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Assessment of Infraspecific Genetic Diversity in *Nepeta kotschyi* Boiss., a Native Iranian Medicinal Plant

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ABSTRACT

The genus *Nepeta* is one of the largest genera in the *Lamiaceae* family, and Iran is one of the main centers of origin of this genus. The genetic diversity estimation of the genus *Nepeta* germplasm provides a basis for future biodiversity conservation efforts and also for the selection of high-productive genotype(s) for the field production improvement. In this work, eleven RAPD primers with suitable banding pattern and prominent polymorphism were used for the estimation of infraspecific genetic diversity of *N. kotschyi* based on 21 populations. The study included 19 and 2 populations belonging to var. *persica* and var. *kotschyi*, respectively. Eleven primers amplified totally 225 scorable RAPD loci, 204 of which were polymorphic. The average number of bands per primer was 20.5, 18.5 of which were polymorphic. Primers *OPF 05, OPB 15, OPT 14, OPO 07,* and *OPF 14* were the most powerful for the detection of the genetic diversity across the samples. Cluster analysis showed six genotypic groups. *N. kotschyi* var. *kotschyi* populations were placed in a group separated from the samples belonging to *N. kotschyi* var. *persica*. The same analysis showed that the genetic diversity pattern corresponds to the geographical distribution of the populations. Of the total variation in the studied germplasm, 32% was related to inter-populations and 68% to intra-populations variation. The results indicated the high potential of RAPD markers to resolve the status of the studied genotypes in regard to inter- and intra-populations diversity and to diversify *N. kotschyi* varieties previously resolved by morphometric methods.

Keywords: Infraspecific variation, *Lamiaceae*, *Nepeta kotschyi* var. *kotschyi*, *Nepeta kotschyi* var. *persica*, RAPD.

INTRODUCTION

Iran is one of the main centers of diversity of the genus *Nepeta* L. (Pojarkova, 1954) with 79 species (Jamzad, 2012). *N. kotschyi* Boiss. is a perennial medicinal plant from *Lamiaceae* family distributed in Iran and Afghanistan. *N. kotschyi* has a wide dispersal in central, western, southern, and south-western parts of Iran. Its habitat is in mountainous regions, on rocks and/or on rocky slopes, mainly inhabiting the oak forests (Jamzad, 2012). This species has a diploid genome (2n= 2x= 34) (Kharazian *et al*., 2013).

The majority of *Nepeta* species are a rich source of nepetalactones and phenolics, which are reported to have a wide range of bioactive and medicinal properties (Jamzad, 2003b; Rabbani *et al*., 2008; Saeidnia *et al*.,

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2008; Bernardi *et al*., 2010; Nestorović *et al*., 2010; Shafaghat and Oji, 2010; Mahboubi *et al*., 2011; Zomorodian *et al*., 2013; Joshi and Sah, 2014; Mišić *et al*., 2015; Hadi *et al*., 2017). Some *Nepeta* species have previously been introduced into cultivation due to their horticultural and medicinal values (Stappen *et al*., 2015). In recent researches, some wild populations of Iranian *Nepeta* species, *N. kotschyi*, *N. menthoides*, *N. crassifolia* and *N. cataria*, were introduced into cultivation under experimental field conditions, and screened for phenolics and essential oil content in order to select the most convenient genotypes for domestication and cultivation programs (Hadi *et al*., 2016, 2017). Based on these results, *N. kotschyi* was highlighted as the most promising candidate species regarding phenolics and essential oil quality and quantity and the most suitable for domestication and cultivation practices compared to other studied species.

Introduction of wild medicinal plants into cultivation represents a great challenge, where selection is the most important and, of course, the most cost-effective step in the domestication of medicinal plants. Moreover, selection among wild populations or landraces is the most commonly used method in herbaceous plants breeding, including medicinal plants, because most of these species have not yet been domesticated and have significant genetic variation (Pank, 2006). Today, in the cultivation and medicinal plants industry, the provision of plant material with a high level of safety in quality and quantity of secondary metabolites, and sustainability and efficiency in production is very much taken into consideration. Therefore, in the event of exploitation and the introduction of a medicinal product into the cultivation and industry, the study of genetic diversity will be very important (Máthé, 1986; Tétényi, 2002). In the last few decades, the study of genetic variation of medicinal plant species has been mainly done using morphological, phytochemical, and recently molecular markers, but combination of these markers yields the most useful results (Bernáth, 2002; Tétényi, 2002). The preference of a molecular marker system should be conciliated between consistency and simplicity of analysis, and assurance of exposing polymorphisms (Gantait *et al*., 2014). The higher heritability and polymorphism of the marker, the more valuable it is in the germplasm survey (Bagheri *et al*., 2002).

In the genus *Nepeta*, information on genome sequences is more closely related to the ITS sequences of nuclear ribosomes and some of the chloroplast markers. Various molecular markers were used in the research of species and populations of this genus: RAPD (Saeidnia *et al*., 2009; Elkholy *et al*., 2011; Baghizadeh *et al*., 2018), ITS (Jamzad *et al*., 2003a; Tjirkalli *et al*., 2006a), ISSR (Smolik *et al*., 2008), nucleotide sequences of the chloroplast *rbc*L and *mat*K genes (Tjirkalli *et al*., 2006a; Al-Qurainy *et al*., 2014) and primers from clones of $(AG)_{12}$ library (Tjirkalli *et al*., 2006b).

RAPD (Random Amplified Polymorphic DNA) is a multilocus arbitrary fingerprinting technique and one of the most efficient molecular methods in terms of its ability to produce abundant polymorphic markers within a short span of time and limited budget. Since its introduction about three decades ago (Williams *et al*., 1990), RAPD has proved to be a valuable tool in various areas of plant research (Caetano-Anolles *et al*., 1991; Aagaard *et al*., 1998; Ramshini *et al*., 2005; Sadder and Ateyyeh, 2006). Among DNA markers, RAPD is generally considered a fast, informative and inexpensive which, despite dominance and low reproducibility, allows analysis of the polymorphism in many individuals with good coverage of the entire genome and only by the use of small amounts of plant material (He *et al*., 1995). RAPD markers can be used to detect DNA polymorphism without previous knowledge of the target genome (Williams *et al*., 1990). The main shortcoming of this approach is that the profiling is contingent on the reaction circumstances, which may fluctuate among

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laboratories. Additionally, since a number of distinct loci in the genome are amplified by each primer, profiles are incompetent to differentiate heterozygous from homozygous individuals (Bardakci, 2001). Careful optimization of each step of the amplification reaction is needed to achieve the satisfactory reproducibility of the RAPD data (Skorić *et al*., 2012). RAPD has been reported as a useful technique for various molecular studies on medicinal plants (Hadian *et al*., 2008; Sonboli *et al*., 2011; Agostini *et al*., 2012; Zhang *et al*., 2012; Rustaiee *et al*., 2013; Khadivi-Khub and Soorni, 2014; Chowdhury *et al*., 2017; Ahmad *et al*., 2018; Gabriel and Chukwudi, 2018; Marzouk and El-Badan, 2018; Panapitiya and Welikala, 2018; Shidfar *et al*., 2018; Ahmed and Al-Sodany, 2019; Jinu *et al*., 2019; Rohela *et al*., 2019; Yadav *et al*., 2019).

The aim of this research was to estimate the extent and patterns of genetic diversity both among and within 21 wild populations of *N. kotschyi* previously studied by Hadi *et al.* (2016, 2017). The present study also represents the initial evaluation of the potential usefulness of RAPD markers as an inexpensive, quick, and efficient tool for preliminary diversity screening, and possible application of MAS (Marker Assisted Selection) in breeding programs of this species.

MATERIALS AND METHODS

Plant Material

Seeds of 21 populations of *N. kotschyi* (Table 1) were provided from the Gene Bank of Natural Resources, Research Institute of Forests and Rangelands (RIFR), Tehran, Iran. Botanical certification of the plant materials was performed by National Herbarium (TARI) of RIFR. Seedlings obtained from seeds were planted in experimental fields of the Department of Horticultural Sciences, Faculty of Agriculture, Tarbiat Modares University, Tehran.

Well-developed leaves from all individuals cultivated in the field were placed in paper envelopes and dried in plastic bags with silica gel. The samples were stored at ambient temperature in dry and dark condition until the time of molecular analyses.

DNA Isolation

The DNA was extracted using a modified CTAB procedure (Doyle and Doyle, 1990) from the dried leaf samples. From each population up to 8 individuals were tested. Dried leaf samples (0.05 g) were pulverized to fine powder using liquid nitrogen and chilled mortar and pestle, and transferred to 1.5 mL cold tubes. The powdered samples were then mixed with 600 µL of CTAB extraction buffer [2% (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 1.4M NaCl, 20 mM NaEDTA (pH 8.0)] containing 2% Polyvinylpyrrolidone (insoluble PVP, Sigma-Aldrich, Steinheim, Germany) and 1% *β*-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany). After vortex-mixing for 3-4 seconds, the samples were incubated in water bath at 56°C for 20 minutes, during which the tubes were gently inverted three times. After incubation, the tubes were kept at ambient temperature for about 20 minutes, then, 600 µL of a chloroform:isoamyl alcohol mixture (24:1, v/v) was added. The tubes were inverted about 30 times horizontally and then centrifuged at 23°C for 10 minutes at 12,000×*g*. The upper phase was collected in fresh tubes. The described step (extraction by chloroform:isoamyl alcohol) was performed twice.

For DNA precipitation, the recovered supernatant was mixed with its half volume (250 µL) of 4M NaCl solution (4°C), and then inverted 15 times horizontally. Next, an equal volume (750 µL) of cold isopropanol (-20°C) was added. Each tube was gently inverted several times, then, the tubes were placed at -20°C for 30 minutes. The tubes

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Table 1. *Nepeta kotschyi* populations obtained from the Gene Bank of Natural Resources, Research Institute of Forests and Rangelands, Tehran, Iran. The origin of plant material and their data are presented. Each population is assigned with corresponding population code.

Species	Population number	Origin of seeds (County, Province, Country)	Longitude (°-'-")	Latitude (°-'-")	Altitude (m)	Year of collection	Population code	No. of individuals tested in RAPD analysis
N. kotschyi var. *persica*	15908	Ardakan, Yazd, Iran	54-39-08	32-24-53	2141	2004	Ardakan1	8
	15857	Ardakan, Yazd, Iran	54-34-42	32-22-49	2187	2004	Ardakan2	7
	13040	Ardakan, Yazd, Iran	54-38-03	32-27-95	2500	2003	Ardakan3	6
	15852	Ardakan, Yazd, Iran	54-31-00	32-19-21	2187	2004	Ardakan4	3
	15853	Taft, Yazd, Iran	53-41-01	31-44-50	2465	2004	Taft1	7
	13061	Taft, Yazd, Iran	54-08-19	31-35-05	3040	2003	Taft2	7
	15827	Taft, Yazd, Iran	53-44-47	31-43-49	2634	2004	Taft3	8
	10350	Taft, Yazd, Iran	53-41-01	31-44-50	2465	2002	Taft4	8
	21048	Taft, Yazd, Iran	53-45-75	31-48-54	2596	2005	Taft5	8
	15828	Taft, Yazd, Iran	54-05-29	31-37-35	3030	2004	Taft6	8
	15850	Mehriz, Yazd, Iran	54-18-00	31-29-00	2387	2004	Mehriz	8
	21027	Saduq, Yazd, Iran	53-47-10	31-54-41	1858	2005	Saduq	7
	21035	Bafq, Yazd, Iran	-	-	-	2005	Bafq	6
	21039	Behabad, Yazd, Iran	56-01-01	31-43-98	2635	2005	Behabad	8
	18697	Yazd, Yazd, Iran	-	-	-	2004	Yazd1	7
	18695	Yazd, Yazd, Iran	-	-	-	2004	Yazd2	8
	28272	Semirom, Isfahan, Iran	51-53-15	31-11-31	2350	2006	Semirom	3
	25740	Khorasan, Khorasan, Iran	-	-	-	2007	Khorasan	5
	29556	Chelgard, Chaharmahal and Bakhtiari, Iran	50-26-40	32-26-40	2341	2009	Chelgard	8
N. kotschyi var. *kotschyi*	31955	Buyer-Ahmad, Kohgiluyeh and Buyer-Ahmad, Iran	-	-	-		Buyer-Ahmad1	8
	29231	Buyer-Ahmad, Kohgiluyeh and Buyer-Ahmad, Iran	-	-	-		Buyer-Ahmad2	8

were centrifuged for 5 min at 8,000×*g* at ambient temperature. The supernatant was discarded and the pellet was washed with 1 mL of 70% cold ethanol (4°C). The tubes were once more centrifuged (5 minutes at 8,000×*g*) and the supernatant was again carefully and thoroughly discarded. Finally, the DNA pellets were air dried in a sterile condition for about an hour, and dissolved in 200 µL TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM NaEDTA (pH 8.0)] for storage at 4°C for 12 hours. The remaining RNA was eliminated by adding 0.5 µL RNaseA with concentration of 10 mg mL^{-1} (Fermentas, Vilnius, Lithuania) and incubating the suspension for one hour at 37°C. After another extraction with an equal volume (200 µL) of chloroform:isoamyl alcohol (24:1), the aqueous phase was conserved at -20°C.

The DNA concentration and purity was estimated by reading absorbance at 260 nm, 280 nm and 230 nm using a UV-visible spectroscopy system (Agilent 8453, Agilent Technologies, Waldbronn, Germany). The integrity of the DNA samples was examined by electrophoresis on a 1% agarose gel in 1X TBE buffer (500 mL 1X buffer contained 5.4 g Tris base, 2.75 g H_3BO_3, 2 mL of 0.5 M NaEDTA (pH 8.0)) supplemented with 0.5 µg mL^{-1} EtBr at 60V. Gels were visualized and photographed using a UV transilluminator and a gel documentation system (ST4 3026-WL/26M, Vilber Lourmat, Torcy, France).

Amplification

Preparation of Polymerase Chain Reaction (PCR) mixture was performed under sterile conditions on ice. After PCR components optimization, all DNA samples were amplified using a 25 µL PCR reaction mixture that contained 200 ng of template DNA, 2.5 mM $MgCl_2$ (Thermo Scientific, Vilnius, Lithuania), 2 U Taq DNA polymerase (Thermo Scientific, Vilnius, Lithuania), 0.2 mM of each dNTPs (Thermo Scientific, Vilnius, Lithuania) with 0.6 µM primer (Metabion, Martinsried, Germany) in $1X(NH_4)_2SO_4$ reaction buffer (Thermo Scientific, Vilnius, Lithuania). A master mix of all components was prepared and only the varying components were adjusted individually so that any experimental error would be evenly distributed throughout all the samples. Reactions without DNA were used as negative controls to check the possible contamination of PCR reactions. Among 23 random 10-mer primers tested, 11 (Table 2) were selected for the analyses based on reproducibility and production of distinguishing banding patterns.

All reactions were done in a thermocycler (2720 Thermal Cycler 96 well, Applied Biosystems®, Foster City, CA, USA) with an optimized cycle profile comprised of one cycle at 95°C for 5 minutes (initial denaturation), 45 cycles of 1 minute at 94°C (denaturation), 1 min at 36°C (annealing) and 2 minutes at 72°C (extension), while the final extension step lasted for 10 minutes at 72°C. To test the reproducibility of the method, PCR was carried out twice for selected samples. Amplified PCR products were separated by horizontal electrophoresis (Compact L, Whatman, Biometra® GmbH, Göettingen, Germany; Power Source: Standard Power Pack P25, Biometra®, Göettingen, Germany) along with standard (100 bp, DNA Ladder, Thermo Scientific, Vilnius, Lithuania) as a fragment size marker on 1% agarose gels in 1X TBE buffer containing 0.5 µg mL^{-1} EtBr at 110V for 75 minutes. DNA banding profiles were visualized and photographed using a UV transilluminator and a gel-doc system. Photographs were further used for PCR product analysis.

Electrophoresis and Statistical Analyses

Banding pattern analysis and binary matrix construction for RAPD data were performed in TotalLab (TL120 1D v. 2009) software (Nonlinear Dynamics Ltd., Newcastle, UK). The presence or absence of fragments was recorded as either 1 or 0.

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Table 2. Primers with their sequences used for the RAPD analysis of 21 populations of *Nepeta kotschyi*.

Primer name	Sequence	Primer name	Sequence
OPA 04	*5'-AATCGGGCTG-3'*	*OPF 14*	*5'-TGCTGCAGGT-3'*
OPB 15	*5'-GGAGGGTGTT-3'*	*OPH 14*	*5'-ACCAGGTTGG-3'*
OPB 17	*5'-AGGGAACGAG-3'*	*OPO 03*	*5'-CTGTTGCTAC-3'*
OPB 18	*5'-CCACAGCAGT-3'*	*OPO 07*	*5'-CAGCACTGAC-3'*
OPT 14	*5'-AATGCCGCAG-3'*	*OPO 15*	*5'-TGGCGTCCTT-3'*
OPF 05	*5'-CCGAATTCCC-3'*		

Only consistently reproducible, well-resolved fragments were included. Monomorphic bands were excluded from analyses. Based on the 1/0 data matrix, the following parameters were calculated for each primer: Number of bands (N) and Polymorphic Bands (NP), Percentage of polymorphism (P), Informativeness of bands (Ib) (Prevost and Wilkinson, 1999) and its Average (AvIb), Resolving power (Rp) (Prevost and Wilkinson, 1999), Polymorphic Information Content per band (PIC) (Roldán-Ruiz *et al*., 2000) and its Average (*AvPIC*) (Lübberstedt *et al*., 2000), and Marker Index (MI) (Tams *et al*., 2005).

Binary matrices were used to construct a genetic distance matrix, according to Nei genetic variation index (1973), and used as the input matrix for Cluster Analysis (CA) using UPGMA algorithm and to draw Polar dendrogram by PowerMarker software (Liu and Muse, 2005). Dendrogram was visualized with FigTree ver. 1.4.3 software (Rambaut, 2016). The relationship of individuals in populations (in Supplementary Figure 4) was visualized based on Jaccard's coefficient and using PAST software ver.1.89 (Hammer *et al*., 2001). To estimate the variance components among and within populations, Analysis of Molecular Variance (AMOVA) (Excoffier *et al*., 1992) was performed using GenAlEx software ver. 6.5 (Peakall and Smouse, 2012). Calculation of the number of loci, the number of private loci, the percentage of polymorphic loci and gene diversity (expected heterozygosity) of each population was performed using GenAlEx software to study the genetic diversity within the populations.

RESULTS AND DISCUSSION

It has been found that there is no significant difference between two former species *N. kotschyi* and *N. persica*, and their only distinct difference was the presence and type of hairs on their stems and floral calyx. Therefore, the taxonomic status of former species *N. persica* has been changed into *N. kotschyi* var. *persica*. In var. *kotschyi*, the stems are covered by leveled felt-like hairs and the floral calyx does not have secretory glands, but in var. *persica*, the stems are covered by simple tall hairs and shorter glandular hairs and the floral calyx has simple hairs and secretory glands (Jamzad, 2012).

Eleven primers that amplified informative banding patterns and showed prominent polymorphism were used to estimate the amount of genetic diversity between and within *N. kotschyi* populations (Table 3). Of the total primers used, 225 bands with high resolution were scored and used for RAPD analysis. Among them, there were 21 monomorphic and 204 polymorphic bands. On average, 20.45 bands were obtained per primer, 18.55 being polymorphic. Primers *OPO 07* and *OPO 15* provided the highest amplified band number (25 bands), while primers *OPF 05*, *OPO 03* and *OPO 07* showed the most polymorphic percentage (100%). An example of a banding pattern amplified with the primer *OPF 05* for individuals of some populations is showed in Figure 1. The highest *AvIb* (0.37) was obtained for primers *OPB 15* and *OPT 14*. Also, the highest *Rp* (8.56), *AvPIC* (0.25)

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Table 3. Total Number of bands (N), Number of Polymorphic bands (NP), polymorphism Percentage (P), Average Informativeness of bands (*AvIb*), Resolving power of the primer (*Rp*), Average Polymorphic Information Content of bands (*AvPIC*) and Marker Index (*MI*) related to the primers used in the RAPD method for investigating the inter-and intra-population diversity of *Nepeta kotschyi*.

Primer	N	NP	P (%)	*AvIb*	*Rp*	*AvPIC*	*MI*
OPA 04	18	16	88.89	0.22	4.02	0.17	2.72
OPB 15	20	19	95.00	0.37	7.34	0.25	4.75
OPB 17	11	8	72.73	0.18	1.93	0.14	1.12
OPB 18	22	19	86.36	0.22	4.88	0.18	3.42
OPT 14	23	19	82.61	0.37	8.56	0.23	4.37
OPF 05	22	22	100.00	0.33	7.23	0.24	5.28
OPF 14	19	17	89.47	0.30	5.78	0.22	3.74
OPH 14	19	14	73.68	0.21	3.90	0.14	1.96
OPO 03	21	21	100.00	0.18	3.86	0.14	2.94
OPO 07	25	25	100.00	0.22	5.56	0.17	4.25
OPO 15	25	24	96.00	0.15	3.77	0.12	2.88
Total	225	204					
Average	20.45	18.55	89.52	0.25	5.17	0.18	

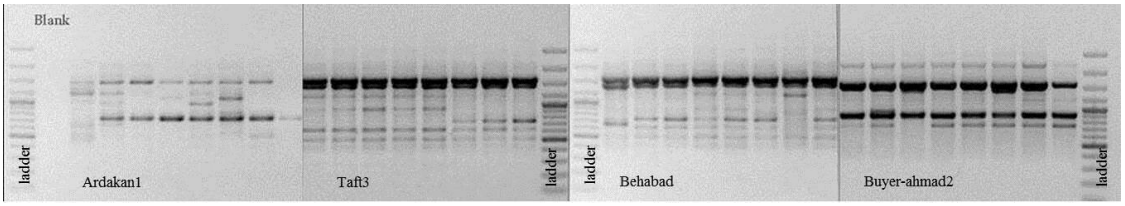

Figure 1. Genomic DNA banding pattern of individuals of some populations of *Nepeta kotschyi* amplified by the primer *OPF 05*.

and *MI* (5.28) were obtained for primers *OPT 14*, *OPB 15* and *OPF 05*, respectively. According to the results, primers *OPF 05*, *OPB 15*, *OPT 14*, *OPO 07* and *OPF 14* due to the better *P*, *AvIb*, *Rp*, *AvPIC* and *MI*, showed a good capability for investigation the genetic variation within the sample set.

Cluster Analysis (CA) was used to visualize genetic relationships among the populations of *N. kotschyi* as the result of RAPD markers data. The matrix of Nei's genetic distances is shown in Table 4 to represent the diversity among the populations. The CA results are shown in the dendrogram (Figure 2; also see Supplementary Figure 4, which represents the relationships of individuals in populations).

The genetic distances coefficients between the populations varied from 0.01 to 0.36 based on the Nei genetic distance (Table 4), such that the least distances and the highest genetic similarities were observed between the populations Yazd1, Behabad and Yazd2. Moreover, the highest distances and the lowest genetic similarities were found between the populations Boyer-Ahmad1/Boyer-Ahmad2 and the population Semirom.

Based on the results of CA, the populations belonging to *N. kotschyi* were placed in six genotype groups (Figure 2 and Table 5). Accordingly, Boyer-Ahmad1 and Boyer-Ahmad2 populations belonging to *N. kotschyi* var. *kotschyi* were separated from the group of the populations made of *N. kotschyi* var. *persica* (In Supplementary Figure 4 we can see a better diversification of the two varieties of *N. kotschyi*). Two populations, namely, Taft1 and Taft4, are in fact one population, sampled in two different years (Table 1) and the presented results

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Table4. The unbiased estimation matrix of the genetic distance between *Nepeta kotschyi* populations based on the Nei's unbiased genetic distance using RAPD molecular markers.

Population		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Ardakan1	1	0.00																				
Ardakan2	2	0.05	0.00																			
Ardakan3	3	0.07	0.07	0.00																		
Ardakan4	4	0.17	0.14	0.14	0.00																	
Semirom	5	0.18	0.15	0.11	0.17	0.00																
Taft1	6	0.09	0.06	0.11	0.09	0.20	0.00															
Taft2	7	0.10	0.11	0.14	0.12	0.22	0.05	0.00														
Taft3	8	0.10	0.08	0.14	0.13	0.23	0.03	0.05	0.00													
Taft4	9	0.10	0.08	0.12	0.15	0.22	0.05	0.06	0.03	0.00												
Taft5	10	0.08	0.10	0.12	0.17	0.24	0.06	0.06	0.06	0.04	0.00											
Taft6	11	0.08	0.11	0.12	0.21	0.24	0.09	0.09	0.09	0.07	0.04	0.00										
Mehriz	12	0.08	0.09	0.13	0.20	0.24	0.07	0.09	0.06	0.07	0.05	0.05	0.00									
Saduq	13	0.12	0.13	0.14	0.21	0.24	0.12	0.12	0.12	0.10	0.11	0.08	0.08	0.00								
Bafq	14	0.11	0.13	0.13	0.16	0.20	0.10	0.12	0.11	0.11	0.11	0.13	0.10	0.14	0.00							
Behabad	15	0.07	0.09	0.12	0.17	0.23	0.08	0.11	0.10	0.08	0.08	0.11	0.08	0.12	0.08	0.00						
Yazd1	16	0.07	0.09	0.11	0.15	0.23	0.08	0.11	0.10	0.09	0.09	0.11	0.08	0.12	0.08	0.01	0.00					
Yazd2	17	0.06	0.09	0.11	0.17	0.23	0.08	0.10	0.09	0.07	0.07	0.10	0.07	0.12	0.08	0.02	0.01	0.00				
Khorasan	18	0.18	0.17	0.17	0.19	0.20	0.16	0.17	0.18	0.17	0.18	0.19	0.18	0.19	0.16	0.15	0.14	0.14	0.00			
Chelgard	19	0.14	0.13	0.14	0.20	0.24	0.12	0.13	0.12	0.10	0.12	0.12	0.11	0.10	0.14	0.12	0.12	0.12	0.14	0.00		
Buyer-Ahmad1	20	0.17	0.16	0.18	0.26	0.31	0.18	0.20	0.19	0.19	0.18	0.16	0.18	0.16	0.19	0.17	0.18	0.18	0.25	0.17	0.00	
Buyer-Ahmad2	21	0.19	0.19	0.21	0.26	0.36	0.19	0.20	0.20	0.19	0.19	0.17	0.18	0.17	0.21	0.17	0.17	0.17	0.26	0.19	0.04	0.00

show slight differences in their RAPD profiles. These differences can be attributed to the cross-pollination nature of the species and different individuals tested between the two years.

The populations clustering show that the pattern of genetic diversity is consistent with the geographical distribution pattern and some of the populations originating in close geographic regions are placed in joint genotype groups (Tables 1 and 5 and Figure 3). For example, the populations Yazd1, Behabad, Yazd2 and Bafq, with the least distances and the highest genetic similarities between them, are located in an adjacent group.

The highest genetic distance between the studied populations of the *N. kotschyi* was 0.36, which is not unexpected because most of the studied populations are from Yazd or nearby provinces, so, the gene flow between them may be substantial, homogenizing their variability and diminishing genetic diversification. Generally, wide geographic distribution of plant species leads to polymorphisms, such that plant populations with more limited geographic distribution have less genetic variation (Hamrick and Godt, 1996). A very small genetic difference was reported for the Egyptian accessions of *N. septemcrenata*, which were collected from a limited geographic area, using the

Table 5. Genotype groups of *Nepeta kotschyi* derived from cluster analysis of RAPD markers.

Genotype group	Populations included
G1	Saduq, Chelgard
G2	Taft1, Taft3, Taft2, Taft4, Taft5, Taft6, Mehriz, Ardakan4
G3	Bafq, Yazd2, Behabad, Yazd1
G4	Ardakan3, Ardakan1, Ardakan2, Semirom
G5	Khorasan
G6	Boyer-Ahmad1, Boyer-Ahmad2

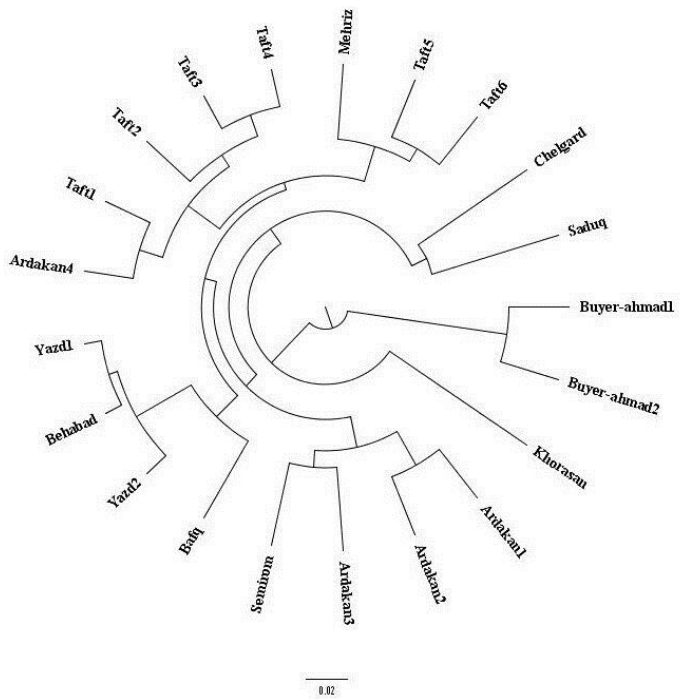

Figure 2. Clustering of *Nepeta kotschyi* populations based on the data of RAPD markers (using Nei's genetic distance coefficient): Two populations of Boyer- Ahmad1 and Boyer-Ahmad2 belong to var. *kotschyi*, and the other populations belong to var. *persica*.

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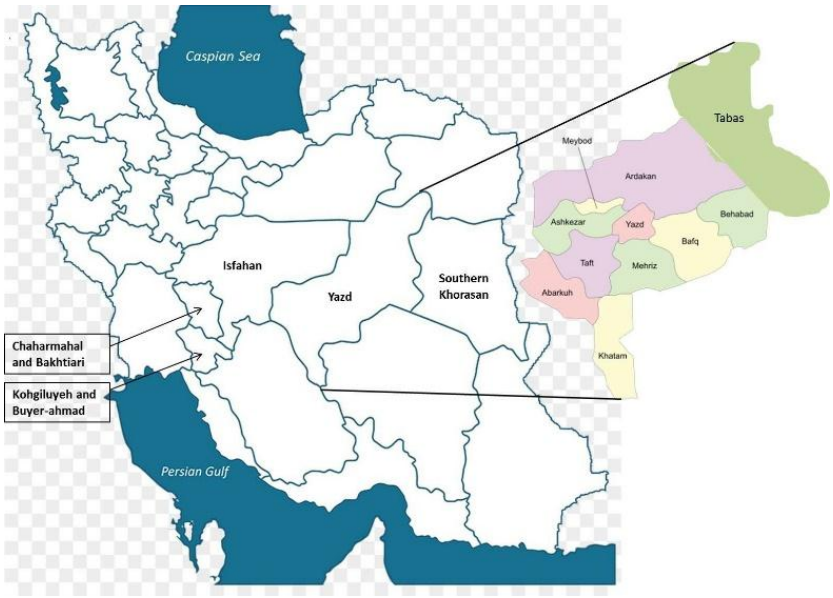

Figure 3. Map of Iran showing provinces where the populations originated in, and map of Yazd Province showing the position of counties.

RAPD marker system (Elkholy *et al*., 2011).

Yazd Province location in the central part of Iran's plateau is characterized by severe winds (from late March to early June) and once in a while dusty storms, high air temperatures and high rainfall fluctuations, mountains and deserts that separate the natural populations, and the presence of various micro-climates. These conditions, as well as the flying range of pollinators and their impressionability from natural factors, make it impossible to make a definite statement about the populations originating from near areas or of similar areas in Yazd Province regarding the level of genetic similarity or distance between them. Such situation, for example, can be seen in Figures 2 and 3 regarding the position of four Ardakan populations.

The results of AMOVA of the studied populations are presented in Table 6. They show that the variation between and within the populations was significant at 1% level. From the whole diversity of the studied germplasm, 32% of the variability was distributed among the populations and 68% of the variation was found within the populations. In wild populations, both the geographical distance and the gene flow between them highly determine the genetic distance. In cross-pollination species, high-rate gene flow decreases the genetic distance between populations and, in contrast, genetic variation is scattered within the populations (Hamrick *et al*., 1991).

Some parameters of genetic diversity including the number of loci, and private loci, the percentage of polymorphism and

Table 6. Analysis of Molecular Variance (AMOVA) of *Nepeta kotschyi* populations based on RAPD markers.

Source	df	SS	MS	Est Var	%	Value	Prob
Among populations	20	1551.89	77.59**	8.59	32%		
Within populations	125	2250.22	18.00**	18.00	68%	0.32	0.001
Total	145	3802.11	95.60	26.59			

** Significant at alpha= 0.01

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Table 7. Some genetic diversity parameters within *Nepeta kotschyi* populations.

Population	No. bands	No. bands freq>= 5%	No. private bands	No. LComm bands (<= 25%)	No. LComm bands (<= 50%)	P (%)	Mean heterozygosity	SE of mean heterozygosity
Ardakan1	126	126	1	4	14	35.5	0.13	0.01
Ardakan2	120	120	0	6	11	34.6	0.14	0.01
Ardakan3	117	117	1	7	14	32.8	0.14	0.01
Ardakan4	116	116	1	3	9	8.44	0.03	0.01
Semirom	120	120	4	8	14	9.33	0.04	0.01
Taft1	119	119	0	3	7	29.7	0.12	0.01
Taft2	117	117	0	4	10	28.0	0.12	0.01
Taft3	120	120	1	4	11	30.6	0.12	0.01
Taft4	122	122	2	7	12	35.1	0.13	0.01
Taft5	119	119	1	5	11	31.5	0.11	0.01
Taft6	121	121	2	4	15	37.7	0.13	0.01
Mehriz	113	113	0	4	11	28.4	0.10	0.01
Saduq	133	133	8	12	25	46.6	0.17	0.01
Bafq	122	122	1	3	14	32.0	0.14	0.01
Behabad	127	127	1	7	15	31.1	0.11	0.01
Yazd1	122	122	1	4	11	28.4	0.12	0.01
Yazd2	118	118	0	4	10	27.1	0.11	0.01
Khorasan	118	118	4	8	13	26.2	0.12	0.01
Chelgard	129	129	1	14	24	41.3	0.16	0.01
Boyer-Ahmad1	120	120	2	20	30	40.8	0.16	0.01
Boyer-Ahmad2	119	119	4	18	25	32.0	0.12	0.01
Mean						30.8		

Nei's gene diversity index in each of the studied populations are presented in Table 7. Based on the results from the study of bands obtained from RAPD markers, the number of loci amplified by the primers used across the populations ranged between 117 (populations Ardakan3 and Taft2) to 133 (population Saduq), and the number of private loci ranged between 0 (populations Ardakan2, Taft1, Taft2, Mehriz and Yazd2) to 8 (population Saduq).

The studied populations were diverse in terms of polymorphism percentage and Nei's gene diversity, which may have variety of consequences such as better adaptation to environmental changes during the evolution, being a rich source of genes for breeding programs. If functionally divergent alleles enable adaptation to different environments, locus-specific polymorphism may be maintained by spatially heterogeneous natural selection (Lee and Mitchell-Olds, 2012). The lowest and highest polymorphism percentage (8.44-46.57%) and Nei's gene diversity index (0.03-0.17) were observed in Ardakan4 and Saduq populations, respectively. The results also indicate a high diversity within the populations Saduq, Chelgard, and Buyer-Ahmad1.

High efficacy of the RAPD markers in evaluating the variation between and within populations of various plant species, including the most important medicinal species of mint family, has been reported as previously mentioned in the Introduction section. The presented study indicates high efficiency of RAPD markers in the classification and evaluation of inter-and intra-population diversity, as well as the separation of the two varieties of *N. kotschyi* (var. *kotschyi* and var. *persica*). The present results provide important information for *N. kotschyi* germplasm characterization, improvement, and conservation. Furthermore, the studied populations exhibited a great deal of genetic variation

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and they seem to have a rich gene pool for breeding programs.

CONCLUSIONS

The current results support previous findings reported by Hadi *et al*. (2016, 2017). Yazd1, Yazd2 and Behabad populations of var. *persica*, and Boyer-Ahmad1 and Boyer-Ahmad2 populations of var. *kotschyi* have been shown as the most promising candidates for domestication and cultivation. RAPD markers placed Yazd1, Yazd2, and Behabad populations in the common group suggesting that 11 selected primers succeeded to cover at least a part of the genetic material coding for some of the enzymes that have roles in the metabolic pathways of the secondary metabolites.

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ارزیابی تنوع ژنتیکی زیرگونه‌ای در *Nepeta kotschyi* Boiss.، یک گیاه دارویی بومی ایرانی

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چکیده

پونه‌سا (*Nepeta*) یکی از بزرگ‌ترین جنس‌های خانواده نعنا (Lamiaceae) است، و ایران، به‌طور ویژه، یکی از خواستگاه‌های اصلی این جنس است. برآورد تنوع ژنتیکی ژرم‌پلاسم جنس پونه‌سا، پایه‌ای برای تلاش‌های آینده حفاظت از تنوع زیستی و همچنین برای انتخاب ژنوتیپ(های) با قابلیت تولیدی بالا برای بهبود تولید زراعی آن فراهم می‌کند. در این تحقیق، برای ارزیابی تنوع ژنتیکی زیرگونه‌ای *N. kotschyi* بر اساس ۲۱ جمعیت، ۱۱ آغازگر RAPD با الگوی نواربندی و چندشکلی مناسب مورد استفاده قرار گرفت. جمعیت‌های مورد مطالعه از دو واریته شامل *persica* (۱۹ جمعیت) و *kotschyi* (۲ جمعیت) بودند. در مجموع، ۱۱ آغازگرRAPD ، ۲۲۵ باند قابل ارزیابی را تکثیر نمودند، که ۲۰۴ تا از آن‌ها چندشکل بودند. میانگین تعداد باندها به ازای هر آغازگر ۲۰/۵ بود، که ۱۸/۵ تا از آن‌ها چندشکل بودند. آغازگرهای *OPF 05*، *OPB 15*، *OPT 14*، *OPO 07* و *OPF 14* برای شناسایی تنوع ژنتیکی نمونه‌ها، کاراتر بودند. تجزیه خوشه‌ای، شش گروه ژنوتیپی را نشان داد. جمعیت‌های *N. kotschyi* var. *kotschyi* در یک گروه مجزا از جمعیت‌های *N. kotschyi* var. *persica* قرار گرفتند. گروه‌بندی جمعیت‌های مورد مطالعه نشان داد که الگوی تنوع ژنتیک با الگوی توزیع جغرافیایی هم‌خوانی دارد. از مجموع تنوع ژرم‌پلاسم مورد مطالعه، ۳۲ درصد به بین جمعیت‌ها و ۶۸ درصد به درون جمعیت‌ها تعلق گرفت. نتایج نشان‌دهنده کارایی بالای نشانگرهای RAPD برای تعیین وضعیت ژنوتیپ‌های مورد مطالعه در خصوص تنوع بین و درون جمعیت‌ها و تفکیک واریته‌های *N. kotschyi* که قبلا توسط روش‌های مورفومتری تعیین شدند، می‌باشد.

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