Isoprenoid emission in hygrophyte and xerophyte European woody flora: ecological and evolutionary implications

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Type of article: **Research paper**

Running title: **Isoprenoid emission in hygrophytes and xerophytes**

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ABSTRACT

Aim. The relationship between isoprenoid emission and hygrophily was investigated in woody plants of the Italian flora, which is representative of European diversity.

Methods. Volatile isoprenoids (isoprene and monoterpenes) were measured or data collected from the literature, on 154 species native or endemic in the Mediterranean. The Ellenberg indicator value for moisture (EIVM) was used to describe plant hygrophily. Phylogenetic analysis was carried out, at a broader taxonomic scale on 128 species, and then refined on strong isoprene emitters (Salix and Populus species) based on isoprene synthase gene sequences (IspS).

Results. Isoprene emitters were significantly more common, and isoprene emission was higher in hygrophilous EIVM classes, whereas monoterpene emitters were more widespread, and monoterpene emission was higher, in xeric classes. However, when controlling for phylogeny, isoprene emission was not associated with EIVM, possibly due to the large presence of Salicaceae among hygrophilous isoprene emitters. Moreover, the distribution of isoprene emitters among EIVM classes was not related to IspS-based phylogenesis in Populus and Salix, suggesting that the gene has not undergone evolution linked to ecological pressure. In contrast, monoterpene emission pattern is independent of phylogeny, suggesting that the evolution of monoterpenes is associated with transitions to more xeric habitats.

Main conclusions. Our results reveal an interesting ecological pattern linking isoprenoids and water availability. The idea is surmised that isoprene is a trait that i) evolved in plants adapted to high water availability; ii) is replaced by more effective protection mechanisms, e.g. more stable isoprenoids, in plants adapting to more xeric environments; iii) being strongly constrained by phylogeny, persists in Salicaceae adapted to more xeric environments.

Keywords: Adaptation, Chemo-taxonomy, Hygrophytes, Isoprene, Monoterpenes, Phylogenies, Salicaceae, Xerophytes, Water stress.

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INTRODUCTION

Leaves of many woody and perennial plants constitutively emit volatile isoprenoids (isoprene and monoterpenes) to the atmosphere, at rates that often exceed 1-2% of the photosynthetic carbon fixation, especially in stressed leaves (Loreto & Schnitzler 2010). Isoprene and monoterpenes are formed from photosynthetic metabolism in the chloroplasts (Loreto & Schnitzler 2010). Generally, either isoprene or monoterpenes are emitted but not both (Harrison *et al*. 2013). However, some species (e.g. Myrtales) show significant storage of monoterpenes in specialized structures and these species can emit both isoprene and monoterpenes (Niinemets *et al.* 2004).

Isoprene is believed to play a role against thermal and oxidative stresses, possibly because of the capacity of this molecule to stabilize thylakoidal membranes (Singsaas *et al.* 1997, Velikova *et al.* 2011), or to remove reactive oxygen or nitrogen species within the mesophyll (Loreto & Velikova 2001; Vickers *et al.* 2009). Light-dependent monoterpenes may play similar roles, but they are also often involved in plant communication with other organisms, especially in multitrophic plant defense and pollination (Dicke & Baldwin 2010).

The emission of isoprene and monoterpenes is widespread across plant families (Harley *et al.* 1999). A recent study has indicated a strong phylogeographic signal for monoterpenes; the emission of monoterpenes is qualitatively different in cork oaks across their distribution range in Europe (Loreto *et al.* 2009). Alien species of Hawaii emit more monoterpenes than native ones, which is also suggested to be an indication of greater evolutionary success of alien species since monoterpene emission is associated with higher stress resistance (Llusiá *et al.* 2010).

However, there seems to be no straightforward relationship between isoprene emission and plant taxonomy or phylogeny. Isoprene emission is absent in herbaceous, annual vegetation, whereas it is widespread in trees and perennial plants (Kesselmeier and Staudt 1999). However, this robust trend may not be associated to phylogeny, as isoprene emission is limited to woody life-forms of families that also include herbaceous species (Fineschi *et al*. 2013). Hanson *et al.* (1999) reported that isoprene emission is more widespread in mosses than in all other taxa, and this is so far the only unambiguous phylogenetic pattern. This finding led to the suggestion that the isoprene emission trait evolved when plants conquered the land and started coping with more severe thermal extremes than in the water-buffered environment (Hanson *et al.* 1999). Similarly, Vickers *et al.* (2009) and Fineschi & Loreto (2012) commented that isoprene could have evolved as a first mechanism to cope with more recurrent and stronger oxidative stress in the terrestrial than in aquatic environments, being then replaced by more effective mechanisms when plants adapted to more

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xeric conditions. No other adaptive relationships are apparent when dealing with volatile isoprenoids emitted from plants that do not have specialized structures to accumulate isoprenoids.

We reasoned that, if the emission of isoprene has evolved in plants conquering the land, then the trait could still be more widespread in hygrophytes than in xerophytes. To test this idea, the emission of isoprene was assessed in the Italian woody flora, which is representative of the Mediterranean eco-region, one of the primary global biodiversity hotspots, and an area of exceptional biodiversity value exhibiting high endemism (Blondel & Aronson 1999; Médail & Quézel 1999; Comes 2004; Thompson 2005; Médail & Diadema 2009). Further, the vast majority of the tree genera of continental and northern-Europe (including Scandinavia and the British Isles) naturally occurs in Italy today, as the Italian peninsula was one of the main Quaternary glacial refugia (Bennet *et al.* 1991). Thus, the Italian woody species account for most of the total European diversity of trees and shrubs.

The Ellenberg indicator values (EIV; Ellenberg 1974; Ellenberg *et al.* 1991) characterize the adaptation of a vascular plant species to edaphic and climatic conditions in comparison to other species: i.e., each plant species is given values denoting the position at which plants reach peak abundance along environmental gradients (Diekmann 2003; Godefroid & Dana 2007). A 9- or 12-point ordinal scale for each of the following parameters is used: moisture, soil nitrogen status, soil pH, soil chloride concentration, light, temperature and continentality. Although EIV were originally designed for Central Europe and assigned to the Central European flora only (Ellenberg 1974; Ellenberg *et al.* 1991), they have been subsequently redefined and calculated for other floras, such as Britain (Hill *et al.* 1999), Southern Greece (Böhling *et al.* 2002) and Italy (Pignatti *et al.* 2005). EIV have been widely used to interpret responses to environmental gradients (Diekmann 2003), and are now used also as an effective tool for applied purposes, such as remotely-sensed vegetation monitoring (Schmidtlein 2005), conservation strategies (Sullivan *et al.* 2010), ecological restoration (Krecek *et al.* 2010), and prediction of pollution effects (Jones *et al.* 2007 ; Dupré *et al.* 2010).

Experimental studies found that EIV ranking within a given flora is a highly reliable indicator of adaptation to environmental conditions (Schaffers & Sýcora 2000; Diekmann 2003; Schmidtlein 2005; Jones *et al.* 2007; Klaus *et al.* 2012): in particular, the index for soil moisture (EIVM) was found to perform the best (Schaffers & Sýcora 2000; Fanelli *et al.* 2007; Krecek *et al.* 2010). The EIVM was therefore used here to rank isoprenoid-emitting species of the Italian woody flora according to an index of hygrophily.

Two phylogenetic analyses were carried out on this dataset at different taxonomic scales. The first analysis was performed at a broad scale on woody species belonging to 31 different orders representing main lineages among woody plant species, to assess whether isoprenoids emissions

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and EIVM show phylogenetic signal (i.e. whether phylogenetically related species tend to have more similar EIVM and/or isoprenoid emission values than more distantly related species). The second analysis was performed on a narrower range of taxa to assess whether changes of the coding sequences of isoprene synthase (IspS), the enzyme responsible for isoprene production (Silver & Fall 1995; Loreto & Schnitzler 2010), are associated with changes of EIVM. To perform the latter test, poplars (*Populus* sp.) and willows (*Salix* sp.), two main genera of isoprene emitters in the Mediterranean area and worldwide (Kesselmeier & Staudt 1999), with plant species spanning several classes of EIVM, were studied in detail.

MATERIALS AND METHODS

Plant material

Constitutive emissions of isoprene and monoterpenes from light-dependent pools that are not concentrated in storage compartments, are found almost exclusively in perennial, woody plants (Loreto & Schnitzler 2010), thus this survey was limited to these plant species. A check-list of woody species (i.e., trees, shrubs and lignified lianas) of the flora of Italy, was compiled using, as a first approximation, the life form assignments made by Pignatti (1982). This preliminary list, only including Phanerophytes (P) and Nano-phanerophytes (NP) life forms, was then complemented with some Chamaephyte (Ch) species that, based on field experience and on species description in regional floras, are in fact lignified shrubs. Further refinement was done by deleting from the check-list: i) all non-native species, as Ellenberg indices can be defined only in comparison to other species growing in natural communities within an homogeneous biogeographical area. Exceptions were possible for those species of very ancient or controversial introduction, such as *Castanea sativa* and *Pinus pinea*, or for alien plants that are now widely naturalized in the Mediterranean vegetation (e.g. *Robinia pseudoacacia*); ii) the micro-species of critical genera such as *Rosa* and *Rubus* (which were then limited to 'main' species; cf. Diekmann 2003); iii) the hybrid taxa and the species of controversial taxonomic value [i.e., those species listed in Pignatti (1982), but rejected or doubtfully accepted in Conti *et al.* 2005]; iv) some species which had an obviously wrong life form in Pignatti (1982). As a result, 323 plant species were considered in the check-list of the Italian woody flora (Appendix S1).

The Ellenberg ecological indicator for moisture

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We used the Ellenberg indicator value for moisture (EIVM) to formalize the ranking of the woody species along a gradient of hygrophily. The ordinal scale defined by Ellenberg (1974) for EIVM is composed of 12 classes; however no woody species of the Italian flora fall within classes 10-12 (i.e. plants with permanently submerged roots and aquatic plants, see Pignatti *et al.* (2005)). Thus, the EIVM of the species included in the present work range from class 1 (plants of extremely arid habitats), to class 9 (species of marshy soils undergoing frequent root submersion).

The EIVM were assigned to plants according to Pignatti *et al.* (2005). However, for 39 species the original attribution by Pignatti *et al.* (2005) was either considered obviously wrong (see Fanelli *et al.* 2007), or missing. In these cases the correct EIVM was attributed according to descriptive vegetation papers and original field data, as recorded during field surveys to collect volatile isoprenoids (Appendix S1).

Volatile isoprenoids

The emission of volatile isoprenoids was reported for 149 species, i.e. about half of the total native woody flora of Italy, and in five exotic species common in the Mediterranean vegetation, that we had considered, without any relevant bias in terms of EIVM class (Appendix S1). No important European tree species is missing from the emission database, whereas, for a small number of common European shrubs or lianas (i.e. *Clematis vitalba, Cornus sanguinea, Crataegus oxyacantha, Euonymus europaeus, Lonicera caprifolium, Rhododendron sp.pl., Viburnum tinus, Vinca sp.pl.*) it was not possible to obtain reliable emission data.

Species were assigned to two Boolean (0/1) categories, emitting or non-emitting, based on the potential emission rate threshold of 1 $\mu g\ g^{-1}\ h^{-1}$ for isoprene and 0.2 $\mu g\ g^{-1}\ h^{-1}$ for monoterpenes, which are known to be emitted 5-10 times less than isoprene. The emission rates actually measured are also presented, to have a quantitative assessment of the relationship between emission and EIVMs.

Plant material was both collected and tested during the summer months (June-August) in a common garden at CNR-Rome, or measurements of isoprenoid emission were made in situ across Italy in periods (June or September) characterized by high temperatures and non-limiting conditions (especially no drought) for the physiology of plants. In all cases, a LI-COR 6400 (LI-COR, Lincoln Nebraska, USA) was used to standardize measurements in its 6 cm^2 gas-exchange cuvette. This leaf area was exposed to 1000 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density, 30°C, and 50% relative humidity, under a flux of 0.5 L min^{-1} of air that was passed through a catalytic converter (Parker Hannifin Corp., ChromGas Zero Air Generator 1001) to filter contaminants and other volatile organic compounds. The released isoprenoids were collected into a cartridge packed with adsorbent

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(200 mg of Tenax; SRA Instruments, Milan, Italy). Tenax has been used in many past experiments for isoprene measurements. Though unable to retain high isoprene concentrations without undergoing breakthrough, Tenax may reveal concentrations as low as < 1 ppb, thus fulfilling the scope of separating non-emitters when loading small volumes of air onto the adsorbent. Two to five L of air were trapped at a flow of 150 ml min^{-1} in the cartridge that was placed at the outlet of the cuvette. Measurements were made when the physiological parameters of the leaf (photosynthesis, transpiration, stomatal conductance; also monitored by the LI-COR 6400 instrument) were stable, and were repeated on at least three different leaves of different plants. The number of replications was increased in presence of large intraspecific variation of the emission, particularly in the case of low monoterpene emitters.

The cartridges were kept refrigerated until desorbed and analyzed with a GC–MS (Agilent 6850; SRA Instruments) using a capillary column (DB-5, Agilent, 30 m × 0.25 mm inner diameter and 0.25 μm film thickness). The actual emissions were positively quantified filling the cartridges with 2 L of air in which 70 ppb of gaseous standards (Rivoira, Milan, Italy) of isoprene or main monoterpenes (α-pinene, β-pinene, sabinene, myrcene, limonene) were mixed.

Broad-scale phylogenetic analysis

We created a composite phylogenetic tree representing the relationships among the studied species (Fig. 1). The tree is based on the Angiosperm Phylogeny Website (Stevens, 2001 onwards) and was further refined based on published molecular phylogenies (Appendix S2). In this way, we could determine the phylogenetic position of 128 species. However, as some of these species tolerate a wide range of moisture conditions (see Appendix S1), the phylogenetic analyses involving EIVM were limited to 119 species.

By using the phylogenetic tree in Fig. 1 we performed an Abouheif (1999) test to assess whether isoprenoids emitters and EIVM show phylogenetic signal at this taxonomic scale. To test the association between phylogenetic signal and hygrophily we used the Ellenberg indicator values for moisture. By contrast, for testing for phylogenetic signal in isoprene and monoterpene emission capability, we performed two distinct tests on the Boolean, emitting/non-emitting (0/1) classes of both isoprenoid emission types.

Next, we used the phylogeny to analyze the relationship between Ellenberg indicator values and isoprenoid emission. Therefore, we built Bayesian Phylogenetic Mixed Models using the MCMCglmm R package (Hadfield 2010, R Core Team 2012), with either isoprene (emitter/non-emitter) or monoterpene (emitter/non-emitter) emission as binary dependent variables and EIVM as explanatory variable. The mixed model implemented in MCMCglmm can incorporate the

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phylogenetic relationships among species as a random factor, thereby controlling for the non-independence of data points due to shared ancestry.

Narrow-scale phylogenetic analysis on Salicaceae

Leaf samples were collected from *Salix* and *Populus* species (Appendix S3) and stored at -80°C until DNA extraction. We selected *Populus* and *Salix* because i) species of these two genera play an important role in the woody Mediterranean and European flora, ii) all species emit isoprene, and iii) species from both genera represent several EIVM classes, spanning from class 3 (e.g. *Salix alpina*) or 5 (*Populus tremula*) to class 8 (e.g. *Salix viminalis* or *Populus nigra*).

Total DNA was extracted using Invitek Invisorb Spin Plant Mini Kit (Stratec GmbH, Berlin, Germany) according to manufacturer's instructions, from approximately 100 mg of material, ground in the automatic grinding mill MM200 (Retsch GmbH, Haan, Germany). Isoprene synthase gene was amplified using Pa*IspS*-Fw2 and Pa*IspS*-Bw3 primers (Fortunati *et al.* 2008; Appendix S4). Polymerase chain reactions (PCRs) were performed in 100 µl containing 30 ng of template DNA, 5x PCR reaction buffer (Promega Corporation, Madison, Wisconsin, USA), 0.2 mM of each dNTPs, 0.2 µM of each primer, 2.0 mM MgCl2, 3.2 U Taq polymerase (GoTaq, Promega). All samples were amplified on a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany), following two touchdown PCR profiles for *Populus* and *Salix* species, respectively: 1) 3 min at 95°C, 15 touchdown cycles of 95°C 30s, 70°C 1' (-1°C/cycle), 72°C 2'; 20 cycles of 95°C 30s, 55°C 1', 72°C 2' and final extension at 72°C 10 min; 2) 3 min at 95°C, 15 touchdown cycles of 95°C 30s, 65°C 1' (-1°C/cycle), 72°C 2'; 20 cycles of 95°C 30s, 50°C 1', 72°C 2' and final extension at 72°C 10 min.

PCR products were purified using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Uppsala, Sweden), and directly sequenced on an ABI 3130 Avant automated sequencer (Life Technologies Corporation, Carlsbad, California, USA) using Pa*IspS*-Fw2 and Pa*IspS*-Bw3 primers and specific internal primers (Appendix S4). Purifications of sequencing reactions products followed the ethanol-sodium acetate precipitation protocol provided with the sequencing kit. Confirmation of sequence identity was performed by BLASTN search against the GenBank non-redundant database using default parameters (Altschul *et al.* 1997). The resulting amino acid sequences were screened for the presence of specific residues that appear to be implicated in reducing active site volume in isoprene synthases relative to monoterpene synthases (Sharkey *et al.* 2013).

The eleven *IspS* coding sequences obtained from poplar and willow species where the EIVM was also identified, together with sequences of the same gene from other plant species (Appendix S3)

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were aligned using ClustalX (Thompson *et al.* 1997). The phylogenetic analyses were conducted using the software MEGA v.5.05 (Tamura *et al.* 2011). Maximum likelihood phylogenetic trees (ML) were reconstructed and the reliability of tree branches was evaluated by using bootstrapping with 9999 pseudo-replicates (Felsenstein 1985). Further, a ds/dn analysis using SNAP (Synonymous (ds) vs Nonsynonymous (dn) Analysis Program) at http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html, which calculates the proportion of synonymous substitutions per potential synonymous site and the proportion of nonsynonymous substitutions per potential nonsynonymous site using the Nei and Gojobori method (Nei and Gojobori 1986), was carried out.

By using the *IspS* phylogenetic tree, an Abouheif test of phylogenetic signal was performed to evaluate whether the EIVM of the *Salix* and *Populus* species were related to changes in *IspS* sequences at this taxonomic scale.

RESULTS

Among the woody species tested for emission of volatile isoprenoids, the proportion of isoprene-emitting species was clearly higher in more hygrophilous EIVM classes, especially in class 8, where about 80% of the plants emit isoprene (Fig. 2a). The trend was opposite for the emission of monoterpenes, with monoterpene-emitters being found more often in the more xeric Ellenberg classes (Fig. 2b). The association between the two classes of volatile isoprenoids and the moisture level that characterize the habitats of the Mediterranean woody species was confirmed by a non-parametric Mann-Whitney Z-test. This test showed that the median EIVM is significantly higher in isoprene-emitting than in non-emitting species, while the EIVM is significantly lower in monoterpene emitters than in non-emitters (Fig. 3, $p < 0.001$ in both cases).

A trend was also found when isoprenoid emission rates were attributed to EIVM classes. Plant species belonging to hygrophilous EIVM classes emitted more isoprene (Fig. 4a, $p = 0.028$), whereas the emission of monoterpenes was generally higher in the xeric EIVM classes (Fig. 4b, $p = 0.030$). However, when differences of emission rates among EIVM classes were assessed statistically, only isoprene was significantly different (Kruskal-Wallis non-parametric test, $p = 0.0042$, followed by post-hoc Dunn's Multiple Comparison Test showing differences between means of EIVM contrasting classes, e.g. 2-6 and 7-8). In the case of monoterpenes, the Kruskal-Wallis test yielded non-significant differences ($p = 0.136$), possibly because of the higher variability of the sampled emissions, and so we did not proceed with statistical mean separation among EIVM

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classes. The presence of monoterpene emitters with and without storage organs among the sampled plant species might have contributed to make more variable the emission. As we only assessed emissions, not contents, we did not separate monoterpene emitters according to the presence of storage organs. The different emission rates of isoprenoids, as highlighted above, were not associated with differences in the rates of photosynthesis among EIVM classes (data not shown).

Since isoprene-emitters of hygrophyte EIVM classes are dominated by Salicaceae, and monoterpene-emitters of xeric EIVM classes mostly belong to Cistaceae and Pinaceae, a phylogenetic analysis was carried out to understand how the phylogeny could have interacted with the ecological signal.

Evidence of evolutionary conservatism in Ellenberg's indicator values was already found by Prinzing *et al.* (2001). In agreement with this former report, the Abouheif test showed significant phylogenetic signal in EIVM in our data set of woody species ($C = 0.380$, $p = 0.001$; 999 permutations, 119 species). Likewise, the species also showed significant phylogenetic signal in both isoprene and monoterpene emitting competence ($C = 0.547$, $p = 0.001$, and $C = 0.276$, $p = 0.001$ for isoprene and monoterpene, respectively; in both cases 999 permutations and 128 species were used). Accordingly, we may hypothesize that, at this broader phylogenetic scale, the species' capability to adapt to more or less xeric terrestrial environments and their isoprenoid emissions are both related to the evolutionary history of plants. In this view, EIVM and isoprenoids emissions refer to large-scale environmental gradients, *sensu* Silvertown *et al.* (2006, Figure 1).

However, when controlling for phylogeny in the MCMCglmm analysis, we found that the presence of isoprene emission is not associated with Ellenberg indicator values (binomial phylogenetic mixed model: posterior mean 2.804, lower 95% credibility interval -2.236, upper 95% credibility interval 7.272, $p = 0.133$). This is most likely due to the overwhelming influence of closely related, hygrophilous isoprene emitters (mainly Salicaceae) in the dataset. To the contrary, monoterpene emission is significantly less frequent in hygrophytes (binomial phylogenetic mixed model: posterior mean -0.465, lower 95% credibility interval -0.882, upper 95% credibility interval -0.124, $p < 0.01$), suggesting that the evolution of monoterpene emission is associated with transitions to more xeric habitats. The two traits (i.e. isoprene and monoterpene emission) were not significantly related to each other (binomial phylogenetic mixed model with isoprene emission as dependent variable: posterior mean: 3.146, lower 95% credibility interval -23.604, upper 95% credibility interval 28.925, $p = 0.711$), which suggests that the two traits are not complementary and their evolution is probably determined by separate ecological factors.

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Since isoprene emitters belonging to *Populus* and *Salix* genera were clearly distributed along the gradient of hygrophily, we further explored whether this distribution was associated with phylogenetic differences, as inferred from differences in the isoprene synthase gene. Eleven partial genomic isoprene synthase sequences were identified in *Populus* and *Salix* species (Appendix S3). All sequences displayed high degree of homology (from 95% to 100%) with already available *IspS* sequences. All the sequences were screened for the presence of two Phe residues that are involved in reducing active site volume in isoprene synthases relative to monoterpene synthases (Sharkey *et al.* 2013). In addition, SNAP analysis demonstrated higher ds than dn in all sequences (average ds/dn pairwise comparison ratio = 8.29).

Phylogenetic analysis was carried out by using the coding sequences of *IspS* of *Populus* and *Salix* species isolated in this study, together with poplar sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (listed in Appendix S3). When using *Vitis vinifera* and *Pueraria montana* as outgroups, the ingroup turned out to be monophyletic even if the relative position of the two outgroups has low bootstrap support. Two main clades were identified within the in-group, one clustering most *Populus* species and the other clustering *Salix* species (Fig. 5a, b). Furthermore, within the *Populus* clade, the species grouped according to section classification based on other markers (Eckenwalder 1996). An exception was represented by *P. nigra*, which was grouped within the *Populus* section in spite of being classified as a member of the *Ageiros* section (Eckenwalder, 1996). The ecological adaptation trait, as marked by the EIVM classes, and the pattern of nucleotide changes in *IspS* were not associated in poplar and willow species (Fig. 5b). This was further confirmed by the non-significant results of the Abouheif test (Abouheif C = 0.033; p = 0.357).

DISCUSSION

An association between isoprene emission and hygrophily was suggested by several independent observations: a) that isoprene is emitted at higher rates in hygrophyte forest plants than in more xeric plants of transitional woodlands and savannahs, e.g. in central Africa (Greenberg *et al.* 1999); (b) that isoprene emission is generally more common in fast-growing, water-spending species (Vickers *et al.* 2009). Perhaps this is in turn related to the phloem-loading mechanism, because isoprene emitters are characterized by symplastic phloem loading (Kerstiens & Possell 2001). Whether this trait is also related to fast-growth and hygrophily should be investigated; c) that isoprene emission is more common in mosses than in other clades of plants (Hanson *et al.* 1999). Hanson *et al.* (1999) suggested that isoprene emission by plants could have been an important

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ancient mechanism of adaptation to terrestrial environment that increased tolerance to thermal stresses in environments not buffered by water. Vickers *et al.* (2009) argued that isoprene could also have helped early land plants cope with high oxidative stress in the atmosphere.

Indeed, isoprene emission is more common and the emission rates are higher in the hygrohytes of the Italian flora that we have tested. Emission rates expressed on a leaf area basis are reported here, but the trend would hold when expressing isoprene emission on a leaf mass basis, as leaves of hygrophytes are generally thinner than in xerophytes. An exception to this trend was found in EIVM class 1. However, this xeric class includes only three species, and only one isoprene-emitting species, *Chamaerops humilis,* which is the sole representative of Arecaceae (the palm family) in mainland-Europe. This taxon evolved in moist tropical climates of the rain-forest biome, where it still harbors its highest diversity; most palms have a very low drought-tolerance, and the few species adapted to dry habitats are probably the result of recent radiation (Eiserhardt *et al.* 2011). Interestingly, a similar reasoning might apply to *Myrtus communis*, the only emitter found in EIVM class 2 (out of 16 tested taxa in this class), as this is the only European member of the tropical family Myrtaceae (Biffin *et al.* 2010).

However, our large-scale phylogenetic analysis does not support the ecological value of these observations, because of the strong phylogenetic signal in isoprene emission; for instance, most isoprene emitting species in the more hygrophilous Ellenberg categories belong to Salicaceae (see Appendix S1). As the evolution of isoprene emission is not associated with evolutionary adaptation to hygrophily, our data suggests that resistance to other environmental factors (such as coping with thermal or oxidative stresses (Vickers *et al*. 2009)) might characterize isoprene emitters. On the other hand, our analysis has shown that, when the phylogenetic relationships are taken into account, monoterpene emission is more common in xeric species of the Italian woody flora, suggesting that monoterpenes evolved in arid habitats, independently on whether the emission of monoterpenes occurs from storage pools or directly from photosynthesis, in a light-dependent way. Thus, different classes of isoprenoids might have evolved in response to different environmental factors, rather than being complementary of each other.

It is unclear why the isoprene emission trait has been lost multiple times in terrestrial plants (Harley *et al.* 1999, Sharkey *et al.* 2005, Sharkey *et al.* 2013). Monson *et al.* (2013) recently noted that the high frequency of loss might indicate that isoprene emission is a favorable trait only in a limited number of environments, or for few plants. As monoterpenes and non-volatile isoprenoids are effective antioxidants protecting plants from many abiotic and biotic stressors (Vickers *et al.* 2009), our observations suggest that isoprene is synthesized and emitted only when more effective mechanisms of stress protection, especially regarding stress conditions associated with xerophily,

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are not active. In fact, as shown in Appendix S1, most plant species emit either isoprene or monoterpenes, not both. The trade-off between isoprene and monoterpenes was also observed by Harrison *et al.* (2013) in a survey that was carried out at worldwide level, and therefore emerges as an important feature, not limited to Mediterranean conditions. The emission of monoterpenes seems to be a successful trait in alien species invading new territories, possibly again due to the ability of monoterpenes of conferring resistance against multiple stresses (Llusia *et al.* 2010).

At a finer taxonomic scale, we then explored whether the hygrophily of isoprene emitters, as indicated by species assignment to the Ellenberg classes, showed phylogenetic signal within Salicaceae. Specifically, we tested whether isoprene emitters, phylogenetically close with respect to *IspS*, also shared similar EIVM classes. However, the distribution of EIVM classes was not associated to the phylogenetic patterns of *IspS*. We therefore hypothesize that *IspS* has not undergone convergent evolution linked to ecological pressure, namely to adaptation to xeric environments. Perhaps genes at earlier stages of the chloroplastic isoprenoid pathway are more pleiotropic and are therefore subjected to heavier selective pressure than *IspS* (Ramsay *et al.* 2009), or regulation of gene expression or enzyme activation, rather than gene sequence, provides sufficient response to changes in hygrophily.

On the other hand, the phylogeny based on *IspS* showed that poplar and willow species could be properly separated, indicating a strong match with taxonomic information (Eckenwalder 1996), and confirming the value of genes underlying volatile isoprenoid biosynthesis as chemo-taxonomical markers (Loreto *et al.* 2009). A relevant exception to the clear match between *IspS* phylogeny and taxonomy in Mediterranean poplar species is represented by *P. nigra*, which grouped within the section *Populus* in spite of being a member of the section *Ageiros*, maybe as a consequence of its hybrid origin (Smith & Sytsma 1990).

The public availability of *IspS* sequences in GenBank made it possible to match the phylogenies of our Mediterranean poplars with those of non-European poplars. The resulting ML tree showed that gene identity between poplars of different regions of the world is higher than the identity between genera sharing the same ecological environment. Moreover, *P. euphratica*, a species adapted to desert conditions (Qiu *et al.* 2011) was phylogenetically very distant from Mediterranean species that are adapted to xeric conditions. Accordingly, changes in *IspS* sequences on poplars of different habitats strongly reflect the species' phylogenetic relationships rather than ecological adaptation. Therefore, the gene evolution and function (i.e. isoprene emission) appears to be a strong phylogenetic trait that did not undergo adaptive modification in recent evolutionary time. This observation is in good agreement with the outlier behavior of *Chamaerops humilis* (Arecaceae) and *Myrtus communis* (Myrtaceae): in spite of their xeric nature, both these plants have retained their

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ancestral isoprene-emission character. A similar conclusion was reached when analyzing isoprenoid emissions in oaks (Loreto *et al.* 1998; Loreto *et al.* 2009). Similarly, also monoterpene-emitting taxa, like e.g. the few *Betula* species that can be found in the Mediterranean area, in clearly hygrophytic habitats, may have retained this trait due to a strong phylogenetic signal rather than with their present-day ecological distribution.

In conclusion, we surmise that biosynthesis and emission of different volatile isoprenoids have likely evolved in response to different stimuli. Isoprene likely has evolved independently many times, characterizing about all vascular plants, from ferns to angiosperms. It might be a primitive adaptive trait to terrestrial life, which might not have further evolved in response to more recent ecological pressures, being rather lost in favor of more effective protective mechanisms, in agreement with the 'opportunistic' hypothesis put forward by Owen & Peñuelas (2005). Monoterpenes might have evolved to adapt to xeric environments and might yet be an important adaptive trait in response to drought in the Mediterranean flora. Further studies are needed to test these conclusions, both completing the current survey of European flora, and, at an even wider level, providing more data about vegetation worldwide.

ACKNOWLEDGMENTS

Mauro Medori, Lucia Michelini and Isabel Nogues helped with sample collection and measurements. Pietro Bianco provided an early version of the data-base of the Italian woody flora. Salvatore Cozzolino provided valuable comments on the manuscript. Costantino Bonomi at the Museo delle Scienze, Trento, and the staff at the Ufficio Territoriale per la Biodiversità, Belluno, are gratefully acknowledged for providing samples of *Salix* species. Arie Altman kindly provided DNA samples of *Populus euphratica*. Riikka Rinnan kindly provided specimens of *Salix reticulata* from boreal latitudes. This work was supported by the European Science Foundation Eurocores programme "Ecology of Plant Volatiles (EuroVOL)", project "Molecular and metabolic bases of volatile isoprenoid emissions (MOMEVIP)", by the European Commission – FP7 project "Development of improved perennial non-food biomass and bioproduct crops for water stressed environments" (WATBIO), and by the Italian Ministry of University and Research PRIN 2011 project: "Going to the root of plant productivity: how the rhizosphere interact with the aboveground armament for indirect and direct defense against abiotic and biotic stressors" (PRO-ROOT).

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BIOSKETCHES

Francesco Loreto is currently the director of the Department of Biology, Agriculture and Food Sciences at the Italian National Research Council. His work spans plant physiology and ecology with a special interest on the functions and metabolism of volatile isoprenoids.

FL conceived and designed the experiments. FB, DC, JT, GS and SF conducted the phylogenetic analysis, CC and GG performed eco-physiological measurements. MDL prepared the first database of isoprenoids emission by woody species, which was revised by GF, and used as a template for ecological assignment of plants by GF and GG. CR performed biostatistics. FL and CR wrote the paper, and all authors contributed to the editing.

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FIGURE LEGENDS

Figure 1. Cladogram describing, for the 128 species subjected to broad-scale phylogenetic analysis, phylogenetic position, isoprenoid emissions capability (black circles; i = isoprene emitter, m = monoterpene emitter), and Ellenberg indicator values for moisture (EIVM).

Figure 2. Fraction of isoprene (A) and monoterpene (B) emitters in the different classes of the woody plant species of the Italian flora as ranked for hygrophily according to the Ellenberg indicator values for moisture (EIVM: 1 = driest; 12 = wettest). Main families of isoprene (Salicaceae) and monoterpene (Pinaceae, Cistaceae and Betulaceae) emitters are shown with different bar patterns, as indicated in the figure legend. Statistical analysis is shown in Figure 3.

Figure 3. Box plots of the distribution in classes of Ellenberg indicator values for moisture (EIVM) of isoprene (grey) and monoterpene (white) emitters versus non-emitters of the woody Italian flora (see Figure 1). Boxes indicate 25-75 percentiles of the collected data. The lines inside boxes indicate the median values. Bars outside boxes indicate the 5-95 percentiles of data, and circles indicate outlier data. A non-parametric Mann-Whitney Z-test was used for comparing median EIVM between emitters and non-emitters. Significant differences with respect to non-emitters were found for both isoprene-emitters ($Z = 3.403$; $p < 0.001$), and monoterpene-emitters ($Z = -4.125$; $p < 0.001$). The latter is significant also after phylogenetic control, confirming the ecological relevance of this finding, while this is not the case for isoprene (see Results).

Figure 4. Emission rates of isoprene (A) and monoterpenes (B) by woody species of the flora of Italy ranked according to the Ellenberg indicator values for moisture (EIVM). The means and standard errors of data collected through field measurements ($n \geq 3$) and surveys of available data sets are shown. Best fits based on linear regressions are shown, together with regression coefficients. The best fit lines showed a statistically significant trend toward higher emission of isoprene in hygrophytes ($p = 0.028$) and higher emission of monoterpenes in xerophytes ($p = 0.030$). Further statistical analysis confirmed isoprene emission rates to be higher in hygrophytes (Kruskal-Wallis non-parametric test, $p = 0.0042$), and statistically significant among EIVM classes (Dunn's Multiple Comparison Test, significantly different means are shown by different letters, $p = 0.05$; class 1 was not included in the post-hoc test due to the low sample size (only one emitting species, as shown in the text)). The Kruskal-Wallis test yielded non-significant differences ($p =$

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0.136) for monoterpenes, and therefore no test was performed to separate EIVM classes of monoterpene-emitters.

Figure 5. Phylogenetic tree based on IspS coding sequences identified in this study for *Populus* and *Salix* species of the Italian flora (A). The numbers close to each species name refer to Ellenberg indicator values for moisture (n.a. = not available). The numbers next to each node are the bootstrap percentages from 10000 pseudo-replicates. Only bootstrap values above 50 % are presented on the tree. In (B) the phylogenetic tree based on available *IspS* coding sequences of *Populus* and *Salix* species is widened to compare with non-European poplar species, and with two outgroup species whose *IspS* sequence is also known. Black dots refer to sequences obtained in this research. The sections *Populus* (P), *Aigeiros* (A), *Tacamahaca* (Ta) and *Turanga* (Tu) are also indicated in (B).

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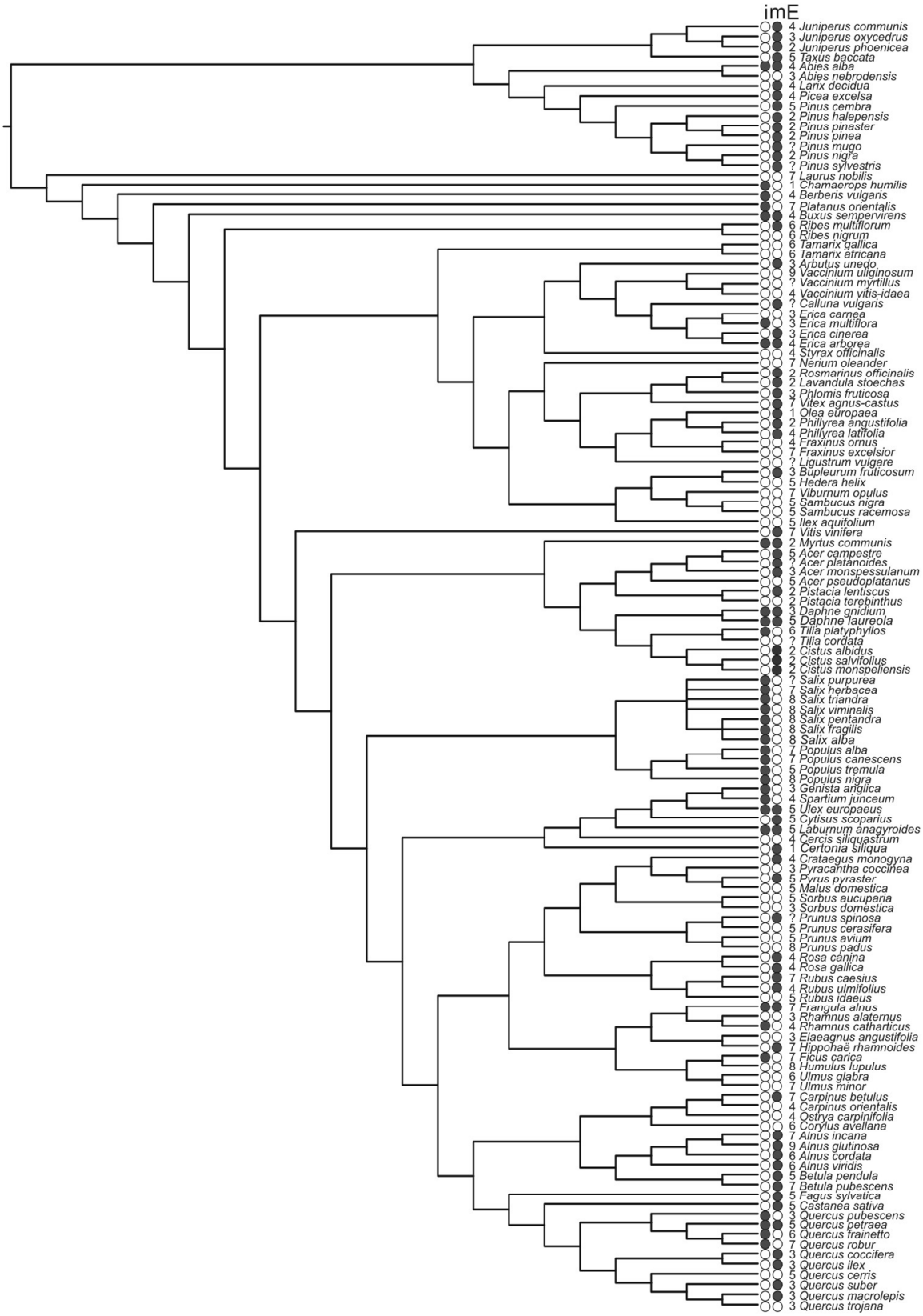

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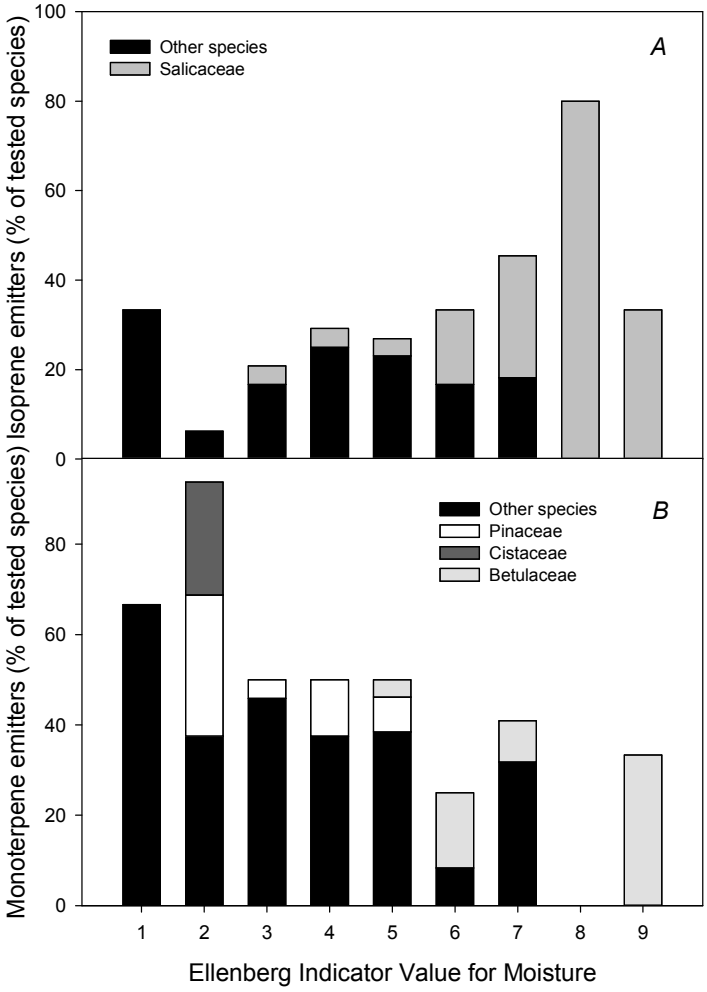

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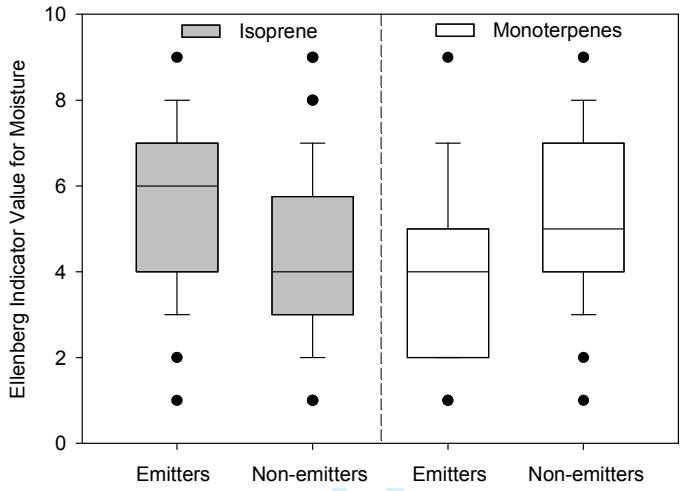

Figure 3.

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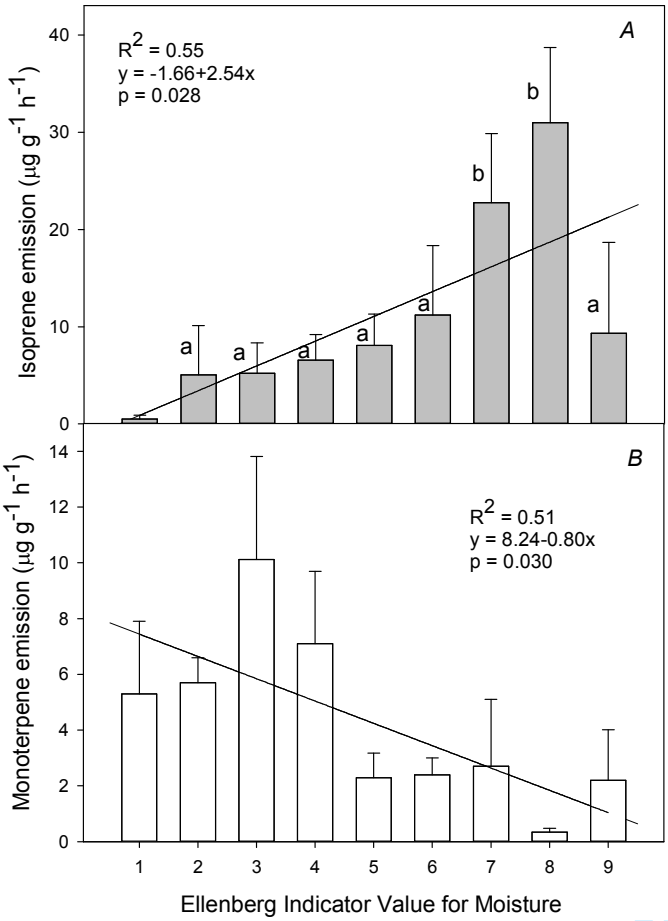

Figure 4.

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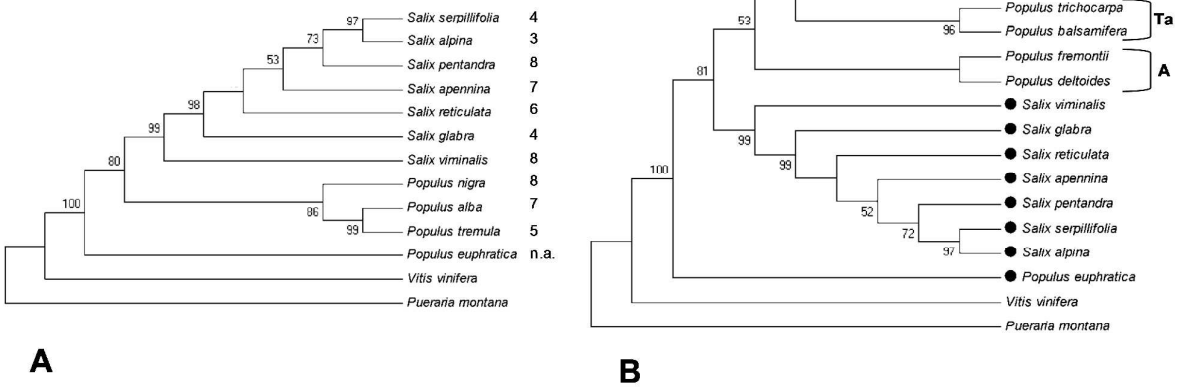

Figure 5.

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Isoprenoid emission in hygrophyte and xerophyte European woody flora: ecological and evolutionary implications

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SUPPORTING INFORMATION

Appendix S1. Attribution of woody species of the flora of Italy to classes of Ellenberg Indicator Values for Moisture (EIVM; 1 to 9 is the incremental scale for moisture, ? indicates species that tolerate a wide range of moisture conditions), and to isoprenoid emission types (I = isoprene; M = monoterpenes; NE = non-emitter; NA = data not available). The superscripted number indicates references are available, as reported at the end of the table.

Family	Species	EIVM	Isoprenoid Emission
Pinaceae	Abies alba Miller	4	I+M [3]
Pinaceae	Abies nebrodensis (Lojac.) Mattei	3	NE [2]
Aceraceae	Acer campestre L.	5	M [3]
Aceraceae	Acer lobelii Ten.	5	NA
Aceraceae	Acer monspessulanum L.	3	M [3]
Aceraceae	Acer obtusatum W. et K.	4	M [1]
Aceraceae	Acer platanoides L.	?	M [3]
Aceraceae	Acer pseudoplatanus L.	5	NE [4]
Fabaceae	Adenocarpus complicatus (L.) Gay	3	NA
Betulaceae	Alnus cordata (Loisel.) Desf.	6	M [3]
Betulaceae	Alnus glutinosa (L.) Gaertner	9	M [1]
Betulaceae	Alnus incana (L.) Moench	7	M [3]
Betulaceae	Alnus viridis (Chaix) DC.	6	M [3]
Rosaceae	Amelanchier ovalis Medicus	3	NA
Fabaceae	Anagyris foetida L.	2	NA
Fabaceae	Anthyllis barba-jovis L.	2	NA
Ericaceae	Arbutus unedo L.	3	M [5]
Asteraceae	Artemisia arborescens L.	2	NA
Fabaceae	Astragalus massiliensis Lam.	2	NA
Fabaceae	Astragalus sempervirens Lam.	4	NA
Berberidaceae	Berberis aetnensis Presl	2	NA
Berberidaceae	Berberis vulgaris L.	4	I [1]
Betulaceae	Betula nana L.	9	NA
Betulaceae	Betula pendula Roth	5	M [3]
Betulaceae	Betula pubescens Ehrh.	7	M [3]
Apiaceae	Bupleurum fruticosum L.	3	M [7]

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Buxaceae	Buxus balearica Lam.	3	NA
Buxaceae	Buxus sempervirens L.	4	I+M [3]
Fabaceae	Calicotome spinosa (L.) Link	2	NA
Fabaceae	Calicotome villosa (Poiret) Link	2	NA
Ericaceae	Calluna vulgaris (L.) Hull	?	M [8]
Capparidaceae	Capparis ovata Desf.	2	NA
Capparidaceae	Capparis spinosa L.	2	NA
Corylaceae	Carpinus betulus L.	7	M [1]
Corylaceae	Carpinus orientalis Miller	4	NE [3]
Fagaceae	Castanea sativa Miller	5	M [3]
Ulmaceae	Celtis aetnensis (Tornabene) Strobl	3	NA
Ulmaceae	Celtis australis L.	3	NA
Fabaceae	Ceratonia siliqua L.	1	M [1,9]
Fabaceae	Cercis siliquastrum L.	4	NE [3]
Arecaceae	Chamaerops humilis L.	1	I [10]
Cistaceae	Cistus albidus L.	2	M [1,9]
Cistaceae	Cistus clusii Dunal	2	NA
Cistaceae	Cistus corsicus Loisel.	2	NA
Cistaceae	Cistus creticus L.	2	NA
Cistaceae	Cistus crispus L.	2	NA
Cistaceae	Cistus incanus L.	2	M [1]
Cistaceae	Cistus laurifolius L.	2	NA
Cistaceae	Cistus monspeliensis L.	2	M [1]
Cistaceae	Cistus parviflorus Lam.	2	NA
Cistaceae	Cistus salvifolius L.	2	M [6]
Ranunculaceae	Clematis alpina (L.) Miller	5	NA
Ranunculaceae	Clematis cirrhosa L.	2	NA
Ranunculaceae	Clematis flammula L.	3	NA
Ranunculaceae	Clematis vitalba L.	5	NA
Ranunculaceae	Clematis viticella L.	4	NA
Cneoraceae	Cneorum tricoccon L.	2	NA
Fabaceae	Colutea arborescens L.	3	NA
Coriariaceae	Coriaria myrtifolia L.	3	NA
Cornaceae	Cornus mas L.	5	NA
Cornaceae	Cornus sanguinea L.	6	NA
Fabaceae	Coronilla emerus L.	4	NA
Fabaceae	Coronilla juncea L.	2	NA
Fabaceae	Coronilla valentina L.	2	NA
Corylaceae	Corylus avellana L.	6	NE [3]
Anacardiaceae	Cotinus coggygria Scop.	3	NA
Rosaceae	Cotoneaster integerrimus Medicus	3	NA
Rosaceae	Cotoneaster nebrodensis (Guss.) Koch	3	NA
Rosaceae	Crataegus laciniata Ucria	3	NA
Rosaceae	Crataegus monogyna Jacq.	4	M [8]
Rosaceae	Crataegus oxyacantha L.	5	NA

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Asclepiadaceae	Cynanchum acutum L.	7	NA
Fabaceae	Cytisus aeolicus Guss.	3	NA
Fabaceae	Cytisus scoparius (L.) Link	5	I [6]
Fabaceae	Cytisus sessilifolius L.	5	NA
Fabaceae	Cytisus villosus Pourret	4	NA
Thymelaeaceae	Daphne alpina L.	3	NA
Thymelaeaceae	Daphne gnidium L.	3	I + M [8]
Thymelaeaceae	Daphne laureola L.	5	I + M [11]
Thymelaeaceae	Daphne mezereum L.	5	NA
Thymelaeaceae	Daphne oleoides Schreber	2	NA
Thymelaeaceae	Daphne sericea Vahl	3	NA
Elaeagnaceae	Elaeagnus angustifolia L.	3	NE [2]
Empetraceae	Empetrum hermaphroditum Hagerup	4	NA
Ephedraceae	Ephedra distachya L.	3	NA
Ephedraceae	Ephedra fragilis Desf.	3	NA
Ephedraceae	Ephedra helvetica C.A. Meyer	3	NA
Ephedraceae	Ephedra major Host	3	NA
Ericaceae	Erica arborea L.	4	I+M [3]
Ericaceae	Erica carnea L.	3	NE [2]
Ericaceae	Erica cinerea L.	3	M [8]
Ericaceae	Erica multiflora L.	3	I [5]
Ericaceae	Erica scoparia L.	3	NE [3]
Ericaceae	Erica sicula Guss.	2	NA
Ericaceae	Erica terminalis Salisb.	2	NA
Celastraceae	Euonymus europaeus L.	5	NA
Celastraceae	Euonymus latifolius (L.) Miller	5	NA
Celastraceae	Euonymus verrucosus Scop.	5	NA
Euphorbiaceae	Euphorbia dendroides L.	2	NA
Fagaceae	Fagus sylvatica L.	5	M [3]
Moraceae	Ficus carica L.	7	I [9]
Rhamnaceae	Frangula alnus Miller	7	I+M [8]
Rhamnaceae	Frangula rupestris (Scop.) Schur	3	NA
Oleaceae	Fraxinus excelsior L.	7	NE [3]
Oleaceae	Fraxinus ornus L.	4	NE [3]
Oleaceae	Fraxinus oxycarpa Bieb.	7	NA
Fabaceae	Genista acanthoclada DC.	2	NA
Fabaceae	Genista aetnensis (Biv.) DC.	3	NA
Fabaceae	Genista anglica L.	3	I [11]
Fabaceae	Genista aspalathoides Lam.	2	NA
Fabaceae	Genista cinerea (Vill.) DC.	3	NA
Fabaceae	Genista corsica (Loisel.) DC.	2	NA
Fabaceae	Genista ephedroides DC.	2	NA
Fabaceae	Genista morisii Colla	2	NA
Fabaceae	Genista salzmannii DC.	2	NA
Cistaceae	Halimium halimifolium (L.) Willk.	2	NA

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Araliaceae	Hedera helix L.	5	NE [4]
Elaeagnaceae	Hippophae rhamnoides L.	7	M [1]
Cannabaceae	Humulus lupulus L.	8	NE [2]
Aquifoliaceae	Ilex aquifolium L.	5	NE [3]
Cupressaceae	Juniperus communis L.	4	M [3]
Cupressaceae	Juniperus oxycedrus L.	3	M [3]
Cupressaceae	Juniperus phoenicea L.	2	M [12]
Cupressaceae	Juniperus sabina L.	3	NA
Cupressaceae	Juniperus thurifera L.	3	NA
Chenopodiaceae	Kochia prostrata (L.) Schrader	3	NA
Fabaceae	Laburnum alpinum (Miller) B. et Presl	6	NA
Fabaceae	Laburnum anagyroides Medicus	5	I + M [11]
Pinaceae	Larix decidua Miller	4	M [3]
Lauraceae	Laurus nobilis L.	7	M [21]
Lamiaceae	Lavandula angustifolia Miller	3	NA
Lamiaceae	Lavandula latifolia Medicus	3	NA
Lamiaceae	Lavandula multifida L.	3	NA
Lamiaceae	Lavandula stoechas L.	2	M [6]
Malvaceae	Lavatera agrigentina Tineo	2	NA
Malvaceae	Lavatera maritima Gouan	2	NA
Malvaceae	Lavatera olbia L.	2	NA
Malvaceae	Lavatera triloba L.	2	NA
Fabaceae	Lembotropis nigricans (L.) Griseb.	4	NA
Oleaceae	Ligustrum vulgare L.	?	NE [2]
Caprifoliaceae	Lonicera alpigena L.	6	NA
Caprifoliaceae	Lonicera caprifolium L.	6	NA
Caprifoliaceae	Lonicera coerulea L.	8	NA
Caprifoliaceae	Lonicera etrusca Santi	3	NA
Caprifoliaceae	Lonicera implexa Aiton	3	NA
Caprifoliaceae	Lonicera nigra L.	5	NA
Caprifoliaceae	Lonicera peryclymenum L.	?	NA
Caprifoliaceae	Lonicera stabiana Pasquale	2	NA
Caprifoliaceae	Lonicera xylosteum L.	5	NA
Rosaceae	Malus domestica Borkh.	5	NE [3]
Rosaceae	Malus florentina (Zuccagni) Schneider	5	NA
Rosaceae	Malus sylvestris Miller	5	NA
Rosaceae	Mespilus germanica L.	4	NA
Myrtaceae	Myrtus communis L.	2	I+M [13]
Apocynaceae	Nerium oleander L.	7	NE [6]
Oleaceae	Olea europaea L. var. sylvestris Brot.	1	M [1,9]
Corylaceae	Ostrya carpinifolia Scop.	4	NE [3]
Santalaceae	Osyris alba L.	3	NA
Rhamnaceae	Paliurus spina-christi Miller	3	NA
Asclepiadaceae	Periploca graeca L.	7	NA
Asclepiadaceae	Periploca laevigata Aiton	2	NA

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Oleaceae	Phillyrea angustifolia L.	2	M [12]
Oleaceae	Phillyrea latifolia L.	4	M [3]
Lamiaceae	Phlomis ferruginea Ten.	3	NA
Lamiaceae	Phlomis fruticosa L.	3	M [1]
Pinaceae	Picea excelsa (Lam.) Link	4	M [3]
Pinaceae	Pinus cembra L.	5	M [3]
Pinaceae	Pinus halepensis Miller	2	M [9]
Pinaceae	Pinus laricio Poiret	3	M [1]
Pinaceae	Pinus leucodermis Antoine	2	M [3]
Pinaceae	Pinus mugo Turra	?	M [3]
Pinaceae	Pinus nigra Arnold	2	M [3]
Pinaceae	Pinus pinaster Aiton	2	M [8]
Pinaceae	Pinus pinea L.	2	M [3]
Pinaceae	Pinus sylvestris L.	?	M [3]
Pinaceae	Pinus uncinata Miller	5	M [3]
Anacardiaceae	Pistacia lentiscus L.	2	M [3]
Anacardiaceae	Pistacia terebinthus L.	2	NE [1]
Platanaceae	Platanus orientalis L.	7	I [3]
Salicaceae	Populus alba L.	7	I [3]
Salicaceae	Populus canescens (Aiton) Sm.	7	I [3]
Salicaceae	Populus nigra L.	8	I [3]
Salicaceae	Populus tremula L.	5	I [3]
Rosaceae	Prunus avium L.	5	NE [3]
Rosaceae	Prunus brigantina Vill.	5	NA
Rosaceae	Prunus cerasifera Ehrh.	5	NE [14]
Rosaceae	Prunus cocomilia Ten.	5	NA
Rosaceae	Prunus fruticosa Pallas	3	NA
Rosaceae	Prunus mahaleb L.	3	NA
Rosaceae	Prunus padus L.	8	NE [3]
Rosaceae	Prunus prostrata Labill.	2	NA
Rosaceae	Prunus spinosa L.	?	M [11]
Rosaceae	Prunus webbii (Spach) Vierh.	2	NA
Rubiaceae	Putoria calabrica (L.fil.) Pers.	2	NA
Rosaceae	Pyracantha coccinea Roemer	3	NE [14]
Rosaceae	Pyrus amygdaliformis Vill.	4	NA
Rosaceae	Pyrus pyraster Burgsd.	5	M [1]
Fagaceae	Quercus cerris L.	5	NE [15]
Fagaceae	Quercus coccifera L.	3	M [3]
Fagaceae	Quercus frainetto Ten.	6	I [3]
Fagaceae	Quercus ilex L.	3	M [3]
Fagaceae	Quercus macrolepis Kotschy	3	M [3]
Fagaceae	Quercus petraea (Mattuschka) Liebl.	5	I+M [3]
Fagaceae	Quercus pubescens Willd.	3	I [3]
Fagaceae	Quercus pyrenaica Willd.	5	I + M [8]
Fagaceae	Quercus robur L.	7	I [1]

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Fagaceae	Quercus suber L.	3	M [9]
Fagaceae	Quercus trojana Webb	3	NE [15]
Fabaceae	Retama raetam (Forsskal) Webb et Berth.	1	NA
Rhamnaceae	Rhamnus alaternus L.	3	NE [16]
Rhamnaceae	Rhamnus alpinus L.	5	NA
Rhamnaceae	Rhamnus catharticus L.	4	I [11]
Rhamnaceae	Rhamnus glaucophyllus Sommier	4	NA
Rhamnaceae	Rhamnus lojaconoi Raimondo	4	NA
Rhamnaceae	Rhamnus oleoides L.	2	NA
Rhamnaceae	Rhamnus persicifolius Moris	3	NA
Rhamnaceae	Rhamnus pumilus Turra	2	NA
Rhamnaceae	Rhamnus saxatilis Jacq.	3	NA
Ericaceae	Rhododendron ferrugineum L.	6	NA
Ericaceae	Rhododendron hirsutum L.	4	NA
Anacardiaceae	Rhus pentaphylla (Jacq.) Desf.	3	NA
Anacardiaceae	Rhus tripartita (Ucria) Grande	3	NA
Saxifragaceae	Ribes alpinum L.	?	NA
Saxifragaceae	Ribes multiflorum Kit.	6	M [1]
Saxifragaceae	Ribes nigrum L.	6	NE [4]
Saxifragaceae	Ribes petraeum Wulfen	4	NA
Saxifragaceae	Ribes rubrum L.	8	NA
Saxifragaceae	Ribes sardoum Martelli	3	NA
Saxifragaceae	Ribes uva-crispa L.	?	NA
Rosaceae	Rosa agrestis Savi	3	NA
Rosaceae	Rosa arvensis Hudson	5	NA
Rosaceae	Rosa canina L.	4	M [1,17]
Rosaceae	Rosa gallica L.	4	M [1]
Rosaceae	Rosa micrantha Sm.	3	NA
Rosaceae	Rosa pendulina L.	5	NA
Rosaceae	Rosa pouzinii Tratt.	3	NA
Rosaceae	Rosa sempervirens L.	3	NA
Lamiaceae	Rosmarinus officinalis L.	2	M [1,13]
Rosaceae	Rubus caesius L.	7	M [11]
Rosaceae	Rubus canescens DC.	4	NA
Rosaceae	Rubus hirtus W. et K.	4	NA
Rosaceae	Rubus idaeus L.	5	NE [4]
Rosaceae	Rubus ulmifolius Schott	4	M [8]
Salicaceae	Salix alba L.	8	I [1]
Salicaceae	Salix alpina Scop.	3	I [1]
Salicaceae	Salix apennina Skvortsov	7	I [1]
Salicaceae	Salix atrocinerea Brot.	7	I [1]
Salicaceae	Salix aurita L.	8	I [1]
Salicaceae	Salix breviserrata Flod.	3	NA
Salicaceae	Salix caesia Vill.	4	NA
Salicaceae	Salix caprea L.	6	I [3]

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Salicaceae	Salix cinerea L.	9	I [1]
Salicaceae	Salix crataegifolia Bertol.	6	NA
Salicaceae	Salix daphnoides Vill.	4	NA
Salicaceae	Salix eleagnos Scop.	7	I [1]
Salicaceae	Salix foetida Schleicher	4	NA
Salicaceae	Salix fragilis L.	8	I [1]
Salicaceae	Salix glabra Scop.	4	I [1]
Salicaceae	Salix glaucosericea Flod.	3	NA
Salicaceae	Salix hastata L.	6	NA
Salicaceae	Salix hegetschweileri Heer	3	NA
Salicaceae	Salix helvetica Vill.	4	NA
Salicaceae	Salix herbacea L.	7	I [1]
Salicaceae	Salix myrsinifolia Salisb.	7	NA
Salicaceae	Salix pentandra L.	8	I [1]
Salicaceae	Salix purpurea L.	?	I [1]
Salicaceae	Salix repens L.	8	I [1]
Salicaceae	Salix reticulata L.	6	I [1]
Salicaceae	Salix retusa L.	6	NA
Salicaceae	Salix serpillyfolia Scop.	4	NA
Salicaceae	Salix triandra L.	8	I [1]
Salicaceae	Salix viminalis L.	8	I [19]
Salicaceae	Salix waldsteiniana Willd.	6	NA
Caprifoliaceae	Sambucus nigra L.	5	NE [11]
Caprifoliaceae	Sambucus racemosa L.	5	NE [11]
Rosaceae	Sarcopoterium spinosum (L.) Spach	2	NA
Smilacaceae	Smilax aspera L.	3	NA
Rosaceae	Sorbus aria (L.) Crantz	4	NE [3]
Rosaceae	Sorbus aucuparia L.	5	NE [3]
Rosaceae	Sorbus chamaemespilus (L.) Crantz	4	NA
Rosaceae	Sorbus domestica L.	3	NE [3]
Rosaceae	Sorbus torminalis (L.) Crantz	4	NE [3]
Fabaceae	Spartium junceum L.	4	I [9]
Staphyleaceae	Staphylea pinnata L.	5	NA
Styracaceae	Styrax officinalis L.	4	NE [2]
Tamaricaceae	Tamarix africana Poiret	6	NE [6]
Tamaricaceae	Tamarix canariensis Willd.	6	NA
Tamaricaceae	Tamarix dalmatica Baum	6	NA
Tamaricaceae	Tamarix gallica L.	6	NE [21]
Taxaceae	Taxus baccata L.	5	M [21]
Fabaceae	Teline monspessulana (L.) Koch	4	NA
Lamiaceae	Teucrium fruticans L.	2	NA
Thymelaeaceae	Thymelaea dioica (Gouan) All.	3	NA
Thymelaeaceae	Thymelaea hirsuta (L.) Endl.	2	NA
Thymelaeaceae	Thymelaea tartonraira (L.) All.	2	NA
Lamiaceae	Thymus capitatus (L.) Hofmgg. et Lk.	2	NA

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Tiliaceae	Tilia cordata Miller	?	NE [3]
Tiliaceae	Tilia platyphyllos Scop.	6	I [11]
Fabaceae	Ulex europaeus L.	5	I + M [11]
Ulmaceae	Ulmus canescens Melville	3	NA
Ulmaceae	Ulmus glabra Hudson	6	NE [3]
Ulmaceae	Ulmus minor Miller	7	NE [3]
Ericaceae	Vaccinium gaultherioides Bigelow	5	NA
Ericaceae	Vaccinium microcarpum (Turcz.) H. Fil.	9	NA
Ericaceae	Vaccinium myrtillus L.	?	NE [19]
Ericaceae	Vaccinium oxycoccos L.	9	NA
Ericaceae	Vaccinium uliginosum L.	9	NE [20]
Ericaceae	Vaccinium vitis-idaea L.	4	NE [19]
Caprifoliaceae	Viburnum lantana L.	4	NA
Caprifoliaceae	Viburnum opulus L.	7	NE [11]
Caprifoliaceae	Viburnum tinus L.	4	NA
Apocynaceae	Vinca difformis Pourret	3	NA
Apocynaceae	Vinca major L.	4	NA
Apocynaceae	Vinca minor L.	5	NA
Apocynaceae	Vinca sardoa (Stearn) Pign.	3	NA
Verbenaceae	Vitex agnus-castus L.	7	M [3]
Vitaceae	Vitis vinifera L.	7	M [8]
Rhamnaceae	Ziziphus lotus (L.) Lam.	1	NA

Common exotic woody plants

Cupressaceae	Cupressus sempervirens L.	3	M [1]
Platanaceae	Platanus x acerifolia (Aiton) Wild.	8	I [1]
Salicaceae	Populus canadensis L.	7	I [1]
Fabaceae	Robinia pseudoacacia L.	4	I [1]
Oleaceae	Syringa vulgaris L.	5	NE [1]

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Appendix S2. List of references used to reconstruct the phylogenetic relationships of species.

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Appendix S3. IspS phylogenetic analysis on Mediterranean species of the genus *Populus* and *Salix* sampled for isoprene emission in this study and belonging to different classes of EIVM (Appendix S1), and on outgroups (North-American species) for which *IspS* accessions are available. *Populus euphratica* is denoted with *, as this species was sampled in this study but does not belong to the flora of Italy and is characterized by extreme adaptation to aridity and salinity (Ding *et al.* 2010).

Species	GenBank accessions	References
Populus grandidentata	JN173038	Gray *et al.* unpublished
Populus fremontii	JN173040	Gray *et al.* unpublished
Populus deltoides	JN173039	Gray *et al.* unpublished
Populus trichocarpa	EU693027	Calfapietra *et al.* (2007)
Populus balsamifera	JN173037	Gray *et al.* unpublished
Populus tremuloides	AY341431	Sharkey *et al.* (2005)
Pueraria montana	AY316691	Sharkey *et al.* (2005)
Populus alba	JQ943922	this study
Populus euphratica (*)	JQ943923	this study
Populus nigra	JQ943924	this study
Populus tremula	JQ943925	this study
Salix apennina	JQ943915	this study
Salix serpyllifolia	JQ943916	this study
Salix alpina	JQ943917	this study
Salix glabra	JQ943918	this study
Salix pentandra	JQ943919	this study
Salix reticulata	JQ943920	this study
Salix viminalis	JQ943921	this study

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Appendix S4. Primers used in amplification and sequencing of *IspS*. Primers used in initial PCR reactions are highlighted in bold.

PRIMER	SEQUENCE 5'-3'
PalSPS-Fw2	gtcgtttggagcattgaagca
ISPS_Nested1-F	gttcgaacctcaatatagtg
ISPS_Nested2-F	gaggcgtgttggtcttgc
ISPS_Nested3_F	cggattatatgaagctctgc
ISPS_Nested4_F	gagttggagctatttacaga
ISPS_Nested5_F	gataccatgtcaaggaacca
ISPS_Nested6_F	gtacagtataaatttcatcag
PalSPS-Bw3	ttatctctcaaagggtagaat
ISPS_Nested1_R	acagaattcgcagtttcacc
ISPS_Nested2_R	caggtttcgtctatcaaattc
ISPS_Nested3_R	ctgaggatgatttccatgca
ISPS_Nested4_R	cttaacaaagccctagaatatg
ISPS_Nested5_R	gagtctcatcatcctcattc
ISPS_Nested6_R	gttggttccttaacaaagccc

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