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PLASTID DNA HOMOGENEITY IN *CELTIS AUSTRALIS* L. (CANNABACEAE) AND *NERIUM OLEANDER* L. (APOCYNACEAE) THROUGHOUT THE MEDITERRANEAN BASIN

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Premise of research. Riparian plants are highly dependent on water sources; consequently, general climatic conditions are less important to these taxa relative to woodland and shrubland species. This leads to interesting research questions regarding riparian plant taxa. Research on phylogeography of Mediterranean riparian tree and shrub species is scarce. In this article, we investigated the plastidial genetic diversity in *Celtis australis* L. (hackberry) and *Nerium oleander* L. (oleander) throughout the Mediterranean Basin. Both species are distributed in gullies, rivers, and stream banks under warm temperate climates.

Methodology. Eighteen cpSSR loci and three noncoding cpDNA regions (*rps*16, *rpl*32-*trn*L, and *trn*Q-5′-*rps*16) were examined to assess the levels and geographic distribution of sequence variation in 41 hackberry and 56 oleander populations. The *rpl*32-*trn*L intergenic region was used for molecular dating analysis.

Pivotal results. The respective 2762- and 3134-bp noncoding cpDNA regions sequenced in *C. australis* and *N. oleander*, as well as the 22 cpSSR analyzed fragments, exhibited the absence of variability in natural populations throughout the Mediterranean Basin. In *N. oleander*, two regions (*rps*16, *rpl*32-*trn*L) exhibited variability in three positions, and four cpSSR microsatellite motifs were polymorphic. The polymorphisms were geographically structured, and three haplotypes were characterized, two from Saharan populations and one from Mediterranean populations. Phylogeny and molecular dating analyses resulted in a tree with high consistency values of posteriori probability (PP = 1) and bootstrap support (98%) from the ingroup (*N. oleander*). The relaxed molecular clock model applied to the calibration of the tree estimates that the diversification of these haplotypes occurred in a range of 7.2–1.2 Ma.

Conclusions. The absence of plastid variability in both hackberry and oleander is explained by a low mutation rate and/or recent recolonization of the Mediterranean Basin. Low temperatures during the Last Glacial Maximum produced freezing water along riparian corridors, with the consequent drastic contraction or even disappearance of both species in the Mediterranean Basin.

Keywords: cpDNA, genetic variability, Mediterranean Basin, phylogeography, riparian plants.

Introduction

The unique aspects of the Mediterranean flora have long drawn the attention of ecologists and biogeographers (Nieto-Feliner 2014). The soil mosaic and varied climatic conditions together with a large number of islands in the Mediterranean and a diverse geologic history of the Mediterranean Basin have resulted in a high degree of endemic species (Thompson 2005; Blondel et al. 2010). Moreover, the Mediterranean flora is represented by floristic elements of different geographic origins, i.e., a mix of relict species of subtropical origin that existed prior to the onset of the current Mediterranean climate (Thompson 2005; Blondel et al. 2010). In addition, long and persistent anthropogenic influences (Rundel et al. 1998; Thompson 2005; Blondel et al. 2010) have modified the Mediterranean flora and are the primary cause of threat and even extinction of many species. Consequently, individual plant populations have experienced repeated isolation, and populations of different origins have undergone admixture over the history of the basin.

Biogeographical studies of the Mediterranean Basin have indicated the marked influence of glaciations on the present distribution of animal and plant species (Comes and Kadereit 1998; Taberlet et al. 1998; Hewitt 2000, 2004). Inasmuch as the southern European peninsulas were less affected by glaciations, many authors unequivocally agree that refugia were important

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during the Last Glacial Maximum (LGM; 20,000–18,000 BP; Comes and Kadereit 1998; Hewitt 2000; Petit et al. 2002; Thompson 2005; Médail and Diadema 2009). In southern regions, Mediterranean species might have persisted in isolated and scattered glacial refugia, similar to cryptic refugia, which are distributed at latitudes or longitudes that differ from what would nowadays be expected and can be described as climatic islands of favorable conditions (Anderson et al. 2006; Cheddadi et al. 2006; Petit et al. 2008). In addition to the effects of Quaternary glaciations, recent studies have emphasized the importance of older geologic events, such as Tertiary plate tectonics in *Quercus suber* L. and *Taxus baccata* L. (Magri et al. 2007; González-Martínez et al. 2010).

Due to the economic and ecological importance of Mediterranean woodlands and shrublands, most phylogeographic research on Mediterranean plant species has addressed how these unique biomes were derived (Taberlet et al. 1998; Magri et al. 2007; Rodríguez-Sánchez et al. 2009); however, riparian plants have been excluded, despite the interesting research questions inherent in riparian plant taxa. Riparian plants exhibit morphologic and physiologic adaptations to water sources; consequently, general climatic conditions are less important to these species relative to woodland and shrubland species. Riparian species are typically distributed along watercourses and margins of lakes or ponds, resulting in increased soil moisture with periods of flooding and inundation. These habitats are buffered to reduce the effects of climatic oscillations, making them good candidates for refugia within the refugia (Gómez and Lunt 2007; Macaya-Sanz et al. 2012).

When riparian plant populations bordering watercourses are isolated, genetic differentiation among them is an expected consequence. Nevertheless, both wind pollination and anemocorous seed dispersal may disperse beyond the floodplain. Results on several European and North American *Populus* (Salicaceae) species (Cottrell et al. 2005; Breen et al. 2009; Fussi et al. 2010; Chenault et al. 2011; Macaya-Sanz et al. 2012), Chinese *Euptelea pleiospermum* (Wei et al. 2013), and Mediterranean populations of *Frangula alnus* Miller (Rhamnaceae; Hampe et al. 2003; Petit et al. 2005) in the southern part of the species range confirmed the isolation of populations among river basins.

In this article, we evaluated chloroplast DNA genetic variability in *Celtis australis* L. (Cannabaceae; hackberry) and *Nerium oleander* L. (Apocynaceae; oleander) populations throughout the Mediterranean Basin. Both species are distributed in warm temperate climates, adjacent to river and stream banks, and in gullies. Birds are the primary mode of dispersal in hackberry, but occasionally mammals play a dispersal role (Traba et al. 2006). Hackberry grows on deep nonhydromorphic soils with high winter moisture levels, becoming superficially dry during summer as well as on rocky slopes. The species is a member of mixed Mediterranean subriparian forests with *Ulmus* and sometimes *Populus* species.

Several species of *Celtis* are known from the Mediterranean Basin (Tutin 1964); however, *C. australis* is the most widely distributed species (Maire 1961). Despite palaeobotanical data (Palamarev 1989; Magyari et al. 2008) indicating the presence since the Oligocene of the *C. lacunose* Kirchh. group, in which *C. australis* is included, the species has not been recognized in the Iberian Peninsula until the recent work of Allué et al. (2015). These authors recorded 91 seed remains placed in the *Aurora stratum* of TD6, which has been dated by different authors in a range of 963–668 ka BP (Bermúdez de Castro et al. 2011; Carrión et al. 2012; Parés et al. 2013). Molecular data on the genus are scarce. Lee et al. (2011) were successful in differentiating between the two morphologically similar species *C. sinensis* Persoon and *C. formosana* Hayata using ISSR and ITS markers. However, de Castro and Maugeri (2006) reported that the Mediterranean *Celtis* species are a rather homogeneous group with low molecular variability.

Nerium oleander belongs to the monospecific genus *Nerium*. The species is so widely cultivated that no precise region of origin has been identified, although southwest Asia has been suggested. The species is considered native to the Mediterranean region, the Indian subcontinent, and western China. The presence of oleander around the Mediterranean Basin has been documented since the Miocene (Palamarev 1989). Oleander grows along temporary streams, primarily ravines, and other highly seasonal streams where flooding is common during spring and autumn; typically, these streams remain dry for several months during summer. Populations are usually distributed below 1000 m in continental Spain and 2000 m and above for North African populations and the Atlas Mountains (Herrera 1991). The only previous research on the species using molecular markers is that of Portis et al. (2004), which successfully distinguished commercial varieties via amplified fragment length polymorphisms.

Based on the previous results on the genetic isolation among individuals of different basins of riparian plant species, the evolutionary histories of the studied species in the Mediterranean Basin, the decreased effects of climate on riparian zones relative to Mediterranean woodland and scrubland biomes, and the role of southern Mediterranean peninsula refugia in genetic diversity/diversification, we aimed to investigate hackberry and oleander plastidial DNA genetic variability throughout the Mediterranean Basin to answer questions about whether genetic variability and structure are similar in both species and whether the populations of different river basins are genetically separate, as previously reported for riparian species.

Methods

Sampling

A broad sampling throughout the Mediterranean Basin was conducted to obtain a representative sample of *Celtis australis* and *Nerium oleander* populations, covering most of the species range in the Mediterranean Basin (fig. 1). Sample population data are provided in tables 1 and 2. A total of 39 populations of *C. australis* and 53 populations of *N. oleander*, with eight individuals per population, were sampled.

DNA Extraction and Sequencing

DNA was extracted from 0.5 g of dried leaf material, following Khanuja et al. (1999). Twenty-two plastid simple sequence repeat (cpSSRs) loci, distributed in different regions along the chloroplast with no overlapping among them or with the sequenced regions, were screened using the universal primer pairs ccmp2, ccmp3, ccmp4, ccmp6, ccmp7 (Weising and Gardner 1999), 4SSR, 5SSR, 6SSR, 7SSR, 9SSR, 12SSR, 13SSR, 14SSR, 15SSR,

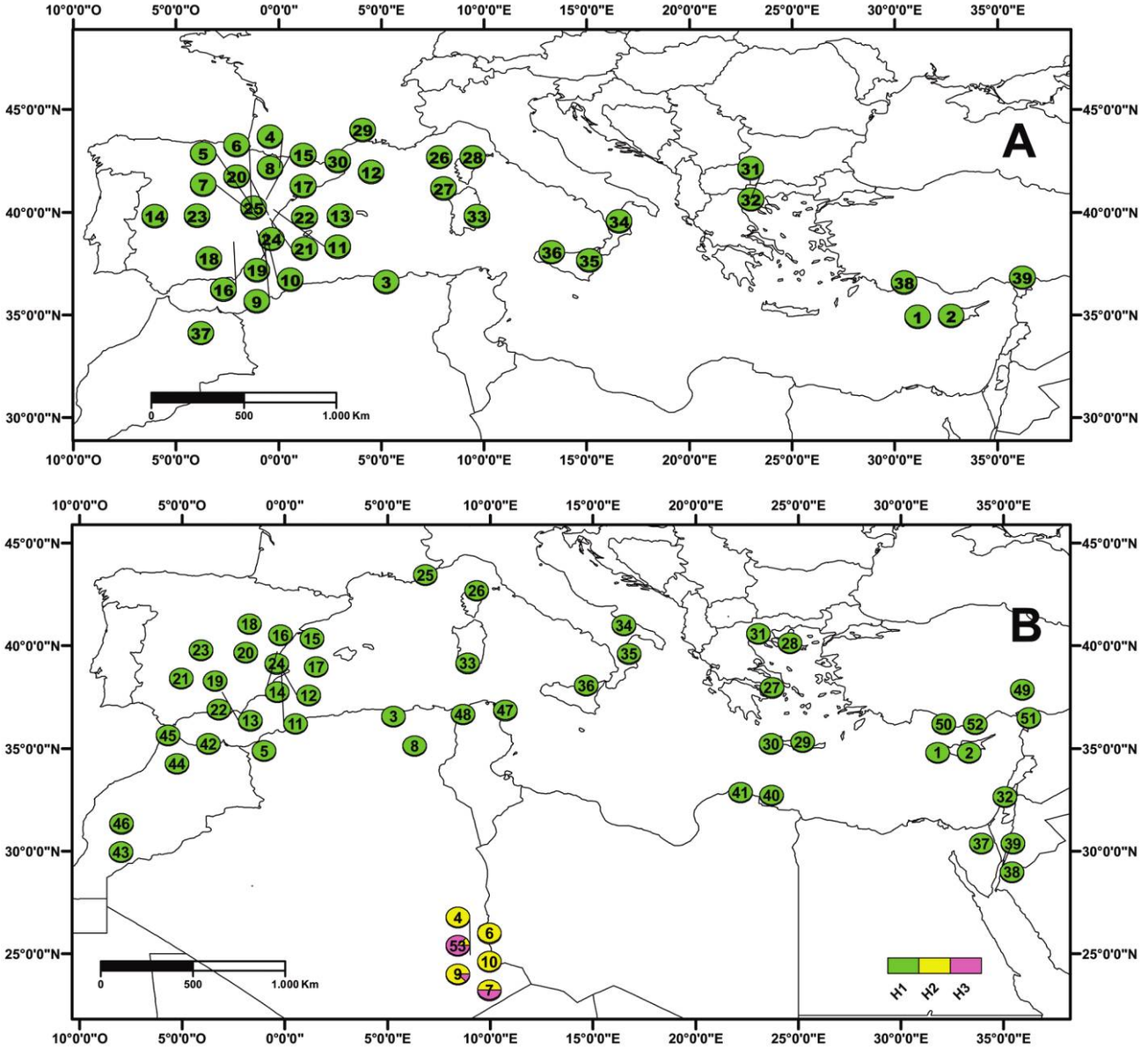

Fig. 1 Geographical distribution of haplotypes identified by cpSSR genotyping among populations of *Celtis australis* (*A*) and *Nerium oleander* (*B*). Numbers correspond to those given in table 5. H1, H2, and H3 designate the three haplotypes of *N. oleander*.

Table 1

Geographical Data of the Sampled Populations of *Celtis australis*

No.	Code	Country	Population	Province	Latitude	Longitude
1	CYDR	Cyprus	Drousha	Paphos	34°56′09″N	32°24′21″E
2	CYKK	Cyprus	Kikkos	Nicosia	34°58′59″N	32°44′29″E
3	DZCA	Algeria	Cap Aokas	Bejaia	36°38′38″N	05°13′55″E
4	ESA	Spain	Alquézar, Sierra de Guara	Huesca	42°10′33″N	00°01′39″E
5	ESAL	Spain	Alpuente	Valencia	39°52′58″N	01°00′42″W
6	ESBH	Spain	Ademuz	Valencia	40°03′24″N	01°18′46″W
7	ESCL	Spain	Calles	Valencia	39°43′25″N	00°58′28″W
8	ESHU	Spain	Castilsabas, Sierra de Guara	Huesca	42°11′56″N	00°18′29″W
9	ESCA	Spain	Enguera-Ayora	Valencia	38°56′48″N	00°43′30″W
10	ESES	Spain	Estubeny	Valencia	39°01′10″N	00°37′25″W
11	ESFI	Spain	Figueroles	Castellón	40°07′12″N	00°14′22″W
12	ESGI	Spain	Colina de Montjuich	Girona	41°59′14″N	02°49′42″E
13	ESLP	Spain	Road Pollença-Lluc	Mallorca	39°52′18″N	02°59′00″E
14	ESMO	Spain	Villareal de San Carlos	Cáceres	39°50′46″N	06°01′34″W
15	ESP	Spain	Pitarque	Teruel	40°38′42″N	00°35′49″W
16	ESPO	Spain	Potiche	Albacete	38°33′15″N	02°10′40″W
17	ESPV	Spain	Picamoixons, Valls	Tarragona	41°18′23″N	01°11′37″E
18	ESRC	Spain	Río Cuadros, Bédmar	Jaén	37°47′17″N	03°24′32″W
19	ESRZ	Spain	Río Zarra, Zarra	Valencia	39°06′04″N	01°03′43″W
20	ESSN	Spain	Salto Novia, Navajas	Castellón	39°52′25″N	00°29′56″W
21	ESSR	Spain	Serra	Valencia	39°41′27″N	00°25′11″W
22	ESTA	Spain	Tales	Castellón	39°57′29″N	00°18′14″W
23	ESTO	Spain	Pinar de la Bastida	Toledo	39°51′26″N	03°58′05″W
24	ESV2	Spain	La Murta, Xátiva	Valencia	39°07′43″N	00°21′40″W
25	ESVT	Spain	Bco. del Tranco, Villel	Teruel	40°15′14″N	01°13′01″W
26	FRCA	Spain	Casta, Agriates	Corsica	42°39′47″N	09°13′11″E
27	FRCO	France	Corte	Corsica	42°18′47″N	09°09′17″E
28	FRCO2	France	Near Bastia	Corsica	42°42′51″N	09°27′17″E
29	FRGA	France	Vic-le-Fesq	Gard	43°51′59″N	04°05′14″E
30	FRPA	France	Montesquieu des Alberes	Pyrénées E	42°31′06″N	02°52′40″E
31	GRFI	Greece	Filiro	Tesalonica	40°39′32″N	22°58′45″E
32	GRRE	Greece	Redziki	Tesalónica	40°39′22″N	22°59′48″E
33	ITBS	Italy	Bari-Sardo	Sardinia	39°50′38″N	09°39′22″E
34	ITCN	Italy	Rossano-Corigliano	Calabria	39°35′15″N	16°34′38″E
35	ITS2	Italy	Zafferana Etnea	Catania	37°41′10″N	15°08′08″E
36	ITSN	Italy	Monreale	Palermo	38°05′29″N	13°18′11″E
37	MOJT	Morocco	Jebel Tazekka	Taza	34°08′56″N	03°47′20″W
38	TRAN	Turkey	Kesmezagaz	Antalya	36°36′44″N	30°26′48″E
39	TRHA	Turkey	Dörtyol	Hatay	36°51′14″N	36°12′48″E

16SSR, 17SSR, 18SSR, 19SSR, 20SSR, 21SSR, 22SSR, and 23SSR (Chung and Staub 2003). PCR amplifications were performed in a 25-μL total reaction volume with a fluorochrome tag at the 5′ end of each forward primer. The amplification conditions were the same as applied in Mateu et al. (2013). All reactions were performed in an Eppendorf Mastercycler-Pro thermalcycler (Hamburg, Germany). Sequences of both fragments and noncoding regions had been deposited in GenBank. Accession numbers are given in table 3.

Resulting fluorescent PCR product sizes were established using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) at the SCSIE DNA and Protein Sequencing unit of the Universitat de València, and cpSSR fragment sizes were determined using the Peak Scanner software package, version 1.0 (Applied Biosystems). Results were repeated at least twice until all loci had been reliably scored in all samples. Variable fragments were studied in eight individuals per population. Data on primer composition, chloroplast genome location, motif, and cpSSR fragment size are provided in table 4.

Three noncoding chloroplast regions (*rps*16, *rpl*32-*trn*L, and *trn*Q-5-*rps*16) described in Shaw et al. (2005, 2007) were sequenced in one individual per population. Primers described in Shaw et al. (2005, 2007) were used to amplify each locus. PCR amplifications were performed in a 20-μL total reaction volume. The amplification conditions were those explained in Shaw et al. (2005, 2007). All reactions were performed in an Eppendorf Mastercycler-Pro thermalcycler. PCR products were purified using the Illustra DNA and gel band purification kit (GE Healthcare). The resulting fragments were sequenced in direct and reverse directions using BigDye terminator cycle sequencing (Applied Biosystems). Sequence reactions were performed in the ABI 3730 analyzer, as mentioned above. Sequence results were edited with BioEdit (available at http://www.mbio.ncsu.edu/BioEdit) and aligned with Clustal W implemented in

Table 2

Geographical Data of the Sampled Populations of *Nerium oleander*

No.	Code	Country	Population	Province	Latitude	Longitude
1	CYKO	Cyprus	Kozan	Kyrenia	35°18′55″N	33°08′21″E
2	CYLK	Cyprus	Likipos-Kornos	Larnaka	34°54′01″N	33°18′53″E
3	DZCK	Algeria	Cascade Kefrida	Bejaia	36°34′14″N	05°17′21″E
4	DZOE	Algeria	Oued Essendilene	Djanet	24°57′32″N	09°00′30″E
5	DZOI	Algeria	Oued Isser	Tlemcen	34°53′07″N	01°01′13″W
6	DZOT	Algeria	Oued Tamrit	Djanet	24°37′45″N	09°38′54″E
7	DZOT2	Algeria	Oued Touia	Djanet	24°27′59″N	09°42′19″E
8	DZOTK	Algeria	Oued T'Kout	Batna	35°08′05″N	06°18′45″E
9	DZS	Algeria	Sefar	Djanet	24°39′36″N	09°44′45″E
10	DZTI	Algeria	Tassili n'Ajjer	Djanet	24°37′45″N	09°38′54″E
11	ESA	Spain	Guadalest	Alicante	38°39′49″N	00°09′39″W
12	ESAC	Spain	Tavernes de Valldigna	Valencia	39°04′42″N	00°16′55″W
13	ESCA	Spain	Ceal	Jaén	37°43′24″N	03°02′42″W
14	ESCG	Spain	Serra	Valencia	39°41′41″N	00°22′32″W
15	ESCS	Spain	Sueras	Castellón	39°56′24″N	00°21′02″W
16	ESCS2	Spain	L'Alcora	Castellón	40°06′30″N	00°12′29″W
17	ESIB	Spain	Santa Eulalia	Baleares-Ibiza	38°58′59″N	01°31′39″E
18	ESRA	Spain	Calles	Valencia	39°44′35″N	00°58′00″W
19	ESRC	Spain	Bedmar	Jaén	37°47′16″N	03°24′30″W
20	ESRJ	Spain	Jalance	Valencia	39°11′56″N	01°04′37″W
21	ESRZ	Spain	Belalcázar	Córdoba	38°40′29″N	05°02′20″W
22	EST	Spain	Torvizcón	Granada	36°54′33″N	03°12′40″W
23	ESTO	Spain	Argés	Toledo	39°48′02″N	04°04′57″W
24	ESV2	Spain	La Murta, Xátiva	Valencia	39°08′19″N	00°23′32″W
25	FRCA	France	Agay	Cote d'Azur	43°27′05″N	06°49′56″E
26	FRCO	France	St. Florent	Capicorsú	42°41′40″N	09°20′07″E
27	GRAC	Greece	Acropolis	Atenas	37°58′12″N	23°43′10″E
28	GRHK	Greece	Halkidiki	Cassandra	40°08′05″N	23°49′36″E
29	GRKU	Greece	Lago Kauna	Creta	35°19′40″N	24°16′44″E
30	GRPA	Greece	Paleophora	Creta	35°14′11″N	23°40′32″E
31	GRPL	Greece	Tessaloniki	Tesalónica	40°35′25″N	23°03′11″E
32	ILNA	Israel	Najal Amud	Tiberiades	32°39′10″N	35°03′46″E
33	ITC1	Italy	Cagliari	Sardinia	39°09′43″N	08°54′26″E
34	ITC2	Italy	Trebisacce	Calabria	39°51′33″N	16°30′25″E
35	ITCN	Italy	Capo Trionto	Calabria	39°37′11″N	16°45′05″E
36	ITSN	Italy	Sta. Agata Militello	Messina	38°04′24″N	14°40′14″E
37	JOOJ	Jordan	Wadi Farasa, Petra	Wadi Musa	30°19′15″N	35°26′42″E
38	JOOS	Jordan	Wadi Siyyagh, Petra	Wadi Musa	30°21′22″N	35°24′12″E
39	JOPP	Jordan	Little Petra	Wadi Musa	30°22′25″N	35°26′49″E
40	LIDE	Libya	Dernah	Cyrenaic	32°43′51″N	22°31′12″E
41	LIRA	Libya	Ra's Al Hilal	Cyrenaic	32°51′58″N	22°10′44″E
42	MOAK	Morocco	Akchoud	W Rif	35°14′20″N	05°10′39″W
43	MOAS	Morocco	Asni	Marrakech	31°14′35″N	07°58′35″W
44	MOCH	Morocco	Chaouen	W Rif	35°09′24″N	05°15′01″W
45	MOT	Morocco	Tanger	Tanger	35°38′57″N	05°41′15″W
46	MOTA	Morocco	Tahnaout	Marrakech	31°21′11″N	07°57′23″W
47	TNCB	Tunisia	Cap Bon	Cap Bon	36°52′13″N	10°43′28″E
48	TNFN	Tunisia	Fernana	Aïn Drahan	36°40′12″N	08°40′49″E
49	TRAD	Turkey	Kozan	Adana	37°10′37″N	35°53′57″E
50	TRAN	Turkey	Kemer	Antalya	36°11′30″N	32°25′35″E
51	TRHA	Turkey	Belen	Hatay	36°29′18″N	36°13′00″E
52	TRIC	Turkey	Ovacik	Mersin	36°11′15″N	33°36′25″E
53	DZIH	Algeria	Iherir	Illizi	25°24′10″N	08°25′31″E

Table 3

GenBank References for the Sequenced Fragments and Regions of the Chloroplast in the Two Species Studied

Fragment	*Celtis australis*	*Nerium oleander*
ccmp2	KJ001189	KJ411985
ccmp3	KJ001190	KJ409556; KJ409557
ccmp4	KJ001191	KP330458
ccmp6	KJ001192	KM924827
ccmp7	KJ001193	KP013123
4SSR	KJ001194	KP013124
6SSR	KJ001195	KJ411986
7SSR	KJ001196	KJ411984
9SSR	KJ001197	KJ413934
13SSR	KJ001198	KJ413935
14SSR	KJ001199	KJ413936; KJ413937
16SSR	KP330459	KJ413938
17SSR	KJ001200	KJ433676
18SSR	KP330460	KJ433675
19SSR	KP330461	KJ438814
20SSR	KP330462	KJ438815
21SSR	KP330463	KJ438813
*rpl*32-*trn*L(V1)	KJ000489	KP330464; KP347983
rpS16(B)	KJ000488	KP347986; KP347987; KP347988
*trn*Q-5′-*rps*16(T)	KJ000490	KP347984; KP347985

BioEdit, with subsequent visual inspection to correct errors. Sequences of both fragments and regions were sent to GenBank. References are given in table 3.

Sequences and Fragments Analysis

The variants of the concatenated DNA sequences will be considered as different haplotypes. Parameters of variability were analyzed with DnaSP, version 5 (Librado and Rozas 2009). In fragments, it was verified by sequencing that different sizes corresponding to different numbers repeat the motif. Haplotype distribution in *N. oleander* study populations was represented on the distribution map using ArcGIS, version 9.3.1 (http://www.esri.com). Due to the absence of detected genetic variability in *C. australis* as well as the low genetic variability in *N. oleander* (three haplotypes resolved), no population structure, admixture, or AMOVA analyses were performed. Phylogenetic relationships among plastid haplotypes were evaluated via a statistical parsimony analysis (fig. 2) with the connection probability set to 95% using TCS, version 1.21 (Clement et al. 2000).

Phylogeny and Molecular Dating Analysis

The *rpl*32-*trn*L intergenic region was used for that purpose. The sequences obtained were aligned with Muscle (Edgar 2004) with default parameters, resulting in a matrix with 1059 bp. There was a total of 81 sequences corresponding to 77 oleander individuals and 4 outgroup species. Models of sequence evolution were obtained with JMODELTEST (Darriba et al. 2012). Model fit was assessed using the Akaike information criterion (AIC; Akaike 1981) to find the best evolutionary model. The AIC model was finally chosen for rpl32-trnL phylogeny.

The evolutionary history was inferred by both Bayesian inference and maximum-likelihood methods. MrBayes 3.2.3 software (Ronquist et al. 2012) implementation was performed using the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985) for Bayesian inference. Four chains were used and two million runs executed, making a 25% burn-in coincident with the point where the convergence of the chain is reached. Maximum-likelihood analysis based on the HKY model was conducted with MEGA 6 (Tamura et al. 2013). The bootstrap consensus tree inferred after 500 replicates was taken (Felsenstein 1985), and branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The initial tree for the heuristic search was obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of estimated pairwise distances and then selecting the topology with superior log-likelihood value.

Molecular dating was performed through a relaxed molecular clock approach (Bayesian MCMC method; Drummond et al. 2006) implemented in BEAST v1.7.4 (Drummond et al. 2012) with the following prior parameters: HKY as evolutionary model, constant size as demographic model (Kingman 1982), and one calibration point in the ingroup root. A random starting tree was used to estimate initial root height. To calibrate the tree, information from Palamarev (1989) dating the probable origin of *N. oleander* in the Upper Miocene (interface Tortonian–Messinian age; 7 Ma) was used.

Results

Following multiple sequence alignment, 2762 bp were resolved for the three noncoding cpDNA regions (*rps*16, *rpl*32-*trn*L, and *trn*Q-5′-*rps*16) in *Celtis australis*. Results showed the absence of sequence variability within and among the three regions. Two (5SSR and 23SSR) out of the 22 screened primer pairs did not amplify fragments. PCR product sequencing of the remaining primer pairs did not show the microsatellite motif in 10 pairs (ccmp3, ccmp4, 4SSR, 12SSR, 15SSR, 16SSR, 18SSR, 19SSR, 21SSR, and 22SSR), while the motif was confirmed in 10 others (ccmp2, ccmp6, ccmp7, 6SSR, 7SSR, 9SSR, 13SSR, 14SSR, 17SSR, and 20SSR). The amplified fragments containing the microsatellite motif did not exhibit variability among the 328 individuals studied.

The multiple sequence alignment resolved 3134 bp for the three noncoding cpDNA regions (*rps*16, *rpl*32-*trn*L, and *trn*Q-5′-*rps*16) in *Nerium oleander*. Two regions (*rps*16, *rpl*32-*trn*L) exhibited variability in three positions; however, polymorphisms

Table 4

Data of cpSSRs Showing Variability and Sequenced Regions in *Nerium oleander*

Primer	Location	Motif	Allele sizes (bp)
ccmp2	5′ to *trn*S	(A)	161–162
ccmp3	*trn*G *intron*	(T)	79–80
7SSR	*psb*C-*trn*S	(T)C(T)	352–356
14SSR	*rps*19-*rpl*2	$(T)_8$ $(T)_9$	206–207
B	*rps*16	…	805–806
V	*rpl*32-*trn*L	…	1118
T	*trn*Q-5′*rps*16	…	1211

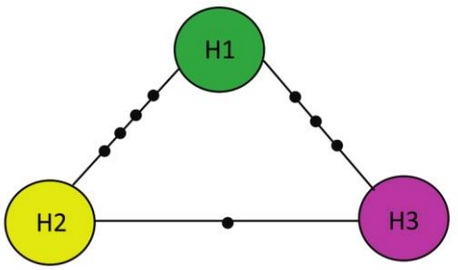

Fig. 2 Haplotype network resulting from the analysis of four polymorphic plastid microsatellites in *Nerium oleander*. Small filled circles = mean mutation changes between haplotypes.

were not detected in the *trn*Q-5′-*rps*16 region. Four variable sites were detected, with one base affected at each position; one transversion (G/T) and three 1-bp indels, two associated with A or T mononucleotide repeats (SSRs) over 10 bp in length. Variability of the sequences studied was combined into three haplotypes (H1, H2, and H3). Analysis of sequences with DnaSP indicated that variable sites were not parsimony informative. Two (5SSR and 23SSR) out of the 22 primer pairs screened for the fragments study did not amplify; 11 out of the 22 primer pairs screened did not show the microsatellite motif (ccmp6, ccmp7, 4SSR, 12SSR, 13SSR, 15SSR, 16SSR, 17SSR, 18SSR, 21SSR, and 22SSR), while the motif was confirmed in nine (ccmp2, ccmp3, ccmp4, 6SSR, 7SSR, 9SSR, 14SSR, 19SSR, and 20SSR), four of them being polymorphic (ccmp2, ccmp3, 7SSR, and 14SSR) with two size variants resolved for each primer (table 4), yielding a total of eight variants in 448 individuals studied. Variation patterns were consistent with the three haplotypes defined by cpDNA. PCR product sequencing confirmed the presence of microsatellite motifs in the amplified regions (table 4) and congruence between SSR repetition number and frequency. Among the three haplotypes, one allele was shared by H1 and H3 (356 in 7SSR), one allele was unique to H2 (352 7SSR), and three alleles were exclusive to H2 and H3 (ccmp2 162, ccmp3 79, and 14SSR 207).

All Mediterranean Basin populations shared the H1 haplotype, while the Saharan populations possessed H2 and H3. Large differences in haplotype frequencies (H1 = 88.7%, H2 = 8.5%, H3 = 2.8%) shown in table 5 are due to the fact that the vast majority of the populations under study are located around the Mediterranean. Relationships between *N. oleander* haplotypes following TCS analysis resulted in a triangular network without a basal haplotype; therefore, genealogical relationships could not be deduced.

Results of molecular dating analyses are shown in a tree (fig. 3) with two sister groups within *N. oleander* representing biogeographically separated populations. All but one of the Saharan populations form a clade (red branch) supported by a posteriori probability of 0.97 (fig. 3), while the other clade (blue branch) includes all the studied individuals from the Mediterranean Basin, plus those from DZOT2. The relaxed molecular clock applied to the tree calibration indicates that the haplotype diversification occurred between 7.2 and 1.2 Ma. The coalescence analysis shows maximum credibility with a posteriori probability of 1 and bootstrap support of 98% for the ingroup (*N. oleander*).

Discussion

Celtis australis and *Nerium oleander* were congruent in the absence of genetic variability for all the Mediterranean Basin populations despite the large number of markers. Similar results had been reported in other Mediterranean plant species. Fernández-Mazuecos and Vargas (2010, 2011) reported the absence of genetic variation in Mediterranean Basin populations of *Cistus monspeliensis* L. (Cistaceae), while the species showed high levels of polymorphisms in the Canary Islands. Guzmán and Vargas (2009) reported low levels of plastidial DNA variability for Iberian and North African *Cistus ladanifer* L. populations; however, cpSSRs were variable and geographically informative (Quintela-Sabarís et al. 2011). Rodríguez-Sánchez et al. (2009) detected two haplotypes in *Laurus azorica* (Seub) Franco and four haplotypes in *Laurus nobilis* L. throughout the Mediterranean Basin. Low levels of chloroplast DNA variation were also reported for *Fraxinus ornus* L. (Oleaceae), a species distributed in the northern Mediterranean Basin and on some main Mediterranean islands. The species exhibited low haplotype numbers (FRAXIGEN 2005; Heuertz et al. 2006), with only two occupying most of the species' range. Vendramin et al. (2008) reported low genetic variation in *Pinus pinea* L. (Pinaceae) throughout the species' geographic range. Several other riparian plants studied to date, e.g., *Populus*, *Salix*, and *Fraxinus*, showed similar results, with more or less geographically structured genetic variability associated with Pleistocene climatic oscillations (Palmé et al. 2003; Petit et al. 2005; Fussi et al. 2010; Macaya-Sanz et al. 2012).

Comparisons of diversity within and among plant species can be affected by the selected cpDNA regions or fragments as well as by sample size. Our broad sampling area, the number of *C. australis* populations sampled, and the variety of fragments analyzed makes it unlikely that the lack of variability observed would be due to a methodological artifact. Plastid markers are maternally inherited and exhibit increased geographic structuring relative to biparentally inherited nuclear markers and are therefore among the markers of choice in phylogeographic research (Navascués and Emerson 2005; Garoia et al. 2007; Fussi et al. 2010; Macaya-Sanz et al. 2012). Despite reported homoplasy in SSRs (e.g., Chen et al. 2002; Curtu et al. 2004), Provan et al. (1999) considered the levels low enough to be informative at the population level, and Quintela-Sabarís et al. (2011) considered homoplasy a problem only in those species with high mutation rates. Barthe et al. (2012) analyzed SSR fragment size and sequence variation in different

Table 5

Allele Size Composition of the Three Haplotypes Identified in *Nerium oleander* and Haplotype Frequencies in Percentage

Haplotype	ccmp2	ccmp3	7SSR	14SSR	Frequency (%)
H1	161	80	356	206	88.68
H2	162	79	352	207	8.49
H3	162	79	356	207	2.83

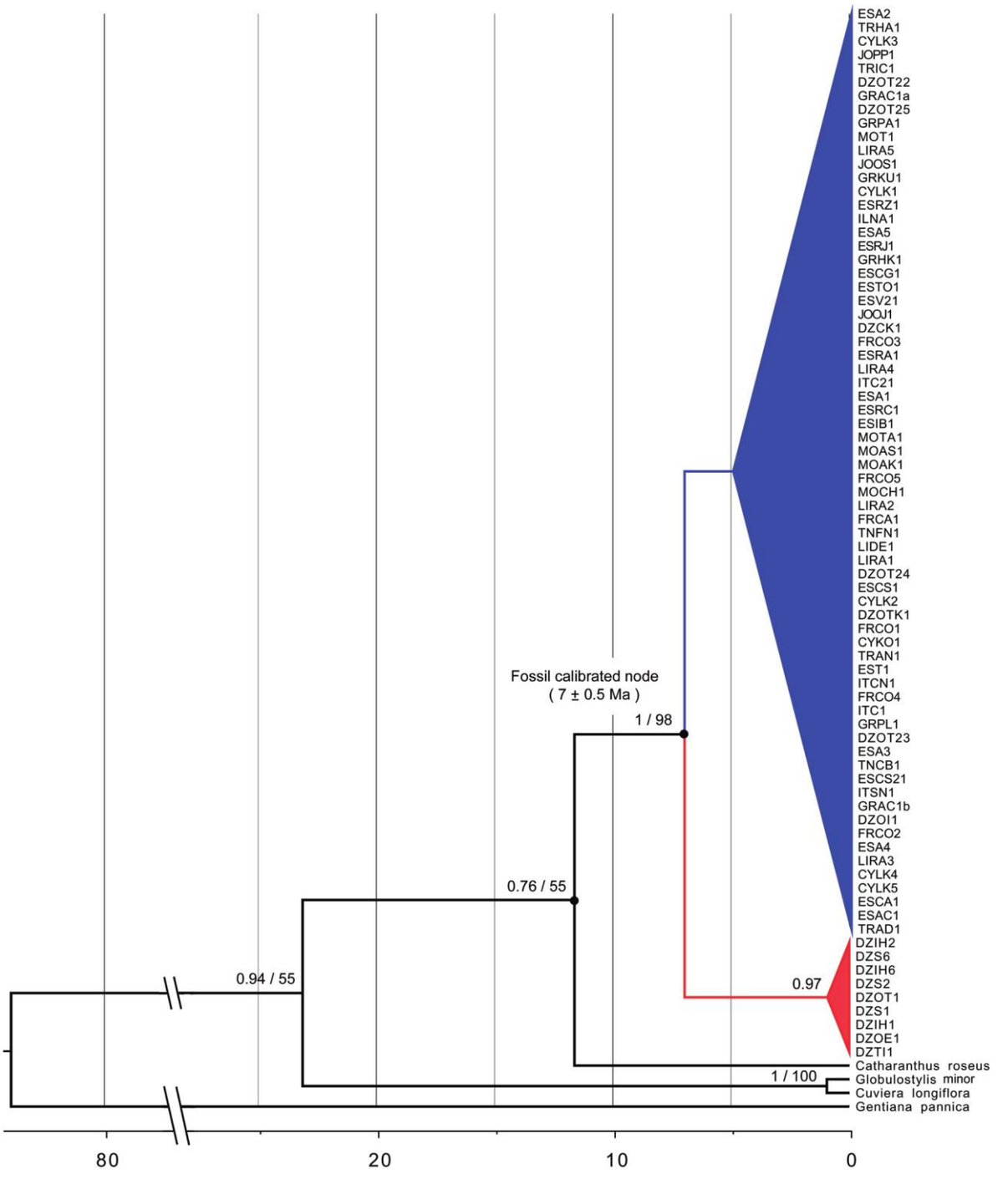

Fig. 3 Maximum clade credibility chronogram of *Nerium oleander* obtained from the *rpl*S32-*trn*L plastid sequence. The first number on the nodes represents the posteriori probability (PP) from 0 to 1; the second number is the bootstrap value in a maximum likelihood analysis. Only values with >5 PP and >50% bootstrap support are placed in the tree.

plant groups, concluding that they add important information for phylogenetic or phylogeographic reconstruction and the combination of sequences and SSR variation provides higher resolution.

Smith and Donoghue (2008) indicated that molecular evolution rates were much lower in trees and shrubs than in herbaceous species. In addition, Aguinagalde et al. (2005) demonstrated that temperature tolerance was an important factor in determining genetic variability, with lower diversity in thermophilous species relative to cold-tolerant species.

C. australis

Due to the absence of polymorphisms among the 41 *C. australis* populations, we used individuals of four other species of *Celtis* (*C. occidentalis*, *C. tetrandra*, *C. sinensis*, and *C. aculeata*) to determine whether there exists variability in these markers. *trn*Q-5′-*rps*16 was the only region that exhibited variability among species, which occurred as mononucleotide transversions (A/G and G/T), and the presence/absence of different-sized nucleotide gaps, confirming a low mutation rate in *Celtis* species.

Our results are congruent with previous results of de Castro and Maugeri (2006), who studied ITS and *trn*L (UAA) sequences in a complex of five Mediterranean *Celtis* species in terms of the low rates of variability as well as the lack of chloroplast DNA variation within and among *C. australis* populations. De Castro and Maugeri (2006) reported two hypotheses, not mutually exclusive, that might explain the low levels of variability observed in *C. australis* and *N. oleander*: (1) very low cpDNA mutation rates, typically associated with the longer life span of perennial tree species (Smith and Donoghue 2008), and (2) a recent spread of the species in the Mediterranean Basin, supported by the results of Carrión et al. (2012), who reported the species' disappearance during the LGM. This recent spreading coupled with the low mutation rates in *Celtis* species can explain such extraordinary low genetic variability over a broad geographic area.

Edibility of the fruits, eaten even by *Homo antecessor* (Allué et al. 2015), and the various uses in the manufacture of agricultural and household utensils (Simchoni and Kislev 2011) makes plausible a progressive human-mediated expansion from the eastern Mediterranean to the western Mediterranean following Neolithic Mediterranean maritime routes (Broodbank 2006; Blondel et al. 2010; Jesse et al. 2011), similar to the Mediterranean Basin migration of other utilitarian plants, including olive (*Olea europaea*: Oleaceae; Besnard et al. 2002) and *Pinus pinea* (Vendramin et al. 2008).

N. oleander

The presence of the *Nerium* genus throughout the Mediterranean Basin has been widely documented since the Oligocene (Quézel and Médail 2003) and Miocene (Palamarev 1989) to the Quaternary (Carrión et al. 2012), consistently associated with riparian species in warm habitats. Persistence time in a region is an important factor affecting a species' or population's genetic variability (Hewitt 2000). Provan et al. (1999) estimated that the mutation rate of plastid microsatellites ranges from 3.2×10^{-5} to 7.9×10^{-5}; therefore, higher levels of variability should be expected in oleander following its more than 7-Myr presence in the Mediterranean Basin. However, Mediterranean *N. oleander* showed absence of variability, while the few Saharan populations studied showed two haplotypes.

Hewitt (2004) provided several lines of evidence for increased genetic diversity and basal haplotype locations associated with diversification centers, and glacial refugia harbored populations with increased genetic diversity (Hewitt 1996, 2000; Médail and Quézel 1997). cpDNA diversity was extremely low in oleander, and a triangle haplotype network was depicted where any haplotype might be basal. The fact that the few Saharan populations show variability while there is none in the Mediterranean allows us to hypothesize that *N. oleander* has persisted in the Sahara longer than in the Mediterranean region.

The onset of recurrent desert conditions in the Sahara was initiated at least 7 Myr ago (Schuster et al. 2006) and has continued with climatic fluctuations in which humid and arid periods have alternated, the former facilitating the formation of biological corridors connecting the Mediterranean and Saharan populations (Larrasoana et al. 2013). Migliore et al. (2012) provided evidence supporting our hypothesis in *Myrtus* (*Myrtaceae*) species, with populations throughout the Mediterranean Basin and the Sahara. Results of the tree calibration indicate a diversification time range of 7.2–1.2 Ma between Saharan and Mediterranean haplotypes, supporting the hypothesis of oleander disappearance in the Mediterranean Basin in the LGM. Despite a single calibration point for tree calibration being inadequate due to probabilistic uncertainty involved, it is useful for our purposes because fossils, both pollen (Jiménez-Moreno et al. 2010) and leaves (Palamarev 1989), are the only data available to date this node, and our interest is in dating two lineages within species with homogeneous replacement rates.

Loss of genetic diversity can be a consequence of several factors, including evolutionary rates, mating systems (Vendramin et al. 2008), or past bottlenecks in the species' history. Smith and Donoghue (2008) demonstrated that rates of molecular evolution are lower in woody plants than in related herbaceous plants and support low polymorphisms, but not enough to explain the lack of genetic variability after 20 Myr in the Mediterranean. A recent recolonization is a plausible hypothesis for the absence of oleander genetic variability. Postglacial recolonization from refugia, including immigration routes and introgression between taxa, was demonstrated in *Populus alba* L., *Populus tremula* L., and *Populus nigra* L. during glacial periods (Cottrell et al. 2005; Fussi et al. 2010, 2012; Macaya-Sanz et al. 2012). Low temperatures during glaciations resulting in freezing water in riparian corridors might explain the disappearance of oleander in the Mediterranean Basin, with a single or few refugia remaining from which the species recolonized following the LGM, and support the homogeneity of populations over the sampled area.

The homogeneity of Mediterranean populations can be explained by a rapid expansion in that area. Rapid colonization of new geographic areas can produce reduced genetic variability (Hewitt 2000), which would support our hypothesis. The recolonization of the Mediterranean Basin could derive either

from any refuge or refugia in the area or from southernmost populations, such as those from Tassili n'Ajjer. Recurrent Green Sahara periods connected the African tropics to African and Eurasian midlatitudes (Migliore et al. 2013; Hely and Lezine 2014), making possible such recolonization.

Our data show the lack of variability in plastidial DNA in the Mediterranean populations of both species studied but not in those Saharan populations studied in *N. oleander*. It would be desirable to have material from areas different than the studied area of *C. australis*, whose results would be compared with Mediterranean materials, similarly to what was done with *N. oleander*. That has not been possible in this work, despite repeated attempts to have Asian or Indian material. Moreover, the study of nuclear DNA could provide more conclusive data on genetic variability since its rate of variability is much greater than that of the plastidial DNA, which is beyond the scope of this work.

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Literature Cited

Aguinagalde I, A Hampe, A Mohanty, JP Martín, J Duminil, RJ Petit 2005 Effects of life-history traits and species distribution on genetic structure at maternally inherited markers in European trees and shrubs. J Biogeogr 32:329–339.

Akaike H 1981 Likelihood of a model and information criteria. J Econom 16:3–14.

Allué E, I Cáceres, I Expósito, A Canals, A Rodríguez, J Rosell, JM Bermúdez de Castro, E Carbonell 2015 Celtis remains from the Lower Pleistocene of Gran Dolina, Atapuerca (Burgos, Spain). J Archaeol Sci 53:570–577.

Anderson LL, FS Hu, DM Nelson, RJ Petit, KN Paige 2006 Ice-age endurance: DNA evidence of a white spruce refugium in Alaska. Proc Natl Acad Sci USA 103:12447–12450.

Barthe S, F Gugerli, NA Barkley, L Maggia, C Cardi, I Scotti 2012 Always look on both sides: phylogenetic information conveyed by simple sequence repeat allele sequences. PLoS ONE 7:e40699.

Bermúdez de Castro JM, M Martinón-Torres, A Gómez-Robles, A Margvelashvili, JL Arsuaga, JM Carretero, I Martínez, S Sarmiento 2011 The Gran Dolina-TD6 human fossil remains and the origin of Neanderthals. Pages 67–75 *in* S Condemi, GC Weniger, eds. Continuity and discontinuity in the peopling of Europe: one hundred fifty years of Neanderthal study. Springer, Dordrecht.

Besnard G, B Khadary, P Baradat, A Bervillé 2002 *Olea europaea* (Oleaceae) phylogeography based on chloroplast DNA polymorphism. Theor Appl Genet 104:1353–1361.

Blondel J, J Aronson, JY Bodiou, G Boeuf 2010 The Mediterranean region: biological diversity in space and time. 2nd ed. Oxford University Press, New York.

Breen AL, DF Murray, MS Olson 2009 Genetic consequences of glacial survival: the late Quaternary history of balsam poplar (*Populus balsamifera* L.) in North America. J Biogeogr 39:918–928.

Broodbank C 2006 The origins and early development of Mediterranean maritime activity. J Mediterr Archaeol 19:199–230.

Carrión JS, S Fernández, P González-Sampériz, L López-Merino, L Peña, F Burjachs, JA López-Sáez, et al 2012 Paleoflora y Paleovegetación de la Península Ibérica e Islas Baleares: Plioceno-Cuaternario. Ministerio de Economía y Competitividad, Madrid.

Cheddadi R, GG Vendramin, T Litt, L François, M Kageyama, S Lorentz, JM Laurent, et al 2006 Imprints of glacial refugia in the modern genetic diversity of *Pinus sylvestris*. Glob Ecol Biogeogr 15:271–282.

Chen Y, YG Cho, SR McCouch 2002 Sequence divergence of rice microsatellites in *Oryza* and other plant species. Mol Genet Genomic 268:331–343.

Chenault N, S Arnaud-Haond, M Juteau, R Valade, JL Almeida, M Villar, C Bastien, A Dowkiw 2011 SSR-based analysis of clonality, spatial genetic structure and introgression from the Lombardy poplar into a natural population of *Populus nigra* L. along the Loire River. Tree Genet Genome 7:1249–1262.

Chung SM, JE Staub 2003 The development and evaluation of consensus chloroplast primer pairs that possess highly variable sequence regions in a diverse array of plant taxa. Theor Appl Genet 107:757–767.

Clement M, D Posada, K Crandall 2000 TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1660.

Comes HP, JW Kadereit 1998 The effect of Quaternary climatic changes on plant distribution and evolution. Trends Plant Sci 3:432–438.

Cottrell JE, V Krystufek, HE Tabbener, AD Milner, T Connoly, L Sing, S Fluch, et al 2005 Postglacial migration of *Populus nigra* L.: lessons learnt from chloroplast DNA. For Ecol Manag 219:293–312.

Curtu AL, R Finkeldey, O Gailing 2004 Comparative sequencing of a microsatellite locus reveals size homoplasy within and between European oak species (*Quercus* spp.). Plant Mol Biol Rep 22:339–346.

Darriba D, G Taboada, R Doallo, D Posada 2012 JModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9:772.

de Castro O, G Maugeri 2006 Molecular notes on the Mediterranean species of the genus *Celtis* L. (Celtidaceae). Plant Biosyst 140:171–175.

Drummond AJ, S Ho, M Phillips, A Rambaut 2006 Relaxed phylogenetics and dating with confidence. PLoS Biol 4:e88. doi:10.1371/journal.pbio.0040088.

Drummond AJ, MA Suchard, D Xie, A Rambaut 2012 Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 29:1969–1973.

Edgar RC 2004 MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinform 5:1–19.

Felsenstein J 1985 Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Fernández-Mazuecos M, P Vargas 2010 Ecological rather than geographical isolation dominates Quaternary formation of Mediterranean *Cistus* species. Mol Ecol 19:1381–1395.

——— 2011 Genetically depauperate in the continent but rich in oceanic islands: *Cistus monspeliensis* (Cistaceae) in the Canary Islands. PLoS ONE 6:e17172.

FRAXIGEN 2005 Ash species in Europe: biological characteristics and practical guidelines for sustainable use. Oxford Forestry Institute, University of Oxford, Oxford.

Fussi B, J Bonello, E Calleja, B Heinze 2012 Combining the use of molecular techniques and archival documentary evidence to trace the origin of *Populus alba* in a central Mediterranean archipelago. Eur J For Res 131:347–354.

Fussi B, C Lexer, B Heinze 2010 Phylogeography of *Populus alba* (L.) and *Populus tremula* (L.) in central Europe: secondary contact and hybridization during recolonisation from disconnected refugia. Tree Genet Genomes 6:439–450.

Garoia F, I Guarniero, D Grifoni, S Marzola, F Tinti 2007 Comparative analysis of AFLPs and SSRs efficiency in resolving population genetic structure of Mediterranean *Solea vulgaris*. Mol Ecol 16:1377–1387.

Gómez A, DH Lunt 2007 Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. Pages 155–188 *in* S Weiss, N Ferrand, eds. Phylogeography of southern European refugia. Springer, Dordrecht.

González-Martínez SC, M Dubreuil, M Riba, GG Vendramin, F Sebastiani, M Mayol 2010 Spatial genetic structure of *Taxus baccata* L. in the western Mediterranean Basin: past and present limits to gene movement over a broad geographic scale. Mol Phylogenet Evol 55:805–815.

Guzmán B, P Vargas 2009 Long-distance colonization of the western Mediterranean by *Cistus ladanifer* (Cistaceae) despite the absence of special dispersal mechanisms. J Biogeogr 36:954–968.

Hampe A, J Arroyo, P Jordano, J Petit 2003 Rangewide phylogeography of a bird-dispersed Eurasian shrub: contrasting Mediterranean and temperate glacial refugia. Mol Ecol 12:3415–3426.

Hasegawa M, H Kishino, T Yano 1985 Dating the human-ape split by a molecular clock of mitochondrial DNA. J Mol Evol 22:160–174.

Hely C, AM Lezine 2014 Holocene changes in African vegetation: tradeoff between climate and water availability. Clim Past 10:681–686.

Herrera J 1991 The reproductive biology of a riparian Mediterranean shrub, *Nerium oleander* L. (Apocynaceae). Bot J Linn Soc 106:147–172.

Heuertz M, S Carnevale, S Fineschi, F Sebastiani, JF Hausman, L Paule, GG Vendramin 2006 Chloroplast DNA phylogeography of European ashes, *Fraxinus* sp. (Oleaceae): roles of hybridization and life history traits. Mol Ecol 15:2131–2140.

Hewitt GM 1996 Some genetic consequences of ice ages, and their role in divergence and speciation. Biol J Linn Soc 58:247–276.

——— 2000 The genetic legacy of the Quaternary ice ages. Nature 405:907–913.

——— 2004 Genetic consequences of climatic oscillations in the Quaternary. Philos Trans R Soc B 359:183–195.

Jesse R, E Véla, M Pfenninger 2011 Phylogeography of a land snail suggests trans-Mediterranean Neolithic transport. PLoS ONE 6:e20734.

Jiménez-Moreno G, S Fauquette, JP Suc 2010 Miocene to Pliocene vegetation reconstruction and climate estimates in the Iberian Peninsula from pollen data. Rev Palaeobot Palynol 162:403–415.

Khanuja SPS, AK Shasany, MP Darokar, S Kumar 1999 Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Mol Biol Rep 17:74.

Kingman JFC 1982 The coalescent. Stoch Proc Appl 13:235–248.

Larrasoana JC, AP Roberts, EJ Rohling 2013 Dynamics of green Sahara periods and their role in hominin evolution. PLoS ONE 8: e76514.

Lee SC, CF Chang, KY Ho 2011 Genetic diversity and molecular discrimination of the closely related Taiwanese Ulmaceae species *Celtis sinensis* Persoon and *Celtis formosana* Hayata based on ISSR and ITS markers. Afr J Agric Res 6:4760–4768.

Librado P, J Rozas 2009 DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Macaya-Sanz D, M Heuertz, U López de Heredia, AI de Lucas, E Hidalgo, C Maestro, A Prada, R Alía, SC González-Martínez 2012 The Atlantic-Mediterranean watershed, river basins and glacial history shape the genetic structure of Iberian poplars. Mol Ecol 21:3593–3609.

Magri D, S Fineschi, R Bellarosa, A Buonamici, F Sebastiani, B Schirone, MC Simeone, GG Vendramin 2007 The distribution of *Quercus suber* chloroplast haplotypes matches the palaeogeographical history of the western Mediterranean. Mol Ecol 16:5259–5266.

Magyari EK, JC Chapman, B Gaydarska, E Marinova, T Deli, JP Huntley, JRM Allen, B Huntley 2008 The "oriental" component of the Balkan flora: evidence of presence on the Thracian Plain during the Weichselian late-glacial. J Biogeogr 35:865–883.

Maire R 1961 Dicotyledonae. Flore de l'Afrique du Nord. Vol 7. Lechevalier, Paris. (In French.)

Mateu I, A Aguilella, F Boisset, R Currás, M Guara, E Laguna, AM Marzo, MF Puche, J Pedrola 2013 Geographical patterns of genetic variation in rosemary (*Rosmarinus officinalis*) in the Mediterranean Basin. Bot J Linn Soc 171:700–712.

Médail F, K Diadema 2009 Glacial refugia influence plant diversity patterns in the Mediterranean Basin. J Biogeogr 36:1333–1345.

Médail F, P Quézel 1997 Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. Ann Mo Bot Gard 84:112–127.

Migliore J, A Baumel, M Juin, B Fady, A Roig, N Duong, F Medail 2013 Surviving in mountain climate refugia: new insights from the genetic diversity and structure of the relict shrub *Myrtus nivellei* (Myrtaceae) in the Sahara Desert. PLoS ONE 8:e73795.

Migliore J, A Baumel, M Juin, F Médail 2012 From Mediterranean shores to central Saharan mountains: key phylogeographical insights from the genus *Myrtus*. J Biogeogr 39:942–956.

Navascués M, BC Emerson 2005 Chloroplast microsatellites: measures of genetic diversity and the effect of homoplasy. Mol Ecol 14: 1333–1341.

Nieto-Feliner G 2014 Patterns and processes in plant phylogeography in the Mediterranean Basin: a review. Perspect Plant Ecol Evol Syst 16:265–278.

Palamarev E 1989 Paleobotanical evidences of the Tertiary history and origin of the Mediterranean sclerophyll dendroflora. Plant Syst Evol 162:93–107.

Palmé AE, V Semerikov, M Lacoux 2003 Absence of geographical structure of chloroplast DNA variation in sallow, *Salix caprea* L. Heredity 91:465–474.

Parés JM, L Arnold, M Duval, M Demuro, A Pérez-González, JMB de Castro, E Carbonell, JL Arsuaga 2013 Reassessing the age of Atapuerca-TD6 (Spain): new paleomagnetic results. J Archaeol Sci 40:4586–4595.

Petit RJ, S Brewer, S Bordács 2002 Identification of refugia and postglacial colonisation routes of European white oaks based on chloroplast DNA and fossil pollen evidence. For Ecol Manag 156:49–79.

Petit RJ, A Hampe, R Cheddadi 2005 Climate changes and tree phylogeography in the Mediterranean. Taxon 54:877–885.

Petit RJ, FS Hu, CW Dick 2008 Forests of the past: a window to future changes. Science 320:1450–1452.

Portis E, C Comino, A Lenzi, P Lombardi, R Tesi, S Lanteri 2004 Amplified fragment length polymorphism for variety identification and genetic diversity assessment in oleander (*Nerium oleander* L.). Euphytica 136:125–137.

Provan J, N Soranzo, NJ Wilson, DB Goldstein, W Powell 1999 A low mutation rate for chloroplast microsatellites. Genetics 153:943–947.

Quézel P, F Médail 2003 Écologie et biogéographie des forêts du bassin méditerranéen. Elsevier, Paris. (In French.)

Quintela-Sabarís C, GG Vendramin, D Castro-Fernández, I Fraga 2011 Chloroplast DNA phylogeography of the shrub *Cistus ladanifer* L. (Cistaceae) in the highly diverse western Mediterranean region. Plant Biol 13:391–400.

Rodríguez-Sánchez F, B Guzmán, A Valido, P Vargas, J Arroyo 2009 Late Neogene history of the laurel tree (*Laurus* L., Lauraceae) based on phylogeographical analyses of Mediterranean and Macaronesian populations. J Biogeogr 36:1270–1281.

Ronquist F, M Teslenko, P van der Mark, D Ayres, A Darling, S Höhna, B Larget, L Liu, MA Suchard, JP Huelsenbeck 2012 MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61:539–542.

Rundel PW, G Montenegro, FM Jaksic 1998 Landscape disturbance and biodiversity in Mediterranean-type ecosystems. Ecological Studies. 136. Springer, Dordrecht.

Schuster M, P Duringer, JF Ghiennes, P Vignaud, HT Mackaye, A Likius, M Brunet 2006 The age of the Sahara Desert. Science 311:821. doi:10.1126/science.1120161.

Shaw J, EB Lickey, JT Beck, SB Farmer, W Liu, J Miller, KC Siripun, CT Winder, EE Schilling, RL Small 2005 The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am J Bot 92:142–166.

Shaw J, EB Lickey, EE Schilling, RL Small 2007 Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare II. Am J Bot 94:275–288.

Simchoni O, ME Kislev 2011 Early finds of *Celtis australis* in the southern Levant. Veg Hist Archaeobot 20:267–271.

Smith SA, MJ Donoghue 2008 Rates of molecular evolution are linked to life history in flowering plants. Science 322:86–89.

Taberlet P, L Fumagalli, AG Wust-Saucy, J-F Cosson 1998 Comparative phylogeography and postglacial colonization routes in Europe. Mol Ecol 7:453–464.

Tamura K, G Stecher, D Peterson, A Filipski, S Kumar 2013 MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 12:2725–2729.

Thompson JD 2005 Plant evolution in the Mediterranean. Oxford University Press, New York.

Traba J, S Arrieta, J Herranz, MC Clamagirand 2006 Red fox (*Vulpes vulpes* L.) favor seed dispersal, germination and seedling survival of Mediterranean hackberry (*Celtis australis* L.). Acta Oecol 30:39–45.

Tutin TG 1964 *Celtis* L. Page 77 *in* TG Tutin, VH Heywood, NA Burgues, DH Valentine, SM Walters, DA Webb, eds. Flora Europaea: Lycopodiaceae to Platanaceae. Vol 1. Cambridge University Press, Cambridge.

Vendramin GG, B Fady, SC González-Martínez, FS Hu, I Scotti, F Sebastiani, A Soto, RJ Petit 2008 Genetically depauperate but widespread: the case of an emblematic Mediterranean pine. Evolution 62:680–688.

Wei X, H Meng, M Jiang 2013 Landscape genetic structure of a streamside tree species *Euptelea pleiospermum* (Eupteleaceae): contrasting roles of river valley and mountain ridge. PLoS ONE 8: e66928.

Weising K, RC Gardner 1999 A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome 42:9–19.