 5 Vale Pincel (Neolithic, Carrión Marco 2005); 6 Pontes de Marchil (Bronze Age, Pinto da Silva 1988); 7 Gorham's and Vanguard Caves (Middle/Upper Palaeolithic, Metcalf 1958, Finlayson 1999; Gale and Carruthers 2000); 8 La Falguera (Mesolithic-Neolithic-Bronze, Carrión Marco 2002); 9 Cova de l'Or (Neolithic, Vernet et al. 1987); 10 La Fonteta Ràquia (3<sup>rd</sup> century BCE, Jardón et al. 2009); 11 Lloma del Betxi (Neolithic, Grau 1991); 12 Fuente Álamo (Bronze Age, Schoch and Schweingruber 1982) 13 Cueva de Nerja (Upper Paleolithic to Neolithic, Badal 1998); 14 El Cigarralejo (5<sup>th</sup>-4<sup>th</sup> c. BCE, Cuadrado 1987, Rivera-Núñez and Obón 2005); 15 El Amarrejo ( 4<sup>th</sup>-2<sup>nd</sup> c. BCE, Broncano 1989); 16 Cabezo del tio Pio (4<sup>th</sup> c. BCE, Rivera-Núñez and Obón 1990); 17 Casa del Monte (4<sup>th</sup> c. BCE; Badal inéd.); 18 Castillo de Doña Blanca (Tartessian Bronze age, Chamorro 1994, Ruiz et al. 1995); 19 Hoyo de la Mina (Neolithic, Uzquiano 2002); 20 Huelva and Punta Umbía (7<sup>th</sup> c. BCE, Sánchez Hernando 2005); 21 Duero Basin (Iron Age, Uzquiano 1995, Rubiales et al. 2011, Hernandez et al. 2011); 22 Maures massif, Durance valley and Crau (Late Würm, Bazile- Robert 1981; 4<sup>th</sup> c. BCE, Henry et al. 2010); 23 Pompeii (Roman, Robinson 2002); 24 Veli Brijun (Roman, Sostaric et al. 2001). Reproduction: courtesy of J.M Rubiales, Technical University of Madrid; references in Rubiales et al. (2011)

From 2<sup>nd</sup> millennium BCE onward, and especially during Classic Antiquity, stone pine items have been present in archaeological sites all over its present geographic range, including the Levant. In Phoenician and Roman times, stone pine was a well-known tree, esteemed and often planted as ornamental (Feinbrun 1959; Thirgood 1981; Martínez and Montero 2004). Although e.g. in Palestine, its introduction seems to have occurred as ornamental only in Hellenistic times (Kislev 1988), stone pine cones have still been found as burial gift in the 12<sup>th</sup> Egyptian dynasty already (19<sup>th</sup> century BCE; Schweinfurth 1884). Cones and pine nuts were traded throughout the whole Imperium, from Syria up to Germania and Britannia, as gourmet food, and for religious uses associated to the cult of the mother goddess Cybele. During the modern era, it was widely used in forestation for soil protection, especially on dunes along Mediterranean coasts and in other similar climate zones in the world. During the troubled re-

ligious persecution times of the 17<sup>th</sup> century, stone pine is said to have been planted as a welcoming sign by Protestants in western France (Feinbrun 1959; Agrimi and Ciancio 1993; Prada et al. 1997; Gil 1999; Scarascia-Mugnozza et al. 2000; Fady et al. 2004; Konstantinidis and Tsiourlis 2011; Yilmaz et al. 2013; Loewe et al. 2012).

Definitively, the timeframe for palaeological references for stone pine, except in the southern coastal Iberia, falls plainly within the Holocene, when long-distance seafaring, trade and transfer of plant species have been documented at east since the spread of Neolithic Cardium Pottery Culture (Fugazzolla Delpino and Mineo 1999; Manen et al. 2007; Linstädter et al. 2012, 2016; Zilhão 2014). In consequence, in absence of further evidence, local pre-historic autochthony of the species is not easy to establish. Moreover, for many historic stone pine forests considered emblematic today, the current stands have clearly been established by man, in some cases only in the late 19<sup>th</sup> or even in the 20<sup>th</sup> century, for instance along the Ligurian and Tyrrhenian littoral or on the slopes of Mount Somma/Vesuvius in Italy, the Strophylia forest west of Patras in Greece, or the Matn and Jezzine areas on the slopes of Mount Lebanon. The single-cohort structure of many of these pine forests still witnesses their origin from planting or sowing, similar to 20<sup>th</sup> century protective forestations, *e.g.* in the Maghreb especially in northern Tunisia (George 1934; Ganatsas 2007; Ganatsas and Thanasis 2010; Mutke 2013).

Approximate current stone pine area is 490,000 hectares in Spain (Alía et al. 2009), 195,000 ha in Turkey (Kilci 2014; Can 2016), 175,000 ha in Portugal (Eira et al. 2010), 46,000 ha in Italy (iStat 2011), 35,000 ha in Tunisia (Ammari et al. 2011), 13,500 in France, 12,700 ha in Lebanon (Stephan 2010; Hamade 2016), and minor areas in Morocco, Greece, Syria, Israel, Croatia or Albania (Mutke 2013). It is noteworthy that due to the presence of stone pine in mixed forests, range area estimates will differ depending on whether only stone-pine dominated stands are computed, or any forest patch with presence of the species is included; for instance, the latter sum nearly 760,000 ha in Spain (Spanish National Forest Inventory, Alía et al. 2009).

The main objective of many stone pine forests is protection rather than production, and their cones are not always collected. This explains why in spite of huge differences in their total stone pine areas, respective annual commercial crops of the main producing countries, Spain, Portugal, Turkey and Lebanon, are quite similar in magnitude, yielding each around 4,000-5,000 t of pine nut in shell, Italy producing only about 1,000 t. Moreover, the actual annual production of each country varies among years from one-half to five times the average yield, owing to masting synchronising regional crop fluctuations mainly by weather cues (Mutke et al. 2005a; Calama et al. 2016). In the whole Mediterranean region, about 0.96 million hectares of stone pine (including recent plantations) yield 16,000-20,000 metric tons of pine nuts annually, *i.e.* 4,000-5,000 tons of shelled kernels (FAO 2010; INC 2012). During the 21<sup>st</sup> century, the increasing frequency of drought events in the Mediterranean has been reducing per-hectare yields (Mutke et al. 2005a; Calama et al. 2008, 2011, 2016). During the last few years, pine nut production has decreased drastically due to the invasion of an exotic seed pest, *Leptoglossus occidentalis*. Predation from this seed bug can make seeds or even whole cones to abort (Bracalini et al. 2013; Lesieur et al. 2014; Farinha et al. 2017).

Despite these hazards, the global pine nut production is expected to increase in the near future, once the aforementioned new plantations in Portugal and Turkey reach their full po-

tential. With more than 250,000 hectares planted since 1990, stone pine has multiplied nearly fourfold its historic area in both countries, about 50,000 ha in Portugal (DGF 1985) and 40,000 ha in Turkey (Acar 1995). Already today, they have relegated Spain from its former role as main cone producer, though part of Portuguese cones are still processed by Spanish and Italian firms (Calado 2012).

Despite the long cultural history of Mediterranean stone pine and its economic relevance, the first steps towards domestication as agronomic nut crop has only been taken during the last decades. This transition has been triggered by the decrease of cone production in natural forests where cones have been traditionally harvested. Causes include climate change, pests and priority shifts in forest ecosystem management (e.g. urban forestry).

Mediterranean pine nuts are today among the most expensive nuts in the world, highly esteemed as gourmet and health food, rich in proteins and unsaturated fatty acids, dietary minerals (phosphorus, iron, zinc, magnesium), vitamin B1 (thiamine), B2 (riboflavin) and E (tocopherols), phytosterols and polyphenols (Nasri et al. 2005, 2007, 2009; Evaristo et al. 2010; Salas-Salvado et al. 2011; Ruiz-Aceituno et al. 2012). Their high price is an opportunity as alternative crop on rain-fed farmland in Mediterranean climates. Stone pine performs well on poor soils even with reduced cultural practices, and it resists well climate adversities such as droughts and extreme or late frosts. Mechanic harvesting and modern automatised cone processing facilities have strongly lowered labour costs (Mutke et al. 2000, 2007a, 2012; Loewe and Delard 2012).

No cultivars or cultivated varieties has been defined for stone pine yet, but recently, several elite clones and a clone mixture, selected for outstanding cone production, have been registered in Spain and Portugal for their grafted use in agroforestry systems or orchards, as discussed in the third section of the chapter (Guadaño and Mutke 2016).

## 2 Molecular diversity

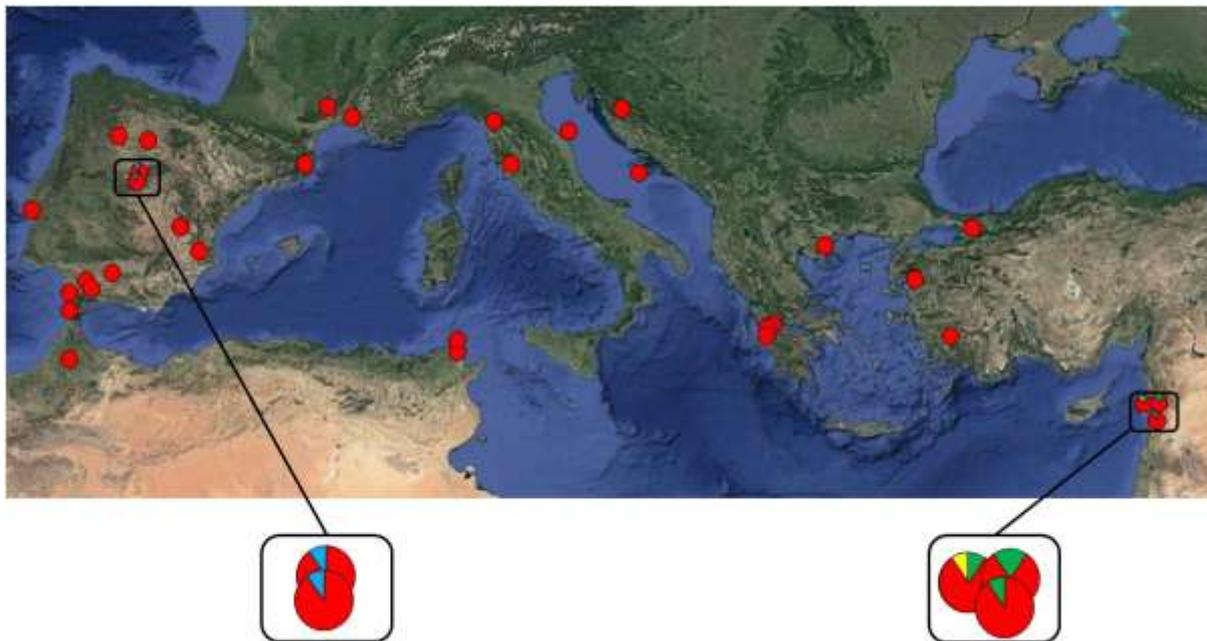
Taxonomically, many authors regarded *P. pinea* as an enigmatic and isolated species (Mirov 1967; Klaus 1989). This species belongs to the so-called Mediterranean pines, which represent a greatly heterogeneous assembly, ranging from shore and island to mountain pines (Fady 2012). They diversified ca. 10 Myr ago, and display distinct biogeographic and demographic histories. Recently, evolution patterns in the Mediterranean pines have been explored by means of a common set of low-copy loci (Grivet et al. 2013). The phylogenetic position of *P. pinea* was the most difficult to assess, but nevertheless the species was placed in the same cluster as *P. canariensis* and *P. roxburghii*, but not far from *P. pinaster*.

Stone pine is characterized by low genetic variation as discussed for different genetic markers in Fallour et al. (1997), Vendramin et al. (2008) and Pinzauti et al. (2012) and by a high degree of phenotypic plasticity (Mutke et al. 2005b, 2010, 2013; Chambel et al. 2007; Carrasquinho and Gonçalves 2013). The forest geneticists have long been confused about this genetically depauperate but widespread species. The absence of genetic variation is regarded negatively, and thus it often remains unreported (Amos and Balmford 2001). Such condition makes it difficult to use the common analytical methods for population genetic studies (Soltis et al. 1992). But most important, the existence of species lacking of genetic variation ques-

tions the common belief that genetic diversity is essential for conservation (Lehman 1998). The absence of genetic variation has been associated with prolonged bottlenecks (O'Brien et al. 1985), and forest trees such as stone pine represent an excellent chance to test such hypotheses (Petit and Hampe 2006).

The genetic diversity of *P. pinea* was first investigated by Fallour et al. (1997) using isozymes, which are genetic markers exhibiting Mendelian inheritance, codominant expression and absence of pleiotropic and epistatic interactions. Seed tissues from 10 populations of *P. pinea* from the northern Mediterranean were assayed with a total of 37 enzyme systems, and only one locus resulted to be polymorphic (with just two alleles). This study put in evidence that the values of genetic diversity in *P. pinea* were an order of magnitude lower than the mean value observed in other species of the genus *Pinus*, and much lower than Mediterranean pines with well-known low diversity such as *P. halepensis* (Fady 2005).

A research, representing to date the most complete survey of molecular diversity in this species, used paternally-inherited chloroplast microsatellites to further analyse the genetic variation and possible phylogenetic origin of *P. pinea* (Vendramin et al. 2008). Chloroplast microsatellites have proven useful to study the geographic distribution of plant genetic diversity (Powell et al. 1995; Vendramin et al. 1999, 2000). Samples were collected from 34 populations distributed across the full range of *P. pinea* (Fig. 5).



**Fig. 5** Chloroplast DNA variation analysed in *P. pinea* populations using 13 microsatellites. Only four haplotypes were identified: H1: red (present in all populations), H2: green (private to Lebanon), H3: blue (private to the central Iberian Peninsula), H4: yellow (private to Lebanon) (Vendramin et al. 2008)

All populations showed the same haplotype with the exception of all three Lebanese and two Spanish populations, where two and one additional low-frequency haplotypes were found, respectively (Fig. 5). Hence, this study extends the previous findings of Fallour et al. (1997) to the chloroplast genome. In this species the chloroplast DNA variation is extremely low: total

haplotype diversity is only 0.019 (0.37 in other conifers) and standardized allelic richness per microsatellite locus was 1.08 (2.58 in other conifers). Sequencing of the cpDNA fragments demonstrated that the absence of polymorphism was not due to loss or interruption of microsatellite repeat motifs. Furthermore, analyses and standardization showed that the almost absent genetic variation of *P. pinea* was neither due to ascertainment bias caused by shorter alleles (i.e., a lower number of repeats microsatellite stretches) nor due to different sample sizes respect to studies in other conifers. Respect to allozymes, chloroplast microsatellites have higher mutation rates (Provan et al. 1999) and are generally highly variable in conifers (e.g., Powell et al. 1995; Vendramin et al. 1996; Petit et al. 2005). Therefore, the very low chloroplast diversity found in *P. pinea* is particularly remarkable. Comparing the level of diversity of *P. pinea* with other plant species, the possible effect of the different attributes (plant size, woody/herbaceous habit, geographic range size, native or introduced status, existence of asexual reproduction -through apomixis or vegetative growth-, and mating system) was tested and taken into consideration. Genetically depauperate plants with characteristics similar to those of *P. pinea* are really remarkable, and the only other conifer with a similar low level of chloroplast microsatellite variation is *P. torreyana*, a Californian narrow endemic (Provan et al. 1999). New nuclear molecular markers (microsatellites and gene sequences) showed a comparable pattern of low genetic variation in stone pine (unpublished data), for example the level of nucleotide diversity at gene level ( $\pi$ ) was one order of magnitude lower and heterozygosity ( $H_E$ ) at microsatellite level significantly lower than in other conifer species. On the other hand, significant genetic differentiation ( $F_{ST}$ ) was found among westernmost and easternmost stone pine origins, with central Spanish and Lebanese populations showing differentiated nuclear polymorphisms too.

The extreme situation of low polymorphism at molecular markers in this species is probably the result of a combination of factors that have acted in the same direction. The three rare cpDNA haplotypes (H2, H3, H4 in Fig. 5) from Lebanon and central Spain, all differing by a single mutation from the widespread haplotype (H1), have as their goal the post-bottleneck mutations in areas of expansion and not the independent survival of haplotypes that existed prior to the bottleneck (Slatkin and Hudson 1991). This suggests that, at some point(s) during the evolutionary history of this species, one or more drastic range-wide decline events occurred, followed by the survival of a single (i.e., geographically circumscribed) population. The prolonged bottleneck experienced by *P. Pinea* could have been the consequence of the natural climatic and sea level fluctuations during the Quaternary. Furthermore, the unusual mating system of the species might have accelerated the loss of genetic diversity. Forest trees normally are subjected to strong inbreeding depression, which causes the elimination of all inbred progeny prior to maturity (Petit and Hampe 2006; Scofield and Schultz 2006). Interestingly, along with *P. resinosa*, another pine species with low genetic diversity (Fowler 1964; Mosseler et al. 1991, 1992; Echt et al. 1998), *P. pinea* is one of the very few tree species that are not subjected to inbreeding depression: in this species height growth is not reduced even by two successive generations of self-pollination (Ammannati 1988). Finally, unlike most of pine species, which are anemophilous, *P. pinea* seeds are dispersed only by means of mutualistic animals, such as birds, often scatter-hoarders that do disperse lots of several seeds from the same tree, resulting in a clustered kin dispersion. The scarcity of seed dispersers during some critical periods of the species history may have prejudiced its ability

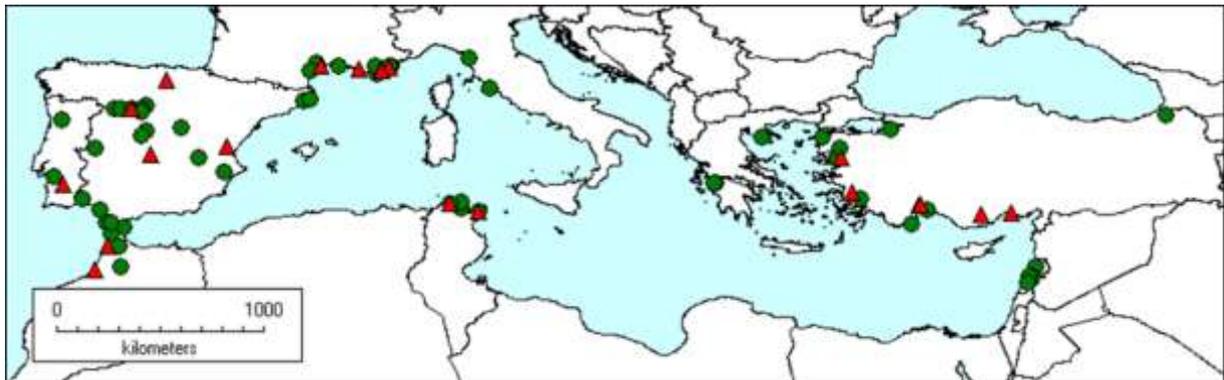
to colonize new territories, thus making stone pine more susceptible to range contractions and affecting its genetic diversity.

However, the most unusual finding is not only the bottleneck and the loss of diversity, but rather the fact that *P. pinea* has maintained a low level of diversity while spreading across a diverse and fragmented region. This spread probably took place during the Holocene, or even earlier, suggesting that stone pine has not been able to regain genetic diversity several hundred or maybe even thousands of generations after its decline. The existence of such genetically depauperate yet widespread species seems to be supported by the reduced evolutionary rates. It is obvious that, after the expansion, the low evolutionary rate maintains a low level of diversity, but at the same time it is not responsible for the success of the expansion itself. A number of not mutually exclusive explanations can be suggested. First, humans have played an important role by spreading the species for at least 3000 years. Second, during the bottleneck phase there might have been a loss of specific parasites and diseases (Amos and Balmford 2001): today *P. pinea* has comparatively few parasites and diseases (Fady et al. 2004). Third, variation at phenotypic traits, and not on marker diversity, may determine the successful adaptation to new environmental conditions. The small but not negligible amount of heritable variation found at adaptive traits (Mutke et al. 2005b, 2010) could have helped in colonizing new environments, although the presence of a reduced genetic variation.

After a bottleneck, epigenetic variation can accumulate quickly (Rapp and Wendel 2005), and it might be responsible for the variation in functional traits of *P. pinea* trees grown in drought stress conditions (Sánchez-Gómez et al. 2011). Epigenetic mechanisms have been proposed to contribute to adaptation in plants (Richards et al. 2010; Bräutigam et al. 2013). Recently, Sáez-Laguna et al. (2014) analysed DNA cytosine methylation in *P. pinea* genome to identify potential epigenetic variability explaining the significant functional variation found in this species. The authors analysed the DNA of 20 vegetatively propagated individuals from five Spanish natural populations by using Amplified Fragment Length Polymorphism (AFLP) and Methylation Sensitive Amplified Polymorphism (MSAP) techniques. No polymorphic marker was identified by means of the AFLP technique, thus confirming the absence of genetic variation displayed by this species. The MSAP technique, however, discovered that approximately 65% of the analysed cytosines at CCGG motifs were methylated. This value is at least 10% higher than estimates indicated annual and other perennial plants (Marfil et al. 2009; Herrera and Bazaga 2010; Li et al. 2011), and is in agreement with hypermethylation of conifer genomes (Nystedt et al. 2013), suggested to underlie genome evolution in conifers. The widespread methylation found in *P. pinea* genome might be closely associated with the repetitive nature of conifer genomes (Nystedt et al. 2013). Furthermore, this study reported a high level of cytosine methylation variability between the analysed trees, that reflect the variation in 42% of the total MSAP markers analysed. Several studies suggest that cytosine methylation variability may be related to the phenotypic plasticity in adaptive traits (Zhang et al. 2013; Herrera and Bazaga 2010). Therefore, in *P. pinea* epigenetic variation could act as a source of variability alternative to genetic diversity, bringing about relevant evolutionary consequences for adaptation (Vendramin et al. 2008; Mutke et al. 2010).

### 3 Phenotypic diversity, selection and breeding

Similarly to the reduced molecular diversity discussed in the previous section, studies of phenotypic variation in quantitative traits, evaluated in common garden tests, have detected only very moderate differences between populations of different geographic origins (provenances) in such adaptive traits as survival, total height and diameter, or phenology. Most of the analysed common gardens were established during the early 1990s as a reciprocal international provenance test. Accessions were exchanged from the full geographic range of stone pine, following an initiative of INRA within the framework of the FAO Committee *Silva Mediterranea*, which involved France, Portugal, Spain, Italy, Turkey, Tunisia, and Morocco, and including further accessions from Greece and Lebanon (Fig. 6; Martín and Prada 1995; Carneiro et al. 2006; Court-Picon et al. 2004; Gordo et al. 2007; Mutke et al. 2008, 2010, 2013; Khaldi et al. 2009; Sbay et al. 2011; Acar et al. 2013; Carrasquinho and Gonçalves 2013a/b; Loewe et al. 2012; Hermida et al. 2016).



**Fig. 6** Mediterranean stone pine provenances tested in FAO provenance trials (circle - provenances; red triangles - test sites Mutke et al., 2013)

For French trial sites, Court-Picon et al. (2004) reported differences in germination capacity among provenances, attributed to differences in seed size and weight that also influenced positively the initial juvenile height growth. Greater vigour was related with earlier bud burst, though those precocious provenances also increased their risk of damages by late frosts. Accessions from limestone origin developed well on siliceous substrate, suggesting no adaptive differentiation regarding soil chemistry. But the variations in soil/micro-ecological conditions were reported to greatly influence the variation in size of the saplings, and the authors suggested that this strong individual variability due to the environment may have masked the differences of growth expected among provenances in their analysis (Court-Picon et al. 2004). Whether the small amount of variability detected in adaptive traits between provenances was only due to seed size, maternal effects, phenotypic plasticity, or also to genetic diversity, remained to be answered by further analyses and monitoring (Fady and Vauthier 2011).

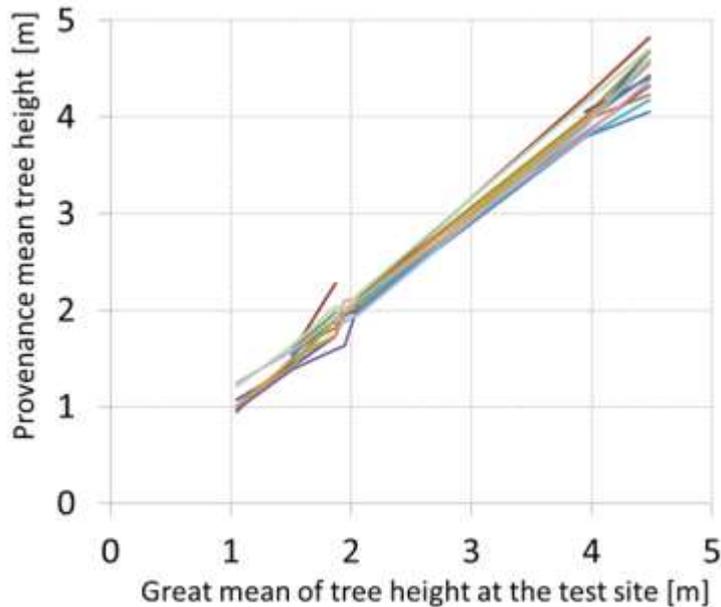
Similarly, Carneiro et al. (2006) and Carrasquinho and Gonçalves (2013a) reported no significant differences between provenances regarding stem diameter at age 13 and strong provenance $\times$ site interactions for two Portuguese test sites. The provenance effect in height growth

was significant, with among-provenance mean heights varying from 1.3 to 1.5 m at age 6 at one site and from 2.2 to 2.6 m at age 11 at the latter. Strong spatial autocorrelations were found within both sites, and this factor had to be taken into account in the models (Carasquinho and Gonçalves 2013b).

Khaldi et al. (2009) reported a lack of significant differences in survival and growth among provenances (mean heights 2.0-2.2 m at age 10) for two Tunisian plots, but they did not take into account spatial effects. In contrast, Sbay et al. (2011) found significant differences in height growth up to age 12-13 at two Moroccan test sites, the best provenance exceeding by 9-10% the mean height (5.8 at age 12 in one site; 6.4 at age 13 in the other site). Two Turkish plots analysed by Acar et al. (2013) revealed also significant differences in height, diameter and conelet number at age 10. The cited studies did not find differences in survival, a trait more closely related to fitness, among provenances.

Analysing the Spanish test sites, Mutke et al. (2010) found strong phenotypic plasticity in response to microsite conditions, prevailing over small, though significant provenances differentiation. The same trend was found when comparing the four Spanish and three French test sites of the international provenance trial, after adjusting mean tree height values to micro-environment factors by iterative spatial analyses (Mutke et al. 2013). Height growth differed significantly between adjusted provenance means, ranging  $\pm 5\%$  the average height at the two French sites, but as much as  $\pm 31\%$  at the worst site in Spain, where mean height was only 1.1 m at age 14, due to the abundance of still juvenile trees without onset of adult whorled stem growth. In average, height growth was lower in trees from warmer (coastal) origins than colder (northern, inland) ones, similar to findings in a recent nursery trial with seedling from Spanish inland or coastal provenances (Pardos et al. 2013). Differences among provenances amounted to 3-12% of the observed phenotypic variance in height among trees at each site, clearly much less than the common, stable reaction norm associated to the microsite that explained 73-78% of the variation observed at each site, or between sites (Figure 7; Mutke et al. 2013).

Another trial in Spain compared accessions from nine populations growing on contrasted soil conditions at seven test sites with an analogous range of geological variation, comprising river terraces with groundwater layers accessible for roots, deep sand deposits, shallow limestone leptosols, and even chalky gypsum marls. The adaptive differentiation in survival, vegetative phase change, height or diameter growth were absolutely non-significant among provenances, contributing only 0.02% of the total observed variation in tree height at 8 years, while variation between sites, as well as microsites within, explained as much as 84% of phenotypic variation (Gordo et al. 2007).



**Fig. 7** Adjusted provenance mean heights plotted against the great mean at each site (15 provenances at 5-7 sites, age 9-16 yr; Mutke et al. 2013)

The reason for this overwhelming impact of microsite variation on stone pine performance is a strong phenotypic plasticity that allows high survival rates even in unfavourable environments, though with strong ontogenic delays as pay-off, such as the described delayed growth due to edaphic restrictions in one of the Spanish test sites. Stone pine is ecologically adapted to drought-prone environments and loose, well-aired soils, such as sand, sandy loam or gravel, where its adult root system can explore more than thirty metres horizontally and several metres in depth in search for water (Montero et al. 2004). In fact, growth can be restrained by too compact soils during the first phase of seedling establishment (less than 40% sand, and more than 40% silt or more than 30% clay), especially if occasional flooding does occur. In these conditions, the plant development can be arrested for many years in a persistent juvenile phase, resulting in a dwarf-juniper-like shrub habit. This juvenile “standby” stage in *Pinus pinea*, also observed in *P. canariensis*, can be seen as an evolutionary advantage to survive under harsh environments, as opposed to other pine species which do not demonstrate this ontogenic plasticity (Climent et al. 2011; Mutke et al. 2012).

Contrasting to the lack of genetic diversity of stone pine at provenance level both at molecular markers and at fitness related phenotypic traits, significant clonal variation has been detected for morphological and functional traits (Carneiro 2005; Mutke et al. 2005b; Carrasquinho et al. 2010). Research on clonal selection and grafting techniques in stone pine has been under way for a few decades in Italy, Portugal and Spain (Magini 1965; Baudín 1967; Magini and Giannini 1971; Balguerías 1971; Gil and Abellanas 1989; Catalán 1990 1998; Abellanas et al. 1997; Prada et al. 1997; Mutke et al. 2000, 2005b, 2007b; Gordo 2004; Castaño et al. 2004). When evaluating the cone productivity of different clones in several comparative grafted field tests, a similar common dependence on microsite was observed for tree growth and cone productivity, given that individual cone production correlates strongly with the crown size of the tree ( $r$  of 0.6-0.9) (Mutke et al. 2007b). The degree of (clonal) genetic determination for cone or nut production was estimated in 7-18 % of overall phenotypic

variation, based on the performance of 462 candidate clones in several grafted comparative trials in Spain, but it increased to as much as 24-57% once estimates were adjusted for variation in tree size. Corresponding estimations for genetic gain would be in the range from +12 to +39% of mean cone yield in each test site by selecting the top 10% (most productive clones), though genotype×site interactions were found to be significant, marking certain agroclimatic gradient from more genuine Mediterranean to colder, more continental climate zones. The genetic gain might be higher if referred to an overall average for the species, because the original selection of the clones compared in the grafted trials included only plus trees for cone production (Mutke et al. 2005b, 2007b).

Within the European Union, superior clones of forest trees can be registered and catalogued officially as *basic materials*, in the categories “*qualified*”, i.e. phenotypically selected at the individual level, or “*tested*” when their superiority has been already demonstrated by comparative testing or estimated from genetic evaluation of their components. Their register is mandatory for admission as donors of commercial graft scions (forest reproductive material) under the frame of European regulation on the marketing of forest reproductive material (Council Directive 1999/105/EC).

For this register, clones must meet a list of requirements, among others the mandatory request to be identifiable by distinctive characters which have been approved and registered with the official body, similarly to markers or descriptors for plant varieties. In the case of stone pine, due to its great phenotypic homogeneity, no morphological or phenological descriptors could be singled out for clones: no clear differences in branching, crown form, needle shape, or phenological calendar do allow for identifying each one of them. Nuclear microsatellite variation (Pinzauti et al. 2012) made the identification of 18 Spanish elite clones possible, while other 31 outstanding clones could not be legally registered as basic materials because their nSSR genotype was not unique compared among 86 sampled clones. In 2015, 15 of these 18 clones were registered in the Spanish National Catalogue of Basic Materials for *Pinus pinea* (Table 1). Five clones, with consistent superiority for cone production at two or three different trial sites, were admitted as basic materials in the category “Tested”; while the other ten clones, having been evaluated only in one test site, were registered temporally in the category “Qualified”, until their further characterisation in other, more recent trials (Guañaño and Mutke 2016).

**Table 1** Stone pine elite clones registered as basic materials in Spain and expected genetic gain estimated from grafted trials

<b>Unit of approval</b>	<b>Genetic gain (+% cone yield)</b>
<b>Category “Tested” (B.O.E., December 12, 2015)</b>	
CL-C-23/Portillo-11	+25-27 %
CL-C-23/Portillo-12	+12-29 %
CL-C-23/La Vega	+12-17 %
CL-C-23/Íscar	+11-20 %
CL-C-23/Valdegalindo	+15-18 %

<b>Unit of approval</b>	<b>Genetic gain (+% cone yield)</b>
<b>Category “Qualified” (B.O.E., May 13, 2015)</b>	
CL-Q-23/Hoyo de Pinares	+ 19 %
CL-Q-23/Almorox	+ 19 %
CL-Q-23/San Martín de Valdeiglesias	+ 24 %
CL-Q-23/El Provencio	+ 31 %
CL-Q-23/Pozoamargo	+ 21 %
CL-Q-23/Casas de Haro	+ 23 %
CL-Q-23/El Picazo	+ 22 %
CL-Q-23/Santa Coloma de Farners	+ 9 %
CL-Q-23/Llagostera	+ 11 %
CL-Q-23/Dosrius	+ 9 %

*B.O.E. - Boletín Oficial del Estado, <http://www.boe.es>*

In Portugal, a clonal mixture has been registered as “Qualified” Basic Material for stone pine, comprising 64 individual clones whose ortets had been phenotypically selected as plus trees for outstanding cone production among 300 candidate trees sampled in the stone pine forests of southern Portugal. In 2004, two mother plant orchard with 50 ramets of each of these clones have been planted in two sites, Coruche (PNMQ01) and Alcácer do Sal (PNMQ02), for producing graft scions, commercialised since 2009 by the Private Forest Owners Associations APFC and ANSUB for in-field grafting of new plantations in the Alentejo region (Carneiro 2005; Carneiro et al. 2007; Carrasquinho et al. 2010; Guadaño and Mutke 2016).

For landowners who cannot count on skilled hands for in-field grafting, the release of the Spanish and Portuguese elite clones is a welcome solution that allows the development of orchard plantations from container-raised trees grafted in nursery. However, cross-border trade of grafted pines is currently hindered, at least partially, by a quarantine restriction for Portuguese forest plant materials due to the high biotic risk of spreading the pine wilt nematode, *Bursaphelenchus xylophilus*, one of the most dangerous threats for European coniferous forests, but that is asymptomatic in *Pinus pinea* (Nunes da Silva 2015; Zas et al. 2015).

Definitively, though current agronomic knowledge about stone pine as orchard nut crop is still limited, and most plantations continue to be managed as extensive forestry or agroforestry systems with trees grown from seeds without selected pedigree, Mediterranean stone pine nuts have lately taken their first steps from a wild-harvested forest commodity to a high value agronomic crop.

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