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Original Article

Phylogenetic relationships of the genus Castanea based on chloroplast rbcL with focusing Irania Chestnu

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ABSTRACT
The aim of this study is the evaluation of chloroplast *rbcl* markers in the genetic differentiation and phylogenetic relationships of European chestnut (*Castanea sativa* Mill.), which is one of the valuable species of endangered *Hyrcanian* Forests .In this study, the use of molecular markers is the easy and fast way in identifying taxonomic position of northern Iranian *Castanea*. Meanwhile, leaf samples were collected from all the habitat of this species in Iran (4 sites listed in the western part of the province of Gilan). Genomic DNA from leaves were extracted by CTAB method.(modified by Janfaza and *et al* inter periation 2016) PCR reaction amplified by universal *rbcl* primers and the fragments were sequenced .The results showed that *rbcl* region in Iranian *Castanea* is about 542 nucleotides .The comparison of the available sequences in GenBank, the numbers of nucleotides adenine and thymine in *Castanea* were more than other nucleotides which was similar with Iranian *Castanea* species in nucleotide composition .Phylogenetic Tree analysis showed that from 542 nucleotide positions, there were 22 conserved sites, 17 variable sites, and 8 parsimony positions. Based on the phylogenetic tree drawn on the base of *rbcl* marker showed that Iranian *Castanea* is in the same group with C.*sativa* species .Also the highest genetic similarity was observed between the *Hyrcanian Castanea* and European *Castanea* .

KEYWORDS: *Iranian Castanea, phylogeny, genetic diversity*

INTRODUCTION

One of the most commercially and valuable trees in Hyrcanian forests is C. *sativa* (Sabeti, 2003) that reported first by Jazayeri (1340).There are only remained four habitats of chestnut in the west of Hyrcanian forest that limited to the province Guilan, named Visrud, Shahbalut mahaleh Lahijan, Ghalerodkhan and Shafarood. (Sabeti 2003). The lack of natural regeneration, collecting seed by villagers for consumption, economic and social problems such as grazing are most important factors to widespread destruction of the habitats of these species. In addition, the emergence of chestnut blight disease (*Cryphonectria parasitica*) is the other reason of Chestnut declining (Wall & Aghayeva 2012; Hyun & Choi 2014; Khodaparast *et al* 2009). The first necessary step to prevent endangering of *Castanea sativa* habitats in Iran and getting a good conservation strategy is to understand the species taxonomy, genetic diversity and structure of the chestnut population.

On the other hand, due to morphological similarities and hybridization between species cause to create ambiguity in taxonomy of the genus *Castanea* (Fineschi *et al* 2009; Lang *et al* 2009). Hence, using new molecular approach approaches such as DNA barcoding can be a good method for solving taxonomic problems.

DNA barcoding is relatively a new concept in achieving the rapid and accurate the identification of species by using DNA (Rechinger 1969) and gradually is increasing in phylogenetic relationships (Hebert *et al.*, 2003; Randal *et al.*, 2005). In plants, 7 DNA barcodes were suggested for taxonomy and phylogeny (Kress *et al* 2005; Chase *et al* 2007; Newmaster *et al* 2006; Hollingsworth *et al* 2011; Dong *et al* 2012). Four are portions of coding genes (matK, *rbcL*, rpoB, and rpoC1), and 3 are noncoding spacers (atpF–atpH, trnH–psbA, and psbK–psbI). The barcode region of *rbcL* is easy to amplify, sequence, and align in most land plants and provides a useful backbone to the barcode dataset, despite it having only modest discriminatory power.

Therefore using chloroplast sequencing widely in phylogenetic studies is *rbcl* sequences in plants (Gismondi *et al* 2015; Manos & Stanford 2001).

Lang *et al* (2006) by using sequences of three chloroplast noncoding trnT-L-F regions showed that the genus *Castanea* is supported as a monophyletic clade, while the section Eucastanon is paraphyletic. C. crenata is the most basal clade and sister to the remainder of the genus and the three Chinese species of *Castanea* are supported as a single monophyletic clade, whose sister group contains the North American and European species. The study of Show *et al* (2012) on three morphologically variable species of *Castanea* consists *C. dentata*, *C. pumila*, and *C. ozarkensis* showed distribution reflects the morphological variation observed in North American *Castanea*. They identified four main lineages. Haplotypes of the fourth lineage are shared among accessions of C. *dentata* and *C. pumila*, and three clades closely correspond to the morphology of North American *Castanea*, and the fourth lineage in the past as the hybrid taxon that is intermediate between *C. dentata* and *C. pumila*. Yousefzadeh *et al* (2014) were investigated *Castanea* specimens of uncertain taxonomic affinity by using sequence data from the chloroplast trnL-F and trnH-psbA intergenic spacer regions. They was detected a low level of haplotype diversity within Hyrcanian samples and the whole species of the genus *Castanea*.

low levels of haplotype diversity was found within small remnant stands of *Castanea* in the Hyrcanian forest, indicating that genetic erosion may increase the extinction risk for these valuable trees.

However, similar successful results was not achieved for the creation of specific *Castanea sativa* molecular databases that don't exist, or still in progress, in the scientific world.

Since, few studies have been done on *Castanea* genus on the base of *rbcl* area, this study tries to sequence the Plastid of *rbcl* region and compare with other species of this genus to find the differences and similarities of Iranian *Castanea* with other *Castanea* in the world.

2. MATERIALS AND METHODS

2.1. Sampling and DNA extraction

Leaf sample was collected from 32 trees from four small isolated population of *Castanea sativa* from *Hyrcanian* forest. The names of populations, geographical positions, size of populations and the number of individuals sampled per population are given in Table 1 and Fig 1. In order to avoid investigating clones or close relatives, sampled individuals within a population were separated by at least 100 m. Total DNA was extracted from fresh leaves using CTAB method (modified by Janfaza and *et al* inter periation 2016).

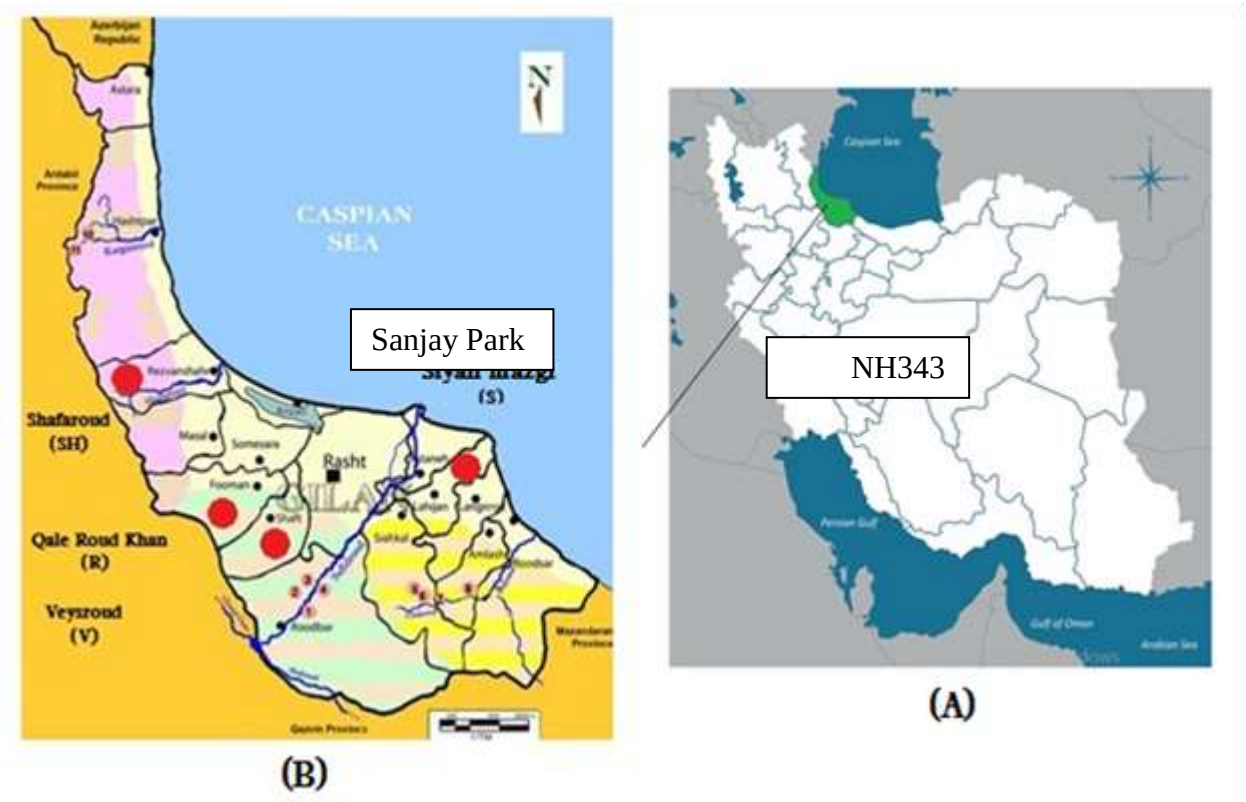

Fig1. (A) map of Iran; Hyrcanian forest are shown with green, (B) Geographical location of studied populations.

Table 1. Populations names, geographical positions and selected tree numbers of *C. sativa* in this study.

Population name	Latitude	Longitude	Altitude	Number of Sample
Veysrud (V)	37˚15′53″	49˚15′03″	211 -711	8
Shafarud(SH)	37˚30′17″	49˚02′24″	200-360	8
Siyah mazdaki(S)	40˚97′14″	35˚49′11″	290-350	8
Qale rudkhan(R)	37˚05′49″	49˚14′43″	200-400	8

2.2. *rbcl* sequences:

Chloroplast DNA was amplified with universal primers *rbcl* regions *rbcl* a-R (5-′ GTAAAATCAAGTCCACCRCG-3′) (*Levin, 2003*) and *rbcl* a-F (5′-TGTCACCACAAACAGAGACTAAAGC-3′) (Kress & Erickson, 2007). PCR amplifications were accomplished in 20 µl reactions with the Accu Power HotStart PCR Premix kit (Bioneer, Korea). The thermal cycling profile consisted of an initial denaturation step of 180 s at 94°C, followed by 36 cycles of 60 s at 94°C, 50 s at 55°C, 90 s at 72°C and a final extension step of 10 min at 72°C. We sequenced PCR products by DNA sequencing 3130-3130 XL.

PCR-based amplification of the purified DNA was carried out in a 50- µL reaction mixture; it contained 50–200 ng template DNA, 2.5 U Jump Start RED Accu Taq LA DNA polymerase (for high fidelity PCR, Sigma-Aldrich), 20 µM of each primers (Sigma-Aldrich), 0.2 mM each dNTP, 1X Taq LA DNA polymerase buffer, 3 mM MgCl2 and 5 % (v.v) DMSO. The reaction was stored at 4 °C. PCR products were fractionated on 1 % (w.v) agarose gel, using 1X TAE buffer (40 mM Tris; 1 mM EDTA; 20 mM acetic acid; pH 8.5) containing 10 mg.mL ethidium bromide, and visualized and photographed under UV light (Gel Doc 2000 BIORAD). All *rbcl* region of *Castanea* species recorded in NCBI were used for drawing phylogenetic

trees. Also, *Fagus orientalis* were selected as out groups in the analysis (Table 2).

Table 2. Geographical characteristics for species and populations of *Castanea* used in study.

Group	Sample	Accession No.	Locality
Iranian	V1, V3, V6		Veysrud (V)
	SH1,SH3,SH7		Shafarud(SH)
	S3,S7		Siyah
	R3, R5, R6		Qale
China	*C. mollissima*	KF418893.1	NCBI
China	*C. henryi*	KJ440003.1	NCBI
American	*C. denta*	KF613012.1	NCBI
European	*C. sativa*	KM360699.1	NCBI
European	*C. sativa*	JN892816.1	NCBI
	Fagus american	L13338.1	NCBI
	fagus orientali	KF418911.1	NCBI

2.3. Phylogenetic analysis:

The electrophenograms of the *rbcl* sequence was further checked by eye using the Chromas software program version 2.33. Mean nucleotide diversity (p) pair-wise genetic distances were calculated following Nei (1987), while phylogenetic inferences were made via maximum likelihood (ML) and the confidence limits of individual clades were estimated as bootstrap supporting values of 1,000 replicates and cutoff value for the consensus tree was 50. Genetic distance among the isolates was evaluated for the selected alignment via the neighbor joining (NJ) algorithm using Kimura-2 parameter distance (Kimura 1980). We used Mega 6 version (Tamura *et al* 2013) for all above parameters.

2.5. Biogeographic analysis and Network analysis

The distribution range of genus *Castanea* was divided into five areas to perform biogeographic analysis. These areas include A (East Asia), B (North America), C (Central Asia), D (Iran) and E (Europe). S-DIVA (Yu *et al* 2010) was applied to get the biogeographic scenario to explain the disjunct distribution pattern in *Castanea*. Phylogenetic network was constructed using the median-joining algorithm with the set of SNPs identified in the *rbcl*. For median-joining (MJ) network analysis, all variable characters of the complete alignment were entered into the program package NETWORK 3.1.1.1 (Bandelt *et al* 1999).

RESULTS AND DISCUSSION

3. Result

3.1. *rbcl* region length and repeats

The total length of the *rbcl* region *Castanea* that their sequences were deriven from gene (NCBI) was 747-542 bp and in Iranian *Castanea* was 542 by omitting 15 bp. The difference in nucleotide diversity in *rbcl* region in Iranian chestnuts was 25.025 while this difference in the total base of studied samples was 0.025 .The nucleotides differentiation of the studied species are shown in Table 3 on the base of regions.

Table 3. Characteristics of the aligned *rbcl* data matrix used for phylogenetic analyses

	A (%)	C (%)	G (%)	T (U)(%)	Total length (bp)	Conserved sites	Variable sites	Parsimony informative sites identical pairs
Iranian populations	27.1	21.6	22.8	28.6	255	0	17	8
All taxa (NCBI)	26.8	21.7	22.8	28.7	270	536	6	7

Results of reading showed that among the 542 positions, only 6 positions were variable and parsimony positions. The first parsimony position was in positions -62,-76,-241 and -538 and belonged to C. *mollissima* species. The second parsimony place was at -124 belonged to C. *henryi* species. The third parsimony variable was at position 258 belonged to species C. *dentata* (Table 4) .

In Iranian *Castanea*, the lowest and highest genetic distances were observed in C. *sativa* and SH1, respectively. The maximum differentiation between the *Castanea* species were between C. *sativa* and C. *mollissima* and the minimum was among C. *sativa* and Iranian taxa. Position of variable and singleton sites in analyzed *Castanea* species .According to the repetitive sequences of *rbcl* region, all the samples studied in this research have TGTGTG and GCGCGC repetitive sequences at positions -177 and -220, respectively (Table 5) .Based on the results of repetitive sequences in this region, *Castanea* were divided in three different groups. Group A included C.*sativa*, C.*henri*, C.*dentata* and Iranian species. Group B, included C. *mollissima* species. The differentiation between groups A and B was repeated sequences of TGTGTG and GCGCGC at positions -177 and -363 in group A and -163 and -349 in group B.

Table 4. Position of variable and singleton sites in analyzed *Castanea* species

	Position																				
Sample	62 C	76 T	124 G	241 G	258 A	376 T	397 T	514 A	515 T	527 T	528 A	529 G	530 G	531 G	532 T	533 A	536 G	537 C	538 A	539 G	540 T
Castanea mollissima	G	A	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-
*Castanea_henryi_*(KJ4	-	-	C	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*Castanea_dentata_*(KF	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*Castanea_sativa_*(KM3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*Castanea_sativa_*(JN89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-		G	G	-	-	-	-
R5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
R6	-	-	-	-	-	-	A	-	-	-	-	-	-	A		-	-	-	-	-	-
SH1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	G
S7	-	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	G	-	-	-	G
V6	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-
V3	-	-	-	-	-	A	-	-	-	A	C	G	-	A	G	-	-	-	-	A	C
S3	-	-	-	-	-	-	-	G	A	A	C	G	T	A	G	-	G	A	-	-	-

Table 5. Sequence repeats found in the *rbcl* region sequence in the genus *Castanea.*

Taxon	*rbcl* Position					
		163	177	349	363	529
	MOTIF	TGTGTG	TGTGTG	GCGCGC	GCGCGC	GAGAGA
	Sample					
IRANIAN	R3		3+		3+	
	R5		3+		3+	
	R6		3+		3+	
	S3		3+		3+	
	S7		3+		3+	
	V1		3+		3+	
	V3		3+		3+	3+
	V6		3+		3+	
	SH1		3+		3+	
	SH3		3+		3+	
	SH7		3+		3+	
NCBI	C. *mollissima*	3+		3+		
	C. *henryi* (KJ440003.1)		3+		3+	
	C. *denta* (KF613012.1)		3+		3+	
	C. *sativa* (KM360699.1)		3+		3+	
	C. *sativa* (JN892816.1)		3+		3+	

3.2. Network and Biogeography analysis

In this study, on the base of *rbcl* region drown according to the phylogenetic tree, 3 haplotypes were identified. Haplotypes diversity was 0.6. Among all 10 studied bases from 4 sites, 4 bases with C. *sativa* formed the first haplotypes. *C. moltissima* Species stayed at the second Haplotypes. The third Haplotypes includes C. *henri and* C.*dentata* .
Phylogenetic tree was drawn on the base of maximum likelihood method and 500 bootstrap replicates. Iranian chestnut is placed in one clade, along with C. *sativa*. In this clade, Iranian samples were divided into two groups. V3, S3 placed in a group and the other species stayed in the second group.

According to the network analysis, C. *dentata* species was the newest species among the studied *Castanea* and C. *Mollissima* species With a mutation at position -124 And C. *henryi* with four mutations at positions -62, -76, -241 and -558. Among the samples, SH3, SH7, and R5, Were quite similar with C. *sativa* and were placed in one central group. V1 and V6 Samples were close to this central group and V3 and SH3 samples were the furthest to this group. According to Network Analysis, we can suppose that S3 and V3 were the oldest species studied in this study, respectively. It should be stated that R3 and R6 with 1 and 4 mutations were Close to the central Iranian group. (Fig 2)

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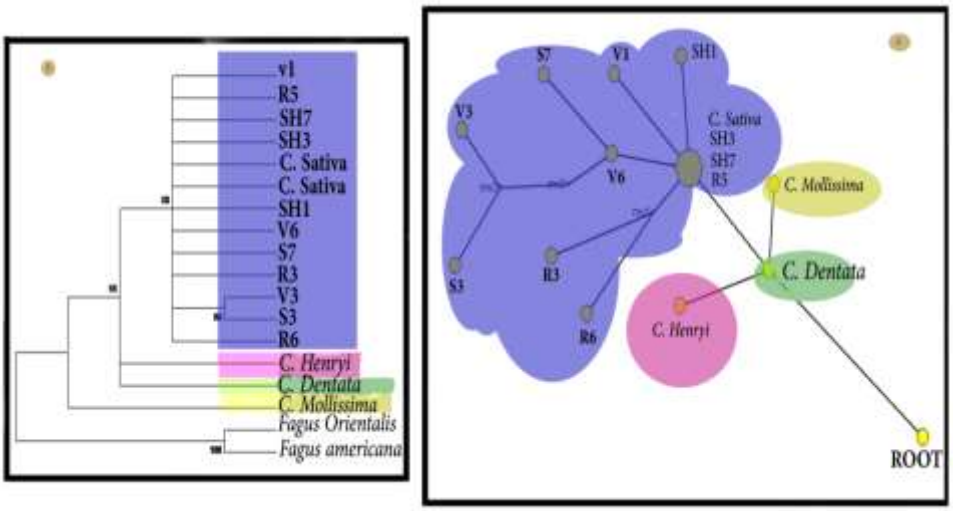

Fig. 2- (a): Taxonomic status of *Iranian Castanea* within the genus *Castanea* by *rbcl* regions, Maximum parsimony consensus original tree (>50%), Bootstrap 500; (b): and Phylogenetic network for the genus *Castanea*.

Table 6. Pairwise genetic distance among analyzed *Castanea* samples

	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Castanea_mollissima*_(KF418893.1)															
2	*Castanea_henryi*_(KJ440003.1)	0.010														
3	*Castanea_dentata*_(KF613012.1)	0.008	0.002													
4	*Castanea_sativa*_(KM360699.1)	0.010	0.004	0.002												
5	*Castanea_sativa*_(JN892816.1)	0.010	0.004	0.002	0.000											
6	SH3	0.010	0.004	0.002	0.000	0.000										
7	SH7	0.010	0.004	0.002	0.000	0.000	0.000									
8	V1	0.010	0.004	0.002	0.000	0.000	0.000	0.000								
9	R3	0.019	0.013	0.011	0.010	0.010	0.010	0.010	0.010							
10	R5	0.010	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.010						
11	R6	0.013	0.008	0.006	0.004	0.004	0.004	0.004	0.004	0.010	0.004					
12	SH1	0.015	0.010	0.008	0.006	0.006	0.006	0.006	0.006	0.013	0.006	0.010				
13	S7	0.015	0.011	0.010	0.008	0.008	0.008	0.008	0.008	0.013	0.008	0.011	0.010			
14	V6	0.011	0.006	0.004	0.002	0.002	0.002	0.002	0.002	0.011	0.002	0.006	0.008	0.006		
15	V3	0.025	0.019	0.017	0.015	0.015	0.015	0.015	0.015	0.019	0.015	0.015	0.015	0.017	0.013	
16	S3	0.029	0.023	0.021	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.025	0.019	0.017	0.015

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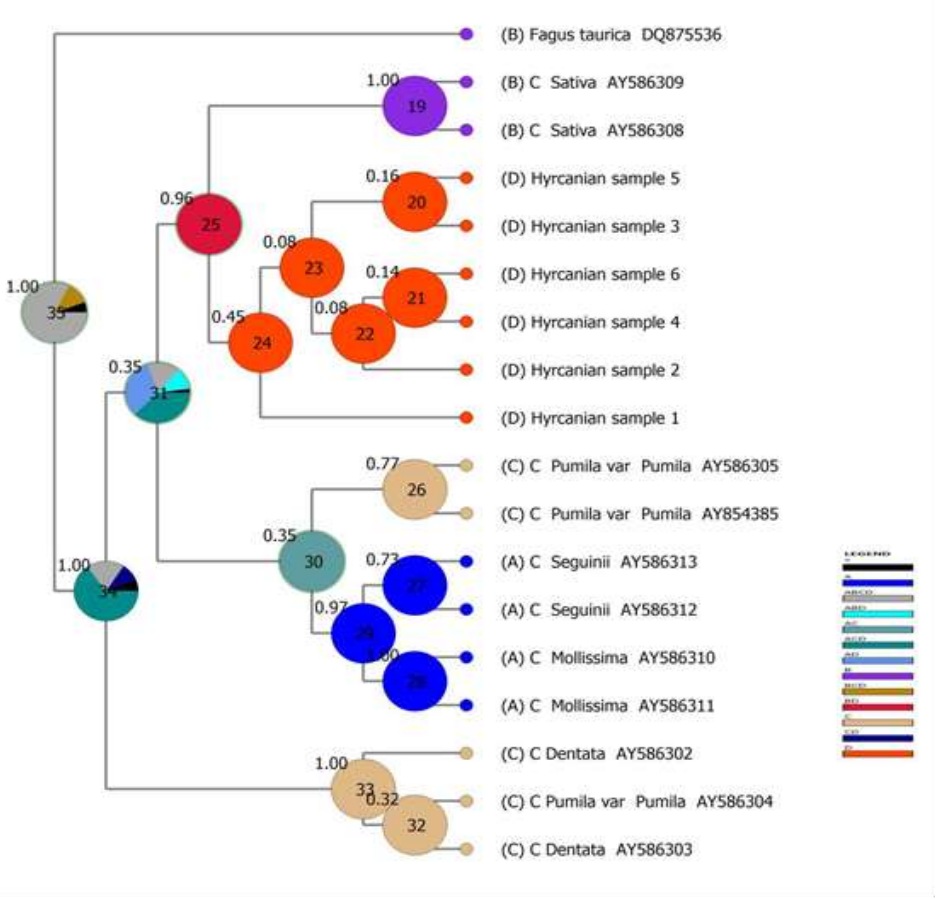

Fig. 3. Ancestral state reconstructions trnL-F region *Castanea* based on statistical Dispersal–Vicariance analysis (S-DIVA) overlaid onto the maximum clade credibility chronogram from BEAST.

Current distributions are indicated before the species names. Pie graphs report relative probabilities from the S-DIVA analysis. Legend- A, East Asia; B, North America; C, Central Asia; D, Iran; and E, Europe.

DISCUSSION

Chestnut genus as an important species is considered by researchers of Asia, US and Europe in the field of economy and plants studies. Obviously, the use of chestnuts in economic activity requires an understanding of existing species of this genus in every natural ecosystem. There are hybrids and high Morphological variation caused differences in diagnosis chestnut species in the world. European chestnut is widespread in all Mediterranean countries. *Castanea* is a genus of deciduous trees found in the temperate forests of eastern North America, southern Europe, and Asia. Hyrcanian forest in North of Iran is the only region in the Middle East that *Castanea* exist there. The Hyrcanian forests in Iran, together with the Colchic forests of Georgia, are the most important relicts of the so called Arcto-Tertiary forests in western Eurasia and an important biodiversity“hotspot” (Scharnweber *et al* 2007). Some researchers believe that the primary sources of South-East Asian chestnuts and chestnut since spread to Europe and America (Lang *et al* 2006؛ Giannini *et al* 2009).

In fact, it uses standard DNA sequence analysis for species identification and classification (Kress *et al*. 2005; Kress and Erickson 2007). Different data demonstrate

how this taxon probably originated in Asia, although contrasting opinions are reported in the literature about the exact place of its inception and the pathway of its geographical diffusion worldwide (Villani *et al* 1994; Manos *et al* 2001; Manos and Stanford 2001; Dane *et al* 2003). Chloroplast genome, with recombination and low substitution and fixed structure in comparision with nuclear genome, have variety of genes with high rate of condensation protection which make easy the possibility of making and designing universal primers, and amplifying by PCR. Also, maternally inherited leads easier analysis. So, cpDNA markers, as a molecular marker, can be confirmed to examine the phylogenetic relationships. Help researchers in identifying species and it is difficult. But due to the increasment in the loss of biodiversity of species, Identification systems based on DNA is have a power to able us in identification of new species (Casiraghi *et al* 2010).

Yousefzadeh *et al* (2014) showed taxonomy of Hyrcanian chestnuts has near to *Castanea sativa* but in *rbcl* there is some differentiation with other. Frascaria *et al* (1993) showed that the rate of evolution of *rbcL* in the family Fagaceae is much slower than that observed for the families of annuals analyzed. The Percentage of (A+T) Nucleotide composition of *rbcl* regions at the studied samples of *Castanea* is more than the percentage of (C+G) Nucleotide composition. This mechanism could further justify the adaptability of *Castanea* species to the adverse climates of past geological eras but it would also explain their hypervariable genetic profiles (Lang *et al* 2006). The results showed that chloroplast region of *rbcl* in Iranian *Castanea* with different population have a nucleotides variety which was accordance with the results of Gismondi *et al* (2015) studies. In this study, the test of phylogenetic showed by omitting 15 bp in *rbcl* area, Iranian chestnut tree of evolutionary relationships, showed that Iranian *Castanea*s (from 4 different sites) were in the same clade with C. *sativa*. And Iranian *Castanea*, in comparison with other species, had the lowest genetic distance with European *Castanea* (Table 6). According to the analysis of S-DIVA, the phylogenic reconstruction of chestnuts starts from the node 33; and we can suppose that all chestnut have a common ancestor from AD area (Asia and Iran). At group 32, variance from AD to D has occured.in groups 22 and 23 of R5 and SH3 species of Iran were in *Castanea sativa* species disperse occurred from BD to D and B (Fiq 3). The our chestnut sequence was very similar to C. *sativa* (KM360699.1)(Pearse *et al* 2015), *C. mollissima* (HQ336406.1)(Jansen *et al* 2011) another member of the family Fagaceae, a DNA homology of 100%, *Quercus robur* (KX163021.1 *Quercus baronii* (KT963087.1) (Jansen *et al* 2011).

In contrary to our result, Gismondi *et al* (2015) Claimed that the nucleotide sequence of C. *sativa* with sequence of *rbcL* gene in possessed 2–3% of variability with respect to those deposited in the scientific database, so they could have been subjected to a past genetic isolation, so they could have been subjected to a past genetic isolation. In conclusion, *rbcl* gene was suggested as the good candidates for intraspecific and population analyses. According to the results obtained from the present work, the genes identified as the best barcoding candidates for C. *sativa* phylogenetic discrimination were *rbcL*, for the plastid genome and ITS for the nuclear one. Finally, we can conclude that against the existence of the morphological and molecular difference between (Akbarinia *et al* 2011) between Iranian and European chestnuts, it is hard to determine the taxonomic position of Iranian chestnut, only, on the base of one marker.

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