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Reticulate evolution patterns in western-Eurasian beeches

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Abstract Reticulate evolution resulting from gene flow among diverged taxa was studied on the example of the *Fagus sylvatica* species complex in Europe and western Asia. *F. sylvatica* L. is one of the most widespread woody species of the Northern Hemisphere with two commonly recognized subspecies (namely, *F. sylvatica subsp. sylvatica* and *F. sylvatica subsp. orientalis*) and two putatively hybridogenous or intermediate taxa (*F. moesiaca* and *F. taurica*). Whereas the distribution of *F. sylvatica* subsp. *sylvatica* in Europe is almost continuous, the Asian range of *F. sylvatica* subsp. *orientalis* is fragmented into several larger regional populations. We studied genetic variation at 12 allozyme loci in 279 local subpopulations to assess whether previous contacts of regional populations left traces in their gene pools. Two approaches were used to infer potential reticulations: the reticulate network (Makarenkov and Legendre in J Comp Biol 11:195–212, 2004) and the neighbor-net network (Bryant and Moulton in Mol Biol Evol 21:255–265, 2004). A UPGMA dendrogram based on genetic distances between local populations revealed a high differentiation level within subsp. *orientalis*, whereby populations from Asia Minor, the Caucasus and the Alborz mts. were placed in different clusters. The reticulated network showed possible connections among adjacent populations, putatively resulting from previous contacts. The neighbor-net network also consisted of two major groups corresponding to *F. sylvatica s. str.* + *F. moesiaca* and *F. orientalis* including *F. taurica*. Regional populations of *F. moesiaca* are placed close to the basis of the European branch and are highly networked, indicating ambiguity in the phylogenetic signal. Similarly, three subgroups can be distinguished within the *F. orientalis* group and correspond well with broad regions of Asia Minor, the Caucasus and the Alborz. The Amanus regional population is intermediary between the subgroup of Asia Minor and the Caucasian subgroup. Crimean populations (i.e., putative hybrids) are placed near the basis of the subgraph. Two explanations of the observed reticulations are discussed: expansions and retreats of the range of beech associated with Pliocene and Pleistocene climatic fluctuations, resulting in contacts and exchanges of gene pools among regional populations, and sharing ancestral polymorphisms due to incomplete lineage sorting.

Keywords Allozymes · *Fagus sylvatica* L. · *Fagus orientalis* Lipsky · Phylogeography · Reticulate evolution

Zusammenfassung Intraspezifische retikuläre Muster der Evolution wurden am Beispiel des taxonomischen Komplexes der Buchen in Europa und Westasien studiert. *Fagus sylvatica* L. ist eine der am weitesten verbreiteten Baumarten der nördlichen Hemisphäre, mit zwei Unterarten und zwei vermutlich hybridogenen Taxa (*F. moesiaca* und *F. taurica*). Während die Verbreitung von *Fagus sylvatica* subsp. *sylvatica* in Europa fast kontinuierlich ist, ist das Areal der asiatischen Unterart *Fagus sylvatica* subsp. *orientalis* fragmentiert. Wir untersuchten die genetische Variation an 12 Isozym-Loci in 279 lokalen Subpopulationen hinsichtlich der Frage, ob frühere Kontakte zwischen heute isolierten Populationen Spuren in deren Genpool hinterlassen haben. Die vermuteten Retikulationen wurden anhand zweier Methoden identifiziert: '*reticulate network*' (Makarenkov

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and Legendre 2004) und '*neighbor-net network*' (Bryant and Moulton 2004). UPGMA Dendrogram zeigte eine große Differenzierung innerhalb von subsp. *orientalis*, wobei die Populationen aus Klein-Asien, dem Kaukasus und dem Alborz gut getrennte Gruppen bildeten. Das '*reticulate network*' zeigte mögliche Querverbindungen zwischen den definierten regionalen Populationen. Auch im '*neighbor-net network*' wurde *F. sylvatica s. str.* und *F. moesiaca* von *F. orientalis* (einschliesslich *F. taurica*) getrennt. Die Populationen von *F. moesiaca* sind durch geringe Distanz zur Gruppe *F. orientalis* + *F. taurica* definiert. Innerhalb von *F. orientalis* kann man drei Hauptgruppen unterscheiden, die gut die Verbreitungsmuster widerspiegeln, wobei die Populationen von Amanus eine intermediäre Position zwischen Kleinasien und dem Kaukasus besetzen. Die Populationen von Krim-Halbinsel (ein potenzieller Hybrid) wurden in Analogie zu *F. moesiaca* im Zentrum des Graphs plaziert. Zwei mögliche Erklärungen der gefundenen retikulaten Muster wurden diskutiert, nämlich Arealverschiebungen im Zuge der Klimaänderungen im Pliozän und Pleistozän, die zum Genfluss zwischen regionalen Populationen führten, und geteilte ancestrale Polymorphismen infolge des unvollständigen Lineage Sorting.

Introduction

A tree has long been assumed to be an appropriate metaphor for the evolutionary process. Evolution has been considered a simple branching process in which individual branches representing taxa or groups of taxa of various ranks diverge through anagenetic processes. Branches can possibly split, become extinct, but cannot be joined again. There are, however, several phylogenetic situations where this metaphor is of no use, because correct displaying of the evolutionary process requires constructing a network rather than a tree. Such processes include homoploid or alloploid hybridization, which is particularly common in angiosperms as almost 25% of plant taxa are suspected to be involved in hybridization (Mallet 2005). As hybrid lineages can be stabilized through apomixis, allopolyploidy or chromosomal rearrangement, hybridization is an important mechanism of speciation (Linder and Rieseberg 2004; Rieseberg and Noyes 1998). Reticulation plays an even more important role at the subspecific level. As criticized by Smouse (2000), trees are used to model the evolution within a species not because the process is necessarily tree-like, but because there are algorithms available for modeling this process as tree-like. However, exchange of genes among regional subpopulations (lateral gene flow), which sometimes may have evolved into separate infraspecific taxa, is absolutely common. Biogeographical reconstructions based on strictly radiating trees may thus often be misleading.

The representatives of the genus *Fagus* in western Eurasia may be a good model to illustrate the reticulation process. In spite of numerous taxonomical studies, there is still a controversy among botanists and forestry practitioners about the number, rank, and distribution of beech taxa. *Fagus sylvatica* L. (common or European beech), occurring in most of Europe except the northern part and East European plains, is generally considered a distinct taxon (species or subspecies) by the vast majority of plant taxonomists. *Fagus orientalis* Lipsky, (Eastern or oriental beech) has been used to address populations distributed from the Rhodopes over Asia Minor and Caucasus to the Alborz Mts. (sometimes including the Crimean populations). It has traditionally been considered a separate species by local botanists (Gulisashvili et al. 1975; Mayer and Aksoy 1986; Mobayen and Tregubov 1970). Greuter and Burdet (1981) reclassified it as a subspecies *F. sylvatica* subsp. *orientalis* and this view became accepted by the recent editions of Flora Europaea (Akeroyd 1993). Recent systematic revisions (Denk 1999a, b), although not confirming it, did not offer alternatives. Moreover, there are at least two additional intermediate taxa within this complex, where the opinions of botanists vary considerably: the Crimean beech, *Fagus taurica* Popl., endemic to the Crimean peninsula, and the Balkan beech, *Fagus moesiaca* (Domin, Maly) Czeczott, occurring in the southwestern Balkans. Both taxa are considered transitional forms (hybrids or phylogenetic links) between *F. sylvatica* and *F. orientalis* (Becker 1981; Boratyńska and Boratyński 1990; Czeczott 1932; Wulff 1932). Most of the taxonomical studies dealing with these taxa were based on morphological or morphometric examination of herbarium or fresh material collected in situ. Although the traits used in taxonomy generally exhibit a high heritability, the effect of site conditions on the classification cannot be fully excluded. Genetic methods were rather scarcely used for this purpose. Detailed range-wide mappings of the gene pools employing isozymes (Comps et al. 2001) and chloroplast DNA markers (Demesure et al. 1996; Magri et al. 2006) were performed in *F. sylvatica s. str.*, but detailed studies in *F. orientalis* are available only for the Alborz Mts. (Salehi-Shanjani et al. 2004). There are only two comprehensive studies comparing representative samples covering the ranges of both taxa, namely that of Gömöry et al. (2007) based on allozymes (the dataset was identical with the present study) and Denk et al. (2002) using ITS (nuclear ribosomal DNA) markers. They both indicated complex relationships among regional populations of *F. sylvatica s.l.*

Gömöry et al. (2007) focused on the description of differentiation patterns in beech of western Eurasia and their different methodological approaches yielded controversial phylogenetic signals. A Bayesian analysis (STRUCTURE, Pritchard et al. 2000) clearly separated *F. sylvatica s. str.* from *F. orientalis*, whereby gene pools of *F. orientalis* were further clustered into three regional groups (Asia Minor, Caucasus and Alborz). On the other hand, a tree-based approach (neighbor-joining tree based on pairwise F_{ST}) showed considerable differentiation within *F. orientalis*, whereas *F. sylvatica s. str.* clustered together with Turkish populations of *F. orientalis*. Based on this approach, *F. sylvatica s. str.* appears to be a paraphyletic taxon. We suspected that this discrepancy may be due to the methodology, as cladistic approach may be inappropriate not only because of the type of gene markers used, but also because evolutionary processes that lead to the formation of differentiation pattern include re-joining of previously separated lineages. Therefore, this study aims at reconstructing the relationships among regional beech populations and previously described taxa using non-tree based approaches for deeper insights into the evolution of this taxonomical complex. In particular, we addressed the following questions: (1) What role have played reticulate events in the evolution of western-Eurasian beech taxa? (2) What is the position of intermediate taxa? Is their delineation as separate taxa genetically justified? (3) Does the present taxonomical concept of two subspecies reflect evolutionary processes within the complex of *F. sylvatica s. l.*?

For simplicity, the traditional concept of two distinct species *Fagus sylvatica* L. and *Fagus orientalis* Lipsky and two intermediate taxa *Fagus moesiaca* Czeczott and *Fagus taurica* Popl. was used throughout this study.

Materials and methods

Within this study, 279 beech stands (local populations) were examined (see Gömöry et al. 2007). The ranges of *Fagus orientalis* as well as intermediate taxa (*F. moesiaca, F. taurica*) were exhaustively sampled. In *F. sylvatica*, the study focused on the Eastern part of the distribution range. Within each population, twigs with dormant buds were collected from 50 adult trees (few populations were represented by larger sample sizes ranging from 100 to 280 individuals) within an area of ~10 ha.

Because we assumed that the genetic differentiation of beech is structured both taxonomically and geographically, we used a three-level hierarchy throughout the study. The term "taxon" refers to four beech taxa described in the Introduction, the term "regional population" to geographically and genetically defined clusters of populations occurring in different orographical units (see Fig. 1), the term "local population" (sometimes only "population") to the units of sampling, i.e. forest stands.

Taxonomical classification of local populations followed local botanical traditions and was done in collaboration with local specialists. Beech populations from Asia, Caucasus (including its European part) and Strandzha mts. (a mountain range in East Bulgaria and the European part of Turkey; this group includes the population Nesebar at the Bulgarian Black-Sea coast) were classified as *F. orientalis*. All Crimean populations were considered to belong to *F. taurica*. The name *F. moesiaca* is traditionally used by Serbian, Macedonian and Bosnian botanists, less frequently in Bulgaria and Greece. For consistency, all populations from the southwestern Balkans (Bosnia, Serbia, Macedonia, Greece, Albania and Bulgaria) were referred to as *F. moesiaca* in our analysis. All the remaining European beech populations were considered *F. sylvatica s. str.* (Fig. 1).

Enzymes were extracted from buds and cortical tissues of each individual, and were separated by means of starch electrophoresis. Protein separation and staining procedures are described in Merzeau et al. (1989), Müller-Starck and Starke (1993) and Thiébaut et al. (1982). Nine isozyme systems coded by 12 loci were scored: isocitrate dehydrogenase (*Idh-A*), malate dehydrogenase (*Mdh-A, Mdh-B, Mdh-C*), menadione reductase (*Mnr-A*), phosphoglucomutase (*Pgm-A*), phosphoglucose isomerase (*Pgi-B*), peroxidase (*Per-A, Per-B*), glutamate–oxaloacetate transaminase (*Got-B*), leucine aminopeptidase (*Lap-A*), and shikimate dehydrogenase (*Skdh-A*).

Based on diploid genotypes, the genotypic and allelic frequencies were calculated. To assess the trends of genetic differentiation, genetic distances (Nei 1978) were calculated between populations. A dendrogram was then constructed using the UPGMA algorithm employing the program PHYLIP v. 3.63 (Felsenstein 2002). For tree visualization, the program TREEVIEW v. 1.6.6 (Page 1996) was used. The resulting tree served for a reasonable subdivision of the whole population set into groups representing regional populations (especially in *F. orientalis*). To visualize the distance matrix without using a tree-like graph, a principal coordinate analysis (PCoA; Gower 1966) was performed on the matrix of genetic distances between regional populations.

Under the assumption that evolutionary processes in *Fagus* in western Eurasia cannot be modeled by a simple branching process because of possible introgressions between beech taxa and/or regional populations, we used two approaches to infer possible reticulations.

Reticulated network approach (Makarenkov and Legendre 2004): A neighbor-joining tree was built based on the matrix of Nei's genetic distances between regional populations. Subsequently, supplementary reticulation branches were step-by-step added to the tree structure. For each new branch, the procedure minimizes the least-squares

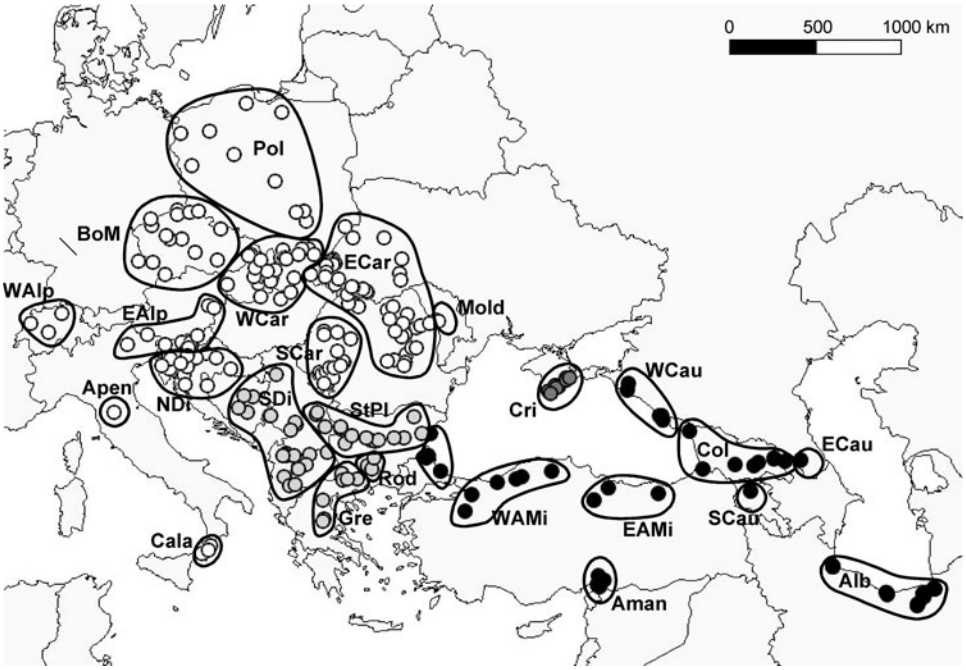


Fig. 1 Distribution of the analyzed stands and delineation of regional populations. *Pol* Polish lowlands, *BoM* Bohemian Massif, *WCar* Western Carpathians, *ECar* Eastern Carpathians (including Galicia), *SCar* Southern Carpathians (including Apuseni Mts.), *Mold* Moldova, *WAlp* Western Alps (including Swiss Jura), *EAlp* Eastern Alps, *Apen* Northern Apennines, *NDi* Northern Dinarians (including Slavonia and west Bosnia), *SDi* Southern Dinarians (including Vojvodina), *StPl* Stara Planina, *Rod* Rhodopes, *Gre* northeastern Greece, *Cala* Calabria, *Cri* Crimea, *WCau* western Great Caucasus, *ECau* eastern Great Caucasus, *SCau* Armenian Small Caucasus, *Col* Colchis (including Adjarian foothills of the Small Caucasus), *Str* Strandzha mts. (including population Nesebar), *WAMi* western Asia Minor, *EAMi* eastern Asia Minor, *Aman* Amanus mts., *Alb* Alborz mts. Symbols: white, *F. sylvatica s. str.*; light grey, *F. moesiaca*; dark grey, *F. taurica*; black, *F. orientalis*

loss function defined as a sum of squared differences between the original distances and the associated reticulogram distances. A goodness-of-fit function is defined, which allows to optimize the number of added branches. The program T-REX v. 3.0 (Makarenkov 2001) was used for the construction of the reticulated network (reticulogram).

Neighbor-net approach (Bryant and Moulton 2004): The method is a generalization of the agglomerative neighbor-joining algorithm of Saitou and Nei (1987). It decomposes the distance matrix into simple components based on weighted splits (split is a partition of the set of classified objects—regional populations in our case—into two subsets). These splits are then represented using a splits graph. In such a network, every edge is associated with a split of the objects, but there may be a number of parallel edges associated with each split. The edges separate objects on one side of the split from the objects on the other side, the length of an edge in the network is proportional to the weight of the associated split. The produced network is planar, i.e. can be represented in two dimensions. The SplitsTree 4 software was employed to construct a neighbor-net graph from the distance matrix.

A three-level AMOVA (Excoffier et al. 1992) was performed to estimate the variation attributable to the differences between taxa (V_a), among local population within taxa (V_b) and among individuals within populations (V_c). The significance of each variance component was tested by the permutation test using 100,000 random permutations against H_0: $V_a = 0$, $V_b = 0$, $V_c = 0$. Because of a supposed strong regional component of differentiation in *F. orientalis*, a similar three-level AMOVA (regional populations, local populations, individuals) was performed separately for both accepted subspecies (for AMOVA, *F. moesiaca* populations were included in *F. sylvatica* and *F. taurica* populations in *F. orientalis*).

Results

For an initial descriptive analysis of genetic differentiation patterns (in terms of both taxonomy and biogeography), a dendrogram based on UPGMA clustering of Nei's genetic distances was used (Fig. 2). As expected, beech populations from Europe, including the Balkans, clustered together. The differentiation within *F. orientalis* shows a strong geographic pattern: at least three distinct clusters can be addressed. Iranian populations (Alb; for acronyms of regional populations, see Fig. 1) are clearly distinct to

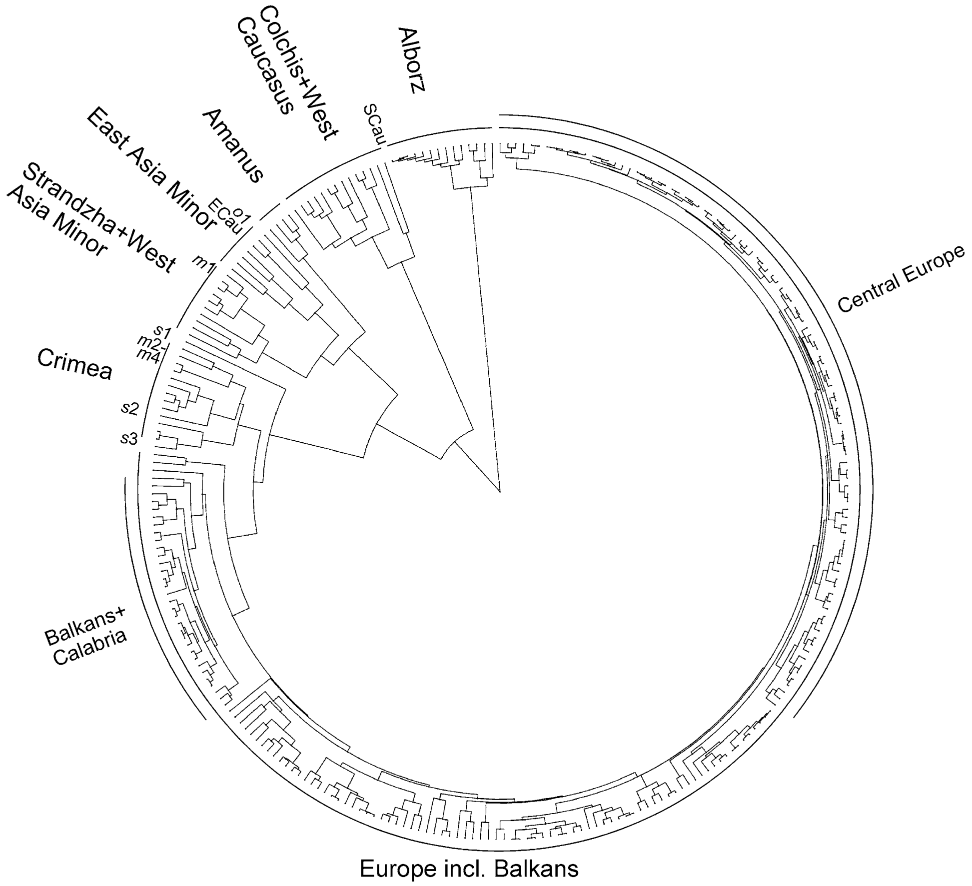


Fig. 2 A UPGMA dendrogram based on Nei's genetic distances among the 279 investigated beech stands. Acronyms of regional populations follow Fig. 1. The cluster 'Central Europe' contains exclusively *F. sylvatica s. str.* Outliers: *o*1 Düzici (Aman), *m*1 Katerini (Gre), *m*2 Shumen (StPl), *m*3 Lailas Serras (Gre), *m*4 Pilyon (Gre), *s*1 Grapa (WCar), *s*2 Neuchatel (WAlp), *s*3 Kodry (Mold)

the rest, forming a very compact cluster. The second cluster comprises populations from the western part of the Great Caucasus and its foothills (WCau), hills and plateaus separating Great and Small Caucasus, designated by the historical name Colchis (Col) and one slightly separated population from the Armenian Small Caucasus (SCau). The third cluster can be further geographically subdivided into three groups, which are quite well-separated: the Amanus Mts. in southeastern Turkey (Aman), the eastern part of Asia Minor (between the 36° and 40° E meridian; EAMi), and the western part of Asia Minor (WAMi) plus the Strandzha mts. in southeastern Europe (Str). The populations from the eastern Great Caucasus (Russian Dagestan republic; ECau) belong also to this group. This is surprising, as they are separated from the easternmost Colchis population Lagodekhi (second cluster) by the main ridge of the Caucasus, but the distance is only few tens of kilometers. The cluster of Crimean populations (Cri; *F. taurica*) is less compact and placed next to the cluster of Balkan and the remaining European beech populations (*F. moesiaca, F. sylvatica s. str.*). Comprised are also some Greek and East-Bulgarian populations of *F. moesiaca* (Gre, StPl) and populations of *F. sylvatica* originating from Moldova (Mold). Clear outliers are the Slovak population Grapa (WCar) and the Swiss population Neuchatel (WAlp). Within the European and Balkan cluster, there is no clear pattern. Most *F. moesiaca* populations (SDi, StPl, Rod, Gre) are placed within a separate cluster, containing no *F. sylvatica s. str.* populations except Calabria (Cala). Another cluster contains *F. sylvatica s. str.* populations from Central Europe, starting from the Bohemian Massif and Polish lowlands (BoM, Pol) over Carpathians (WCar, ECar, SCar) to northwestern Balkans and eastern Pre-Alps (NDi, EAlp). But, all the other comprise both *F. sylvatica s. str.* and *F. moesiaca* populations.

Since no clear substructure was identified within the cluster containing European beech populations, we defined regional populations simply based on orographical or phytogeographical units. For *F. orientalis*, geographical subdivision readily coincided with genetic differentiation patterns. For both subspecies, they respected major discontinuities within the range (see beech distribution maps on http://www.euforgen.org). Regional populations defined in this way were subsequently used as basic units for further analyses.

The reticulated network based on Nei's genetic distances among regional populations shows essentially the same relationships as the single-population-based UPGMA tree (Fig. 3). Eight branches were added to the neighbor-joining tree. Some of these reticulation branches connect

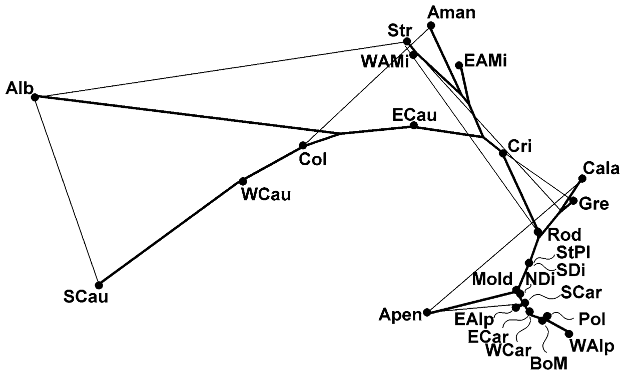
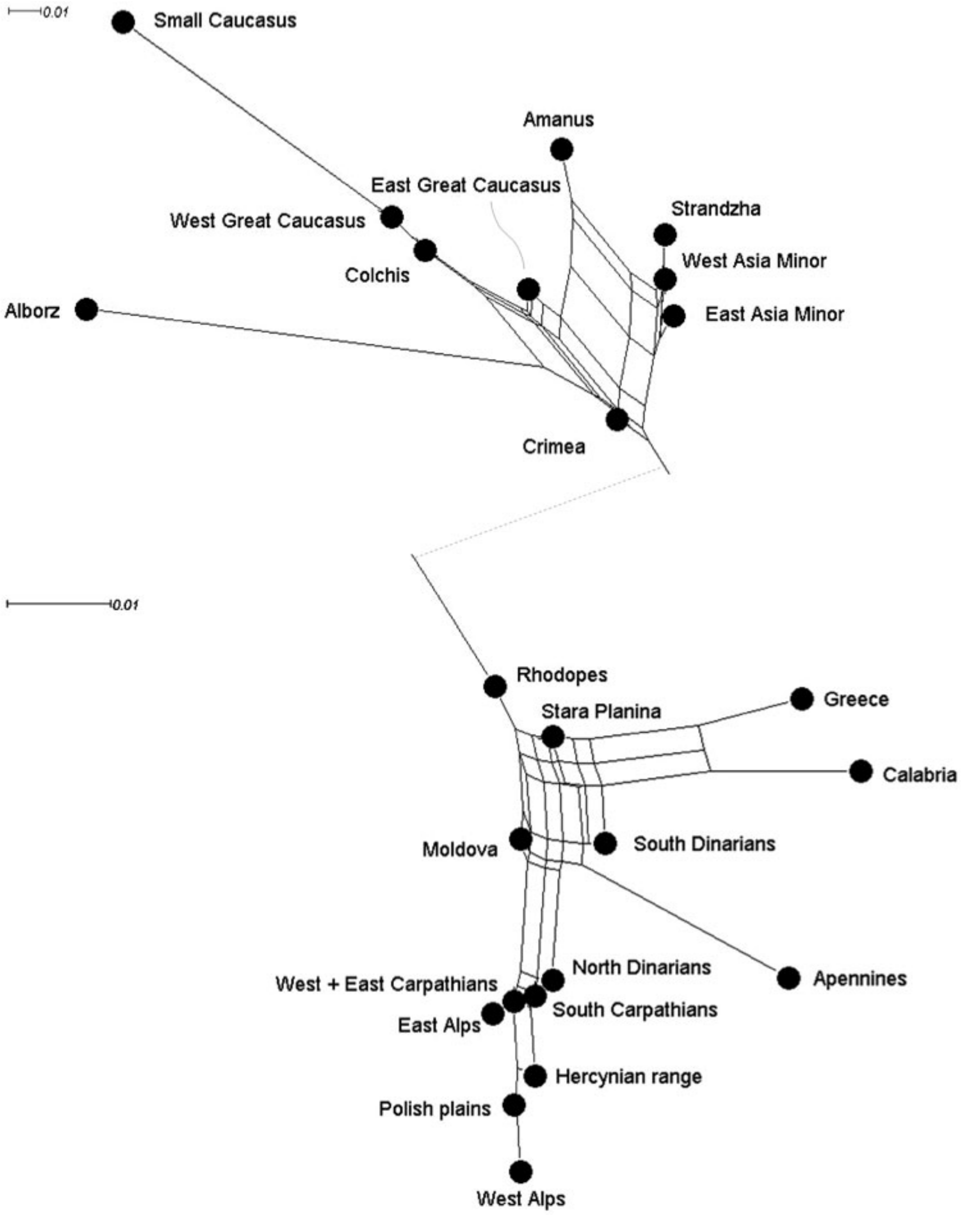


Fig. 3 A reticulated network based on the matrix of genetic distances among regional beech populations. *Thick lines* neighbor-joining tree, *thin lines* added reticulation branches

geographically proximate regional populations (Alb–SCau, Aman–Col, Rod–Str, Apen–Cala). In some cases, however, they connect geographically distant regions, where a contact in the past was hardly possible and which are interspersed by other regional populations (Alb–Str, Cri–Gre).

In the neighbor-net network (Fig. 4), European populations are clearly separated from *F. orientalis* and *F. taurica* (since the European cluster is very compact, it is displayed on a larger scale). Within the cluster of *F. sylvatica s. str.*–*F. moesiaca*, three "tips" can be identified, one comprising regional populations from the Carpathians and adjacent territories (Pol, BoM, WCar, ECar, SCar), the second one Cala and Gre, and the last one Apen. Balkan beech regional

Fig. 4 Neighbor-net graph based on the matrix of genetic distances among regional beech populations



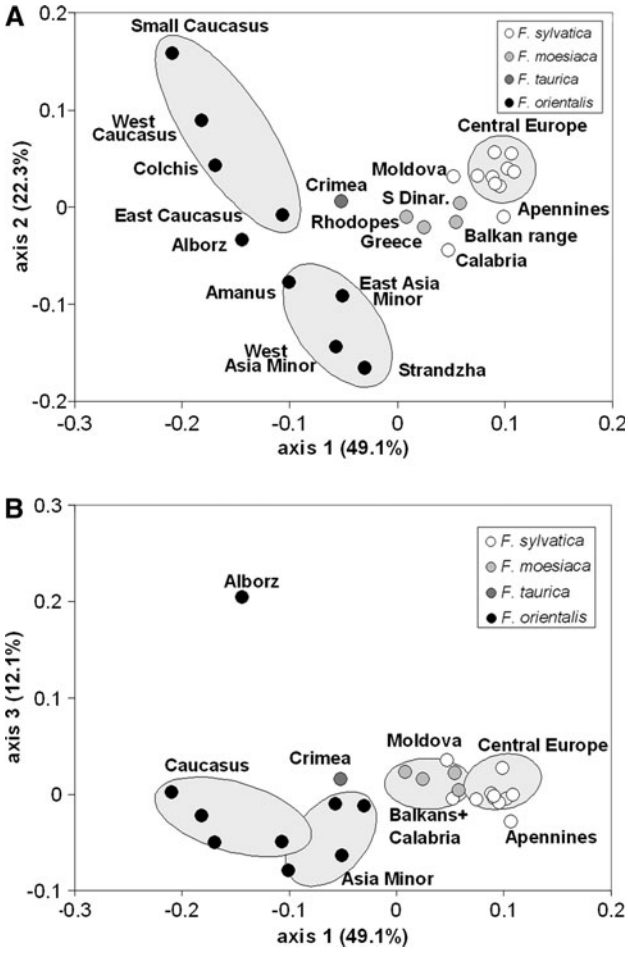


Fig. 5 Principal coordinate analysis based on genetic distances among regional populations. **a** Projection into the plane of the first and second axis, **b** projection into the plane of the first and third axis. The cluster 'Central Europe' includes regional populations WAlp, EAlp, BoM, Pol, WCar, ECar, SCar and NDi

Table 1 Analysis of molecular variance for the whole *F. sylvatica/F. orientalis* complex and within subspecies

	Variance component (%)	*F*	*P*
F. sylvatica vs. *F. orientalis*			
Between taxa	10.24	0.10239	<0.0001
Among populations within taxa	4.30	0.04788	<0.0001
Within populations	85.46		
Total	100.00	0.14537	<0.0001
F. sylvatica			
Among regions	1.29	0.01286	<0.0001
Among populations within regions	1.39	0.01409	<0.0001
Within populations	97.32		
Total	100.00	0.02677	<0.0001
F. orientalis			
Among regions	12.18	0.12177	<0.0001
Among populations within regions	2.61	0.02967	<0.0001
Within populations	85.22		
Total	100.00	0.14783	<0.0001

populations (SDi, StPl, Rod, Gre) are highly networked, indicating ambiguity of the phylogenetic signal in the data. Similarly, within the *F. orientalis*–*F. taurica* group, three subgroups are visible, one formed by Alb, the second one comprising WCau, SCau and Col (ECau placed proximally) and the last comprising Str, WAMi and EAMi. Aman shows affinities to both the second and the third group. As in Fig. 3, the Crimean populations (Cri; *F. taurica*) are placed close to the centre of the graph, mirroring the placement of Rod and StPl in the *F. sylvatica s. str.*–*F. moesiaca* subgraph.

The principal coordinate analysis showed a similar structure. The projection into the plane of the first two principal axes (explaining cumulatively 71.4% of the variation) showed three clusters corresponding with the regional populations of *F. orientalis* (Fig. 5a). Alb is completely separated from the remaining *F. orientalis* along the third principal axis (Fig. 5b). *F. sylvatica s. str.* populations form one compact cluster with few outliers: Mold, Apen and Cala. Both *F. taurica* and *F. moesiaca* occupy intermediate positions (Fig. 5a).

AMOVA results indicate that variance components at all levels (taxon, region, population) are significantly different from zero (Table 1). More than 10% of variation is attributable to differences between the two main taxa. However, the regional component strongly differs between *F. sylvatica* and *F. orientalis*: whereas only 1.3% of variation can be attributed to differences between regional populations in European beech, the regional component exceeds 12% in eastern beech.

Discussion

The patterns of genetic variation within the *F. sylvatica* complex can be associated with its history. The origin of the genus *Fagus* is supposed to be located in the Eocene of the Northern Pacific area (Denk and Grimm 2009; Manchester and Dillhoff 2004; Manos and Stanford 2001). The representatives of the genus appeared in Europe and western Asia as early as in the Oligocene and a continuous presence of beech until the end of the Tertiary in this area is documented by rich fossil material (Denk 2004; Kvaček and Walther 1991). Variation in fossil material is, however, enormous and fossil remnants from Europe exhibit a morphological affinity to several extant beech species, including North American and East Asian ones (*F. grandifolia, F. longipetiolata, F. crenata, F. hayatae*; cf. Denk

2003, 2004). Since the Pliocene, all beech in western Eurasia belonged to polymorphic fossil species *Fagus haidingeri* Kovats, but the absence of clear geographical trends and a great variation in the late Tertiary beech fossils indicate a high initial genetic diversity (Denk 1999c). With the onset of climate change at the end of the Pliocene and during the Pleistocene, the range of beech became smaller and fragmented (Atalay 1998; Gulisashvili et al. 1975; Tumajanov 1971). This reduction of population sizes and increase of genetic isolation may have contributed to the formation of the present patterns of variation.

F. sylvatica and *F. orientalis*: one or two taxa?

A previous study based on the same material (Gömöry et al. 2007) showed that *F. sylvatica s. str.* and *F. orientalis* share a substantial part of their nuclear gene pools, but can be quite clearly distinguished. Moreover, they possess almost completely different sets of chloroplast haplotypes (Demesure et al. 1996; Gailing and von Wuehlisch 2004; Salehi-Shanjani et al. 2004; Vendramin personal communication). Nevertheless, *F. orientalis* populations are richer in isozyme alleles and incomparably more differentiated (Gömöry et al. 2007). This confirms the concept of *F. orientalis* as an ancient taxon and *F. sylvatica s. str.* as a relatively recently diverged branch.

The problem of the taxonomy of beech in Europe and the Near East is partly associated with a wide variation of phenotypic traits used to discriminate between the taxa. A survey of taxonomical studies (mainly from Balkan countries, Central Europe and the U.S.S.R.) made by J. Vyšný (unpublished data) showed that there is no unanimity among different authors concerning the means and ranges of quantitative leaf and fruit traits characterizing individual taxa. Moreover, although the means or modal values of these traits may differ among taxa within a study, the ranges are generally very broad and overlap considerably (cf. Denk 1999a, b). Denk et al. (2002) showed relationships among seven morphotypes of beeches in West-Eurasia defined by Denk (1999a, b). Differentiation patterns identified within this study correspond with the distribution and relationships of morphotypes at the large scale. Within *F. orientalis*, all the presented analyses (Figs. 2, 3, 4, 5) show the existence of three large genetic clusters: the Caucasus, Asia Minor and the Alborz mts., identical with clusters produced by Bayesian analysis of genetic structure (Gömöry et al. 2007). As shown by Denk et al. (2002), *F. orientalis* morphotypes can also be grouped in identical way, again with the most pronounced divergence of the Alborz mts. The East–West cline-like trend of morphological change reported by Denk et al. (2002) can also be observed in our genetic data to some degree (cf. Figs. 3, 5b). However, at a more detailed scale, there are already differences. Genetic separation of eastern and western Turkish populations (reflected not only in gene frequencies, but also in presence of private alleles) or affinity between East-Caucasian and East-Turkish beech populations are not observed in morphotype distribution (Denk 1999a). Anyway, neither the genetic nor the morphological data seem to justify the current taxonomical concept, which considers almost all European beech populations as one subspecies and unifies all the diverse and highly differentiated West-Asian populations into another one.

Origin and evolution of *F. moesiaca* and *F. taurica*

Both *F. moesiaca* and *F. taurica* are considered transitional taxa between *F. orientalis* and *F. sylvatica*. However, the opinions about the meaning of the term “transition” diverge. Borza (1965) or Diduch (1992) considered both taxa to be identical, what implies origin from a common source population and a subsequent fragmentation of its range. Other studies suggested that they represent phylogenetic links between *F. orientalis* (generally considered a more ancient taxon) and *F. sylvatica s. str.* (Czeczott 1932, 1933; Tutin 1964; Wulff 1932), i.e. intermediate stages of an anagenetic process leading from an ancestral taxon to a successor. According to Becker (1981) or Boratyńska and Boratyński (1990), they are hybrids, what means that they were formed by interbreeding between *F. sylvatica* and *F. orientalis*, after the basic taxa diverged. Finally, Stănescu (1979) or Svoboda (1953) treated both intermediate taxa as ecotypes (i.e. products of adaptation to specific environments).

A recent study of Magri et al. (2006) combining paleobotanic and genetic approaches demonstrated that *F. moesiaca* survived the last (Würm/Vistulian) glacial in local refugia, expanding independently from the main refugial population located in Istria and at the eastern foothills of the Alps (*F. sylvatica s. str.*). Consequently, populations in the very southern part of the Balkan Peninsula differ from the Central-European ones by allozyme gene frequencies (Gömöry et al. 1999, 2007) and contain a different mixture of chloroplast haplotypes. On the other hand, they share cpDNA haplotypes quite extensively both with Italian populations of *F. sylvatica* and with West-Turkish *F. orientalis* (Hatziskakis et al. 2009; Magri et al. 2006; Paffetti et al. 2007).

The southeastern boundary of *F. moesiaca* is not problematic and the taxon is well delimited from ssp. *orientalis*. In Bulgaria, populations of *F. orientalis* are geographically isolated from *F. moesiaca*. In eastern Greece, the ranges overlap and even mixed stands occur, but the taxa still can be distinguished by morphology (cf. Papageorgiou et al. 2007 and the citation therein). In the Northeast, *F. moesiaca* is separated from *F. sylvatica* by the Danube valley (Wallachian lowland). This wide geographic barrier is not impermeable to gene flow, as genes in wind-pollinated forest trees can be transported viable over large distance by pollen (Lindgren et al. 1995). In contrast, the boundary is unclear in the Northwest, where the Balkan and East-Alpine refugial populations met during the Holocene expansion and exchanged genes, resulting in a smooth clinal variation of gene frequencies (Gömöry et al. 1999). The expansion of *F. moesiaca* refugia does not seem to have continued towards the East and South-East. Gene exchange by pollen between *F. orientalis* and Bulgarian *F. moesiaca* seems to be largely unidirectional: nuclear gene frequencies in European populations of *F. orientalis* (Str) do not essentially differ from those of the populations in the western part of the Ponthic mountain range in Asia Minor (WAMi), whereas gene frequencies as well as patterns of rare alleles occurrence in the easternmost populations of *F. moesiaca* in StPl and Rod resemble *F. orientalis* and change smoothly towards the West (Gömöry et al. 1999, 2007; see also Fig. 5a, b).

Peculiar is a genetic proximity and even partial sharing of chloroplast haplotypes between south-Italian and Balkan populations (Gömöry et al. 1999; Vettori et al. 2004), reflected also in the direct relationship between Calabria and Balkan populations (Gre, Rod) shown in the reticulogram (Fig. 3) and the position of both groups in the neighbor-net network (Fig. 4). A similar relationship was found in the orchid *Anacamptis palustris* (Musacchio et al. 2006) and was explained by a land-bridge covered by marshes over the Otranto strait. This land-bridge disappeared at the beginning of the Pliocene and accepting this view here would mean that the *F. sylvatica s. str.*–*F. moesiaca* complex diverged from *F. orientalis* very early. The spread of *F. moesiaca*-like beech around the Adriatic coast during the Pleistocene (or even earlier) with a later extinction of connecting populations and a subsequent recolonization of the North-Adriatic area by *F. sylvatica s. str.* is a more plausible explanation. It is supported also by a discovery of fossil pollen with chloroplast haplotype identical with the extant *F. moesiaca* (not distinguishable from *F. orientalis* at the analyzed loci) in the Venice lagoon, originating from the end of the last interglacial (Paffetti et al. 2007).

The intermediate position of *F. moesiaca* (SDi, StPl, Rod, Gre) between subsp. *sylvatica s. str.* and western-Turkish populations of subsp. *orientalis* (Str, WAMi; see Fig. 5a, b) could also indicate that *F. sylvatica* diverged from the regional population of the West Asia Minor. However, there exists another transitional pathway between both subspecies of beech, namely that of *F. taurica,* which is intermediate in respect to morphology as well as frequencies of allozyme genes (Gömöry et al. 1998, 2007). Interestingly, Moldovan populations of beech (Mold; geographically most proximate to Crimea) are among the *F. sylvatica s. str.* populations those that are most proximate to *F. taurica* (Cri) genetically. Such a relationship is hard to comply with both speciation and introgression. Although no pollen data are available directly from Moldova, all evidence indicates that the Carpathian as well as Moldovan populations are late newcomers after the last glaciation (Magri et al. 2006). Moreover, Moldova is separated from Crimea by a wide zone of a hot steppe, which has not been suitable for the survival of beech since the onset of Holocene.

Genetic data do not indicate that *F. moesiaca* is more than a group of populations originating from different glacial refugia than the majority of *F. sylvatica s. str.* populations in Europe and influenced by gene flow from the neighboring West-Turkish populations of *F. orientalis*. For *F. taurica*, there is not enough data to conclude about its evolution. Further paleobotanical and paleontological research focusing on southern Ukraine and Moldova (where this sort of data is really scarce) and analyses of ancient DNA from fossil samples could shed light on the transition between *F. sylvatica s. str.* and *F. orientalis*.

Hybridization or lineage sorting?

There are two explanations for genetic similarity of a pair of populations: incomplete lineage sorting and contemporary or past gene flow (Joly et al. 2009; Kubatko 2009). Actually, only the latter represents a reticulate mechanism in the strict sense, i.e. divergence of lineages followed by their re-joining, but their effect on gene frequencies is more or less identical. Accordingly, the interpretation of the reticulogram is not straightforward. A strong differentiation in *F. orientalis* indicates a long-lasting isolation and, in some cases, fragmentation of beechwoods in the three large regions (East Bulgaria + Turkey, Great and Small Caucasus, Alborz). As documented by the fossil record as well as the presence of Tertiary relics in the extant beechwoods, beech has probably been continuously present in all three regions since the Miocene (Gulisashvili et al. 1975; see also Denk et al. 2002 and the citations therein). In contrast to Europe, the range of *F. orientalis* was much less affected by glaciations during the Pleistocene and mixed forests survived continuously even during the glacial maxima (Walter and Straka 1970). Naturally, this does not exclude expansions and retreats of the range of eastern beech associated with Pliocene and Pleistocene climatic fluctuations, by which gene flow between regional populations was established. Such events could explain several reticulation branches, e.g., Alb–SCau or Aman–Col. Similar periodic changes of the range size may explain reticulation branches between Cala, Apen and EAlp. As mentioned above, also reticulation branches between Str and Gre and/or Rod may result from gene flow between these regions. As some connections in the original neighbor-joining tree are biogeographically unrealistic and not supported by cytoplasmic marker data, reticulation lines may represent a more plausible evolutionary alternative in some cases. For instance, there can hardly be any direct evolutionary relationship between Apen and Mold, and this connection may have resulted from inflow of genes and/or immigration from the same source population in the southern Balkans.

On the other hand, gene flow cannot explain the proposed reticulation branch Alb–Str. Therefore, incomplete lineage sorting must be considered. This explanation requires large effective population sizes and a relatively recent speciation event (Muir and Schlötterer 2005), which both seems to apply in the case of beech. Western-Eurasian beech descended probably from a highly polymorphic Miocene-Pliocene *F. haidingeri* that was distributed over Europe and west Asia during the Pliocene. However, the ancestry is by far not without ambiguities as this species exhibits affinities to the extant East-Asian species rather than to *F. sylvatica* and includes types previously classified as *F. kraeuselii*, similar to both extant subspecies of *F. sylvatica* (Denk 1999c, 2004). Nevertheless, there is no proof of the extension of *F. haidingeri* range into Iran, where the extant populations (genetically most divergent from the remainder) show morphological similarities to the North-American *F. grandifolia* (Denk 1999c). Moreover, the role of another Miocene species, *F. gussonii*, in the phylogeny of *F. sylvatica* is still unclear (Denk and Grimm 2009). Morphological diversity and absence of geographic trends until the beginning of the Pleistocene point to high levels of gene flow, possibly maintaining low geographic differentiation despite a high initial genetic diversity in the probably continuous ancestral population, from which *F. sylvatica s. l.* derived (Denk 1999c, Denk and Grimm 2009). Fragmentation of the range due to the Pleistocene climate changes, associated with local extinctions and recolonizations, may have disrupted the previous continuity of genetic structures. Accordingly, a pair of continuously surviving populations retaining the original gene pool may have become interspersed by newcomers originating from a different source population (resulting in connections between distant non-adjacent populations in the reticulogram). In particular, this might be the case for the Crimean populations (*F. taurica*), which show affinity to both *F. sylvatica s. str.* and *F. orientalis* from Asia Minor and the Caucasus (Figs. 2, 3, 4, 5, see also Gömöry et al. 2007), or Amanus mts. placed in the intermediate position between Asia Minor and the Caucasus (Fig. 4).

Gene frequency data alone do not allow explicit testing of hybridization versus incomplete lineage sorting hypotheses. However, both phenomena can actually be responsible for the observed reticulations. We tended to explain reticulations by gene flow in the case of geographically proximate adjacent regional populations, while sharing of ancestral polymorphisms was suggested for pairs of distant regions.

Implications for taxonomy

Our data confirm the opinion of Denk et al. (2002) that the Greuter and Burdet's (1981) concept of two subspecies (subsp. *sylvatica* vs. subsp. *orientalis*) does not reflect evolutionary processes within the complex of *Fagus sylvatica s. l.* However, the alternatives proposed by Duty (1985) and Shen (1992) do neither fully correspond to differentiation patterns presented in this study. Duty's concept of three subspecies of *F. orientalis* within the Asian part of the range conforms to our data. However, the classification of the whole southern Balkans as *F. orientalis* subsp. *balcanica* is out of reality, as well as the extension of the range of subsp. *moesiaca* deep into Central Europe. Shen (1992) distinguishes three subspecies of *F. sylvatica*, two of them in Asia, but includes both Caucasian and Iranian populations into subsp. *hohenackeriana*. Our data indicate that *F. sylvatica* does not represent a single biological species, as beechwoods in the three parts of the Asian range (Asia Minor, Caucasus, Alborz) are reproductively isolated today, and apparently sufficiently distinct in morphology to justify delineating them as separate taxa. This applies primarily to populations in the Alborz, which are highly divergent, morphologically distinct (Denk et al. 2002) and maybe have a different ancestor than the remainder of the species (*F. castaneifolia*?; cf. Denk and Grimm 2009). On the other hand, distinguishing *F. moesiaca* as a separate taxon (whatever is the rank) does not seem justified. If such name should be used for European populations originating from the Balkan glacial refugia of beech, then it must be reserved for populations in the very southern part of the Balkans, as shown by Magri et al. (2006).

Reticulate evolution in woody species has most frequently been documented by sharing chloroplast haplotypes or lineages among different taxa within a genus (Jackson et al. 1999; McKinnon et al. 2003; Petit et al. 2002). The example of *Fagus* in western Eurasia has shown that ancient reticulation events leave their traces in the nuclear genome as well.

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