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

N. Hadi¹, A. Shojaeiyan²*, F. Sefidkon¹, A. A. Jafari³, D. Mišić⁴, T. Banjanač⁴, and B. Šiler⁴

**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., 2013).

¹ Medicinal Plants and By-Products Research Division, Research Institute of Forests and Rangelands, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Islamic Republic of Iran.  
² Department of Horticultural Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Islamic Republic of Iran.  
*Corresponding author; e-mail: shojaeiyan@modares.ac.ir  
³ Rangelands Research Division, Research Institute of Forests and Rangelands, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Islamic Republic of Iran.  
⁴ Institute for Biological Research "Siniša Stanković" – National Institute of Republic of Serbia, University of Belgrade, Belgrade, Serbia.
has been mainly done using morphological, genetic variation of medicinal plant species (Máthé, 1986; Tétényi, 2002). Among DNA markers, RAPD is the most cost-effective 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 (Saedinia 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 rbcL and matK 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., 2005). 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

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.
Infraspecific Genetic Diversity in Nepeta kotschyi

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; Rustaeie 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
Table 1. *Negata 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Population number</th>
<th>Origin of seeds (County, Province, Country)</th>
<th>Longitude (°,.’”)</th>
<th>Latitude (°,.’”)</th>
<th>Altitude (m)</th>
<th>Year of collection</th>
<th>Population code</th>
<th>No. of individuals tested in RAPD analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>15908</td>
<td>Ardasan, Yazd, Iran</td>
<td>54-39-08</td>
<td>32-24-53</td>
<td>2141</td>
<td>2004</td>
<td>Ardasan1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>15857</td>
<td>Ardasan, Yazd, Iran</td>
<td>54-34-42</td>
<td>32-22-49</td>
<td>2187</td>
<td>2004</td>
<td>Ardasan2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>13040</td>
<td>Ardasan, Yazd, Iran</td>
<td>54-38-03</td>
<td>32-27-95</td>
<td>2500</td>
<td>2003</td>
<td>Ardasan3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>15853</td>
<td>Taft, Yazd, Iran</td>
<td>54-41-01</td>
<td>31-44-50</td>
<td>2465</td>
<td>2004</td>
<td>Taft1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>13061</td>
<td>Taft, Yazd, Iran</td>
<td>54-08-19</td>
<td>31-35-05</td>
<td>3040</td>
<td>2003</td>
<td>Taft2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>15827</td>
<td>Taft, Yazd, Iran</td>
<td>54-44-47</td>
<td>31-43-49</td>
<td>2634</td>
<td>2004</td>
<td>Taft3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>10250</td>
<td>Taft, Yazd, Iran</td>
<td>54-41-01</td>
<td>31-44-50</td>
<td>2465</td>
<td>2002</td>
<td>Taft4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>21048</td>
<td>Taft, Yazd, Iran</td>
<td>54-45-75</td>
<td>31-48-54</td>
<td>2596</td>
<td>2005</td>
<td>Taft5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>15828</td>
<td>Taft, Yazd, Iran</td>
<td>54-05-29</td>
<td>31-37-35</td>
<td>3030</td>
<td>2004</td>
<td>Taft6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>15850</td>
<td>Mehriz, Yazd, Iran</td>
<td>54-18-00</td>
<td>31-29-00</td>
<td>2387</td>
<td>2004</td>
<td>Mehriz</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>21207</td>
<td>Saduq, Yazd, Iran</td>
<td>53-47-10</td>
<td>31-54-41</td>
<td>1858</td>
<td>2005</td>
<td>Saduq</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>21035</td>
<td>Bafq, Yazd, Iran</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2005</td>
<td>Bafq</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>21039</td>
<td>Behabat, Yazd, Iran</td>
<td>56-01-01</td>
<td>31-43-98</td>
<td>2635</td>
<td>2005</td>
<td>Behabat</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>18697</td>
<td>Yazd, Yazd, Iran</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2004</td>
<td>Yazd1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>18695</td>
<td>Yazd, Yazd, Iran</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2004</td>
<td>Yazd2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>28272</td>
<td>Semirom, Isfahan, Iran</td>
<td>51-53-15</td>
<td>31-11-31</td>
<td>2350</td>
<td>2006</td>
<td>Semirom</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>25740</td>
<td>Khorasan, Khorasan, Iran</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2007</td>
<td>Khorasan</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>29556</td>
<td>Chelgard, Chaharmahal and Bakhtian, Iran</td>
<td>50-26-40</td>
<td>32-26-40</td>
<td>2341</td>
<td>2009</td>
<td>Chelgard</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>31955</td>
<td>Buyer-Ahmad, Kohgiluyeh and Buyer-Ahmad, Iran</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2004</td>
<td>Buyer-Ahmad1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>29231</td>
<td>Buyer-Ahmad, Kohgiluyeh and Buyer-Ahmad, Iran</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2007</td>
<td>Buyer-Ahmad2</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>
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 H₃BO₃, 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 MgCl₂ (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 1XNH₄₂SO₄ 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, Göttingen, Germany; Power Source: Standard Power Pack P25, Biometra®, Göttingen, 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 documentation 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.
Table 2. Primers with their sequences used for the RAPD analysis of 21 populations of *Nepeta kotschyi*.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA 04</td>
<td>5'-AATCGGGGCTG-3'</td>
<td>OPF 14</td>
<td>5'-TGCTGACGTG-3'</td>
</tr>
<tr>
<td>OPB 15</td>
<td>5'-GGAGGTTGTT-3'</td>
<td>OPH 14</td>
<td>5'-ACCAAGTTGG-3'</td>
</tr>
<tr>
<td>OPB 17</td>
<td>5'-AGGGAAAGGAG-3'</td>
<td>OPO 03</td>
<td>5'-CTGTGCATC-3'</td>
</tr>
<tr>
<td>OPB 18</td>
<td>5'-CCACACGCTG-3'</td>
<td>OPO 07</td>
<td>5'-CAGCAGTGAC-3'</td>
</tr>
<tr>
<td>OPT 14</td>
<td>5'-AATGCCGAG-3'</td>
<td>OPO 15</td>
<td>5'-TGCGGTCTCT-3'</td>
</tr>
<tr>
<td>OPF 05</td>
<td>5'-CCGAATTCCC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 shown 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)
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.

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

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...
Table 4. The unbiased estimation matrix of the genetic distance between *Nepera 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|     |     |     |     |     |     |     |     |
| Sadoq        | 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| 0.13| 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| 0.18| 0.19| 0.16| 0.15| 0.14| 0.14| 0.00|     |     |     |
| Cheigard     | 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| 0.14| 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| 0.26| 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.

<table>
<thead>
<tr>
<th>Genotype group</th>
<th>Populations included</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Saduq, Chelgard</td>
</tr>
<tr>
<td>G2</td>
<td>Taft1, Taft3, Taft2, Taft4, Taft5, Taft6, Mehriz, Ardakan4</td>
</tr>
<tr>
<td>G3</td>
<td>Bafq, Yazd2, Behabad, Yazd1</td>
</tr>
<tr>
<td>G4</td>
<td>Ardakan3, Ardakan1, Ardakan2, Semirom</td>
</tr>
<tr>
<td>G5</td>
<td>Khorasan</td>
</tr>
<tr>
<td>G6</td>
<td>Boyer-Ahmad1, Boyer-Ahmad2</td>
</tr>
</tbody>
</table>

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

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.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Est Var</th>
<th>%</th>
<th>Value</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>20</td>
<td>1551.89</td>
<td>77.59</td>
<td>8.59</td>
<td>32%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>125</td>
<td>2250.22</td>
<td>18.00</td>
<td>18.00</td>
<td>68%</td>
<td>0.32</td>
<td>0.001</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>3802.11</td>
<td>95.60</td>
<td>26.59</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Significant at alpha= 0.01

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.
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 Boyer-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.
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.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Ziba Jamzad from the Research Institute of Forests and Rangelands, Iran for botanical certification of the plant materials. Molecular analyses were financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia - Grant No. 173024. The first author benefited from funding support by Tarbiat Modares University, Iran.

REFERENCES


47. Rambaut, A. 2016. FigTree Ver. 1.4.3 Software. Institute of Evolutionary Biology, University of Edinburgh.


Infraspecific Genetic Diversity in Nepeta kotschyi


ارزیابی تنوع زننیکی زیرگونه‌ای در 
بومی ایرانی

نویسنده‌ها: هادی، ع. شجاعیان، ف. سفیدکن، ع. جعفری، د. میشه، ت. بانیانک، و ب.

چکیده

پوشا (Nepeta) یکی از بزرگترین جنس‌های خانواده نما (Lamiaceae) است، و ایران، به‌طور ویژه، یکی از خواسم‌های اصلی این جنس است. بر اساس توصیف وزننیکی زرمپلاسم جنس پوشا، پایه‌ای برای تلاش‌های آینده حفاظت از تنوع زننیکی و همچنین برای انتخاب زننیکی (نیا) با قابلیت تولیدی

ن. کاکری بهبود تولید زراعی آن فراهم می‌کند. در این تحقیق برای ارزیابی تنوع زننیکی زیرگونه‌ای 

ن کاکری RAPD با الگوی نواربندی و چندشکلی مناسب مورد استفاده قرار گرفت. جمعیت‌های مورد مطالعه از دو واریته شامل ن. کاکری persica (21 جمعیت) و ن. کاکری kotschyi (24 جمعیت) بودند. در مجموع، 11 آغازگر RAPD 225 باندل ارزیابی را تکثیر نمودند. که 204 تا از آنها چندشکل بودند. میانگین تعداد باندلی به ازای هر آغازگر 20/5 بود، که که 18/5 تا از آنها

بنشک شدند. آغازگرها 505 نیا، 14 OPF 14 OPO 07، 14، 15 OPF 0، 14، 15 شناسایی تناوب زننیکی نمو‌های کارا بودند. تجزیه واردهای، شش گروه وزننیکی را نشان داد.

ن. kotschyi var. kotschyi در یک گروه جدا از جمعیت‌های 

فناوت. جمعیت‌های مورد مطالعه نشان داد که الگوی تونوی نیا، کاکری از جمعیت‌های persica توزیع جغرافیایی هم‌خوانی دارد. از مجموع تونوی زرمپلاسم مورد مطالعه، 32 درصد به بین جمعیت‌ها و 68 درصد به درون جمعیت‌ها تعلق گرفت. نتایج نشان دهنده کارایی بسیاری نیاگرها 

برای تعیین وضعیت زننیکی مورد مطالعه در خصوص تونوی بین و درون جمعیت‌ها و تفکیک واریته‌های 

که قبل توسط روش‌های مورفومتری تعیین شدند، می‌باشد. N. kotschyi

1342