Late Neogene history of the laurel tree (*Laurus* L., Lauraceae) based on phylogeographical analyses of Mediterranean and Macaronesian populations

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ABSTRACT

Aim The post-glacial range dynamics of many European plant species have been widely investigated, but information rapidly diminishes as one moves further back in time. Here we infer the historical range shifts of *Laurus*, a paradigmatic tree of the Tethyan flora that has covered southern Eurasia since the Oligo-Miocene, by means of phylogenetic and phylogeographical analyses.

Location Mediterranean Basin, Black Sea and Macaronesian archipelagos (Azores, Madeira, Canary Islands).

Methods We analysed plastid DNA (cpDNA) sequence (*trnK–matK*, *trnD–trnT*) variation in 57 populations of *Laurus* and three Lauraceae genera. Phylogenetic methods (maximum parsimony and Bayesian inference) and statistical parsimony networks were used to reconstruct relationships among haplotypes. These results were contrasted with the fossil record and bioclimatic niche-based model predictions of past distributions to infer the migration routes and location of refugia.

Results The phylogenetic tree revealed monophyly for *Laurus*. Overall sequence variability was low within *Laurus*, but six different haplotypes were distinguished and a single network retrieved, portraying three lineages primarily related to geography. A strongly divergent eastern lineage occupied Turkey and the Near East, a second clade was located in the Aegean region and, lastly, a western clade grouped all Macaronesian and central and western Mediterranean populations. A close relationship was observed between the Macaronesian populations of *L. azorica* and the western populations of *L. nobilis*.

Main conclusions The phylogeographical structure of *Laurus* preserves the imprints of an ancient contraction and break-up of the range that resulted in the evolution of separate cpDNA lineages in its western- and easternmost extremes. Intense range dynamics in the western Mediterranean and multiple glacial refugia contributed to the generation and long-term conservation of this phylogeographical pattern, as shown by the fit between the haplotype ranges and past suitable areas inferred from bioclimatic models. Finally, our results challenge the taxonomic separation of *Laurus* into two distinct species.

Keywords

Long-distance dispersal, Macaronesia, Mediterranean, Neogene, phylogeography, plastid sequences, range dynamics, refugia, relict, Tertiary.

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INTRODUCTION

The history of the Mediterranean flora and vegetation has fascinated biogeographers ever since it was formally described as a floristic region (for a botanical account see Takhtajan, 1986). In particular, the prevalence of ancient subtropical taxa (e.g. *Myrtus*, *Laurus*, *Olea*, *Phillyrea*) in the Mediterranean flora has long been recognized. The ancestors of extant species of these genera occupied a wide region around the former Tethys Sea during most of the Palaeogene and early Neogene (Mai, 1989; Palamarev, 1989), when low and middle latitudes of the Northern Hemisphere were largely covered by broadleaved evergreen vegetation dominated by taxa of tropical affinities (e.g. Lauraceae, Myrtaceae, Palmae). This so-called 'Madrean–Tethyan' flora (Axelrod, 1975) suffered widespread range contractions and extinctions since the middle Miocene due to large-scale climatic and tectonic changes (Mai, 1989). In particular, the Tethys Sea was greatly reduced by the progressive proximity of the Eurasian and African plates, in conjunction with a cooler and drier climate (Krijgsman, 2002). The onset of the Mediterranean climate in the mid-Pliocene, as well as the increased aridity and cold temperatures brought about by Pleistocene glaciations, ultimately resulted in the extinction of most relict populations of Tethyan plants in southern Europe and North Africa (Kovar-Eder *et al.*, 2006).

The late Neogene range dynamics of those members of the former Tethyan flora that have survived to the present remain largely unknown. In contrast with increasingly available knowledge of the Quaternary range dynamics of temperate plant taxa (e.g. Hewitt, 2004), few studies have been conducted on species of Tethyan origin (Petit *et al.*, 2005). Based on a comparison of fossil and extant floras from the Western Palaearctic, both Asia Minor and the Macaronesian Islands (Azores, Madeira and the Canaries) have traditionally been regarded as outstanding refugia for relict Tertiary lineages (Engler, 1879; Axelrod, 1975; Bramwell, 1976; Sunding, 1979; Cronk, 1992; Denk *et al.*, 2001). Mesic areas within the Mediterranean Basin also enabled long-term survival of some species in pocket refugia (Thompson, 2005; Mejías *et al.*, 2007). In the particular case of Macaronesia, close to the western end of the Mediterranean, recent phylogenetic studies have confirmed the existence of ancient (Tertiary) lineages, as well as close relationships with related Mediterranean taxa (Andrus *et al.*, 2004; Carine *et al.*, 2004; Vargas, 2007). Yet molecular studies have also shown that several presumed Macaronesian relict lineages have a recent, derived origin (reviewed by Emerson, 2002; Vargas, 2007). Thus, as different taxa usually show unrelated evolutionary patterns, generalizations are problematic. Moreover, most molecular studies of the Macaronesian flora have focused on phylogenetic (macroevolutionary) or within-archipelago phylogeographical patterns, and few have been undertaken at a population level encompassing both range-wide Mediterranean and Macaronesian areas (Comes, 2004). Thus, the migration dynamics responsible for those biogeographical patterns have rarely been explored.

The laurel tree (*Laurus* L.) is the only member of the Lauraceae that has persisted to the present in southern Eurasia, despite a considerable number of genera (*Neolitsea*, *Lindera*, *Persea*, *Cinammomum* and others) recorded in the Mio-Pliocene (Mai, 1989; Barrón & Peyrot, 2006). After considerable range reductions throughout the Neogene, its current distribution is limited to relatively mesic areas in the Mediterranean Basin, the Pontic region (southern Black Sea) and the Macaronesian archipelagos (Santos, 1990). *Laurus* is a dioecious tree with entomophilous pollination and fleshy-fruited seeds dispersed by birds (Forfang & Olesen, 1998; Hampe, 2003). Given its long-standing presence, *Laurus* represents an excellent model for exploring the evolutionary history of ancient Mediterranean–Macaronesian lineages. Indeed, several authors have emphasized the need for molecular studies involving extant Lauraceae in order to ascertain the biogeographical origin of the Macaronesian laurel forests (Emerson, 2002; Comes, 2004). Of the four genera of Lauraceae currently inhabiting Macaronesia, namely *Apollonias*, *Ocotea*, *Persea* and *Laurus*, the latter is the best suited with regard to testing Mediterranean–Macaronesian biogeographical connections, as it is the only one still persisting in the Mediterranean Basin.

Two species of *Laurus* have been traditionally recognized: *Laurus nobilis* L., distributed across the Mediterranean Basin and southern Black Sea, and *Laurus azorica* (Seub) Franco, endemic to Macaronesia and southern Morocco (Barbero *et al.*, 1981; Jalas & Suominen, 1991; Fig. 1). Key characters of the species, however, have been questioned in relation to morphological (Ferguson, 1974; Marques & Sales, 1999) and genetic (Arroyo-García *et al.*, 2001) data. Inference of genetic relatedness in the whole range of *Laurus* is needed to identify the level of evolutionary differentiation within the genus, past population dynamics, and the potential role of human-mediated introductions.

A previous study (Rodríguez-Sánchez & Arroyo, 2008) based on bioclimatic niche modelling showed that *Laurus* experienced remarkable range retreat and fragmentation driven by climatic changes since the late Tertiary. The species may have persisted at small, isolated refugia in the Mediterranean Basin and Macaronesia during Pleistocene glaciations. Here we attempt to combine these three sources of information (fossil records, bioclimatic models and phylogeography) to infer the historical range dynamics and lineage evolution in *Laurus* through the late Neogene. Additionally, we discuss the taxonomic implications of our phylogenetic results for species delimitation within the genus *Laurus*.

MATERIALS AND METHODS

Sampling strategy and DNA sequencing

We sampled 57 populations throughout the natural range of *Laurus* (Fig. 1; for population data see Appendix S1). Fresh

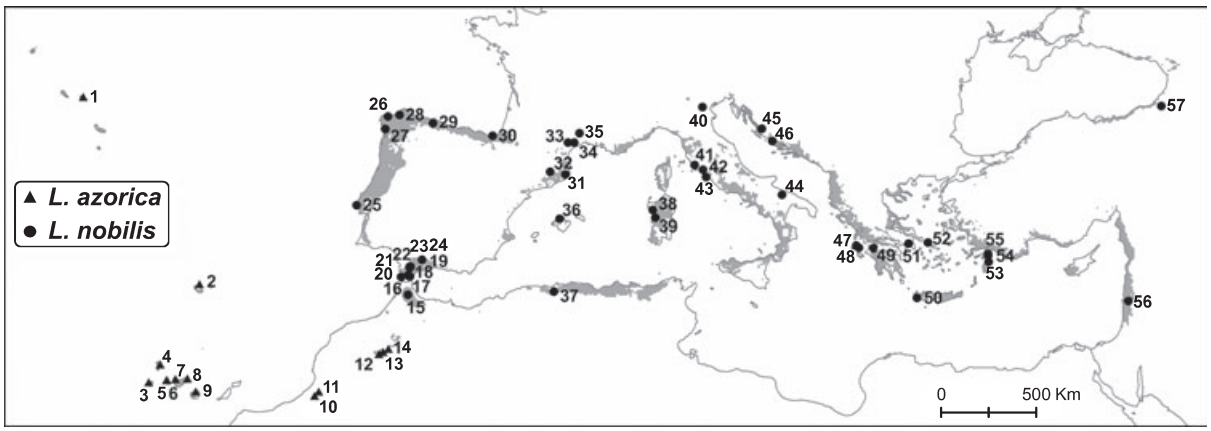

Figure 1 Map showing locations of *Laurus* populations sampled in this study. The current distribution of both species (*L. nobilis* and *L. azorica*) is shown in grey (after Rodríguez-Sánchez & Arroyo, 2008).

leaves were collected and stored in silica gel until processing in the laboratory. Total genomic DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

We sequenced plastid DNA (cpDNA) to obtain haplotypes suitable for phylogeographical analyses. Assuming the standard maternal inheritance of plastids in angiosperms, any phylogeographical pattern should arise exclusively from successful seed-dispersal events. As we were concerned about previous findings of extremely low cpDNA variability in the Lauraceae (Rohwer, 2000; Chanderbali *et al.*, 2001), we first performed a pilot study of sequence variability within *Laurus* at 11 cpDNA regions (Appendix S2). Polymerase chain reaction (PCR) amplifications were performed on a Perkin-Elmer PCR System 9700 (Waltham, MA, USA) or an MJ Research thermal cycler. The PCR procedure included a denaturation step of 1–4 min at 94°C, followed by 24–35 cycles of 1 min at 94°C, 0.5–1 min at the annealing temperature of the respective DNA region (Appendix S2), and 1–2 min at 72°C. One microlitre of dimethyl-sulfoxide (DMSO) was included in each 25 μL reaction. Amplified products were cleaned using spin filter columns (PCR Clean-up kit; MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocols. Cleaned products were then directly sequenced by means of dye terminators (Big Dye Terminator ver. 2.0; Applied Biosystems, Little Chalfont, UK) following the manufacturer's protocols, and run into polyacrylamide electrophoresis gels (7%) with an Applied Biosystems Prism model 3700 automated sequencer (Applied Biosystems, Foster City, CA, USA). PCR primers were used for cycle sequencing. We then assembled and edited the sequenced data using the program Seqed (Applied Biosystems). The limits of the regions were determined by the position of flanking primers. Finally, we selected the two spacers (*trnK–matK*, *trnD–trnT*) rendering a higher number of polymorphisms and extended the sequencing to one randomly chosen individual from each of the 57 sampled populations for both DNA regions. Given the low overall variability and the clear geographical segregation of the haplotypes found, we increased the population-sequencing effort only in those regions that contained more than one haplotype (eastern Mediterranean, southern Iberia and northern Morocco; Fig. 1). In these regions, up to three individuals per population were sequenced. In total, we obtained sequences from 88 individuals of *Laurus* (for further information, including GenBank accession numbers, see Appendix S1). We also included one sample of *Lindera benzoin* Blume, a close relative of *Laurus* (Li *et al.*, 2004), and two other Lauraceae species (*Ocotea foetens* Benth. & Hook. and *Persea indica* Spreng) from the Canary Islands and Madeira, respectively, as outgroup accessions (GenBank codes FJ408866/67/68 and FJ408955/56/57). Sequences were aligned using ClustalW 1.83 (Chenna *et al.*, 2003), with further adjustments by visual inspection.

Phylogenetic and phylogeographical analyses

Maximum parsimony (MP) and Bayesian inference (BI) analyses were performed on the combined *trnK–matK*/*trnD–trnT* matrix. We conducted all parsimony analyses using Fitch parsimony (as implemented in paup*; Swofford, 1999) with equal weighting of all characters and of transitions/transversions. Heuristic searches were replicated 1000 times with random taxon addition sequences, tree bisection–reconnection (TBR) branch swapping, the options MulTrees and Steepest Descent in effect and holding 100 trees per replicate. We performed a full heuristic bootstrap analysis using 10,000 replicates with random taxon addition, TBR branch swapping, and the options Multrees and Steepest Descent in effect, and saving 10 trees per replicate.

In order to determine the simplest model of sequence evolution that best fits the sequence data, the Akaike information criterion was implemented in each data set using MrModeltest 1.1b (Posada & Crandall, 1998; Nylander, 2002). A BI analysis was conducted in MrBayes 3.0b4 (Ronquist & Huelsenbeck, 2003) by means of two identical searches with three million generations each (four Markov chain Monte Carlo, chain temperature = 0.2; sample frequency = 100). In both runs, probabilities converged at the same stable value after approximately generation 45,000. A 50% majority-rule consensus tree was calculated using the *sumt* command to yield the final

Bayesian estimate of phylogeny. We used the posterior probability as an estimate of robustness.

Phylogeographical relationships of haplotypes were inferred by statistical parsimony (Posada & Crandall, 2001) using TCS 1.21 (Clement *et al.*, 2000). We ran separate analyses on the combined *trnK–matK*/*trnD–trnT* matrix with indels either coded as single-site substitutions or treated as missing characters. Length variations in mononucleotide repeats (Table 1) were kept for the analysis, as they provided a phylogeographically coherent signal without signs of homoplasy. Nonetheless, both haplotype networks had the same structure, differing only in the number of mutations connecting some haplotypes. Only the former network (with indels coded) is described for the sake of brevity.

RESULTS

The aligned length of the combined *trnK–matK* and *trnD–trnT* sequences was 2562 bp. Thirty-nine of the 66 total polymorphic sites were single-site substitutions, while mononucleotide repeats and insertions/deletions accounted for the remaining mutations (Table 1). Fourteen variable characters were parsimony-informative. The MP analysis generated two trees of 39 steps with a consistency index (CI) of 1.00 and a retention index (RI) of 1.00. The strict consensus tree (not shown) was identical to the BI tree using the simplest model of evolution (*trnK–matK*: KHY; *trnD–trnT*: GTR; Fig. 2), but displayed different support values. These phylogenetic analyses revealed the monophyly of *Laurus* and identified several clades within the genus, closely related to their geographical distribution (Fig. 2). However, *L. nobilis* appeared paraphyletic to *L. azorica*, the status of which remained equivocal.

Genetic variability within *Laurus* was remarkably low. However, six different cpDNA haplotypes, differing by up to nine mutations, were found. The phylogeographical analysis produced a single network of six extant *Laurus* haplotypes connected with no loops (Fig. 3a). This, together with high CI and RI values in the phylogenetic analysis, indicates no homoplasy signal of our molecular markers. All connections in the network were within the 95% parsimony limit, including those of *Laurus* haplotypes with the outgroup samples (*L. benzoin*, *O. foetens*, *P. indica*). There was a clear geographical structure of haplotypes (Fig. 3b; Appendix S1), and most haplotypes were confined to specific regions. However, one haplotype (H6) was widely distributed throughout the Mediterranean Basin, with the exception of the easternmost populations. Southern Iberia and the Aegean region were the only areas that contained more than one single haplotype (two and three, respectively), and even within these areas we found only one south-western Turkish (Marmaris Peninsula) population harbouring more than one haplotype (Fig. 3b).

The phylogeographical network (Fig. 3a) showed three different lineages primarily related to an east–west geographical gradient. All three lineages derived from an unsampled ancestor, which differed in only one mutation step from the extant Aegean *Laurus* populations (haplotype H1). A second lineage contained one remarkably divergent haplotype (H2, separated by five mutations from the hypothetical ancestor haplotype) that was distributed from northern Turkey to Israel. The third lineage included all Macaronesian and western Mediterranean populations of *L. nobilis* and *L. azorica*. This western lineage comprised one central haplotype (H3), found in southern Morocco, Madeira and the Canary Islands, and three derived haplotypes observed in the Azores (H4), in southern Iberia (H5), and throughout much of the Mediterranean Basin from northern Morocco to the western Aegean Sea (H6).

DISCUSSION

We found low levels of cpDNA variation within *Laurus* at the 11 DNA regions tested (Appendix S2). Only two spacers (*trnK–matK* and *trnD–trnT*) displayed a considerable number of nucleotide substitutions. Although low within-population variation might be the result of our limited sample, the same pattern was obtained across populations and geographical regions, and even between taxa. One might be surprised by this scarce genetic variation, considering the ancient origin and long evolutionary history of *Laurus* (Palamarev, 1989; see Appendix S3 for a compilation of Neogene fossil records of *Laurus*), which would have provided ample opportunities for lineage divergence. Nevertheless, low cpDNA variation has also been found in the Lauraceae as a whole (Rohwer, 2000; Chanderbali *et al.*, 2001), and in other tree species of the Lauraceae (Wu *et al.*, 2006) and other families (e.g. *Olea europaea*, Besnard *et al.*, 2007; *Quercus suber*, Magri *et al.*, 2007; *Pinus pinea*, Vendramin *et al.*, 2008; see also Shaw *et al.*, 2005, for lineage comparison of these cpDNA regions across Spermatophyta). Reliable explanations for this low cpDNA variation include low mutation rates and long generation times, the demographic stability of most populations, or their relatively high gene flow (Petit *et al.*, 2005; Petit & Hampe, 2006; Smith & Donoghue, 2008). In the particular case of *Laurus*, the phylogeographical pattern depicted here and the low level of DNA variation in the Lauraceae suggest that the limited haplotype diversity stems from historically low mutation rates. In addition, the dioecious character of *Laurus* should have increased the rate of cpDNA lineage sorting, as the effective population size is reduced relative to hermaphrodite species (Cruzan & Templeton, 2000). A higher number of populations and haplotypes is needed to test the hypothesis of range expansion following demographic bottlenecks (Vendramin *et al.*, 2008).

The six distinct haplotypes found showed a clear geographical structure across the range of *Laurus*, pointing to a limited influence of historical human-mediated translocations, despite the long history of cultivation. Three cpDNA lineages were detected (Fig. 3), one distributed around the Aegean Sea (hereafter termed the Aegean lineage), one across Asia Minor and the Near East (the eastern lineage), and the third through the western Mediterranean and Macaronesia (western lineage). All three lineages are derived from an unsampled – probably

Table 1 Polymorphic sites of the two plastid DNA fragment sequences (*trnK–matK*, *trnD–trnT*) in *Laurus* and related species of the Lauraceae (*Lindera benzoin*, *Ocotea foetens*, *Persea indica*); sequences are numbered from 5′ to 3′ in both data sets.

		Nucleotide position																					
		trnK–matK																					
Species	Haplotype	42	47	128	283	335	382	383	384	385	463	464	526	538	622	821	878	1022	1041	1249	1291	1307	
Lindera benzoin		T	A	T	A	G	–	–	–	–	C	A	T	A	–	A	C	G	G	T	C	C	
Ocotea foetens		T	G	G	A	A	T	–	–	–	A	A	T	A	–	A	C	T	G	T	C	A	
Persea indica		T	A	T	T	A	T	–	–	–	C	C	T	A	C	A	T	T	A	C	T	C	
Laurus nobilis	H1	C	A	T	A	A	T	T	T	–	C	A	T	A	–	A	C	T	G	T	C	C	
Laurus nobilis	H2	C	A	T	A	A	T	T	T	T	C	A	C	A	–	A	C	T	G	T	C	C	
Laurus azorica	H3	C	A	T	A	A	T	T	–	–	C	A	T	C	–	A	C	T	G	T	C	C	
Laurus azorica	H4	C	A	T	A	A	T	T	–	–	C	A	T	C	–	A	C	T	G	T	C	C	
Laurus nobilis	H5	C	A	T	A	A	T	T	–	–	C	A	T	C	–	A	C	T	G	T	C	C	
Laurus nobilis	H6	C	A	T	A	A	T	T	–	–	C	A	T	C	–	G	C	T	G	T	C	C	

		trnD–trnT																																
		7	44	84	85	109	223	224	259	273	373	679	690	727	729	735	751	761	773	796	799	892	893	894	895	939	958	1073	1078	1108	1115
Lindera benzoin		C	A	–	–	G	A	G	A	G	A	T	A	T	C	T	A	G	G	T	C	T	T	–	–	T	G	C	A	C	A
Ocotea foetens		C	A	–	–	T	A	A	A	G	G	G	A	G	C	C	C	G	G	T	C	T	T	T	–	T	G	T	A	C	C
Persea indica		T	–	–	–	T	A	G	A	G	G	T	A	G	C	C	A	G	A	C	C	–	–	–	–	T	G	C	C	C	A
Laurus nobilis	H1	C	A	G	*	T	A	G	A	A	A	T	G	G	C	C	A	A	G	T	C	T	T	T	–	T	A	C	A	A	A
Laurus nobilis	H2	C	A	A	*	T	–	G	G	A	A	T	G	G	C	C	A	A	G	T	C	T	T	T	–	C	A	C	A	A	A
Laurus azorica	H3	C	A	G	*	T	–	G	A	A	A	T	G	G	C	C	A	A	G	T	C	T	T	T	–	T	A	C	A	A	A
Laurus azorica	H4	C	A	G	*	T	–	G	A	A	A	T	G	G	C	C	A	A	G	T	T	T	T	T	–	T	A	C	A	A	A
Laurus nobilis	H5	C	A	G	*	T	–	G	A	A	A	T	G	G	T	C	A	A	G	T	C	T	T	T	T	T	A	C	A	A	A
Laurus nobilis	H6	C	A	G	*	T	–	G	A	A	A	T	G	G	C	C	A	A	G	T	C	T	T	T	–	T	A	C	A	A	A

*CTGTTACAAGAAAG.

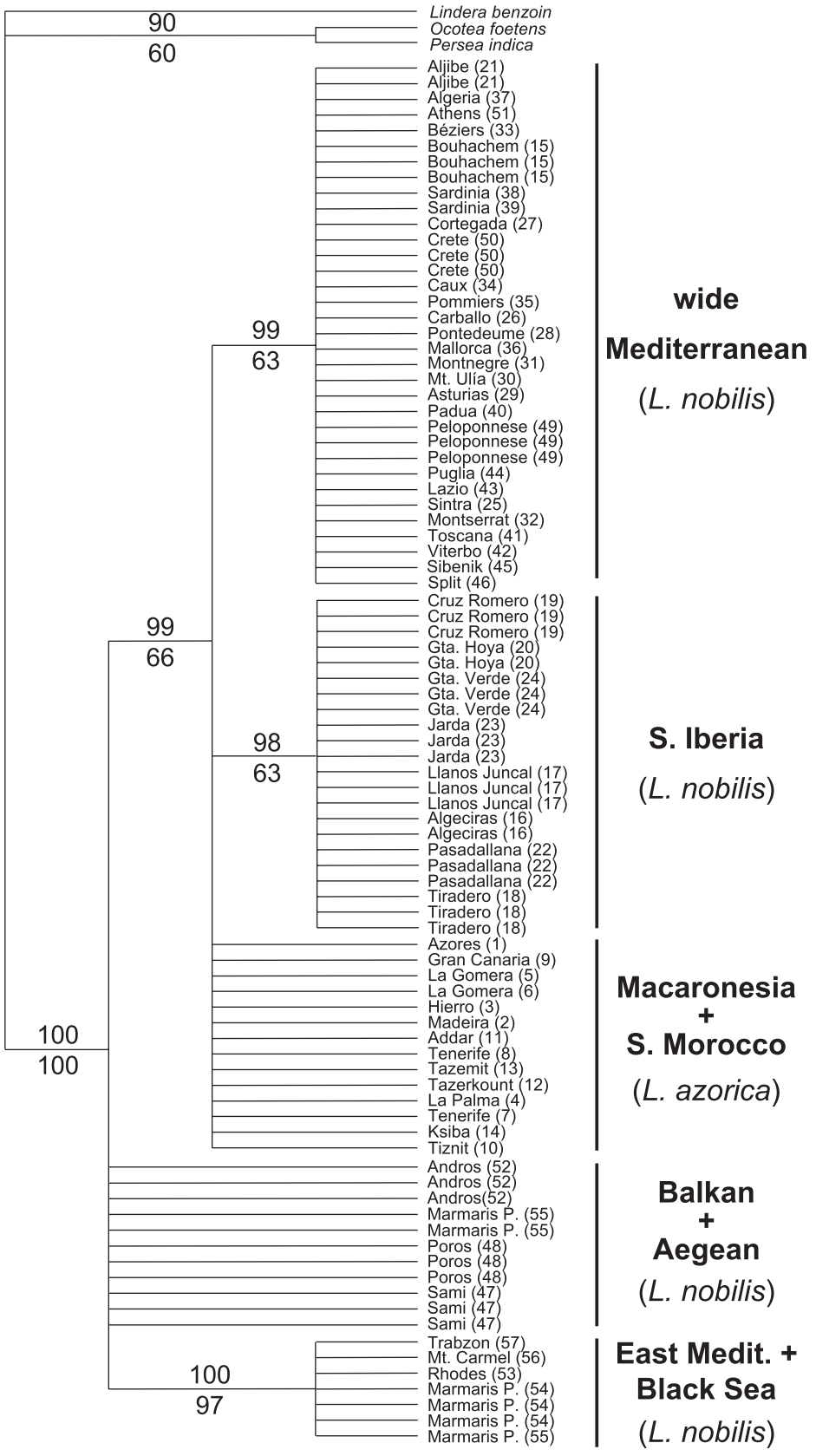

Figure 2 Consensus Bayesian inference tree based on the combined data sets of *trnK–matK* and *trnD–trnT* sequences. Numbers above and below branches are Bayesian posterior probabilities and bootstrap values, respectively. *Laurus* population coding as in Fig. 1 and Appendix S1.

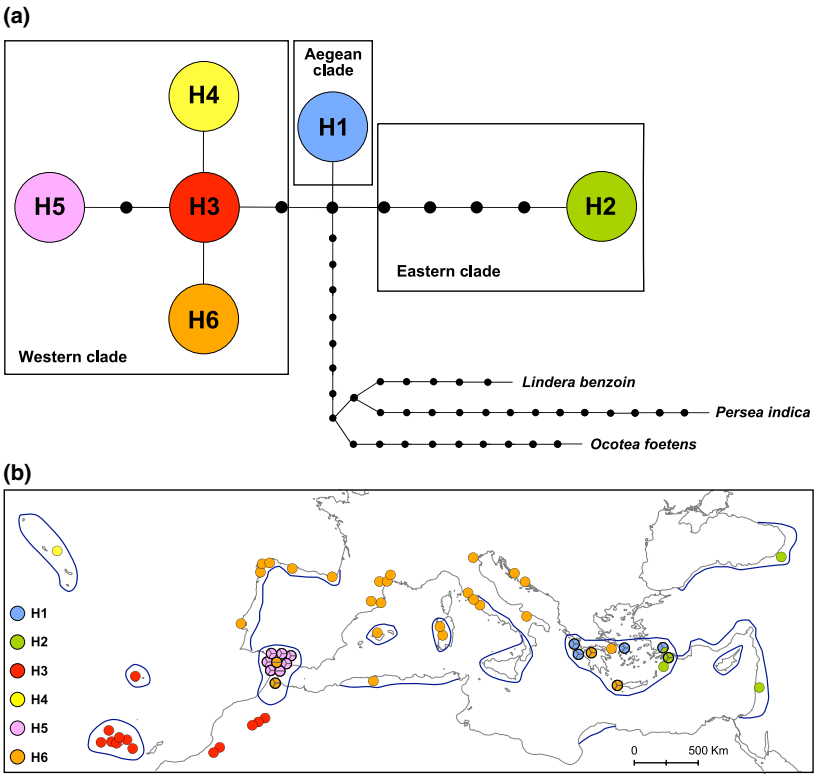

Figure 3 (a) Phylogeographical relationships among *Laurus* haplotypes, as inferred by statistical parsimony. Black circles indicate missing intermediate haplotypes. (b) Geographical distribution of *Laurus* plastid haplotypes. Sectors within circles represent number of individuals sampled and haplotypes found in each population. Dotted lines enclose main areas suitable for the persistence of *Laurus* populations during the last glacial period, as predicted by bioclimatic niche-based models (after Rodríguez-Sánchez & Arroyo, 2008).

extinct – ancestor. The haplotype network suggests an eastern Mediterranean diversification of extant *Laurus*, followed by subsequent westward expansion by a single haplotype, which colonized and diversified across the western Mediterranean and Macaronesia. Such an east–west vicariance across the Mediterranean region has been described for several other plant (e.g. Lumaret *et al.*, 2002, 2005; Hampe *et al.*, 2003; Fady-Welterlen, 2005; Besnard *et al.*, 2007) and animal taxa (e.g. Oosterbroek & Arntzen, 1992), and has often been interpreted as the genetic footprint of old (pre-Quaternary) range dynamics coupled with changes in the Tethys–Mediterranean palaeogeography (Oosterbroek & Arntzen, 1992; Petit *et al.*, 2005). In particular, the continuous movement of microplates and the sporadic appearance of water barriers throughout the Neogene would favour both migration and differentiation within the Basin (Steininger & Rögl, 1984; Rosenbaum *et al.*, 2002). Few studies, however, have tested the temporal matching of significant palaeogeographical events with the divergence between lineages (through molecular dating; but cf. Comes & Abbott, 2001; Oberprieler, 2005; Mansion *et al.*, 2008). Despite the relatively abundant fossil record for *Laurus* since the early Miocene (Appendix S3), the lack of distinctive characters in their macrofossils unfortunately precludes calibration of a relaxed molecular clock that could inform on likely divergence dates for haplotypes (Renner, 2005; Ho *et al.*, 2008). Nonetheless, fossil records may help us to interpret the past range dynamics of *Laurus* through the Neogene, although the inherent limitations of such inferences should be recognized. First, the sampling effort in palaeobotanical studies is not homogeneous, and is clearly limited in some areas (e.g. Macaronesia, North Africa); thus, the lack of fossil evidence should not be equated with the absence of the species in that area. Moreover, the information on past distributions provided by the fossil record might be poorly correlated with the actual palaeodistribution of low-density species (McLachlan & Clark, 2004). Lastly, extinctions followed by subsequent recolonization are difficult to infer from the fossil record. Unfortunately, in the particular case of *Laurus*, the poor state of conservation of most fossil records precludes the application of the cuticular analyses needed to confirm genus identification (Ferguson, 1974). Nevertheless, the abundant fossil evidence for other broadleaved evergreen species, including several genera of the Lauraceae, indicates the presence of extensive lauroid forests in the Mediterranean Basin – and probably also Macaronesia – throughout the Miocene and early Pliocene (Heer, 1857; Schmincke, 1968; Axelrod, 1975; Velitzelos & Gregor, 1990; Barrón & Peyrot, 2006; Kovar-Eder *et al.*, 2006; Utescher *et al.*, 2007).

In addition to fossil evidence, models that take into account the environmental requirements of species can alternatively be

used to predict their potential distributions at different time stages (e.g. Cheddadi *et al.*, 2006), providing independent evidence that may help to reduce the uncertainty associated with the fossil record. Rodríguez-Sánchez & Arroyo (2008) used such a framework to reconstruct *Laurus* range dynamics over the past 3 Myr, documenting a process of range retreat and fragmentation driven by harsh climatic changes, but also outlining multiple isolated regions in the Mediterranean Basin and Macaronesia that could have acted as long-term refugia for the species. Interestingly, most of those regions (Macaronesian Islands, southern Iberia and North Africa, the Aegean and Black Seas, and the Near East; Fig. 3b) harbour distinct cpDNA haplotypes, supporting the notion that these regions have sustained populations long enough to allow their genetic divergence. It seems noteworthy in this context that most of the genetic distinctiveness of *Laurus* exists in those areas that are considered to be most vulnerable to predicted climate change (Rodríguez-Sánchez & Arroyo, 2008). Considering the evidence as a whole (i.e. the low mutation rate of cpDNA, particularly slow in trees; the stability of climatic refugia in the Mediterranean region; and the sustained presence of *Laurus* fossil records throughout the Neogene, Appendix S3), together with some life-history characteristics of *Laurus* (long life span, remarkable sprouting ability and shade tolerance, bird-dispersed seeds) that confer high resilience to extinction (Bond & Midgley, 2001), we hypothesize that the phylogeographical structure of *Laurus* might be of ancient origin. Further evidence, particularly that from the integration of phylogenetic dating and appropriate fossil records, is needed to provide accurate dates.

Inferred range dynamics of *Laurus* across the Mediterranean and Macaronesia

Despite the relative geographical proximity, the eastern lineage present in Turkey, Rhodes and the Near East is strongly differentiated from nearby Aegean populations by six changes in the cpDNA sequences. This sharp phylogeographical break across the Aegean has been found for other taxa (e.g. *Nigella*; Bittkau & Comes, 2005) and roughly coincides with the well established floristic Rechinger's line (Strid, 1996). The existence of this barrier to plant migration and gene flow appears to stem from the palaeogeographical evolution of the region through the Miocene and early Pliocene (Greuter, 1979; see also Bittkau & Comes, 2005, and references therein). Indeed, our phylogeographical reconstruction (Fig. 3a) agrees with an ancient split and posterior differentiation of the H1 and H2 lineages in the Balkan–Aegean and easternmost Mediterranean domains, respectively. In addition, historically low population sizes and limited seed dispersal across populations might have contributed to the maintenance of this phylogeographical break (Irwin, 2002). A fine-scaled sampling of populations across both regions, combined with appropriate molecular dating, should help to ascertain the role of those palaeogeographical changes on lineage divergence. We note, however, the presence of haplotypes of the two lineages in one single population from south-western Turkey (Fig. 3b), which points out that Rechinger's line may have been crossed in more recent times.

The western Mediterranean acted as a remarkable centre of diversification for *Laurus*, generating four of the six haplotypes detected. This pattern of greater differentiation in the west resembles that of other Mediterranean taxa of ancient origin, such as *Frangula alnus* (Hampe *et al.*, 2003), *Hedera* (Valcárcel *et al.*, 2003) and *Olea europaea* (Besnard *et al.*, 2007). The ancestral haplotype (H3) for this western clade of *Laurus* is currently present in Madeira, the Canary Islands and southern Morocco, the other three western haplotypes (those of the Azores, southern Iberia and the widely distributed Mediterranean haplotype H6) deriving from it. Colonization of the western Mediterranean Basin by *Laurus* might have occurred as early as the middle Miocene, as suggested by fossil evidence in north-east Iberia, although subsequent recolonizations cannot be discounted. Similarly, southern Iberian populations of *Laurus* (haplotype H5) may already have been established in the Pliocene, considering the occurrence of fossil records of *Laurophyllum* (probably attributable to *Laurus*) from that period in nearby areas (Barrón *et al.*, 2003) and the long-term environmental suitability of this region for *Laurus* persistence (Rodríguez-Sánchez & Arroyo, 2008; Rodríguez-Sánchez *et al.*, 2008).

The current geographical pattern of haplotypes suggests a complex history of range fragmentation and retreat, coupled with certain long-distance dispersal events. The latter are necessary to explain at least the presence of *Laurus* in the Macaronesian archipelagos. *Laurus* seeds are dispersed by medium- to large-sized birds (Hampe, 2003; F.R.S., unpublished data), and are therefore capable of long-distance dispersal to oceanic islands. Recent long-distance dispersal, probably favoured by human translocations, might also explain the wide distribution of the H6 haplotype across the Mediterranean. This haplotype should have experienced the most intense range shifts as driven by the Quaternary glacial cycles, although several glacial refugia have been proposed based on their climatic suitability (Rodríguez-Sánchez & Arroyo, 2008). In agreement with this, the amplified fragment length polymorphism (AFLP) study by Arroyo-García *et al.* (2001) detected some degree of genetic differentiation among populations fixed for our H6 haplotype, which suggests the persistence of *Laurus* populations at multiple isolated refugia (e.g. Cantabrian Range, southern and north-east Iberia, Sardinia, Italian peninsula) during the glacial periods. Nonetheless, some extant populations may result from recent colonizations, such as those in south-east France, which appear more related to Italian *Laurus* populations than to nearby populations from north-east Iberia (Arroyo-García *et al.*, 2001).

The ancestral position of the Moroccan–Macaronesian haplotype H3 in the western clade, although somewhat anticipated by Bramwell (1972), is remarkable (see also Carine *et al.*, 2004). Further evidence from chromosome counts in both species ($2n = 24$, 48 in *L. nobilis*, and $2n = 36$, 48 in

L. azorica; Ehrendorfer *et al.*, 1968; Jalas & Suominen, 1991) suggests a derived polyploid origin for western *Laurus* populations. If the multiple descriptions of *L. azorica* from Neogene deposits in western Europe (Barbero *et al.*, 1981) are considered to be reliable, the current distribution of this haplotype (H3) would imply an extraordinary range retreat towards southern locations, followed by extinction in northern countries and colonization of Madeira and the Canaries. Alternatively, North Africa might have been colonized early by an extinct ancestor, and from there a formidable range expansion of the species could have occurred towards Macaronesia, Iberia and the entire western Mediterranean. A similar colonization pattern was found in *Quercus ilex* (Lumaret *et al.*, 2002), yet the species apparently did not reach Macaronesia (but see de Nascimento *et al.*, 2009). In fact, the former tropical climate of North Africa enabled the presence of extensive lauroid forests throughout much of the Miocene (Axelrod, 1975).

Unfortunately, the spatial and temporal realms of the colonization of Macaronesia from *Laurus* continental populations cannot be fully inferred with the available evidence. Whereas our population sample from the Macaronesian islands is relatively exhaustive, the lack of haplotype variability and reliable fossil records on the islands preclude any plausible reconstruction. Arroyo-García *et al.* (2001) used a more appropriate marker set (AFLP) at this scale, but their sampling included very few Macaronesian populations and none from Morocco. Thus, colonization events to and within Macaronesia could not be clearly inferred. However, results from this AFLP study suggested recent introductions into Madeira and the Canary Islands from Iberian populations (Arroyo-García *et al.*, 2001). Given the distribution of extant cpDNA haplotypes found here, North Africa emerges as the most likely source (see also Axelrod, 1975). Although neither process excludes the other, a North African–Canarian connection has been found in many other plant and animal taxa (Hess *et al.*, 2000; Carranza *et al.*, 2002; Valcárcel *et al.*, 2003; Juste *et al.*, 2004; Guzmán & Vargas, 2005; Besnard *et al.*, 2007; see also Médail & Quézel, 1999).

How many species within *Laurus*?

Our results correspond with those of Arroyo-García *et al.* (2001) in that they do not support the current delimitation of species within the genus *Laurus*. Both genetic analyses show that western Mediterranean and particularly Iberian laurel populations (considered as '*L. nobilis*') are more closely related to Macaronesian '*L. azorica*' than to other '*L. nobilis*' populations from the eastern Mediterranean. Analyses of morphological characters (Ferguson, 1974; Marques & Sales, 1999) point in the same direction. No reliable taxonomic key characters have been proposed, considering the remarkable leaf plasticity (Franco, 1960; see also Giacomini & Zaniboni, 1946). Moreover, both species are interfertile and their hybrid progeny grows well (Todua, 1988). Given all this evidence, we argue that the current taxonomic status of *Laurus* species requires a critical re-evaluation based on solid criteria. For instance, the recent classification of Madeiran, Canarian and southern Moroccan populations of *Laurus* as a third species, *L. novocanariensis* (Rivas-Martínez *et al.*, 2002), appears to be wanting with regard to morphological and molecular data (Franco, 1960; Marques & Sales, 1999; Arroyo-García *et al.*, 2001). Although there is a need for further studies, including detailed morphological analyses and more genetic markers, the current evidence appears to support the existence of only one species of *Laurus*.

We are now beginning to understand the range dynamics of European plants since the Last Glacial Maximum (*c.* 21 ka), but information diminishes rapidly as one moves further back in time, and to lower latitudes. Studies that apply integrative approaches to suitable model organisms are needed in order to reveal the more complex and much older range dynamics experienced by plants in the Mediterranean Basin and Macaronesia (Petit *et al.*, 2005; Vargas, 2007). Here we have shown that phylogeographical patterns are better explained when independent evidence from other fields is brought to bear, such as the fossil record or bioclimatic modelling. These joint analyses will ultimately throw light on one of the most recurrent questions in historical biogeography, the origin of the Mediterranean flora and vegetation.

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SUPPORTING INFORMATION

Additional Supporting Information is available for this article:

Appendix S1 Data on sampled *Laurus* populations: locality, species assignation, number of samples included (with their corres ponding GenBank accession numbers) and haplotypes found.
Appendix S2 Plastid DNA regions sampled and primers used for the study of sequence variability in *Laurus*.
Appendix S3 Map of Neogene fossil records of *Laurus* and source references.

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BIOSKETCH

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Appendix S1 Additional information on the *Laurus* populations sampled in this study: population code, locality, species assignation, geographical coordinates, number of samples included in the molecular analyses, haplotypes found in each population and GenBank accession numbers.

No.	Country	Locality	Species	Latitude (º N)	Longitude (º E)*	No. of samples	Haplotype	*trn*D-*trn*T accession no.	*trn*K-*mat*K accession no.
1	Portugal	Terceira (Azores)	*L. azorica*	38.7500	-27.2100	1	H4	FJ408965	FJ408876
2	Portugal	Madeira	*L. azorica*	32.7768	-16.9963	1	H3	FJ409002	FJ408913
3	Spain	Mencafete, El Hierro (Canary I.)	*L. azorica*	27.7424	-18.1006	1	H3	FJ408994	FJ408905
4	Spain	Barranco Seco, La Palma (Canary I.)	*L. azorica*	28.7418	-17.7860	1	H3	FJ409035	FJ408946
5	Spain	Acebiños, La Gomera (Canary I.)	*L. azorica*	28.1384	-17.2269	1	H3	FJ408987	FJ408898
6	Spain	Cedro, La Gomera (Canary I.)	*L. azorica*	28.1500	-17.2001	1	H3	FJ408988	FJ408899
7	Spain	Cuevas Negras, Tenerife (Canary I.)	*L. azorica*	28.3370	-16.8090	1	H3	FJ409036	FJ408947
8	Spain	Taganana, Tenerife (Canary I.)	*L. azorica*	28.5513	-16.2053	1	H3	FJ409032	FJ408943
9	Spain	Los Tilos, Gran Canaria (Canary I.)	*L. azorica*	28.0892	-15.5933	1	H3	FJ408986	FJ408897
10	Morocco	Anezi, Tiznit (Anti-Atlas)	*L. azorica*	29.6600	-9.3600	1	H3	FJ409040	FJ408951
11	Morocco	Oumarhouz, Addar (Anti-Atlas)	*L. azorica*	29.7500	-9.2550	1	H3	FJ409007	FJ408918
12	Morocco	Jb. Tazerkount (Middle Atlas)	*L. azorica*	32.1667	-6.4667	1	H3	FJ409034	FJ408945

13	Morocco	Jb. Tazemit (Middle Atlas)	*L. azorica*	32.3000	-6.2667	1	H3	FJ409033	FJ408944
14	Morocco	Jb. Ksiba (Middle Atlas)	*L. azorica*	32.5000	-6.0000	1	H3	FJ408998	FJ408909
15	Morocco	Jb. Bouhachem (Rif)	*L. nobilis*	35.2333	-5.4500	3	H6	FJ408967/68/69	FJ408878/79/80
16	Spain	Río Miel, Algeciras (Cádiz)	*L. nobilis*	36.1110	-5.5035	2	H5	FJ409004/05	FJ408915/16
17	Spain	Llanos del Juncal (Cádiz)	*L. nobilis*	36.1078	-5.5344	3	H5	FJ408999/9000/9001	FJ408910/11/12
18	Spain	Tiradero (Cádiz)	*L. nobilis*	36.1725	-5.5978	3	H5	FJ409037/38/39	FJ408948/49/50
19	Spain	Cruz del Romero (Cádiz)	*L. nobilis*	36.1756	-5.6083	3	H5	FJ408979/80/81	FJ408890/91/92
20	Spain	Gta. Hoya (Cádiz)	*L. nobilis*	36.205	-5.6325	2	H5	FJ408989/90	FJ408900/01
21	Spain	Aljibe (Cádiz)	*L. nobilis*	36.5300	-5.6300	2	H6	FJ408958/59	FJ408869/70
22	Spain	Pasadallana (Cádiz)	*L. nobilis*	36.5186	-5.5983	3	H5	FJ409024/25/26	FJ408935/36/37
23	Spain	La Jarda (Cádiz)	*L. nobilis*	36.5678	-5.5903	3	H5	FJ408995/96/97	FJ408906/07/08
24	Spain	Gta. Verde (Cádiz)	*L. nobilis*	36.8143	-5.40475	3	H5	FJ408991/92/93	FJ408902/03/04
25	Portugal	Sintra (Lisbon)	*L. nobilis*	38.7821	-9.4225	1	H6	FJ409027	FJ408938
26	Spain	Carballo (Galicia)	*L. nobilis*	43.2172	-8.7822	1	H6	FJ408984	FJ408895
27	Spain	Cortegada (Galicia)	*L. nobilis*	42.6167	-8.7667	1	H6	FJ408975	FJ408886
28	Spain	Pontedeume (Galicia)	*L. nobilis*	43.4153	-8.1026	1	H6	FJ408985	FJ408896
29	Spain	Sta. Mª del Naranco (Asturias)	*L. nobilis*	43.3881	-5.8680	1	H6	FJ409010	FJ408921
30	Spain	Monte Ulía (Basque Country)	*L. nobilis*	43.3328	-1.9525	1	H6	FJ409009	FJ408920

31	Spain	Montnegre (Catalonia)	*L. nobilis*	41.9500	2.9333	1	H6	FJ409006	FJ408917
32	Spain	Montserrat (Catalonia)	*L. nobilis*	41.613	1.799	1	H6	FJ409031	FJ408942
33	France	Béziers (Languedoc)	*L. nobilis*	43.4547	2.9347	1	H6	FJ408966	FJ408877
34	France	Caux (Languedoc)	*L. nobilis*	43.4944	3.3503	1	H6	FJ408982	FJ408893
35	France	Pommiers (Languedoc)	*L. nobilis*	43.9575	3.6122	1	H6	FJ408983	FJ408894
36	Spain	Tramuntana, Mallorca (Balearic Islands)	*L. nobilis*	39.8245	2.8288	1	H6	FJ409003	FJ408914
37	Algeria	Algiers	*L. nobilis*	36.4000	2.8500	1	H6	FJ408963	FJ408874
38	Italy	Villanova Monteleone (Sardinia)	*L. nobilis*	40.5045	8.5000	1	H6	FJ408974	FJ408885
39	Italy	Santulussurgiu (Sardinia)	*L. nobilis*	40.1300	8.6458	1	H6	FJ408973	FJ408884
40	Italy	Rovolon, Padua	*L. nobilis*	45.3666	11.6670	1	H6	FJ409011	FJ408922
41	Italy	Monti dell'Uccellina (Toscana)	*L. nobilis*	42.6333	11.0836	1	H6	FJ409041	FJ408952
42	Italy	Fiora River (Viterbo)	*L. nobilis*	42.4165	11.6350	1	H6	FJ409043	FJ408954
43	Italy	Marangone Valley (Lazio)	*L. nobilis*	42.0500	11.8170	1	H6	FJ409020	FJ408931
44	Italy	Bari (Puglia)	*L. nobilis*	41.0300	16.4900	1	H6	FJ409018	FJ408929
45	Croatia	Sibenik	*L. nobilis*	43.7409	15.8943	1	H6	FJ487607	FJ487609
46	Croatia	Split	*L. nobilis*	43.5066	16.4421	1	H6	FJ487608	FJ487610
47	Greece	Sami (Kefalonia)	*L. nobilis*	38.2531	20.6606	3	H1	FJ409021/22/23	FJ408932/33/34
48	Greece	Poros (Kefalonia)	*L. nobilis*	38.1489	20.7929	3	H1	FJ409015/16/17	FJ408926/27/28

49	Greece	Mainalo (Peloponnese)	*L. nobilis*	37.6666	22.2501	3	H6	FJ409012/13/14	FJ408923/24/25
50	Greece	Crete	*L. nobilis*	35.3469	23.7376	3	H6	FJ408976/77/78	FJ408887/88/89
51	Greece	Athens	*L. nobilis*	37.9802	23.7398	1	H6	FJ408964	FJ408875
52	Greece	Andros (Cyclades)	*L. nobilis*	37.8455	24.8817	3	H1	FJ408960/61/62	FJ408871/72/73
53	Greece	Rhodes (Dodecanese)	*L. nobilis*	36.3215	28.1544	1	H2	FJ409019	FJ408930
54	Turkey	Sögutköy (Marmaris Peninsula)	*L. nobilis*	36.6380	28.1892	3	H2	FJ409028/29/30	FJ408939/40/41
55	Turkey	Bayirköy (Marmaris Peninsula)	*L. nobilis*	36.7046	28.2233	3	H1, H2	FJ408970/71/72	FJ408881/82/83
56	Israel	Mt. Carmel	*L. nobilis*	32.6771	35.3019	1	H2	FJ409008	FJ408919
57	Turkey	Akçaabat (Trabzon)	*L. nobilis*	40.6836	40.7538	1	H2	FJ409042	FJ408953

* Western longitudes are negative

Appendix S2 Plastid DNA regions sampled and primers used for the study of sequence variability in *Laurus*.

Plastid region	Forward primer	Forward primer sequence	Reverse primer	Reverse primer sequence	References
trnH-trnK	trnH_GUG	ACG GGA ATT GAA CCC GCG CA	trnK_UUUr	CCG ACT AGT TCC GGG TTC GA	Demesure *et al*. (1995)
psbC-trnS	psbC	GGT CGT GAC CAA GAA ACC AC	trnS_UGAr	GGT TCG AAT CCC TCT CTC TC	Demesure *et al*. (1995)
trnS-trnfM	trnS_UGA	GAG AGA GAG GGA TTC GAA CC	trnfM_CAUr	CAT AAC CTT GAG GTC ACG GG	Demesure *et al*. (1995)
trnQ-trnR	trnQf	GGG ACG GAA GGA TTC GAA CC	trnRr	ATT GCG TCC AAT AGG ATT TGA A	Dumolin-Lapegue *et al*. (1997)
trnK-matK	3914F	TGG GTT GCT AAC TCA ATG G	1470R	AAG ATG TTG AT(CT) GTA AAT GA	Johnson & Soltis (1994)
trnD-trnT	trnD_GUC	ACC AAT TGA ACT ACA ATC CC	trnT_GGUr	CTA CCA CTG AGT TAA AAG GG	Demesure *et al*. (1995)
trnK-trnK	trnK_UUU	GGG TTG CCC GGG ACT CGA AC	trnK_UUUr2	CAA CGG TAG AGT ACT CGG CTT TTA	Demesure *et al*. (1995)
trnT-trnL	TRN A	CAT TAC AAA TGC GAT GCT CT	TRN B	TCT ACC GAT TTC GCC ATA TC	Taberlet *et al*. (1991)
trnM-rbcL	trnM_CAU	TGC TTT CAT ACG GCG GGA GT	rbcl_r	GCT TTA GTC TCT GTT TGT G	Demesure *et al*. (1995)
psaA-trnS	psaA	ACT TCT GGT TCC GGC GAA CGA A	trnS_GGAr	AAC CAC TCG GCC ATC TCT CCT A	Demesure *et al*. (1995)
trnC-trnD	trnC_GCA	CCA GTT CAA ATC TGG GTG TC	trnD_GUCr	GGG ATT GTA GTT CAA TTG GT	Demesure *et al*. (1995)

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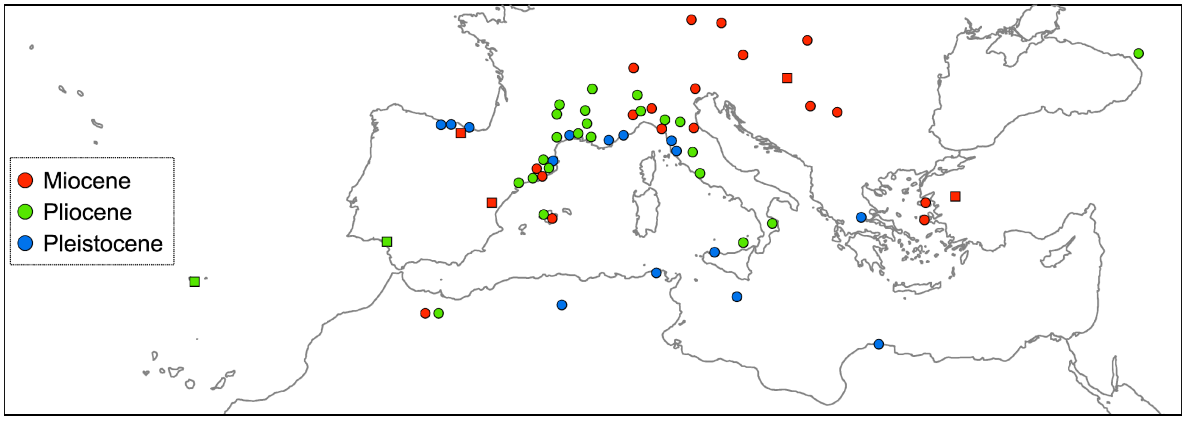
Appendix S3 Map of *Laurus* fossil records for the Neogene and source references. Square symbols represent uncertain records (classified as *Laurophyllum*).



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