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ORIGINAL ARTICLE

From Mediterranean shores to central Saharan mountains: key phylogeographical insights from the genus *Myrtus*

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ABSTRACT

Aim The Mediterranean region is often regarded as a crossroads where species of various origins meet. However, the biogeographical relationships between this region and contiguous Saharan, Macaronesian and Irano-Turanian regions have not been investigated in detail. The aim of this study was to characterize the phylogeography of the circum-Mediterranean species *Myrtus communis* and to investigate the origin of isolated central Saharan populations of *Myrtus nivellei*.

Location The distribution ranges of *M. communis* from Macaronesia to the Irano-Turanian region (173 sampled populations) and of *M. nivellei* in the mountains (Hoggar, Tassili n'Ajjer, Immidir, Tibesti) of the central Sahara (23 populations).

Methods Nuclear ribosomal DNA (nrDNA) sequences of Myrtaceae were used to root the phylogeny of *Myrtus*, and to date its crown node, according to a detailed review of the palaeobotanical records used for multiple fossil calibration. Chloroplast DNA (cpDNA) sequences were analysed through the determination of genetic diversity indices and by statistical phylogeography.

Results Both cpDNA and nrDNA markers indicated east–west genetic differentiation within *M. communis*. During the late Miocene, a key vicariance event affected the previous circum-Mediterranean distribution of *Myrtus*, leading to the isolation of eastern populations. During the late Miocene or early Pliocene, two clades diverged: one is now scattered in the Mediterranean Basin and adjacent regions, whereas the other evolved in the western Mediterranean region. The differentiation of lineages during the Plio-Pleistocene occurred mainly in the western part of the Mediterranean Basin, which has been at the origin of migrations towards Macaronesian islands and Saharan mountains. This is one of the first plant phylogeographical studies to report migrations from the Mediterranean to the Sahara.

Main conclusions The genus *Myrtus* has persisted in the Mediterranean region since at least the Neogene and its biogeography reflects the cumulation of the species' responses to successive palaeoenvironmental changes. The current distribution of the genus *Myrtus* in the Mediterranean Basin and in isolated areas, such as the Macaronesian islands and Saharan mountains, can be explained by the striking ability of this plant not only to persist locally in various refugia, but also to migrate.

Keywords

Ancestral range reconstruction, chloroplast DNA, endemism, genetic diversity, molecular dating, Myrtaceae, palaeoecology, phylogeny, phylogeography, ribosomal DNA.

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INTRODUCTION

Phylogeography combines the fields of phylogenetics, population genetics and biogeography. In Europe and North America, genetic surveys of current populations have detected the genetic imprints of shifts in range that occurred in response to the climatic oscillations during the Quaternary, in particular in response to the Last Glacial Maximum, which occurred *c.* 20,000 years ago (Hewitt, 2004; Soltis *et al.*, 2006). Molecular phylogeographical approaches can also act as a bridge between historical biogeography and palaeoecology, enabling refugia and pathways of post-glacial migration to be identified (Hu *et al.*, 2009).

In the Mediterranean Basin, as compared with more northern temperate regions, biodiversity is moulded by more complex patterns that are linked to the heterogeneous historical biogeography of this region (Woodward, 2009; Blondel *et al.*, 2010). The current Mediterranean flora has been shaped by three different factors: (1) geological history and climate change since the early Cenozoic, (2) environmental constraints linked to aridity during the summer, and (3) ancient human activity (Thompson, 2005; Médail & Diadema, 2009; Nieto Feliner, 2011). Within the patchwork of Mediterranean insular and continental ecosystems, there is an extreme diversity of ecological processes across both space and time. With respect to space, for example, migration routes for flora from the European, Saharan and Irano-Turanian regions meet within the Mediterranean region (Quézel, 1985). With respect to time, some taxa have resulted from long-term *in situ* evolution, whereas various periods of colonization from adjacent or far-distant regions have been hypothesized for other taxa. For example, the study of Mansion *et al.* (2008) has demonstrated the heterogeneous origin of Mediterranean Araceae, which have arisen from two episodes of migration: one from an area that connected North America and Eurasia (Late Cretaceous, around 82.7 ± 6.8 Ma) and the other from Anatolia (late Eocene to middle Oligocene, 37.8 ± 7.2 Ma). The Mediterranean region can indeed be regarded as a biogeographical crossroads for species of various origins (Thompson, 2005; Blondel *et al.*, 2010). The history of many plant populations is much longer than that of populations that arose from post-glacial recolonization at higher latitudes, and can span the period up to the Neogene (Petit *et al.*, 2003). Some important palaeogeographical events, such as the tectonic break-off of the Tyrrhenian plate from the Iberian Peninsula during the late Oligocene (*c.* 30 Ma), have influenced modern patterns of genetic structure in plants strongly, as suggested for *Quercus suber* and *Pinus pinaster* in the western Mediterranean (Magri *et al.*, 2007). Another key event that had a definite influence on plant diversification in the Mediterranean was the Messinian salinity crisis, which was induced by the desiccation of the Mediterranean Sea due to the closing of the Strait of Gibraltar between 5.96 and 5.33 Ma (CIESM, 2008). Climatic oscillations during the Quaternary (Tzedakis, 2009) also affected the patterns of genetic diversity of Mediterranean plant taxa (Fady & Conord, 2010) with persistence being exhibited within various refugia, located mainly on islands and mountains (Médail & Diadema, 2009). As a consequence, when analysing patterns of genetic diversity, it is important to consider the timing of key biogeographical processes, especially for those lineages that are thought to be the most ancient, namely, the Tertiary Mediterranean palaeoflora (*sensu* Palamarev, 1989).

To date, very few studies have examined the genetic structure of widely distributed representative Mediterranean trees of Neogene origin (Rodríguez-Sánchez *et al.*, 2010) because phylogeographies exist only for sclerophyllous *Quercus* species (López de Heredia *et al.*, 2007), *Olea europaea* (Rubio de Casas *et al.*, 2006; Besnard *et al.*, 2007a), *Buxus balearica* (Rosselló *et al.*, 2007), *Laurus* species (Rodríguez-Sánchez *et al.*, 2009), and *Erica arborea* (Désamoré *et al.*, 2011). Furthermore, the eastern Mediterranean region and North Africa have often been neglected by researchers, despite the fact that they constitute hotspots for biodiversity and contain refugia for many species (Médail & Quézel, 1997; Médail & Diadema, 2009). From the perspective of biogeography and conservation, there is a strong need to conduct studies on widespread and key taxa of the Mediterranean palaeoflora in order to examine the genetic imprints that have been left by: (1) climatic changes during the Cenozoic period and the onset of the Mediterranean climate, (2) palaeogeographical events that pre-dated the Pleistocene, such as the Messinian salinity crisis, and (3) the highly complex geography of the Mediterranean which drives migration processes. Molecular dating techniques, complemented by the use of fossil data, can reveal the correspondence between the phylogeography of Mediterranean plants and geological or palaeontological events, as in the integrative studies of Boraginaceae (Mansion *et al.*, 2009) and Rutaceae (Salvo *et al.*, 2010).

One of the most interesting species that can be used to reconstruct Mediterranean phylogeography is *Myrtus communis* L. (Myrtaceae). The Myrtaceae family includes *c.* 5600 tropical species, but only the ditypic genus *Myrtus* has reached the northern temperate region. *Myrtus communis*, which is representative of the Mediterranean thermophilous matorral, is widespread throughout the Mediterranean region, whereas there are only scattered populations in Macaronesia and in the Irano-Turanian region. Among Mediterranean model species that can be used for phylogeography, *Myrtus* has the advantage of having valuable fossil records available. Palaeobotanical studies based on plant macrofossils have revealed that *Myrtus* does indeed belong to the Palaeo-Mediterranean flora and have suggested that it was present before the Plio-Pleistocene development of the Mediterranean climate (de Saporta, 1863; Palamarev, 1989; Allen, 2003). It is likely that the woody ancestors of *Myrtus* grew within the Palaeogene/Neogene broadleaved evergreen vegetation (laurisilva), which was dominated by taxa of tropical affinities during the Oligocene and has persisted until today, notably in the Macaronesian

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islands (Fernández-Palacios *et al.*, 2011). According to the phylogenetic and molecular clock analyses of Sytsma *et al.* (2004), Lucas *et al.* (2007) and Biffin *et al.* (2010), the lineage of the genus *Myrtus* diverged at the base of the Myrteae tribe during the Eocene. As a consequence, the study of *M. communis* might provide useful insights into the nature of the establishment of mediterranean-type ecosystems and their response to climatic changes.

Interestingly, the genus *Myrtus* is characterized by a disjunct distribution between *M. communis* and the only other species within the genus, *Myrtus nivellei* Batt. & Trab. The latter species is endemic to the central Saharan mountains, where it grows in rocky and sandy wadis and gorges, at high elevation (Battandier & Trabut, 1911; Quézel, 1958). Two different hypotheses have been proposed to account for the divergence of these taxa, which are found in two disjunct areas and in contrasting environments. The first hypothesis is that both taxa have Palaeogene/Neogene origins, with *M. nivellei* being the Saharan vicariant of *M. communis*. The second is that, after a relatively recent period of isolation (late Neogene or Pleistocene), one of these two taxa migrated from the area in which the ancestral taxon was distributed. For example, *M. communis* might have been derived from *M. nivellei* through migration along a hypothetical African migration route, as was the case for *Olea europaea* and *Erica arborea* (Besnard *et al.*, 2007a; Désamoré *et al.*, 2011).

In the study described herein, we used both chloroplast and nuclear markers to investigate the temporal and geographical diversification of *Myrtus*. By analysing genetic and fossil data, we addressed the following questions: (1) What are the extent and distribution of the genetic variation of the genus *Myrtus* throughout its whole distribution range? (2) What can be inferred about the divergence times and migration pathways of this disjunct genus? (3) What is the phylogenetic relationship between the Saharan endemic species *M. nivellei* and its Mediterranean sister species *M. communis*?

MATERIALS AND METHODS

Biological models and sampling strategy

Myrtus communis is an evergreen shrub that is widespread in the Mediterranean Basin. It has been reported as far west as Macaronesia (Madeira and the Azores), and as far east as western Asia (Iran and Afghanistan) (Meusel *et al.*, 1978; Browicz, 1984; de Bolòs & Vigo, 1984; Strid, 1996; Güner *et al.*, 2000; Fig. 1). It grows to 0.5–3 m in height and can be found at elevations up to *c.* 500 m a.s.l. This diploid species is allogamous and self-compatible. The fruits of *M. communis* are berries; these are dispersed mainly by frugivorous passerine birds and some mammals (Traveset *et al.*, 2001; González-Varo, 2010).

Myrtus nivellei is endemic to the central Saharan mountains and grows in scattered populations (Quézel, 1965). This species is found in Algeria (Hoggar, Tefedest, Tassili n'Immidir and Tassili n'Ajjer) and in Chad (Tibesti), where the subspecies *M. nivellei* subsp. *tibesticus* has been reported (Quézel, 1958) (Fig. 1). *Myrtus nivellei* grows in rocky and sandy wadis where subterranean waterpoints exist, and generally at an elevation above 1400 m. It grows to 1–2 m in height and is morphologically close to *M. communis*, but differs in that it has lanceolate and very linear leaves (4–5 cm in length) (Battandier & Trabut, 1911).

Sampling locations were selected to cover the entire distribution range of the two species. We collected samples from 173 populations of *M. communis* and from 23 populations of *M. nivellei* throughout their natural ranges (see Appendix S1 in the Supporting Information); numerous botanists and scientists helped us with this sampling (see Acknowledgements). At each site, one to two individuals were sampled, sufficiently distant from each other to sample physically isolated individuals. Fresh leaf material was dried in silica gel and then conserved at −34 °C.

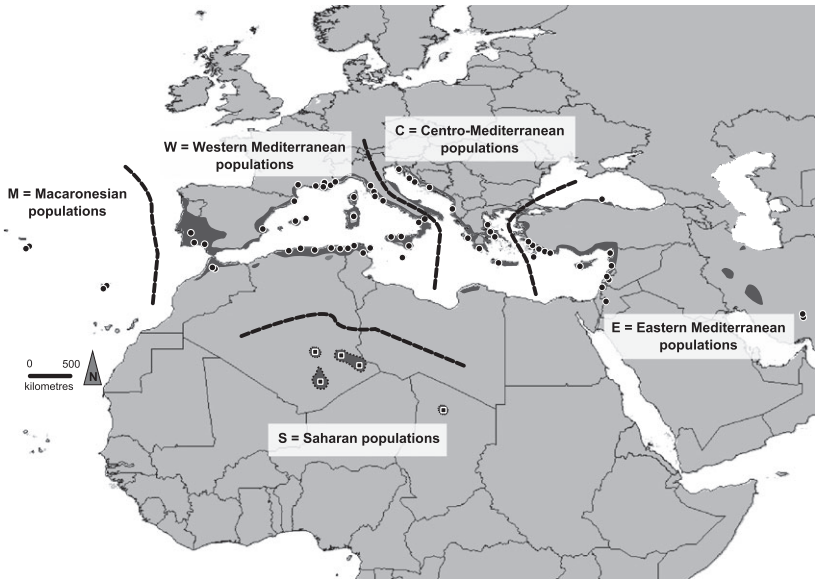


Figure 1 Distribution map of *Myrtus communis* (dark grey areas) and *Myrtus nivellei* (dark grey areas bordered by dotted line), with the sampled areas represented by circles for *M. communis* and squares for *M. nivellei*. Dashed lines represent the borders that divide the five biogeographical regions defined for the analyses of genetic diversity and reconstructions of ancestral area.

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Palaeobotanical data

The palaeobotanical evidence for occurrences of *Myrtus* species was reviewed using the European Pollen Database, the PANGEAE, and the Paleodiversitas databases, in addition to information supplied by palaeobotanists (see Appendix S2 and Acknowledgements). The palaeoecological literature was analysed with a focus on pollen and plant macrofossils. Fossil pollen of *M. communis* can be identified to the species level, but it does not appear very frequently within the cores of sediments.

DNA extraction, PCR parameters and sequencing

Ribosomal DNA sequences of Myrtaceae were used to root the phylogeny of *Myrtus* and to date its crown node. Chloroplast DNA sequences were analysed through the determination of genetic diversity indices and by statistical phylogeography.

Total DNA was extracted from the samples dried in silica gel and from herbarium specimens of *M. nivellei* subsp. *tibesticus*, using the Doyle & Doyle (1987) procedure with the following modifications: 1.4 mM NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8 and 4% hexadecyltrimethyl-ammonium bromide. The concentration and quality of the DNA were determined using a photometer (BioPhotometer, Eppendorf, Hamburg, Germany).

Polymerase chain reactions (PCR) were performed in a volume of 50 μL that contained 1× buffer, 3 mM $MgCl_2$, 0.6 mM dNTPs, 0.2 μM each primer, and 2.5 units of *Taq* polymerase (Q-Biogen, Illkirch, France) in a PTC-200 Gradient Thermal Cycler (MJ Research, Watertown, MA, USA). First, we performed a pilot study to analyse sequence variability within *Myrtus* in 12 regions of chloroplast DNA and ribosomal DNA [*mat*K, *ndhf*, *psb*A–*trn*H, *rbc*L, *rp*L20–5′*rps*12, *trn*S–*trn*G, *trn*T–*trn*D, *trn*T–*trn*L, *rpl*2–*trn*H, *trn*L–*trn*F, external transcribed spacer (ETS) region and internal transcribed spacer (ITS1–5.8S–ITS2) region] among 10 individuals with distinct origins throughout the entire geographical range of *Myrtus*. Subsequently, we selected the pairs of primers that yielded the highest levels of polymorphism. Together, these primers amplified 1111 bp of nuclear ribosomal DNA (nrDNA: ETS and ITS) and 932 bp of chloroplast DNA (cpDNA: *trn*L–*trn*F and *rpl*2–*trn*H intergenic regions). In general, two individuals were analysed per location for cpDNA (n = 366 sequences). For the nrDNA analysis, only one individual per location was analysed (n = 196 sequences). Specifically, the four pairs of primers that were used to amplify and sequence the nrDNA and cpDNA regions were as follows: for the ETS region: ETS/18S (Lucas *et al.*, 2007); for the ITS1–5.8S–ITS2 region: ITSa/ITSb (Wendel *et al.*, 1995); for the intergenic spacer *trn*L–*trn*F: *trne*/*trnf* (Taberlet *et al.*, 1991); and for the *rpl*2–*trn*H region: J_{LA}^{+} *euro_rpl2*/*eucpsbA* (Payn *et al.*, 2007). The following amplification conditions were used: an initial denaturation for 2 min at 94 °C, followed by 32 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 52 °C, and elongation for 2 min at 72 °C, and a final 5-min elongation step at 72 °C. Direct sequencing was carried out by AGOWA genomic services (LGC Genomics, Berlin, Germany).

New sequences were checked, edited, and assembled using MEGA4 (Kumar *et al.*, 2008) and submitted to GenBank (Appendix S3). Within the nrDNA sequences, a few sites were detected to be polymorphic at the intra-individual level: i.e. more than one peak was present in the electrophoretogram and the weakest signal reached at least 25% of the strength of the strongest signal. These sites were coded using IUPAC codes in accordance with Fuertes Aguilar & Nieto Feliner (2003).

Phylogeny and molecular dating analyses from nrDNA regions

Phylogenetic relationships between nrDNA ETS and ITS haplotypes were inferred using a Bayesian approach, performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Twenty-two supplementary Myrtaceae sequences, obtained from GenBank (Appendix S3), were added to the alignment of *Myrtus* sequences to serve as outgroups and to provide calibrated nodes for the molecular dating of the *Myrtus* crown node (reviewed in Biffin *et al.*, 2010). Two different general time reversible + Gamma models of nucleotide substitution were selected with the Akaike information criterion as implemented in jModeltest 0.1 (Posada, 2008). As a consequence, the ETS and ITS data were partitioned to allow different parameters to be set for each molecular model. Markov chain Monte Carlo (MCMC) analyses were run for 5,000,000 generations with MrBayes. Trees and model parameters were sampled every 100 generations. The majority rule consensus tree produced by MrBayes was drawn with FigTree 1.3.1 (Rambaut, 2009).

A multiple fossil calibration approach was chosen to estimate the node ages of the *Myrtus* phylogeny that was obtained from nrDNA sequences and MrBayes. The following Myrtaceae fossils were used: (1) well-preserved fruits of *Paleomyrtinaea princetonensis* from the Palaeocene of North America [normal truncated calibration for node C: mean = 56 Ma, standard deviation (SD) = 6 Ma, min = 55 Ma; Pigg *et al.*, 1993], (2) fossil leaves and fruits of *Metrosideros* from the early Miocene of New Zealand (normal calibration for node B: mean = 20 Ma, SD = 6 Ma; Pole *et al.*, 2008), and (3) the earliest Mediterranean and most precisely dated macrofossil of *Myrtus* in France from the early Miocene (lognormal calibration to impose a minimum age for node D: mean = 23 Ma, SD = 1, min = 20 Ma; de Saporta, 1863; Bessedik, 1984). In BEAST 1.6 (Drummond & Rambaut, 2007), a Bayesian MCMC method was applied with the following prior parameters: a constant population size for the coalescent model and an uncorrelated log normal rate variation among branches in the tree for the relaxed clock model. Ten independent runs were performed with an MCMC chain that was 20,000,000 in length (topology and parameter values sampled every 2,000 steps). Tracer 1.4 (Rambaut & Drummond, 2007) was used, first to check the convergence and mixing of each parameter between runs, then to estimate

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the mean and 95% highest posterior density (HPD) of age nodes, sampled from the posterior distribution of the combined runs. The effective sample size (ESS) of each parameter was sufficient to provide reasonable estimates of the variance in model parameters (i.e. ESS values > 200, after excluding a burn-in fraction of 10%). Only the tree obtained with MrBayes is shown because of the congruence of this tree with the maximum clade credibility trees provided by beast.

Diversity and phylogeographical analyses from cpDNA regions

To analyse genetic diversity, we grouped the samples into five biogeographical regions, which were constructed on the basis of the findings of a number of previous studies (Zohary, 1973; Takhtajan, 1986; Strid, 1996). These regions were the following: (1) Macaronesian region (the Azores and Madeira; symbol: M), (2) western Mediterranean region (Morocco, Portugal, Spain, the Balearic Islands, France, Corsica, Sardinia, Italy, Sicily, Tunisia and Algeria; W), (3) central Mediterranean region (Croatia, Albania, Greece and Crete; C), (4) eastern Mediterranean region (Turkey, Rhodes, Cyprus, Syria, Lebanon, Israel, Jordan and Iran; E), and (5) Saharan region (southern Algeria and Chad; S).

Chloroplast DNA data were used to calculate the following indices of intraregion diversity using Arlequin 3.1 (Excoffier *et al.*, 2005) and GenAlEx 6.2 (Peakall & Smouse, 2006): the number of haplotypes, the percentage of private haplotypes, the percentage of variable loci (*P*), the gene diversity (*H*, the probability that two randomly chosen haplotypes are different), and the mean number of pairwise differences between all pairs of haplotypes (π). Polymorphic sites within nrDNA sequences, which were assumed to denote allelic differences in heterozygous individuals, were also used to describe the genetic diversity of *Myrtus* in more detail.

For the two cpDNA regions considered in this study, there were no GenBank accessions available for Myrtaceae species. Therefore, the cpDNA chronogram was calibrated using the nrDNA calibrated phylogeny previously obtained: the node E was calibrated with a normal distribution of the node age (mean = 6.7 Ma, SD = 1). An extended Bayesian skyline plot (for mitochondrial DNA with stepwise changes) was assumed for the coalescent model and an uncorrelated lognormal-distributed relaxed molecular clock was employed. One run of 20,000,000 MCMC steps was performed; parameter values were sampled every 2000 cycles. Convergence and acceptable mixing of the sampled parameters were checked using Tracer; all ESS values were > 200.

To undertake an ancestral area reconstruction approach, each of the cpDNA sequences was scored according to its observed presence or absence in each of the five biogeographical regions that were specified above. Following a statistical phylogeographical approach, the maximum clade credibility (MCC) tree produced by beast was used to map the ancestral distributions reconstructed for each node, by a statistical dispersal–vicariance analysis (S-DIVA, implemented in the program rasp 1.1; Yu *et al.*, 2010, 2011) conducted on the 10,000 trees sampled by beast. The S-DIVA analysis was performed without restriction about the maximum range areas.

RESULTS

Palaeobotanical data

In our review of the palaeobotanical occurrences of *Myrtus*, we found that 10 macroflora fossils were discovered in central and southern Europe and dated to the Oligocene and Miocene (Fig. 2; Appendix S2). The earliest Mediterranean and most precisely dated macrofossil of *Myrtus* was found in France, and dated to the early Miocene (minimum age calibration for the node D of nrDNA chronogram, Fig. 3). The most ancient occurrences of pollen of *Myrtus* were dated to the Miocene in Spain (Bessedik & Cabrera, 1985; Rivas-Carballo, 1991). Sixty-five of the 88 sites, where occurrences of *Myrtus* were recorded, were dated to the late Pleistocene and the Holocene. During these periods, even before the Neolithic period, the large range of palynological records attests to the circum-Mediterranean distribution of *M. communis* in approximately the same areas as today (Fig. 2). To our knowledge, the most ancient occurrence of *Myrtus* in the central Sahara was dated to 27,000 (uncalibrated) yr bp, near Lake Chad (Maley, 1981).

Phylogeny and molecular dating analyses from nrDNA regions

The alignment of the *Myrtus* nrDNA sequences was 1111 bp long and represented 102 haplotypes, namely 71 ITS haplotypes and 46 ETS haplotypes. The alignment for the *Myrtus* ingroup was characterized by 38 variable sites, with 19 single-site substitutions in each of the nrDNA regions examined. The percentage of individuals that were characterized by at least one polymorphic step appeared to indicate that heterozygosity was the highest for populations from the eastern Mediterranean (62.5%) and the lowest for Saharan populations (8.0%), with a mean of 37.7%.

In the nrDNA phylogeny, *Myrtus* was connected to the genera *Rhodamnia* and *Rhodomyrtus*, which are native to Asia, Malesia, Melanesia and Australia (Fig. 3). The nrDNA sequences of *Myrtus* are mainly structured in three clades. The first clade to diverge (node E) is restricted to the eastern Mediterranean region (Fig. 1, group E). The second major divergence led to the separation of two clades: one is now scattered in different parts of the *Myrtus* distribution (peri-Mediterranean, Macaronesian and Saharan regions; Fig. 1, groups M, W, C and E) and the second remains almost exclusively isolated in the western part of the distribution (Fig. 1, groups M and W).

The posterior mean rates of evolution that were calculated by beast for the nrDNA sequences were as follows: 1.66×10^{-9} substitutions per site per year for ITS and 1.76×10^{-9} substitutions per site per year for ETS. In respect of fossil data that have suggested the early Miocene origin of

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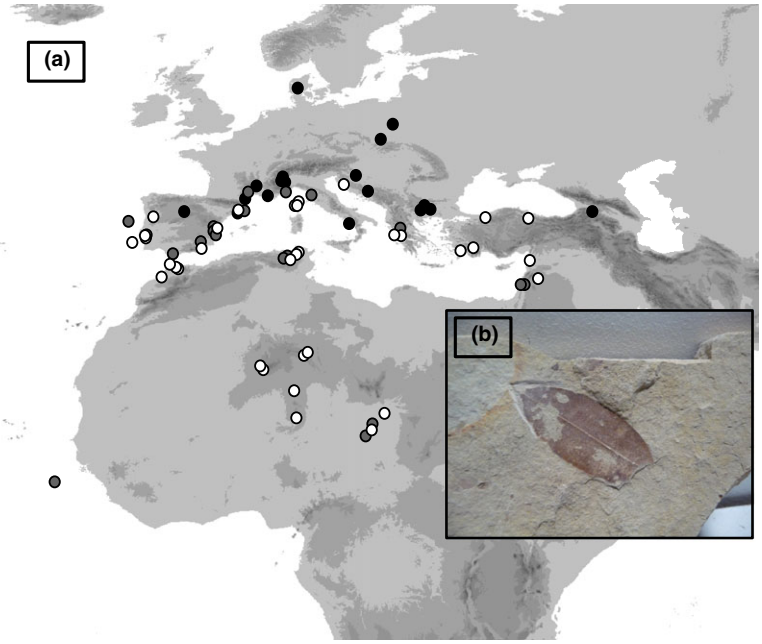


Figure 2 (a) Location of plant fossils and pollen records of *Myrtus* species for the Holocene (white circles), Pleistocene (grey circles), and Neogene/Palaeogene until the Eocene (black circles) (references are listed in Appendix S2). (b) A fossil leaf of *Myrtus aptensis* Sap. from the Ducos collection of the Natural History Museum of Aix-en-Provence.

the *Myrtus* lineage (node D), the extant *Myrtus* lineages started to originate during the late Miocene (Messinian) or the early Pliocene: the result of BEAST from the nrDNA data showed that the age of the basal crown node (node E) ranged from 3.86 to 9.82 Ma (95% HPD) with a mean of 6.69 Ma (Table 1). This node age and its 95% HPD were then used to calibrate the cpDNA chronogram.

Diversity and phylogeographical analyses from cpDNA regions

The length of the alignment of the *trn*L–*trn*F intergenic spacer and the *rpl*2–*trn*H region was 932 nucleotides. This alignment contained 14 cpDNA haplotypes and was characterized by 17 variable sites (Appendix S3), with a mean genetic diversity (H) of 0.666 ± 0.091 and a mean nucleotide number of pairwise differences (π) of 1.827 ± 1.062 (Table 2). Eleven variable sites were single-site substitutions, whereas five mononucleotide repeats and one insertion/deletion accounted for the remaining mutations. In particular, cpDNA haplotype 14, which included samples from Spain and Morocco, was characterized by a 24-bp insertion, which was coded as a single variable site.

For the five biogeographical regions, the haplotypic and genetic diversity indices that were calculated for cpDNA (Table 2) revealed several interesting patterns. Firstly, regions located at the periphery of the range exhibited a higher genetic diversity than those located more internally (π = 3.905 ± 2.225 and π = 1.677 ± 0.994 for Macaronesian and eastern Mediterranean regions but π = 0.981 ± 0.668 and π = 1.242 ± 0.807 for western and central Mediterranean regions, respectively). Secondly, the mean proportion of private cpDNA haplotypes was quite high (64.3%) but, in the central Mediterranean region, this proportion was well below the mean value (no private haplotypes).

Several cpDNA haplotypes were confined to particular areas and, on a broader scale, an east–west Mediterranean differentiation was evident (Fig. 4). Haplotypes 1 and 2 were found among the extreme eastern Mediterranean populations (Iran, Syria, Lebanon, Israel, Jordan, Turkey and Crete), whereas haplotypes 6 and 7 were found in the Azorean populations. Haplotypes 4 and 12 were widespread, being located in both western and eastern parts of the sampling area; haplotype 3 was more centrally distributed in the Mediterranean (Sicily and Greece). Haplotypes 8, 9, 10, 13 and 14 were found exclusively in western Mediterranean and Macaronesian populations of *M. communis*. Two private haplotypes were detected for *M. nivellei* in the Sahara (haplotypes 5 and 11).

The cpDNA sequences, as the nrDNA, revealed phylogenetic information mainly structured in eastern, western and peri-Mediterranean clades (Figs 3 & 4). However, the two data sets were not congruent because a major difference between the nrDNA and cpDNA phylogenies concerned *M. nivellei*. Although the Saharan nrDNA sequences were monophyletic (Fig. 3), *M. nivellei* was characterized by four distinct cpDNA haplotypes (4, 5, 11 and 12), which were disseminated in the cpDNA phylogeny, and most of these haplotypes were shared with *M. communis* (Fig. 4). Haplotype 4 was observed in both the Tassili n'Ajjer and the Tibesti mountain ranges, haplotype 5 in the Tassili n'Ajjer, haplotype 11 in the Hoggar, and haplotype 12 in the Tassili n'Immidir. The Saharan populations were found to be connected closely with the peri-Mediterranean (4), western Mediterranean (10), and central Mediterranean (12) cpDNA haplotypes of *M. communis*.

The posterior mean rate of evolution that was calculated by BEAST for the cpDNA reaches 3.59×10^{-10} substitutions per site per year. This value is congruent with the average absolute rate of substitution for cpDNA across land plants with relatively long generation times (Smith & Donoghue, 2008; Désamoré *et al.*,

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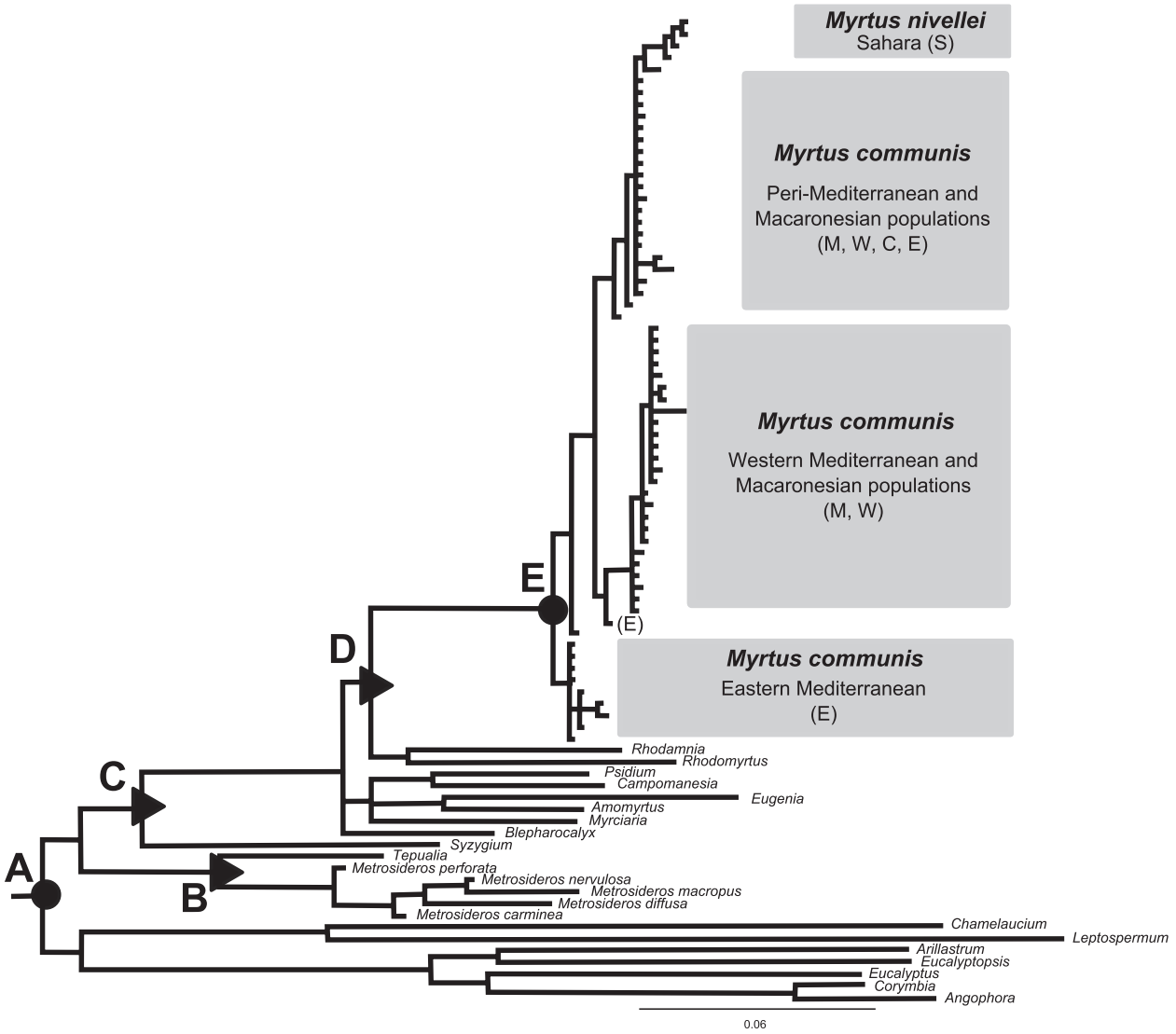


Figure 3 Majority rule consensus tree generated by Bayesian analysis (MrBayes 3.1.2) of combined ITS and ETS sequence data of *Myrtus*. The tree shows the major clades identified by the analyses together with their presence within the five biogeographical regions defined in Fig. 1. Letters characterize the nodes studied by molecular dating (triangles refer to the nodes used to calibrate the molecular clock; see Table 1). Myrtaceae sequences are detailed in Appendix S3. The scale bar represents the expected substitutions per site.

Table 1 Estimated ages of diversification and their 95% highest posterior density (HPD) for the main nodes defined in the nrDNA phylogeny (Fig. 3), using BEAST 1.6 software.

Node characteristics		Mean divergence time estimate (Ma)	95% HPD	Epoch
A	Myrtaceae	134.20	71.24–210.54	Early Cretaceous
B	Metrosidereae (macrofossil calibration)	21.29	13.56–29.56	Neogene–Early Miocene
C	Myrteae (macrofossil calibration)	58.49	55.00–64.14	Palaeogene–Late Palaeocene
D	*Rhodomyrtus–Rhodamnia–Myrtus* (macrofossil calibration)	21.87	20.03–25.84	Neogene–Early Miocene
E	Genus *Myrtus* (divergence of eastern Mediterranean populations)	6.69	3.86–9.82	Neogene–Late Miocene

2011). Taking into account the mean node ages and their 95% HPD from the BEAST analysis (Table 3), the first genetic divergence (node E, calibrated from nrDNA analyses), dated to the late Miocene, led to the separation of an eastern lineage of *M. communis*. This was followed by the divergence of the two main clades that encompass most of the genetic diversity in *Myrtus*, which occurred 3.98 Ma (node F, with a 95% HPD of 0.93–6.61 Ma). This divergence gave rise to the peri- and

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Table 2 Genetic and haplotypic diversity of the cpDNA sequences of *Myrtus communis* and *M. nivellei* examined for the five biogeographical regions defined in Fig. 1. Standard deviations are given in brackets.

Biogeographical regions	Number of individuals	Number of haplotypes	Percentage of private haplotypes	Percentage of polymorphic loci (*P*)	Mean gene diversity (*H*)	Mean number of pairwise differences (π)
Macaronesian populations	7	4	75.0	41.18	0.810 (0.130)	3.905 (2.225)
Western populations	190	7	42.9	35.29	0.565 (0.036)	0.981 (0.668)
Central populations	33	4	0	23.53	0.623 (0.075)	1.242 (0.807)
Eastern populations	81	4	25.0	23.53	0.677 (0.021)	1.677 (0.994)
Saharan populations	55	4	50.0	29.41	0.655 (0.032)	1.332 (0.841)
Total/Mean	366	14	64.3	30.59 (3.43)	0.666 (0.091)	1.827 (1.062)

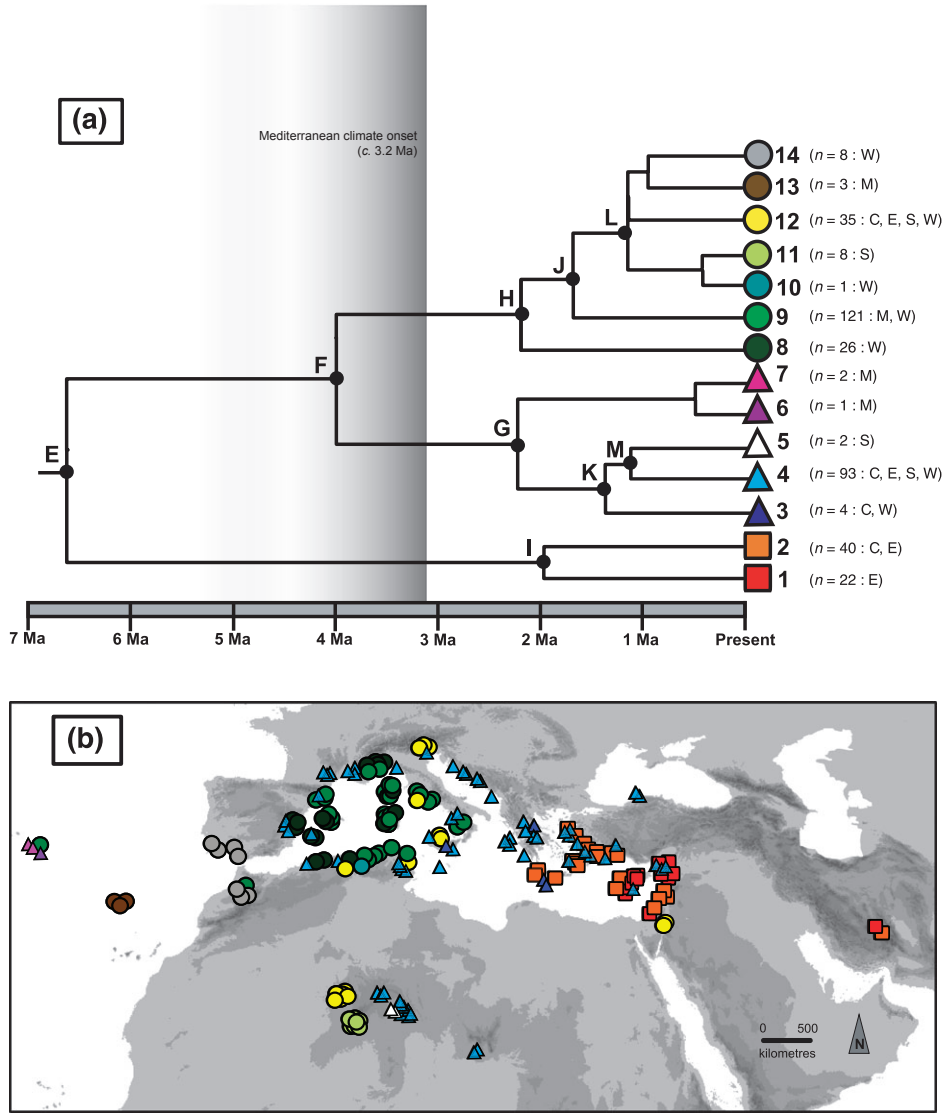


Figure 4 (a) Chronogram based on a Bayesian analysis of cpDNA *trn*L–F and *rpl*2–*trn*H haplotypes of *Myrtus*, using a Bayesian skyline plot coalescent model of demographical history, and a relaxed and uncorrelated log normal molecular clock calibrated according to the nrDNA phylogeny (BEAST 1.6). Only the 14 cpDNA haplotypes are represented, with their sizes and their ranges of distribution within the five biogeographical regions defined in Fig. 1 indicated in brackets. The letters on each node refer to the molecular datings and areas of ancestral reconstruction detailed in Table 3. (b) The geographical distribution of cpDNA haplotypes using the same symbols shown in the chronogram in panel (a).

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Table 3 Estimated ages of diversification for the main nodes of *Myrtus communis* and *M. nivellei* defined in the cpDNA chronogram (Fig. 4), and their 95% highest posterior density (HPD), using BEAST 1.6 software. Hypothetical ancestral areas were reconstructed from the statistical dispersal–vicariance analysis (S-DIVA) method implemented in RASP, with the support percentages of different hypothetical ancestral areas where several possibilities were detected.

Node characteristics		Mean divergence time estimate (Ma)	95% HPD	Epoch	Hypothetical ancestral area determined with RASP (S-DIVA method)
E	Genus *Myrtus* (macrofossil calibration)	6.62	4.33–8.36	Neogene–Late Miocene	C, E, M, S, W
F	Divergence haplotypes 3–7 vs. haplotypes 8–14	3.98	0.93–6.61	Neogene–Early Pliocene	C, M, S, W
G	Divergence haplotypes 3–5 vs. haplotypes 6–7	2.22	0.24–4.13	Pleistocene	C, M, S, W (43%) or C, M, S (38%)
H	Divergence haplotype 8 vs. haplotypes 9–14	2.19	0.36–4.17	Pleistocene	W
I	Divergence haplotypes 1 and 2	1.96	0.15–4.42	Pleistocene	E
J	Divergence haplotype 9 vs. haplotypes 10–14	1.68	0.16–3.35	Pleistocene	W
K	Divergence haplotype 3 vs. haplotypes 4–5	1.36	0.13–2.52	Pleistocene	C, W, S
L	Divergence haplotypes 10–14	1.14	0.11–2.29	Pleistocene	W (53%) or W, S (38%)
M	Divergence haplotypes 4 and 5	1.11	0.15–1.97	Pleistocene	W, S (57%) or S (34%)

C, Centro-Mediterranean; E, Eastern Mediterranean; M, Macaronesia; S, Sahara; W, Western Mediterranean.

western Mediterranean lineages. Within these three major lineages (western, peri- and eastern Mediterranean lineages), differentiation events seem to have occurred during similar periods in the early Pleistocene (see nodes G, H and I in the cpDNA chronogram). The more recent Pleistocene differentiation events (nodes J–M, Fig. 4) gave rise to the present cpDNA haplotype phylogeny and diversity.

As noted above, the cpDNA chronogram reports multiple origins rather than a single origin for *M. nivellei*. The cpDNA haplotypes observed for *M. nivellei* populations diverged around 1.36–1.14 Ma (node K: 95% HPD of 0.13–2.52 Ma, and node L: 95% HPD of 0.11–2.29 Ma), and the node M led to the divergence of the private Saharan haplotype 5 around 1.11 Ma (node M: 95% HPD of 0.15–1.97 Ma), suggesting that *M. nivellei* was present in the Sahara during the Pleistocene (but see the S-DIVA results below).

The ancestral ranges that were inferred using the S-DIVA method implemented in RASP are summarized in Table 3. A probable ancient widespread distribution of *Myrtus* (node E) was revealed, with a vicariance event separating an eastern Mediterranean lineage (node I) from the rest of the distribution (node F). Given that the ancestral ranges of node H were distributed in the western Mediterranean region, migration events must have occurred from the west to central and eastern Mediterranean regions (cpDNA haplotype 12), to Macaronesian islands (haplotypes 9 and 13), and also to Saharan mountains (haplotypes 11 and 12). Ancestral ranges of node G were distributed everywhere except in the eastern part of the Mediterranean Basin, therefore another migration event can be inferred from the west to the east of the Mediterranean region, according to the widespread distributed haplotype 4.

To summarize, the S-DIVA analysis suggested: (1) the vicariance of western and eastern Mediterranean populations, (2) ancient occurrences of *Myrtus* before the node E for Saharan mountains and Macaronesian archipelagos (but this could be a bias of the method, see the Discussion), with also recent immigrations of new cpDNA haplotypes from the western Mediterranean regions, and (3) recent west-to-east migrations through the Mediterranean Basin.

DISCUSSION

Persistence over the Cenozoic and east–west differentiation of *Myrtus communis*

Macrofossils of ancestral *Myrtus* are dated from the Miocene and are derived from the Mediterranean Basin (de Saporta, 1863), although some macrofossils have also been discovered in the Eocene–Oligocene deposits from central Europe (Palamarev, 1989; Bozukov *et al.*, 2009). The Miocene origin of *Myrtus* appears to be a conservative hypothesis, which would involve its long-term persistence in the Mediterranean Basin and adjacent regions (Macaronesian and Irano-Turanian regions) over the Cenozoic era. The molecular dating, which was based on the nrDNA phylogeny calibrated with various fossils, predicted the origin of the extant lineages of *Myrtus* in the late Miocene (*c.* 6.69 Ma: node E, Fig. 3 and Table 1). This hypothesis is supported by the most ancient pollen fossils of *M. communis* dated from the Miocene, and described in Spain (Bessedik & Cabrera, 1985; Rivas-Carballo, 1991).

Reconstructions of ancestral ranges on the basis of the cpDNA chronogram suggested that the widespread distribution of *Myrtus* occurred from the late Miocene (Table 3, node E). Some taphonomical and sampling biases, linked to fragmentary palaeoecological records, limit the number of fossil data that cover the entire Mediterranean region throughout the time period in question. However, palaeobotanical occurrences of *M. communis* testify to its widespread distribution (from the Iberian Peninsula, Ligurian Alps, northern

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Italy, southern Balkan Peninsula, to the Kroumirie region in north-western Tunisia, and the Palestinian region; Fig. 2) even during the Pleistocene climatic oscillations. This ancient and continuous circum-Mediterranean distribution of *Myrtus* differs from that described for *Erica arborea*, which was affected by two waves of expansion of its range from eastern Africa/Arabia (Désamoré *et al.*, 2011).

The cpDNA chronogram (Fig. 4) and genetic diversity patterns (Table 2) revealed a strong degree of genetic differentiation between the eastern and western Mediterranean populations of *M. communis*. An east–west structure of intraspecific genetic diversity across the Mediterranean region has been described in most of the phylogeographical studies of species with Neogene origin, such as *Olea europaea* (Besnard *et al.*, 2007a, 2009) and *Laurus* spp. (Rodríguez-Sánchez *et al.*, 2009). This bipolarization of genetic diversity across the Mediterranean region has been interpreted to be the result of significant changes during the Cenozoic in the palaeogeography and palaeoclimatology of this region (Oosterbroek & Arntzen, 1992; Petit *et al.*, 2005). Substantial environmental changes occurred during this period, which caused the 'serial extinction' of numerous palaeotropical taxa and the expansion of sclerophyllous and xerophytic assemblages (e.g. Suc *et al.*, 1999; Svenning, 2003; Postigo-Mijarra *et al.*, 2009; Tzedakis, 2009). During the Neogene, the progressive climatic transition from the warm conditions that characterized the early Miocene to the cooler Pleistocene period (Fauquette *et al.*, 1999; van Dam, 2006) led to a decrease in habitat suitable for plants with 'tropical affinities' (Herrera, 1992; Svenning, 2003). In the recent review by Postigo-Mijarra *et al.* (2009), the disappearance of *c.* 100 plant taxa in Iberia was attributed largely to climatic changes that led to a continuous fragmentation of range during the Neogene period. The demise of the tropical–subtropical climate was intensified by the Messinian salinity crisis, which occurred between 5.96 and 5.33 Ma (CIESM, 2008). This event, which was associated with huge changes in connections of land masses, coastlines and hydrography of the Mediterranean, induced well-established processes of speciation and differentiation (e.g. *Androcymbium*: Caujapé-Castells & Jansen, 2003; *Stauracanthus*: Pardo *et al.*, 2008; *Ranunculus*: Emadzade *et al.*, 2011). Interestingly, the Messinian salinity crisis occurred during the period limited by nodes E and F within the cpDNA chronogram of *Myrtus*. A tempting hypothesis would be that this crucial event has caused the vicariance, and isolation of western and eastern Mediterranean populations of *Myrtus*. However, the molecular dating methods are still too imprecise (see the 95% HPD of nodes E and F) to precisely infer the evolutionary importance of such a brief geological event.

Despite the drastic palaeoenvironmental changes that have occurred, relict populations of flora of the ancient subtropical laurel forest have persisted up to the present day in geographically restricted areas, which act as climatic refugia (Mejías *et al.*, 2007). Indeed, the fragmented distributions of *Buxus balearica*, *Prunus lusitanica* and *Rhododendron ponticum*, which are found within narrow ecological niches in palaeorefugia on both sides of the Mediterranean Basin, represent the disruption of previously continuous distribution ranges (Milne, 2004; Mejías *et al.*, 2007; Calleja *et al.*, 2009). In contrast, populations of *M. communis* have probably persisted in a greater range of refugia, from moist gorges and small valleys to riparian or even marshy habitats, where the species still grows today. The less strict ecological and climatic requirements of *M. communis* are also supported by its capacity to colonize Mediterranean matorrals.

Plio-Pleistocene differentiation and migrations of *Myrtus communis*

A second phase of differentiation occurred during the Plio-Pleistocene, after the onset of the Mediterranean climate 3.2 Ma (Suc, 1984). The branching patterns of *Myrtus* cpDNA haplotypes reveal that, during this phase, at least seven differentiation events occurred within the three major *Myrtus* lineages (nodes G–M, from 2.22 Ma with a 95% HPD of 0.24–4.13 Ma; Table 3). The reconstruction of ancestral areas supports the hypothesis that the western Mediterranean Basin has acted as the main centre of recent differentiation for *M. communis* because six of the seven differentiation events occurred in this area (Table 3). It was also evidenced with the lowest values of pairwise differences (π) (Table 2) observed in the western Mediterranean region despite the highest sampling effort and the high number of cpDNA haplotypes in this region. Surprisingly, the eastern lineage did not experience such differentiation (node I only).

The cpDNA phylogeny and the reconstruction of ancestral areas suggest that distinct migration events started from the western part of the Mediterranean Basin (Fig. 4 and Table 3). A west-to-east migration is the likely cause of the high frequency and wide distribution of *Myrtus* carrying haplotype 4, in particular in the Balkan Peninsula. Distinct migration events from western Mediterranean populations to the Macaronesian islands are also well supported (Fig. 4), as it is the case for *Erica arborea* (Désamoré *et al.*, 2011). *Myrtus* occurred in the Macaronesian archipelagos before node E (Table 3), but it is perhaps a bias of the S-DIVA method for this basal node because S-DIVA is conservative for the hypothesis of vicariance. The connection of haplotypes 6 and 7 on node G could also support the hypothesis that the migration event to the Azores of their ancestral haplotype occurred between nodes F and G, and then was followed by their divergence. A third hypothesis could be that this dispersal event occurred after node G and that their continental ancestor went extinct after this. Although the Azorean flora exhibits some affinities with the Eurosiberian region, our data did not provide a clear cut off between these hypotheses. In any case, our results highlight the hypothesis that migrations of *Myrtus* towards the Azores and Madeira islands occurred repeatedly. The Macaronesian islands are indeed characterized by the coexistence of ancient lineages inherited from the Tethyan–Tertiary ecosystems and more recent lineages coming from western Mediterranean regions or further north European regions where *Myrtus* is now extinct.

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We found that the Strait of Gibraltar does not constitute a barrier to gene flow for *M. communis* because cpDNA haplotype 14 was present in the populations of both the Iberian Peninsula and the Maghreb. Similar results have been found for *Quercus suber* (Magri *et al.*, 2007) and several other plants (review of Rodríguez-Sánchez *et al.*, 2008). There was also a close phylogeographical link between Tunisia, Sicily and the Italian Peninsula (haplotypes 4 and 12), which could correspond to a confluence of migratory routes for different birds. The involvement of birds in the distribution of *Myrtus* is supported by the known ornitochory dispersal of the fleshy fruits of *Myrtus*; this dispersal is one of the most successful means of diaspore movement for Mediterranean shrubs (Hess *et al.*, 2000; Valcárcel *et al.*, 2003). For *Myrtus*, ornitochory is facilitated by the persistence of fleshy fruits over several weeks, and by the increased rate of the germination of seeds after ingestion by birds (Traveset *et al.*, 2001). The importance of ornitochory is supported by the existence of gene flow between populations in the fragmented landscape of the Guadalquivir river valley in southern Spain (Albaladejo *et al.*, 2008). The homogeneity of certain insular (Corsica and Sardinia) and Balkan populations are likely to be the result of very recent expansions, which provide another support for the capacity of *Myrtus* to migrate across the Mediterranean Basin.

Origins of *Myrtus nivellei* in the Saharan mountains

Phylogenetic inferences from nrDNA and cpDNA sequences are incongruent regarding the origin of *M. nivellei*. According to the nrDNA phylogeny, the current *M. nivellei* populations have a single origin, and became isolated well after the origin of *M. communis*. The S-DIVA analysis performed on the cpDNA phylogeny supports an ancestral Saharan distribution of *Myrtus* lineages (see nodes E, F, G and K; Table 3). But, Tertiary fossil data are too scarce to reject this hypothesis (Maley, 2010). Both nrDNA and cpDNA phylogenies revealed that the history of *M. nivellei* is included in the one of *M. communis*. Besnard *et al.* (2007a) also identified this type of close phylogenetic link between two disjunct subspecies of *Olea europaea*, the central Saharan subsp. *laperrinei* and the Mediterranean subsp. *europaea*, using chloroplast markers, but incongruencies between cpDNA and nrDNA markers, and hybridization phenomena distorted the phylogeographical signal. The cpDNA phylogeny also revealed at least two migration events from Mediterranean to the Sahara. To the best of our knowledge, this phylogeographical analysis of *Myrtus* constitutes one of the first molecular studies to demonstrate the migration of plants from Mediterranean shores to the central Saharan mountains. Therefore, *M. nivellei* can be regarded as a subset of *M. communis* populations that have become isolated well beyond the limits of the Mediterranean range. These results point to a striking ability of a Mediterranean plant to migrate and persist locally despite extreme changes in environment.

The presence of cpDNA haplotypes that are restricted to single parts of the Saharan mountain ranges (Fig. 4) indicates that the current populations of *M. nivellei* have been isolated from each other since at least the late Pleistocene (cf. node M, *c.* 1.11 Ma, Table 3). The monophyly of nrDNA sequences in *M. nivellei* and the occurrence of fossil pollen outside its current distribution range (notably further south near Lake Chad during the late Pleistocene; Maley, 1981; Fig. 2) can be explained by the succession of pluvial and arid phases in the Sahara that have punctuated the climatic history of this desert (Szabo *et al.*, 1995; Maley, 2010) since its establishment 7 Ma (Schuster *et al.*, 2006). Pluvial humid periods have favoured the establishment of biological corridors between Mediterranean and central Saharan ecosystems (Quézel & Martinez, 1960; Osborne *et al.*, 2008), and certainly between Saharan mountains. These corridors have promoted gene flow between populations of *M. nivellei*, and probably genetic admixture that could explain the homogeneity of its nrDNA sequences. In contrast, the arid phases and desertification processes that have occurred might have reduced the distribution range of *M. nivellei*. Such a reduction in range has been modelled from the distribution of the fossil pollen of several tropical plants from the end of the last Pluvial period (6000 years ago), a period better known as the 'Green Sahara' (Watrin *et al.*, 2009).

The surprising absence of differentiation between Algerian populations of *M. nivellei* and the Tibesti mountain population (Chad: subsp. *tibesticus*; Quézel, 1958) reinforces the possibility of past habitat connectivity or long-distance gene flow between isolated parts of the Saharan mountain ranges. In the same way, rare and long-distance gene flow for the endozoochorous *Olea europaea* subsp. *laperrinei* was supported by nrDNA data, despite the fact that the analysis of cpDNA markers suggested that Saharan mountain populations of this species are isolated genetically (Besnard *et al.*, 2007b). The analysis of these central Saharan populations of *Myrtus*, with AFLP (amplified fragment length polymorphism) markers could help to better understand the dynamics of gene flow between these populations (J. Migliore, A. Baumel, M. Juin, B. Fady, A. Roig & F. Médail, submitted article).

It may be concluded that the central Saharan mountains constitute crucial biogeographical refugia for Mediterranean plants that are unable to survive in arid habitats below 1500 m a.s.l. Several other plants with Mediterranean relatives persist within the central Saharan mountains: the Duprez cypress (*Cupressus dupreziana*), the tree heath (*Erica arborea*), the shrubby globularia (*Globularia alypum*), Laperrine's olive (*Olea europaea* subsp. *laperrinei*), *Nerium oleander* and *Rhus tripartita* (Quézel, 1965). In the future, comparative phylogeographical studies on Saharan–Mediterranean taxa should help us to understand better the biogeography of these central Saharan refugia.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Review of all the sampled populations of *Myrtus communis* and *M. nivellei*, with their precise location and their GPS coordinates.

Appendix S2 (a) List of the sites where *Myrtus* palaeobotanical occurrences were found, with associated references, and (b) chronozone reminder based on the geological timescale edited by The Geological Society of America (2009).

Appendix S3 (a) List of haplotypes of *Myrtus communis* and *M. nivellei* obtained by performing cpDNA and nrDNA sequencing, together with their GenBank accession numbers, (b) references and GenBank accession numbers of Myrtaceae sequences included in the matrix of alignment for the nrDNA phylogenetic study, and (c) table of cpDNA haplotypes of *Myrtus communis* and *M. nivellei* with the variable sites detected.

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BIOSKETCH

Jérémy Migliore's research interests include phylogeography and population genetics of Mediterranean and Saharan plant species. He received his PhD at Aix-Marseille University Mediterranean Institute for Biodiversity and Ecology (IMBE) in 2011 focusing on the imprints of environmental changes on the evolution and persistence of the genus *Myrtus*, with a perspective of conservation biogeography.

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Author contributions: J.M., A.B. and F.M. planned this study and were responsible for the writing of the article, and also participated to the collection of the field data; J.M. and M.J. performed the molecular biology work, after which J.M. and A.B. focused on the molecular analyses.

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