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RESEARCH ARTICLE

Assessment of genetic diversity in some of black and brown Iranian truffles by ISSR markers

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ABSTRACT: Tuber species are edible fungi and plant-symbiotic microorganisms that form a beneficial relationship with the roots of certain trees and plants (ectomycorrhizae). After interaction with a plant host, tuber species produced hypogeous fruit bodies of great economic value known as forest truffles. There are different species of truffles, but based on species and place of origin varied their quality and market price. Truffle identification is based on morphological analysis maybe fail to distinguish them due to highly susceptible to environmental conditions. But using molecular markers to identify truffles can be more accurate, less expensive and reliable monitoring. In this context, twelve inter-simple sequence repeats (ISSR) primers were chosen for amplifying the genetic materials of black and brown truffles. In this study, a total of 57 polymorphic bands were amplified (an average of 5.18 bands). The Polymorphism Information Content (PIC) value and gene diversity (H) was with an average 0.37 and 0.50, respectively. During the ISSR screening good amplification products were obtained from primers based on GA, (AG) G, (AG)T, and GAC repeats. The population analysis result revealed that there are three main clusters A, B and C. Four strains Ardabil, Khalkhal, Zanjan and Urmia were identified to be in the group A cluster. The strains of at second and third groups were black and brown truffles respectively. The results indicated that truffles had two separate speciation events (DK = 2). According to DK = 2, the samples of Ardabil, Khalkhal, Zanjan and Urmia grouped in the same group and rest of truffles in other groups.

KEYWORDS: Cluster analysis, Genetic variations, Iranian truffles, *Tuberaceae*

INTRODUCTION

Truffles (*Tuber* spp.) are belonging to the genus *Tuber*, *Tuberaceae* family and class of the Ascomycetes. The geographic distribution of truffle species mainly covers the temperate zones of the northern hemisphere [12]; [15]. They are edible subterranean ascomycete fungus with a complex life cycle. The hyphae of truffles latched in close association with roots of trees to create symbiotic relationships called mycorrhizae. From this step, hyphae aggregate and grow what form a truffle, an aromatic fruiting body [19]. Some species of this genus are also economically important since they produce high-value edible mushrooms, known as truffles, with unique organoleptic qualities and also have distinct aromatic properties [15]; [2]. The truffle-fruiting bodies emit hundreds of volatiles terpenoids [19]; [3] and many bioactive compounds including phenols, nucleotides, polysaccharides, steroids [13]. For example, six volatile aromas compounds have been reported for *T. aestivum* included alcohols, ketones and sulfur containing compounds [10]. Among the tuber species, *T. magnatum* and *T. aestivum* have interesting organoleptic qualities [11]. The evaluation of the intraspecific genetic diversity and population genetic structure of a species is crucial to understand its biology and ascertain its origin, history and

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evolution [2]. Few studies have focused on the genetic variability of truffle populations [9]. Molecular markers are useful in detecting genetic diversity and identify differences between closely related strains in many species. Inter simple sequence repeats (ISSR) molecular markers works based on polymerase chain reaction (PCR) [21]. This marker has advantages such as using small amounts of DNA sample without radioactive labels, powerful, rapid, simple, reproducible and inexpensive [7]. Using ISSR fingerprints, Garcia-Cunchillos and colleagues (2014) confirmed high levels of genetic diversity in Spanish black truffle populations. *Tuber magnatum* have very restricted geographical distribution as this truffle is harvested only in Italy and in some Balkan areas, although it has been reported from some other countries [20]; [16]. Rubini et al. 2004 carried out genetic diversity of *T. magnatum* and identified geographically structured populations in this specie. Moreover, these studies indicated in the last ice age *T. magnatum* experienced a population bottleneck [17]. Weden et al. (2004) utilizing dominant molecular markers such as inter simple sequence repeat (ISSR) markers and sequencing of a few genes suggested the existence of genetic diversity between *T. aestivum* populations. In this study we attempted to identify some of truffles from different geographical regions of Iran, using molecular assays.

MATERIALS AND METHODS

plant material

Six strains of black and brown truffles (Fig. 1) belong to four regions of Iran including Zanjan, Urmia, Ardabil and Golestan were collected in 2019 (Table 1).

Table 1. The geographical location of sampling site

Regions	No of Ascocarp	Latitude Longitude	Elevation (m)	Mean Temperature (C°)	Annual rainfall (mm)
Ardabil	10	38° 51'N 47° 73'E	1750	12	400
Khalkhal	11	37° 35'N 48° 63'E	1830	10	320
Zanjan	8	36° 17'N 48° 82'E	1650	13	350
Urmia	10	38° 06'N 45° 15'E	1230	13	560
Golestan-brown	12	37° 18'N 55° 36'E	700	18	700
Golestan-black	10	38° 57'N 48° 10'E	710	18	700

Table 2. The ISSR primers used for PCR amplification in truffles

ISSR Markers	Repeat motif	Ta (C°)	Reference
UBC807	(AG)8T	50	[1]
UBC881	(GGGT)4	58	[2]
UBC846	(CA)8RY	50	[13]
UBC873	(GACA)4	48	[1]
ISSR 11	(GAC)5	51	[1]
UBC841	(GA)8YC	44	[1]
ISSR 23	(GA)8C	54	[3]
UBC826	(AC)8C	50	[1]
UBC809	(AG)8G	48	[1]
UBC825	(AC)8 T	51	[1]
UBC810	(GA)8 T	50	[1]

R = (A, G); Y = (C, T);

DNA extraction

Genomic DNA was extracted from 100 mg of hymenium from -80 C° stored material of black and brown truffles by following method [21]. The hymenium was ground under liquid nitrogen into a sterile prechilled mortar and pestle. After the addition of 1 ml of extraction buffer [100 mM Tris-HCl, 20 mM EDTA, 2 M NaCl, 2% (w/v) PVP, pH 8], transferred the homogenate mixture to a 65°C water bath for 20 minutes. After incubation, the tubes were cooled on ice for 15 min and then, spin the CTAB/plant extract mixture at 10,000 g for 10 min at 4°C, the supernatant was separated and transferred to a clean tube. The Precipitated DNA was mixed with 350 µl (0.7 (v/v)) volumes of cold isopropanol (-20°C) and then centrifuged at 9,000 g for 11 min. The supernatant was separated. The precipitated DNA was washed with an equal volume of 70% (v/v) ethanol. Then pellets were dried and dissolved in 50 µl of Double-distilled water. DNA quality was assessed by Nanodrop and 0.8 % Agarose gel electrophoresis. Before using DNA samples 2 µl RNase was added at 37 °C.

Molecular Fingerprinting

Twelve inter-simple sequence repeats (ISSR) primers were chosen for amplifying the genetic materials of black and brown truffles (Table 2). The choice was based on the degree of polymorphism, as well as reproducibility of amplified DNA fragments. DNA amplification was carried out in 10 µl reactions. The 10 µl volume PCR reactions contained 50 ng of genomic DNA, 5 µl of a PCR

Figure 1. Some of black and brown truffles from different regions of Iran; including Khalkhal, Urmia, Black Golestan, Ardabil, Zanjan, and Brown Golestan. The circle above of each picture showed texture of truffles by 10X light microscopy.

kit (Sigma, St. Louis, MO, USA), 1.1 µl of primer, and 2.5 µl of double-distilled water. The amplification was performed by using Q-cycler thermocycler (HainLifescience, UK). The Thermocycler Set-up consisted of an initial 5 min denaturation step at 94 ˚C, followed by 35 cycles, each of which had a denaturing step at 94 ˚C for 45 s, primer annealing at 52 ˚C for 30 s (depending on the primers) and elongation step at 72 ˚C for 1 min. The final extension was at 72 ˚C for 10 min. The enhancement of amplified products was separated on agarose gel 1.3%, stained with fluorescent dye in 1x TBE buffer. DNA fingerprints were visualized using a digital camera under UV. DNA ladder of 100bp (New England Bio Labs, USA) has been used for determining the molecular weight.

Data analysis

The observed bands in the gel was scored as the present (1) and absent (0) for each entry. The non-reproducible bands were taken out from scoring. The genetic parameters such as; Number of scored band (NSB), Number of polymorphic (NPB), observed number of alleles (Na), effective number of alleles (Ne), gene diversity (H), and Shannon's Information index (I) (Lewontin 1972) was calculated by PopGene program version 1.31. Cluster analysis was performed by WARD (minimum spherical cluster) dissimilarity index using Windows (DARwin5) software. For the population structure, a model analysis was fulfilled to infer the genetic structure and to clarify the number of sub-populations using the software STRUCTURE (2.3.4) (Pritchard et al. 2000). The algorithm structure was run using with K value from 1 to 10. Each run included a burning period of 50,000 iterations. The number of supposed populations (K) was set from one to ten. The run with the maximum likelihood was employed to set genotypes into subpopulations.

RESULTS AND DISCUSSION

The only bright amplified products were scored as present (1) or absent (0). In this study, a total of 57 scorable and polymorphic bands (NPB: Number of polymorphic bands) were generated, an average of 5.18 bands per primer. The number of DNA amplified fragment revealed by the ISSR varied from ranging from 6 to 23 bands per primer (Table 3). The maximum and lowest numbers of amplified bands were for UBC807 with 8 bands and UBC810 with 2 bands, respectively (Fig. 2). The sizes of DNA bands shown in amplification products ranged from 100-800bp. According to results, there is a high level of genetic diversity with high polymorphism ratio among the black and brown truffles belong to six regions of Iran from Zanjan, Urmia, Khalkhal, Golestan and Ardabil. The PIC value varied from 0.53 (UBC809) to 0.20 (UBC810), with

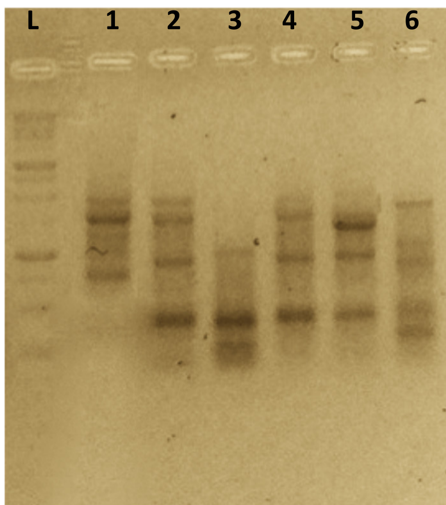


Figure 2. Patterns bands of truffles amplified by UBC807, L: 100 bp DNA size marker.

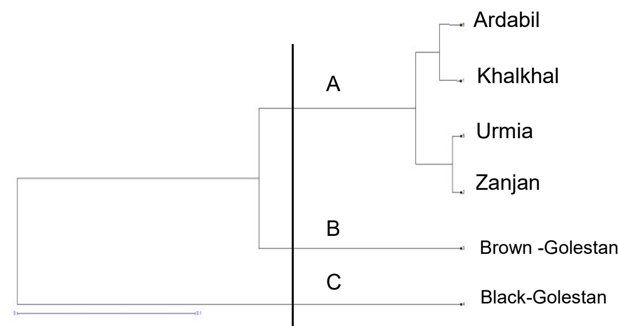


Figure 3. Dendrogram of truffles based on Dice's similarity coefficient and WARD method.

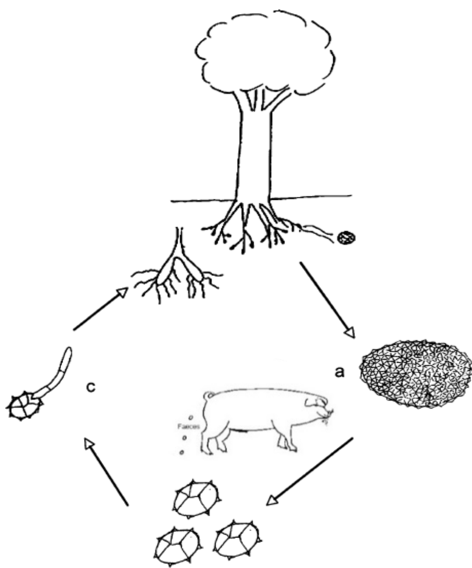


Figure 4. Tuber life cycle: a) underground ascocarp (fruiting body); b) production of ascospores and spread by animals; c) ascospore and infection of roots (mycorrhizal symbiosis).

an average of 0.37 (Table 3). This value is a reflection of allele diversity and frequency among the varieties. The high values of PIC parameter exhibited a high polymorphism and variety; therefore, high PIC markers are useful to separate genotypes with close relationship [8].

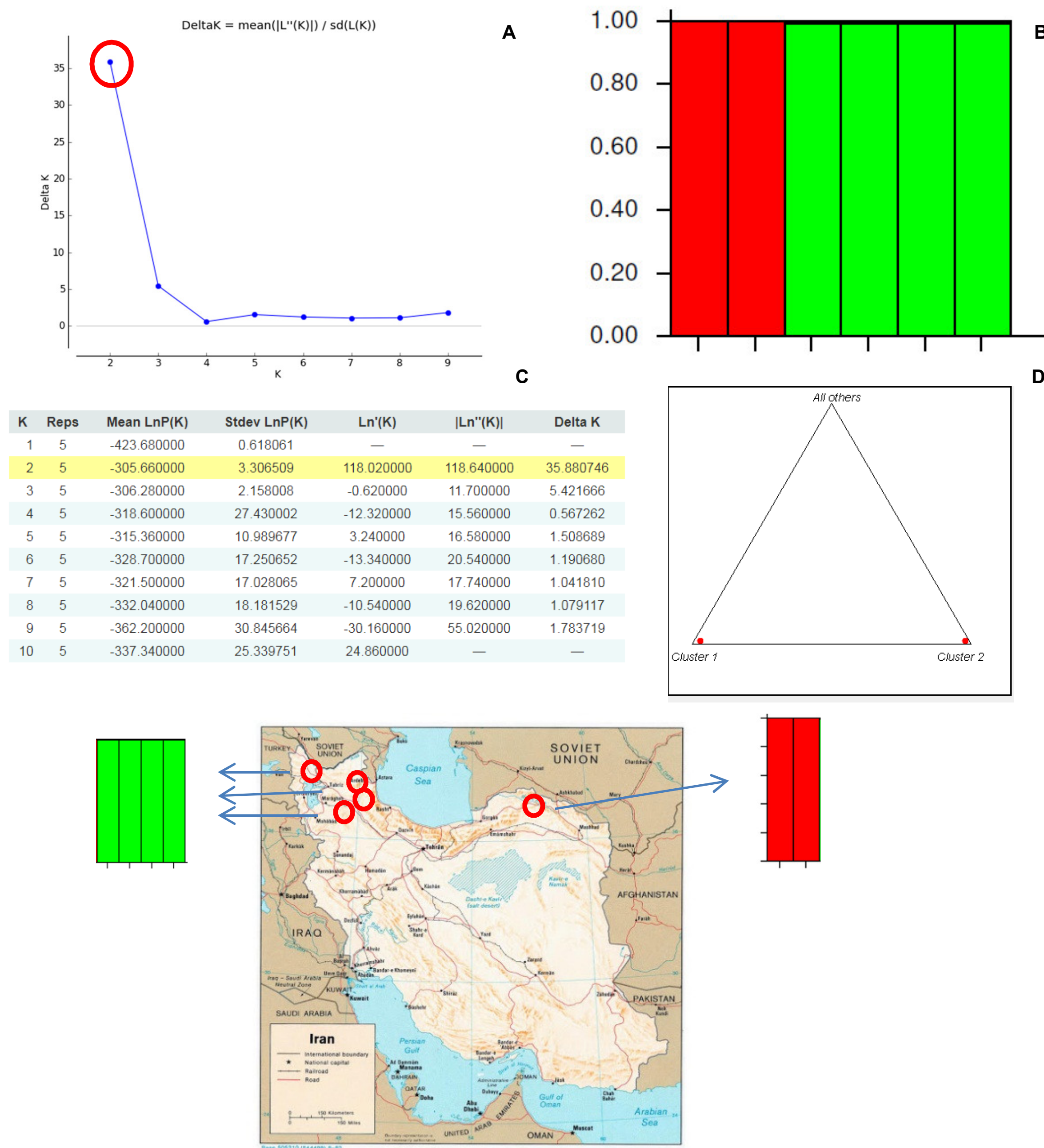
The mean value of observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H) and Shannon index (I) were 10, 8.02, 0.33 and 0.50 respectively (Table 2). The PIC value will be zero if there isn't any allelic variation (monomorphic) and it can reach to maximum (or 1.0) if an individual has only new allele [11]. Good amplification products were obtained from primers based on GA, (AG) G, (AG)T, and GAC repeats. One of most abundant classes of microsatellites are GT and CT motifs in higher organisms [3]. Recently work on black truffle genome revealed a significant wealth in repeated motifs such as microsatellites [12]

Truffle identification based on morphological analysis, may fail to distinguish their ectomycorrhizae, as some of them are very similar morphologically and highly susceptible to environmental conditions for example, *T. indicum* species (a Asiatic truffle) has low-priced ascocarp, which are morphologically very similar to high-priced ascocarp of *T. melanosporum*, using species-specific ITS primers, the main species can be identified [14] . Kumar & Agrawal (2019) cited that ISSR markers was more efficient than SCoT primers in genetic diversity, as well as produced more number of polymorphic bands and percentage polymorphism.

Cluster analysis

A WARD algorithms produced by Dice coefficients revealed the relationships among six strains of black and brown truffles are shown in Fig. 3. The phylogenetic analysis result revealed that there are three main clusters A, B and C. According to results, four strains Ardabil, Khalkhal, Zanjan and Urmia were identified to be in group A cluster. The strains of at second and third groups were black and brown truffles respectively.

According to the results of cluster analysis, Truffles samples from close geographical areas had a slight genetic distance from each other. Since truffles grow underground, they spread spores in the way different from other mushrooms. Truffles attract 'fungivores' by their unique aroma, to that enjoy snacking on them [8], include mammals like mice, squirrels and rabbit, pigs and bears. The most of the flesh is digested, when consumed; the



K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-423.680000	0.618061	—	—	—
2	5	-305.660000	3.306509	118.020000	118.640000	35.880746
3	5	-306.280000	2.158008	-0.620000	11.700000	5.421666
4	5	-318.600000	27.430002	-12.320000	15.560000	0.567262
5	5	-315.360000	10.989677	3.240000	16.580000	1.508689
6	5	-328.700000	17.250652	-13.340000	20.540000	1.190680
7	5	-321.500000	17.028065	7.200000	17.740000	1.041810
8	5	-332.040000	18.181529	-10.540000	19.620000	1.079117
9	5	-362.200000	30.845664	-30.160000	55.020000	1.783719
10	5	-337.340000	25.339751	24.860000	—	—

Figure 5. Population structure of Truffles accessions using STRUCTURE software version 2.3.4 and Structure Harvester v6.0 program, (A) DK values for different numbers of populations assumed (K) in the STRUCURE analysis, (C) Evanno table output, (B) bar plot representation for K=2, each vertical bar represents one individual strain, (D) triangle plot representation for K=2.

spores pass without scathed by animal's body. The animal's feces, causes spores get back into the soil, which is usually deposited in a near-by area. The gastrointestinal tract disperses feces, to germinate and form new fungal colonies (Fig. 4) [16]. Therefore, truffle spores pass through the, often near distances away and close geographical areas are similar to each other. Because of sexual reproduction system and self-fertilizes, there is little genetic diversity between the sampled populations [12]. The genetic diversity observed in this study refers to haploid maternal of truffles. Rubini et al. (2004) cited that, because the ascocarp of truffle is related to the maternal tissue so, would be haploid. For the reason that, parent DNA would be present in ascocarps only and they are not easily broken during the typical DNA extraction process. The parent DNA might not be as easily recoverable. Thus, haploid maternal tissue will be the conquering component of the truffle and presumably represents.

STRUCTURE analysis

Structural genetic analysis was carried out on the six samples of truffles with the STRUCTURE and Structure Harvester programs on the similar data set to better discovery substructures of population. This clustering method is based on an algorithm that confers genotypes to analogous groups, given a number of clusters (K). The most plausible number (K) of subpopulations was identified following Evanno et al. (2005). The results indicated that samples of truffles had two separate speciation events (The highest Delta K value occurred at DK = 2) (Fig. 5). According to DK = 2, the samples of Ardabil, Khalkhal, Zanjan and Urmia grouped in the same group and brown and black truffles of Golestan were in second group. According to structure analysis, the samples of Golestan had different genetic structures rest of genotypes (Fig. 4). One of the important factors for this reason is difference geographical distance of these regions. The distance from Golestan (located in the east) and rest of samples (located in the west) on average, it was about 1000 km. As maintained by Nowak (2015) truffles grow completely underground. Whereas mushrooms routinely spread their spores by wind, truffles require the help of animals to disperse their spores. Most truffles are consumed by forest animals as food. When mature, truffles produce these odor signals, the aromas released only when at least some spores are mature. Duo to method spread of truffles, it is clear that far distance areas will have different genetic pool because the spores are not transmitted by animals at far distances.

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Conflict of interest

The authors declare that they have no conflict of interest.

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بررسی تنوع ژنتیکی در برخی ترافل‌های قهوه‌ای و سیاه ایران با نشانگر ISSR

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چکیده

گونه‌های Tuber، شامل قارچ‌های خوراکی و ارگانیسم‌های همزیست گیاهی هستند که دارای ارتباط سودمند با ریشه درختان و گیاهان خاصی (اکتومایکوریزا) می‌باشند. این قارچ‌ها بعد از واکنش با گیاه میزبان، اندام‌های زیرزمینی تولید می‌کنند که دارای ارزش اقتصادی بالا بوده و به عنوان ترافل شناخته می‌شود. گونه‌های متفاوتی از ترافل‌ها وجود دارند که براساس گونه و منشائ، کیفیت و ارزش تجاری آنها متفاوت است. شناسایی ترافل‌ها براساس آنالیز مورفولوژیکی ممکن است به دلیل تحت تاثیر محیط قرار گرفتن دقیق نباشد. اما استفاده از نشانگرهای مولکولی می‌تواند دقیق‌تر، ارزان‌تر و بررسی آن نیز آسان‌تر باشد. در این پژوهش، بیست نشانگر ISSR برای بررسی ژنتیکی ترافل‌های سیاه و قهوه‌ای مورد استفاده قرار گرفت. در این مطالعه، در مجموع ۵۷ باند چندشکل تکثیر شدند (متوسط ۵/۱۸ باند). مقدار PIC و تنوع ژنتیکی (H) بطور متوسط به ترتیب ۰/۳۷ و ۰/۵۰بود. در طی آنالیز باندها، مشخص شد که نشانگرهایی با توالی GA، (AG)G، (AG)T و GAC باندهای بهتری تولید کردند. نتایج آنالیز جمعیت نشان داد سه کلاستر اصلی A، B و C وجود داشت. چهار سویه اردبیل، خلخال، زنجان و ارومیه در گروه اول و سویه‌های قهوه‌ای و سیاه گلستان نیز در گروه‌های دوم و سوم قرار گرفتند. نتایج آنالیز جمعیت بیانگر آن بود که ترافل‌های مورد بررسی دو خزانه ژنتیکی مجزا داشتند. براساس DK = 2، نمونه‌های اردبیل، خلخال، زنجان و اورمیه در گروه یکسان قرار گرفتند و بقیه ترافل‌ها در گروه دیگر بودند.

کلمات کلیدی: تنوع ژنتیکی، دنبالان قهوه‌ای، دنبالان سیاه، مارکر ISSR