IDENTIFICATION OF THE *FAGUS SYLVATICA* L. AND *FAGUS ORIENTALIS* LIPSKY SPECIES AND INTRASPECIFIC VARIABILITY[1]

Cristina Vettori [1], Donatella Paffetti [2], Ladislav Paule [3] & Raffaello Giannini [1, 2]

[1)] Institute of Plant Genetics Division of Florence, National Research Council, Via Madonna del Piano, I-50019 Sesto Fiorentino (FI), Italy
[2)] Department of Forest Environmental Science and Technologies, University of Florence, Via San Bonaventura 13, I-50145 Florence, Italy
[3)] Faculty of Forestry,Technical University, SK-96053 Zvolen, Slovakia
Corresponding author: Giannini R, Institute of Plant Genetics Division of Florence, National Research Council, Via Madonna del Piano, I-50019 Sesto Fiorentino (FI), Italy. Fax number: 0039 055 5225729, e-mail: raffaello.giannini@igv. cnr.it

ABSTRACT

Fagus sylvatica L. and *Fagus orientalis* Lipsky are very close species, and an open question is if the two species are really separated. The main goal of this research was to clarify the presence of an unique *F. sylvatica-orientalis* complex or two distinct species by studying the sequence of the *trn*L-*trn*F region of chloroplast DNA (cpDNA). Twenty-nine *F. sylvatica* and twenty-two *F. orientalis* populations have been sampled, and to better delineate the systematic position in genus *Fagus*, *Fagus taurica* Popl., *Fagus moesiaca* Czecz., *Fagus grandifolia* Ehrh., *Fagus crenata* Bl., *Fagus japonica* Bl., and *Fagus hayatae* Palibin were also considered.

The sequence analysis permitted to resolve distinct groups corresponding to taxa such as *F. sylvatica*, *F. moesiaca*, *F. taurica*, and *F. orientalis*. Moreover within the European beech, *F. orientalis* seems to be the ancestral species. This data is corroborated by a higher variability found in *F. orientalis* than in *F. sylvatica* (9 and 6 haplotypes, respectively.

Key words: *Fagus* spp., *Fagus orientalis, Fagus sylvatica*, *trn*L-*trn*F, cpDNA region, phylogeny, genetic variability

INTRODUCTION

Fagus sylvatica L. and *Fagus orientalis* Lipsky are very close species. The first one is distributed in western, central and southern Europe with individual occurrences in England, and Scandinavia. The natural range of the second one is Asia Minor, the Amanus Mts. (Syria), the Elburz Mts. (Iran), and Caucasus. Contact zone between the natural ranges of both species are in northern Greece and Bulgaria (PAULE 1995). Different authors are not in agreement concerning the taxonomic position of *Fagus taurica* Popl. and *Fagus moesiaca* Czecz. The entity *F. taurica* is by some authors considered as the intermediate form between *F. sylvatica* and *F. orientalis*, and by the others as an independent species. Similar dubious regard the taxonomic unit *F. moesiaca* considered an independent species or most frequently the subspecies of *F. sylvatica* (PAULE 1995). The description of these taxa has been done mainly on the morphological traits of leaves. Recently, DENK *et al.* (2002) tried to combine morphological and molecular data to resolve this controversy without succeed to resolve the relationships within the western Eurasiatic taxa. From these statements it is clear that an open question is if the two species (*F. sylvatica* and *F. orientalis*) are really separated.

The main goal of this research was to clarify the presence of an unique *F. sylvatica-orientalis* complex or two distinct species by studying the sequence of the *trn*L-*trn*F region of chloroplast DNA (cpDNA). The effectiveness of this region in distinguishing the systematic entities in *Quercus* spp. has been previously demonstrated (PAFFETTI *et al.*, 2001). This cpDNA region is constituted by the *trn*L intron, the second *trn*L exon, and the *trn*L-*trn*F intergenic spacer. The intron region permits to identify the species, and the intergenic

[1] This paper has been presented as invited paper at the IUFRO Symposium on Population and Evolutionary Genetics of Forest Trees held in Stará Lesná, Slovakia, on August 25–29, 2002.

Table 1a. Taxonomic position and geographic origin of plant material of *Fagus sylvatica* L.

Geographic origin	Country	Latitude	Longitude
Annunziata	Sicilia, Italy	37° 53' N	14° 57" E
Gullmarsberg	Sweden	58° 22' N	11° 39 ' E
Westfield	Scotland	57° 40' N	3° 25 ' W
Lowther E.	England	54° 37' N	2° 44 ' W
Alsted	Denmark	55° 24' N	11° 39 ' E
Tomaszow Lubelski	Poland	50° 37' N	23° 23 ' E
Tatra National Park	Slovakia	49° 16' N	20° 10 ' E
Tharandt	Germany	48° 83' N	10° 25 ' E
Slovany	Slovakia	48° 59' N	18° 44 ' E
Horna Suca	Slovakia	48° 56' N	18° 00 ' E
Zamutov	Slovakia	48° 52' N	21° 34 ' E
Vihorlat	Slovakia	48° 50' N	22° 02 ' E
Zvolen	Slovakia	48° 40' N	19° 02 ' E
Polana	Slovakia	48° 37' N	19° 28 ' E
Sitno	Slovakia	48° 24' N	18° 50 ' E
Lozorno	Slovakia	48° 20' N	17° 08 ' E
Beius Bihor	Romania	46° 68' N	22° 26 ' E
Des Colletes	France	46° 18' N	2° 95 ' E
Limitaciones	Spain	42° 81' N	2° 25 ' E
Kladska	Czech Republic	50° 05' N	14° 26 ' E
Glarus	Switzerland	47° 30' N	9° 40 ' E
Nevrokopi	Greece	41° 11' N	23° 57 ' E
Pylion	Greece	39° 35' N	22° 14 ' E
Passo San Boldo	Veneto, Italy	46° 01' N	12° 13 ' E
Monte Soro Nebrodi	Sicilia, Italy	37° 50' N	14° 43 ' E
Medingen	Germany	53° 05' N	10° 57 ' E
Prati di Tivo	Abruzzo, Italy	42° 31' N	13° 32 ' E
Pian di Novello	Toscano, Italy	44° 07' N	10° 42 ' E
Bosco della Martese	Abruzzo, Italy	42° 41' N	13° 30 ' E

The ranking of populations follow the haplotype ranking as indicated in Table 3.

region, having a high variability at the intraspecific level, is useful to determine the variability inside the species (PAFFETTI *et al.*, 2001).

MATERIAL AND METHODS

Plant material

29 and 22 populations (one individual each) of *Fagus sylvatica* L. and of *Fagus orientalis* Lipsky, respectively, were sampled across their native range for a total of 51 individuals (Table 1). Young leaves or dormant buds were taken from each individual and stored at –20 °C.

In addition, *Fagus taurica* Popl., *Fagus moesiaca* Czecz., *Fagus grandifolia* Ehrh., and *Fagus crenata* Bl. were also considered as outgroup.

DNA extraction

Total DNA was extracted from young leaves or dormant buds (100 mg as starting material) using the NucleoSpin® Plant kit (Macherey-Nagel, Germany) following the manufacturer's specifications.

Polymerase Chain Reactions (PCR) conditions

Amplifications were performed in a 20 µl volume as reported by PAFFETTI *et al.* (2001) using the universal primers (c and d) for the *trn*L-*trn*F cpDNA region described by TABERLET *et al.* (1991). A Perkin Elmer 9700 thermocycler was used.

Amplification products (10 µl per lane) were analyzed by gel electrophoresis on 1% (w/v) agarose gel (GIBCO BRL, USA) at 10 $V \cdot cm^{-1}$ for 2 h in Trisacetate-EDTA buffer containing 0.5 $\mu g \cdot ml^{-1}$ (w/v) of ethidium bromide (SAMBROOK *et al.* 1989). The gels were photographed and analyzed with an UVP gel scanner (Photo-Capt,

Table 1b. Taxonomic position and geographic origin of plant material of *Fagus orientalis* Lipsky

Geographic origin	Country	Latitude	Longitude
Sukhansk	Cherek river, Russia	43° 21' N	43° 48' E
Devrek	Turkey	41° 10' N	32° 16' E
Eregli	Turkey	37° 03' N	34° 17' E
Vanadzor	Armenia	40° 48' N	44° 30' E
Vanadzor 2	Armenia	40° 48' N	44° 30' E
Sochi	West Caucasus, Russia	43° 38' N	39° 42' E
Zorkum	Ammanus, Turkey	36° 58' N	36° 24' E
Neka	Iran	36° 22' N	53° 33' E
Shilda 1	Georgia	42° 06' N	45° 50' E
Bakuriani	Georgia	41° 44' N	43° 32' E
Nichbisi	Georgia	41° 50' N	44° 33' E
Duzce	Turkey	45° 28' N	34° 07' E
Dortyol	Turkey	36° 47' N	36° 36' E
Batumi / Keda	Georgia	41° 40' N	41° 55' E
Akkus	Turkey	40° 50' N	37° 05' E
Inegoel	Turkey	39° 53' N	29° 36' E
Karabuk	Turkey	41° 16' N	32° 32' E
Arboretum Vallambrosa	Italy	43° 44' N	11° 32' E
Sovetskoe	Cherk river, Russia	43° 21' N	43° 48' E
Catalca	Turkey	41° 28' N	28° 21' E
Izmit	Turkey	40° 34' N	29° 57' E
Asalem (1900 m)	Iran	37° 38' N	48° 46' E

The ranking of populations follow the haplotype ranking as indicated in Table 4.

Vilbert Coormat, France).

DNA cloning and sequencing

The amplification products were gel purified using the NucleoSpin® Extract kit (Macherey-Nagel, Germany) following the manufacture's specifications. Direct sequencing of amplified DNA was done in both directions from independent amplification reactions using the dideoxy-chain termination method (SANGER *et al.* 1977) and the Sequenase kit (USB) with the necessary modifications for direct PCR sequencing. The *F. sylvatica*, *F. orientalis*, *F. taurica*, *F. moesiaca*, *F. grandifolia*, and *F. crenata* sequences generated in this study have been submitted to Gen- Bank with the following accession numbers, respectively: AF133654, AF533691, AF533690, AF533692, AF533694, and AF533693.

Data analysis

DNA sequences from the *Fagus* spp. of this work were multiply aligned, using the Clustal X program (THOMPSON *et al.* 1997), among them and with sequences of *Quercus* spp. (AF268937, AF133652, AF268938, AF133648, AF133647, AF268939, AF133650, and AF133649), *Castanea sativa* (AF344183), *F. japonica* Bl. (AB046521) and with *F. hayatae* Pablin (AB046522) present in GenBank, and these alignments were used for further analysis. Phylogenetic analysis was performed by two methods: maximum parsimony (FITCH 1971), and maximum-likelihood (FELSENSTEIN & CHURCHILL 1996) phylogeny estimation using the DNAPARS, and DNAML programs, respectively, of the PHYLIP version 3.5c package (FELSENSTEIN 1993). Numbers of substitutions were also estimated using the one-parameter method of JUKES and CANTOR (1969), and the 2- parameter method of KIMURA (1980). The matrices of pairwise substitution rates were then submitted to the neighbor-joining method of phylogenetic tree reconstruction. In all cases, bootstrap procedure was calculated from 500 replicates. Bootstrap values were calculated from 500 replicates using SEQBOOT program in PHYLIP version 3.5c package, and a consensus tree was obtained by CONSENSE program in PHYLIP version 3.5c package. A maximum likelihood tree was inferred using a transition/ transversion ratio of 0.5, global rearrangement, and randomising the sequence addition order (JUMBLE).

Table 2. Variable informative sites (exclusive sites of genus *Fagus* in red; exclusive of the species in blue).

trnL intron region (position 1-506)	trnL exon (position 507-556)	trnL-trnF intergenic region (position 557-873)
111111 1111112223	3333344444 5555555556	6666666666 6667777777 7777788888 8888888888 8888888888 8888888
5779011345 6678891781	1224736679 0034779990	1111224555 5566777777 7890001122 3444600011 1112222333 3344444455 5566667
4180979313 0793554230	4578295939 2380273569	1237258124 5607023567 8872456858 1458278901 5691246234 5712578901 7817781
F. sylvatica CAGAATTAAA	TGACACCTGA --CGAATTAT	AGTACTTTTT TTAT------- ---------- ---------- ----TTCAA GGTCGTTTCA ACATTCTCTT AA-CTA
F. taurica .....AA...	....	.....C.C. ....
F. orientalis .....AA...	....	....
F. moesiaca .....AA...	....C....	....
F. hayatae .....AA.C.	....	....
F. japonica .....AAGC.	....	....
F. crenata ....-AA.C.	....	....
F. grandifolia ...GG.C.	....	....
Q. robur AGC..AA.CG	GTCACAGA.. AAT..GCC.G	...GTC.CGC AA.GTCACAC TCTTACGGGT TTC.TTTATT GCGTCACTGT AACTAAACTT TTGAGAGAAA GGCAAC
Q. pubescens A.C..AA.CG	GTCACAGA.. AAT...CC.G	...GTC.CGC .A.GTAACTC TCTT.CTA.T TTC.TTTATT ACGTCACTGT AACTAAACTT TTGAGAGAAA GGCAAC
Q. suber A.C..AA.CG	GTCACAGA.. AAT...CC.G	...GTC.CGC AA.GTCACTC TCTTACTA.T TTC.TTTATT GCGTCACTGT AACTAAACTT TTGACAGAAA GGCAAC
Q. trojana A.CG.AA.CG	GTCACAGA.. AAT...CC.G	...GTC.CGC AA.GTAACAC TCATACTA.T TTC.TTTATT GCGTCACTGT AACTAAACTT TTGAGAGAAA GGCAAC
Q. macrolepis A.CG.AA.CG	GTCACAGA.. AAT...CC.G	...GTC.CGC ...-GTAACTC TCTTACTA.T TTC.TTTATT GCGTTACTGT AACTAAACTT TTGAGAGAAA GGCAAC
Q. ilex A.CG.AA.CG	GTCACAGA.. AAT...CC.G	...GTC.CGC GA.GGAATTT T.ATACGA.T TCC.TTTATT GCATCACTGT AACTAAACTT TTGACAGAAA GGCAAC
Q. coccifera A.CG.AA.CG	GTCACAGA.. AAT...CC.G	...GTC.CGC .A.GTAACTC TCTTACTA.T TTC.TTTATT GCGTCACTGT AACTAAACTT TTGAGAGAAA GGCAAC
Q. calliprinos A.CG.AA.CG	GTCACAGA.. AAT...CC.G	...GTC.CGC .A.GTAACTC TCATACTA.T TTC.TTTCTT GCATCACTGT AACTAAACTT TTGACAGAAA GGCAAC
C. sativa A.CG.AA.CG	.TCACAG.C. CAT.C.CCTG	GAGGTC.CGC ...GTCACAC TCTTACTATT GTTTTTTATT GCGTCACTGT AACTAAACTT TTGAGAGAAA GGCAAC

RESULTS

Sequence analysis of the *trn*L-*trn*F cpDNA region

The alignment of the *trn*L-*trn*F cpDNA region of *Fagus sylvatica* L. (29 populations, one individual each) and *Fagus orientalis* Lipsky (22 populations, one individual each) (Table 1) indicated that the entire region, including the *trn*L intron, the second trn*L* exon, and the partial sequence of the *trn*L-*trn*F intergenic spacer, ranged from 660 bp in *F. sylvatica* and *F. grandifolia*, to 655 bp in *F. crenata*. After multiple alignment of the *trn*L-*trn*F region towards *F. grandifolia*, the entire region alignment had a total length of 873 bp, the *trn*L intron an average length of 506 bp, the second *trn*L exon a length of 50 bp, and the *trn*L-*trn*F partial intergenic spacer an average length of 317 bp.

Several mutations belonging to individuals of the same species are fixed and permit to identify each genus and species: (i) a 40 bp insertion (between positions 388 and 428 in the intron), and a 183 bp deletion (between positions 622 and 805) in the intergenic region characterise the genus *Fagus*; (ii) two transversions (site 117 and site 119) in the intron identify *F. sylvatica*; (iii) two transitions (site 117 and 119) in the intron identify *F. grandifolia*; (iv) one transition (site 133) in the intron identifies *F. japonica*; (v) one transversion (site 439) in the intron identifies *F. moesiaca*; and (vi) two transitions (site 593 and site 596) in the intergenic identify *F. taurica* (Table 2). The transversion site 141 is particularly interesting as it discriminate between the European species from the other.

Phylogenetic analysis

One most parsimonious trees of 500 steps was found with parsimony analysis (Figure 1A). An identical topology was obtained with the 50%-bootstrap consensus tree, by maximum-likelihood phylogeny estimation using a transition/ tras-version ratio of 0.5 and a ln-likelihood of –575.30020 (Fig. 1B), in total 2449 dendrograms were examined.

Intraspecific variability

Intraspecific variability was also found in *F. sylvatica* and *F. orientalis*, and a total of 15 cpDNA haplotypes (Haps) has been identified. The geographic distribution of the haplotypes is reported in Figures 2A and 2B.

Six Haps have been determined among the 29 populations identified as *F. sylvatica*, and the Haps differ from each other for mutation events

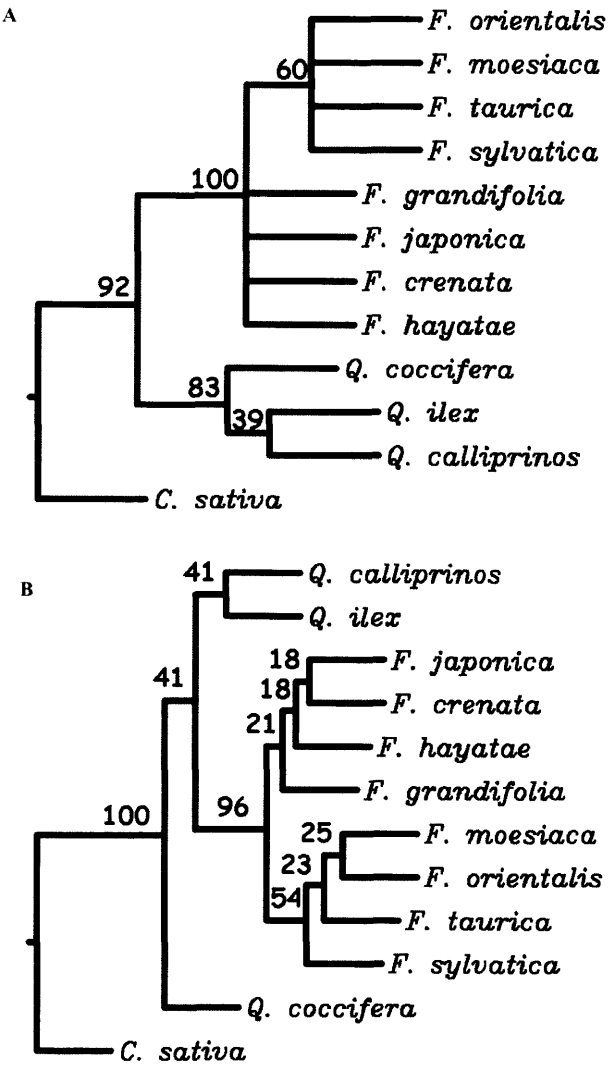

Figure 1. A (upper figure): strict consensus of ****minimal trees (**** steps) obtained from parsimony analysis of *trn*L-*trn*F cpDNA region sequences of *Castanea*, *Quercus*, and *Fagus*. Numbers on branches indicate bootstrap values from 500 replicates (in %). **B:** (lower figure) Maximum-likelihood tree. All nodes, and branch lengths appear to be significantly positive ($p < 0.01$).

occurred in the intron microsatellite (ins/del: site 107 and site 108), and in the intergenic regions (ins/del: site 668, site 703, and site 707) characterized by repeated zones (Table 3). The combination of variable sites found in the intron microsatellite (del: site 104, and site 105), intron region (transversions: site 166, and site 328), and intergenic spacer (dels: site 659 and site 668), has given 9 Haps among the 22 populations of *F. orientalis* analysed (Table 4).

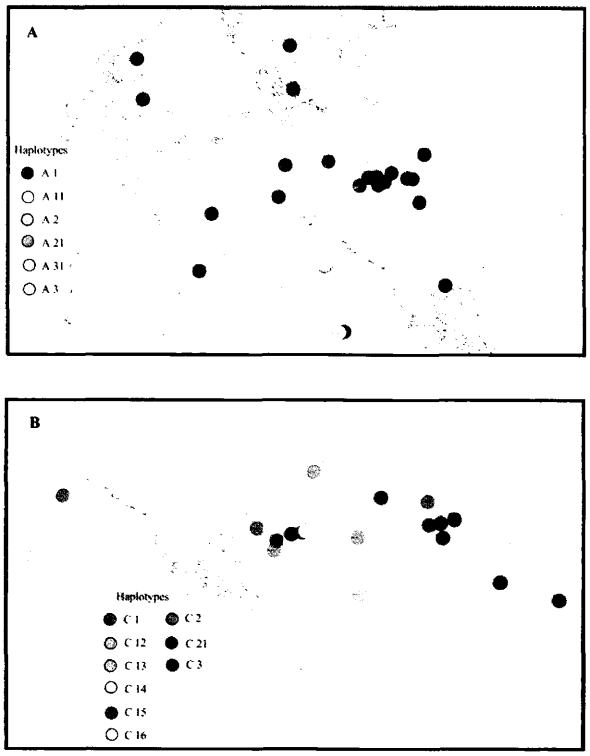

Figure 2. Geographical distribution of chloroplast haplotypes in *Fagus sylvatica* (**A**) and in *Fagus orientalis* (**B**).

DISCUSSION

The phylogeny in the genus *Fagus* was analysed by studing the *trn*L-*trn*F cpDNA region. The variable and informative sites at the interspecific level were localized in the sequences of the *trnL* intron and identified each *Fagus* entity studied. The intergenic region had a higher variability at the intraspecific level. These data confirm the results of previous studies, conducted in the genus *Quercus*, in the potential of this sequence in phylogenetic study (PAFFETTI *et al.* 2001). The trees obtained with the different methods of phylogenetic tree reconstruction (parsimony, and maximum-likelihood) show two separated clusters of the *Fagus* spp., one clustering with *F. grandifolia* and the other with *F. sylvatica*. This clear separation among European and Asian *Fagus* spp. is also indicated by the A/C transversion founded in the *trn*L intron (site 141). All the European species have an A, and the Asiatic spp. have a C. As also the other genus *Quercus* and genus *Castanea* have a C in the site 141, this indicates that this nucleotide was present in the common ancestor of the genus *Fagaceae*, and that its mutation to A occurred in European *Fagus*

Table 3. Haplotypes found in *Fagus sylvatica* L.

Geographic origin	Country	Variable sites	Haplotype
		11677 00600 78837	
Annunziata	Sicilia, Italy	A-AT-	A1
Gullmarsberg	Sweden	A-AT-	A1
Westfield	Scotland	A-AT-	A1
Lowther E.	England	A-AT-	A1
Alsted	Denmark	A-AT-	A1
Tomaszow Lubelski	Poland	A-AT-	A1
Tatra National Park	Slovakia	A-AT-	A1
Tharandt	Germany	A-AT-	A1
Slovany	Slovakia	A-AT-	A1
Horna Suca	Slovakia	A-AT-	A1
Zamutov	Slovakia	A-AT-	A1
Vihorlat	Slovakia	A-AT-	A1
Zvolen	Slovakia	A-AT-	A1
Polana	Slovakia	A-AT-	A1
Sitno	Slovakia	A-AT-	A1
Lozorno	Slovakia	A-AT-	A1
Beius Bihor	Romania	A-AT-	A1
Des Colletes	France	A-AT-	A1
Limitaciones	Spain	A-AT-	A1
Kladska	Czech Republic	A-AT-	A1
Glarus	Switzerland	A-AT-	A1
Nevrokopi	Greece	A- -T-	A11
Pylion	Greece	- - AT-	A2
Passo San Boldo	Veneto, Italy	- - AT-	A2
Monte Soro Nebrodi	Sicilia, Italy	- - AT-	A2
Medingen	Germany	- - AT-	A21
Prati di Tivo	Abruzzo, Italy	- - A - -	A31
Pian di Novello	Toscano, Italy	AAATA	A3
Bosco della Martese	Abruzzo, Italy	AAAT -	

occurred in European *Fagus* more recently. Therefore, the Asiatic species are more ancient than the European ones. Moreover, *F. orientalis* seems to be originated earlier than the other European groups, as it presents an identical sequence to the Asiatic ones except for the site 114. Successively, for one transversion (site 439), for two transitions (site 593 and site 596), and for two transversion (site 117 and 119), *F. moesiaca*, *F. taurica* and *F. sylvatica* originated from *F. orientalis*, respectively. This fact could corroborate the hypothesis according to which *F. orientalis* is the ancestral species in European beech. This is also supported by the higher genetic variability recorded in *F. orientalis* (9 hapltypes *vs* 6 haplotypes in *F. orientalis* and in *F. sylvatica*, respectively). In fact, the reduction in genetic variability is considered to be a consequence of migration of the original species (NEI & CHAKRABORTY 1975; DOEBLEY 1989). These data are congruent with previous studies performed with ITS gene and cpDNA regions (MANOS & STANFORD 2001) in suggesting an Asiatic origin for the genus *Fagus*. Moreover, GÖMÖRY *et al.* (1999) stated that probably *F. moesiaca* belongs to the rank of subspecies. In our work we have succeeded to distinguish it as a true species phylogenetically linked to *F. orientalis*. This apparent difference between the two work is only to be ascribed to the different sensibility of the molecular method used. The innovative aspect is that we succeed to resolve distinct groups corresponding to taxa such as *F. sylvatica*, *F. moesiaca*, *F. taurica*, and *F. orientalis* that were not resolved using only ITS gene regions by DENK *et al.* (2002).

Table 4. Haplotypes found in *Fagus orientalis* Lipsky

Geographic origin	Country	Variable site	Haplotype
		1112366 0060256 4568898	
Sukhansk	Cherek river, Russia	A-CGT-A	C1
Devrek	Turkey	A-CGT-A	C1
Eregli	Turkey	A-CGT-A	C1
Vanadzor	Armenia	A-CGT-A	C1
Vanadzor 2	Armenia	A-CGT-A	C1
Sochi	West Caucasus, Russia	A-CGT-A	C1
Zorkum	Ammanus, Turkey	A-CGT-A	C1
Neka	Iran	A-CGT-A	C1
Shilda 1	Georgia	A-CGT-A	C1
Bakuriani	Georgia	A-CGT-A	C1
Nichbisi	Georgia	A-CGT-A	C1
Duzce	Turkey	A-CGTCA	C11
Dortyol	Turkey	A-GGT-A	C12
Batumi / Keda	Georgia	A-CGG-A	C13
Akkus	Turkey	AACGT-A	C15
Inegoel	Turkey	AACGT-A	C15
Karabuk	Turkey	AACATCA	C16
Arboretum Vallambrosa	Italy	A-CGT- -	C2
Sovetskoe	Cherk river, Russia	A-CGT- -	C2
Catalca	Turkey	A-CGT- -	C2
Izmit	Turkey	A-CGTC -	C21
Asalem (1900 m)	Iran	- -CGT-A	C3

ACKNOWLEDGEMENT

We express all our gratitude to A. M. Proietti (University of Florence, Italy), the Italian Forest Service, M. Liesebach (Germany), P. Salehi Shanjani (Iran), Z. Vardanyan (Armenia), M. Akhalkatsi (Georgia), A. Prokazin (Russia), M. Pridnya (Russia), I. Shvadchak (Ukraine), P. Zhelev (Bulgaria), D. Gömöry (Slovakia), S. McKeand (USA), J. Beaulieu (Canada), K. Nagasaka (Japan), and Z.-S. Kim (Korea) for assistance during the material collection. Valuable comments on the manuscript provided by anonymous reviewers are appreciated.

REFERENCES

DENK, T., GRIMM, G., STÖGERER, K., LANGER, M. & HEMLEBEN, V. 2002: The evolutionary history of *Fagus* in western Eurasia: Evidence from genes, morphology and the fossil record. *Plant Syst. Evol.* **232:** 213–236.

DOEBLEY, J. 1989: Isozymic evidence and the evolution of crop plants. *In:* Isozymes in plant biology. (eds. D. E. Soltis, P. S. Soltis). pp. 165–186. Discorides Press, Portland, Oregon.

FELSENSTEIN, J. 1993: PHYLIP (Phylogen Inference Package), version 3.5c. Distributed by theauthors, Departement of Genetics, University of Washington, Seattle.

FELSENSTEIN, J. & CHURCHILL, G.A. 1996: A hidden Markoff model approach to variation among sites in rate of evolution. *Mol. Biol. Evol.* **13:** 93–104.

FITCH, W. M. 1971: Toward defining the course of evolution: minimum change for a specified tree topology. *Syst. Zool.* **20:** 406–416.

GÖMÖRY, D., PAULE, L., BRUS, R., ZHELEV, P., TOMOVIĆ, Z., & GRAČAN, J. 1999: Genetic differen-tiation and phylogeny of beech on the Balkan peninsula. *J. Evol. Biol.* **12:** 746–754.

JUKES, T. H. & CANTOR, C. R. 1969: Evolution of protein molecules. *In:* Mammalian Protein Metabolism (H. N. Munro, Ed.), pp. 21–132. Academic Press, New York.

KIMURA, M. 1980: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16:** 11–120.

MANOS, P. S. & STANFORD, A. M. 2001: The historical biogeography of Fagaceae: tracking the tertiary history of temperate and subtropical forests of the northern hemisphere. *Internatl. J. Plant Sci.* **162**(6 Suppl.): S77–S93.

NEI, T. M. & CHAKRABORTY, R. 1975: The bottleneck effect and genetic variability in populations.

Evolution **29:** 1–10.

PAFFETTI, D., VETTORI, C. & GIANNINI, R. 2001: Relict populations of *Quercus calliprinos* Webb on Sardinia island identified by chloroplast DNA sequences. *For. Genet.* **8:** 1–11.

PAULE, L. 1995: Gene conservation in European beech (*Fagus sylvatica* L.). *For. Genet.* **2**(3): 161–170.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. 1989: Molecular Cloning. A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory, New York.

SANGER, F., NICKLEN, S. & COULSON, A. R. 1977: DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463 –5467.

TARBELET, P., GIELLY, L., PAUTOU, G. & BOUVET, J. 1991: Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* **17:** 1105 –1109.

THOMPSON, J. D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D. G. 1997: The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* **25**(24): 4876–4882.