

Intraspecific variation and geographic patterns of *Fagus orientalis* Lipsky chloroplast DNA

By P. SALEHI SHANJANI¹, C. VETTORI², R. GIANNINI² and R. A. KHAVARI-NEJAD³

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Abstract

CpDNA variation in Iranian beech, *Fagus orientalis* Lipsky (Fagaceae) was studied in 14 populations distributed throughout the species range in the Hyrcanian zone. Two cpDNA intergenic regions were analyzed: (i) one in the DT region between *trnD* (tRNA-Asp) and *trnT* (tRNA-Thr) genes, and (ii) one in the OA region between the *orf184* and *petA* genes. The restriction fragments of the region DT did not show polymorphism among individuals within any population analyzed. However, among individuals within analyzed populations of Asalem region and Neka-1400 population, polymorphism in the restriction fragments of the OA region were found. A total of 3 different chloroplast (cp) haplotypes were scored. The distribution of the cpDNA haplotypes revealed a geographical structure of the genetic differentiation with $Gst = 68.7\%$ and $Nst = 70.3\%$. The distribution pattern of *F. orientalis* cpDNA haplotypes may reflect environmental differences and migration history of beech during historical distribution in Tertiary from Asalem (most polymorphic region) to East of Hyrcanian forests.

Key words: cpDNA, *Fagus orientalis* Lipsky, Hyrcanian zone, Iranian beech, intraspecific diversity, genetic patterns.

Introduction

Chloroplast DNA (cpDNA) is highly conserved and has a lower mutation rate than sequences present in the plant nuclear genome (WOLFE *et al.*, 1987). This can be a serious limitation for studies at low taxonomic levels. Despite the conservative nature of cpDNA evolution, restriction site and length variation has been detected within many species. (SOLTIS *et al.*, 1989, 1991, 1992; ARNOLD *et al.*, 1991; BYRNE and MORAN, 1994).

Intraspecific phylogeography, defined by AVISE *et al.* (1987) as the study of the relationship between the phylogeny of variants and their geographic distribution, is more and more becoming of interest in evolutionary science (DUMOLIN-LAPÈGUE *et al.*, 1997). Intraspecific cpDNA variation has also facilitated studies of population-level phenomena, such as gene flow, long-distance seed dispersal, and genetic diversity within and among populations. These processes bear directly on the interpretation of cpDNA evolution with regard to phylogenetic studies (SOLTIS *et al.*, 1992).

Several studies of geographical variation in the Fagaceae family have been done using molecular markers of cpDNA (DUMOLIN-LAPÈGUE *et al.*, 1997, 1998; TARBELET *et al.*, 1998; WHITTEMORE and SCHAAL, 1991). DEMESURE *et al.* (1996) studied the polymorphism in the chloroplast genome of European beech

(*Fagus sylvatica* L.) by relative restriction site studies of PCR-amplified fragments. In a large survey (399 individuals in 85 populations), they found eleven haplotypes, which could be phylogenetically ordered, encompassing most of the natural range of the species. According to their results the northern most populations of Europe are genetically uniform, suggesting a bottleneck at the time of postglacial recolonisation, a scenario which fits with paleobotanical reconstructions. They infer a correlation between geographic distribution of cpDNA haplotypes and their phylogenetic relationships.

Among the broad-leaved temperate forests on Northern Hemisphere, Hyrcanian forest types are very special. These forests with total area of 1.5 million ha are located at southern costs of Caspian Sea and on the northern slopes of Alborz Mountain in the northern part of Iran. The Hyrcanian forest ecosystem is considered to be one of the last remnants of natural deciduous forests in the world. In comparison to European broad-leaved forests from Paleobotanical point of view, which has been influenced by the glacial and human activities, the Hyrcanian forests seem to have remained from the Tertiary and to be relic ecosystem (Sagheb-Talebi, 2000). The watershed of the Alborz on the Caspian coast is characterized by mesophilous forest vegetation, originating from the Tertiary, therefore being very ancient. Beech in this zone survived intense climate and geological changes during the Quaternary because the populations lived in this area were not in contact with glacial periods, but influenced indirectly (MOBAYEN and TREGUBOV, 1969). From the point of view of its floristic composition, the beech belt is linked with European forests. This level particularly has some affinities with the beech forests of the Balkans. The lower levels, on the contrary, are much more specific and include subtropical elements.

Pure and mixed beech (*Fagus orientalis* Lipsky) forests are the most important, making up the richest and the most beautiful forests of Iran containing trees up to 50 m high and 2 m in diameter. From the economical point of view, they are the most important and commercial forests in the Caspian zone. Beech forests cover around 17.6% of Hyrcanian forests surface and form around 25% of forest trees volume in Iran. Therefore, from the economical point of view, beech stands are the most valuable stands and allocate the most rate of timber production in Iran.

It is the objective of our study to better understand genetic diversity and phylogenetical relations of oriental beech populations.

Material and Methods

Population characteristics and sampling

The studied beech forests are located on the northern slopes of Alborz Mountains, within an altitude of about 600–2000 m above sea level. They have formed a forest strip with 600 km length that is located in 3 provinces of Gilan, Mazandaran and Golestan. We sampled a total of 72 trees from 14 autochthonous beech populations representing the entire geographical range of *Fagus orientalis* in Iran (Table 1, Fig. 1).

¹ Dr. PARVIN SALEHI SHANJANI, Member of scientific board at Forest Research Division Research Institute of Forests and Rangelands, P.O. Box 13185-116, Tehran, Iran. Tel.: 0098 21 419 5901, 5902, 5903, 5904, 5905. Fax: 0098 21 419 6575, 6571. E-mail: psalehi@rifr-ac.ir and psalehi2@yahoo.com. http://www.riffr-ac.org/research_divisions/forest/salehi.htm

² Institute of Plant Genetic, CNR, via Madonna del Piano, I-50019 Sesto Fiorentino, Firenze, Italy.

³ Biology Department, Faculty of Science, University of Teachers Education, Tehran, Iran.

Table 1. – Site characteristics of the studied populations of *Fagus orientalis*.

Region	Altitude (m)	Abbreviation	Latitude (N)	Longitude (E)	Exposition	Forest composition	Canopy (%)
Gorgan	2000	G-2000	36° 45"	54° 07"	N, NW	Beech 90%	95
“	1400	G-1400	36° 41"	54° 05"	N, NW	Beech 90%	90
“	600	G-600	36° 42"	54° 06"	N, NW	Beech 90%	90
Neka	1400	N-1400	36° 22"	53° 33"	N, NW	Beech 90%	80
“	900	N-900	36° 29"	53° 27"	N, NW	Beech 90%	90
Sangdeh	1900	S-1900	36° 00"	53° 12"	N, NW	Beech 90%	95
“	1400	S-1400	36° 03"	53° 14"	SW, W	Beech 70%	70
“	900	S-900	36° 06"	53° 16"	N, NW	Beech 90%	65
Kheirud	2000	K-2000	36° 28"	51° 40"	N	Beech 90%	90
“	1200	K-1200	36° 32"	51° 39"	SE	Beech 90%	90
“	600	K-600	36° 35"	51° 33"	SE	Beech 90%	90
Asalem	1900	A-1900	37° 38"	48° 46"	N	Beech 90%	80
“	1200	A-1200	37° 38"	48° 48"	NW	Beech 90%	90
“	600	A-600	37° 41"	48° 48"	N	Beech 90%	70

Laboratory analysis

DNA was isolated from dormant buds (100 mg as starting material) using Nucleospin plant kit (Macherey Nagel, Germany).

The intergenic spacers between the *trnD* (tRNA-Asp)-*trnT* (tRNA-Thr) and *orf184-petA* were amplified via PCR using spe-

cific pairs of chloroplast primers (Table 2). PCR reactions were carried out on a template of genomic DNA (10 ng for each reaction). The reaction mixture was composed by 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ (Gibco BRL, Life Technologies), 0.001% (w/v) gelatin, 250 µM of each deoxynucleoside triphosphate (dNTP), 1 µM of each Primer, 1 U of Ampli Taq DNA polymerase (Perkin Elmer, USA) for a final volume of 20 µl. After incubation for 60 s at 90°C, the reaction mixtures were subjected to different temperature profiles: (1) 95°C for 30 s, 60°C for 30 s, 72°C for 4 min, for 5 cycles; (2) 95°C for 30 s, 55°C for 30 s, 72°C for 4 min, for 5 cycles; and (3) 95°C for 30 s, 50°C for 30 s, 72°C for 4 min, for 25 cycles. Amplification products were then incubated at 72°C for 10 min. (SAMBROOK *et al.*, 1989).

The DT and OA amplification products (15 µl containing, 1 µg of DNA) were treated with 5 U of restriction enzyme *Hae* III and *Hinf* I (Takara, Japan), respectively, in a total volume of 20 µl at 37°C for 4 h (Table 3). The mixtures (20 µl per lane)

Table 2. – Details on primers used for the amplification of DT and OA cpDNA regions.

Gene	Primer pairs and sequence	Code	Reference
<i>TrnD</i>	ACCAATTGAACTACAATCCC	DT	Demesure <i>et al.</i> , 1995
<i>TrnT</i>	CTACCACTGAGTTAAAAGGG		
<i>Orf184</i>	TGGCGATCAGAACATATATGGATAG	OA	Grivet <i>et al.</i> , 2001
<i>petA</i>	CCCTCGAAACAAGAAGTT		



Figure 1. – Location of studied regions.

Table 3. – Restriction endonuclease number individualized in DT and OA sequences in *Nicotiana tabacum* and *Fagus sylvatica* and number of restriction sites found in related intergenic spacer.

Endonuclease	Recognition sequence	Restriction site	
		<i>N. tabacum</i> ²	<i>Fagus sylvatica</i> ³
Hae III	5' GG/CC 3'	3	2
Hinf I	5' G/AN ¹ TC 3'	15	13

¹ N = A, C, T or G

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³ C. Vettori Personal Communication

were analysed by gel electrophoresis on 8% (w/v) sequencing non-denaturing polyacrylamide gels (SAMBROOK *et al.*, 1989). The gels were poured and run in 1 x TBE at a constant current of 166 mA for 9 hours and were stopped 2 h after the xylene cyanol FF (this dye co-migrates with the fragment of 160 bp of double stranded DNA) reached the edge of the gel and completed gushed from the gel (S2001 Life Technologies).

The gels were stained with ethidium bromide and scanned (Photo-Capt, Vilbert Loomat, France). A 100 bp ladder from Genenco (Germany) was used as size marker.

Statistical analysis

Polymorphism was scored as point mutations or insertion/deletions characters and used to define chloroplast haplotypes (according to different combination length variants).

Parameters of diversity (h_s and h_t) and differentiation (G_{st} and N_{st}) in the chloroplast genome were estimated following the methods described by PONS and PETIT (1995, 1996) using the software HAPLODIV. G_{st} is based only on the haplotype frequencies, whereas N_{st} takes into consideration genetic similarities between haplotypes (proportion of shared fragments). These two parameters were compared (PONS and PETIT, 1996). The contribution of a given population to total diversity (C_i) was calculated as reported by PETIT *et al.* (1997).

A ratio of seed flow to pollen flow was calculated using the equation of ENNOS (1994) in which F_{ST} was substituted by G_{ST} :

$$\frac{\text{pollen flow}}{\text{seed flow}} = \frac{\left(\frac{1}{G_{stb}} - 1\right) - 2\left(\frac{1}{G_{stc}} - 1\right)}{\left(\frac{1}{G_{stc}} - 1\right)}$$

Where G_{STb} and G_{STc} indicate the level of population subdivision based on nuclear and cytoplasmic markers respectively.

G_{stc} from this study was used as an estimator of population subdivision in maternally inherited genomes, whilst the value of G_{stb} for allozyme from F_{st} in SALEHI SHANJANI (2002).

Results

No differences in sizes of the DT and OA amplified products were found among Iranian populations with agarose gel electrophoresis. The digestion profiles of the two fragments were compared simultaneously with the digestion fragments of control DNA of known haplotype (kindly provided by R. GIANNINI, IGV Sec. CNR, Florence, Italy) to identify Iranian haplotypes. The restriction fragments of the region DT did not show polymorphism among individuals within any population analysed. However, among individuals within analysed populations of Asalem region and Neka-1400 population, polymorphism in the restriction fragments of the OA region were found. By the simultaneous comparison of the digestion profiles of the DT and OA fragments with the corresponding digestion fragments of Italian control DNA of known sequences and haplotypes, it has been possible to determine the DT and OA variants present

in the Iranian populations. In the DT region, except the Kheirud-600 population, which had the polymorphic fragment, variant 4, the same fragment of variant 1 was found in all the other populations of Hyrcanian forests. The length of polymorphic fragment analyses at OA region indicate that the variant 4 was present in one individual of Neka-1400 population, in two individuals of Asalem-1900 and Asalem-1200 populations, and in three individuals of Asalem-600 population, and the variant 3 in all the individuals of the other populations (Table 4, 5).

On the other hand restriction analysis identified 2 variants for each region, which combine into 3 cpDNA haplotypes. The distribution of the observed haplotypes in each population is reported in Table 5.

Most haplotypes differ from each other for one mutation event. The geographic distribution of the cpDNA haplotype (Hap) is Hap 2 with a frequency of 0.79, and presents in all the natural range of Hyrcanian forests but one (Kheirud-600). One relatively rare haplotype with a frequency of 0.103 (Hap 3) is present at the level of single individual (indiv) in few populations of West Hyrcanian forests (Asalem-600, Asalem-1900 and Neka-1200) or in one population in Neka-1400 the Center of Hyrcanian). Hap 14 was peculiar of Kheirud-600 population (6 indiv). Most of the cpDNA differentiation is distributed among populations ($G_{st} = 68.70\%$).

The total diversity (h_t) was 0.364. The Kheirud-600, Asalem 1900, Asalem 1200 and Asalem 600 populations, having a C_i of 0.290, 0.026, 0.260 and 0.027, respectively, have contributed

Table 4. – Number of PCR-RFLP bands and type of variants detected in DT and OA regions of *F. orientalis*.

Primer code	Total RFLP bands ¹	Total number of variants ²	Mutation type of each variant	
			Code of each variant	variant
DT	3	2	1 & 4	In/del ³
OA	14	2	3 & 4	In/del

¹ Indicates the total number of bands detected by electrophoresis after digestion of each amplified fragment.

² Indicates the number of electrophoretic variants detected for each single polymorphic band.

³ In = Insertion; del = deletion.

Table 5. – Details on sample size, location, and number of individuals per haplotype and haplotype frequencies in *F. orientalis* populations.

Population	Variation		Haplotypes PCR-RFLP			Sample size	Haplotype frequency		
	DT	OA	2*	3**	14***		2*	3**	14***
G-2000	1	3	5	0	0	5	1.0	0.0	0.0
G-1400	1	3	5	0	0	5	1.0	0.0	0.0
G-600	1	3	5	0	0	5	1.0	0.0	0.0
N-1400	1	3, 4	4	1	0	5	0.8	0.2	0.0
N-900	1	3	5	0	0	5	1.0	0.0	0.0
S-1900	1	3	5	0	0	5	1.0	0.0	0.0
S-1400	1	3	5	0	0	5	1.0	0.0	0.0
S-900	1	3	5	0	0	5	1.0	0.0	0.0
K-2000	1	3	5	0	0	5	1.0	0.0	0.0
K-1200	1	3	5	0	0	5	1.0	0.0	0.0
K-600	4	3	0	0	6	6	0.0	0.0	1.0
A-1900	1	3, 4	3	2	0	5	0.6	0.4	0.0
A-1200	1	3, 4	3	2	0	5	0.6	0.4	0.0
A-600	1	3, 4	3	3	0	6	0.5	0.5	0.0
Total			58	8	6	72	0.79	0.102	0.08

*: Most frequent haplotype in North of Europe and North of Italy + 2 populations of Bulgaria

** : Most frequent haplotype in North of Europe and North of Italy + 2 individuals of one population in Bulgaria

***: one population in Iran

much more to the total diversity. In some populations intrapopulation polymorphism is also found (Neka-1400, Asalem-1900, Asalem-1200 and Asalem-600). According to these results one of the populations of Kheirud (as unique haplotype), and the Asalem region (the most polymorphic) are the most particular populations.

The N_{st} (70.3%) is similar to G_{st} (68.7%) indicating that the haplotypes are phylogenetically equivalent [U test (N_{st}/G_{st}) = 1.02], and that only a geographical structure is evident.

Results obtained from the application of ENNOS's formula (pollen flow/seed flow ratio) indicated that interpopulation pollen flow is 44 times greater than interpopulation seed flow.

Discussion

We were able to recognize 3 distinct cpDNA haplotypes in *F. orientalis*. The PCR-RFLP haplotypes distribution showed that: i) the most common haplotype (2) is present in all but one population, ii) 10 of 14 populations are monomorphic (fixed haplotypes 2 and 14). These observations show that the genetic diversity of *F. orientalis* in Iran follows a geographic pattern.

Many studies have reported geographic pattern in cpDNA variation below the species level (MAYER *et al.*, 1994; SEWELL *et al.*, 1996; DUMOLIN-LAPÈGUE *et al.*, 1997, 1998; VAN DIJK and BAKX-SCHATMAN, 1997; WOLF *et al.*, 1997; KING and FERRIS, 1998; TREMBLAY and SCHOEN, 1990; ABBOTT *et al.*, 2000). Furthermore, intra-specific cpDNA variation has been observed among *Fagus* species including *Fagus sylvatica* (DEMESURE *et al.*, 1996), *F. crenata* (FUJII *et al.*, 2002). Results of most of these studies suggest that key historical events (e.g. glacial events) have profound effects on the geographic structure of cpDNA variation.

DEMESURE *et al.* (1996) investigated the cpDNA diversity and geographic structure of the European beech (*Fagus sylvatica*). In Northern Europe, widespread distribution of a single cpDNA haplotype was observed, on the other hand, many haplotypes were recognized in Southern Europe. Although clear-cut variation in cpDNA distribution has been observed, no such clear-cut patterns have been observed by FUJII *et al.* (2002) in Japanese beech. In the present analysis, however, one haplotype (type 2) was widely distributed in the all studied populations except population Kheirud-600. The second frequent cpDNA haplotype (type 3) was found to be basically segregated between the western most and remind parts of Hyrcanian forests. It has been hypothesized that Hyrcanian forests were formed by environmental differences, especially in the amount of precipitation, air humidity, temperature, humus and bed rock kind from East to West (MARVIE-MOHADJER, 1976; PARSAPAJOUH, 1976; HABIBI, 1985). An alternative explanation is that the distribution pattern of *F. orientalis* cpDNA haplotypes may reflect migration history of beech during historical distribution in Tertiary from Asalem (most polymorphic region) to East of Hyrcanian forests.

Differences between Kheirud-600 and other populations of Iranian beech were evident from the cpDNA analysis. This population was also characterized by a high level of genetic variability from allozyme analysis, mainly because of abundant rare alleles (SALEHI-SHANJANI, 2002).

The level of population subdivision was higher for the other European *Fagaceae* investigated so far: $G_{STc} = 0.902$ for *Fagus sylvatica* (DEMESURE *et al.*, 1996), $G_{STc} = 0.829$ for *Quercus petraea*, $G_{STc} = 0.907$ for *Q. pubescens*, and $G_{STc} = 0.782$ for *Q. robur* (DUMOLIN-LAPÈGUE *et al.*, 1997). The only related *Fagaceae* of the southern hemisphere studied so far, *Nothofagus nervosa*, showed a very clear geographical pattern of

cpDNA polymorphism, revealing a geographical divide across its distribution area in Argentina (MARCHELLI *et al.*, 1998).

Iranian beech does not show a geographically structured distribution of the diversity using nuclear isozyme markers (SALEHI SHANJANI, 2002) but the level of cpDNA differentiation among populations in beech is not low ($G_{STc} = 68.7\%$): the most common haplotype (2) is widely dispersed in almost all distribution areas but haplotype 3 is distributed principally in the west of Hyrcanian forests.

Analysis of the cpDNA variation within species has demonstrated that the majority of the variation is located among populations, with very low levels of intrapopulation variation (SOLTIS *et al.*, 1989, 1991; KIM *et al.*, 1992; PETIT *et al.*, 1993a, b). In a comparative study of population subdivision measured for 97 plant species was $G_{st} = 0.70$; in particular the G_{st} value was 0.73 for angiosperm tree species. Species belonging to the family of *Fagaceae* and producing heavy seeds, like *F. sylvatica*, *Quercus robur* and *Q. petraea* are characterized by higher values of G_{st} ranging between 83% and 90% (DEMESURE *et al.*, 1996; DUMOLIN-LAPÈGUE *et al.*, 1997). Even for *Alnus glutinosa*, a species characterized by wind dispersal of tiny winged seeds, a high value of genetic differentiation estimated using chloroplast markers was reported ($G_{st} = 87\%$, KING and FERRIS, 1998).

The pollen/seed flow value for Iranian beech is 44. This value can be explained by wind-pollination in concordance with other species of *Fagaceae* (*Quercus robur* = 286, *Q. petraea* = 500 and *F. sylvatica* = 84, PETIT, 1999) but is in contrast with chestnut (=1, FINESCHI *et al.*, 2000), which maybe explained by the insect contribution to the pollination and the active role of humans in moving and transferring not only fruits but also propagation material. On the other hand, the pollen/seed flow ratio is low when calculated for tree species characterized either by insect pollination or by very efficient seed dispersal mechanisms (FINESCHI *et al.*, 2000).

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