6. Genetic variation and relationship of tetraploid alfalfa populations: Application in breeding

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Abstract. Alfalfa is an autotetraploid, allogamous and heterozygous forage legume, whose varieties are synthetic populations. Due to the complex nature of the species, information about genetic diversity of germplasm, used in any alfalfa breeding program, is most beneficial. The genetic diversity of five alfalfa varieties and their offspring, involved in breeding program at Institute of Field and Vegetable Crops, Novi Sad was characterized based on RAPD markers. RAPD analysis was performed on individual and bulked DNA samples of five alfalfa parental varieties and bulked DNA samples of their progenies: 20 F1 populations from reciprocal diallel crosses and five S1 populations from self-pollination. Parental variety Zuzana had the highest values for all tested parameters, exhibiting the highest level of variation, whereas variety RSI 20 exhibited the lowest. The cluster analysis for individual samples from parental varieties revealed differences in their population structures: variety Zuzana showed a very high level of genetic variation, Banat and Ghareh were divided in subpopulations, while Pecy and RSI 20 were relatively uniform. Ways of exploiting the investigated germplasm in the breeding programs are suggested,

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depending on their population structure and diversity. Genetic variation in bulked samples of parental populations and S1 progenies was similar, while in F1 progeny populations was observed higher level of diversity. Cluster analysis revealed that self-pollinated progenies, as well as the hybrid offspring where Zuzana, and RSI 20 were maternal parents, were placed in separate groups. The results indicate that use of RAPD markers on bulked DNA samples can be fast and cost-effective way for differentiation of alfalfa parental populations and their offspring, as well as for evaluation of their genetic relationships.

1. Introduction

Alfalfa (*Medicago sativa* L.) is extensively used as forage legume, with over 32 million hectares grown worldwide. It is one of the most important perennial legume crops and a superior source of forage due to its high nutritional quality and herbage yield (Riday & Brummer, 2002; Deshpande et al., 2002; Li & Brummer, 2009). In addition, the ability of alfalfa to fix atmospheric nitrogen makes it valuable in crop rotations for higher productivity of crops (Barnes, 1993; Sandoval et al., 2007). Also alfalfa contains phytoestrogens which should be used for medical purposes in production of pharmaceutical products with high level of estrogens for human consumptions (Seguin et al 2004). Alfalfa as perennial legume can play important role in phytoremediation i.e. in soil recultivation (Schwab et al 2006). The benefits of alfalfa are not restricted to agriculture and animal husbandry. Alfalfa has the potential for applications in molecular farming for monoclonal antibody (Bardor et al., 2003) and vaccine production (D’Aoust et al., 2004; Dus Santos et al., 2005). The recent technological progress in harvesting technology and lignin modification further enhance the potential of alfalfa as a key biofuel feedstock (McCaslin and Miller 2007)

Breeding programs aimed to genetically improve alfalfa started at the beginning of 20th Century, but the progress of alfalfa breeding has been slow, compared to other important food and feed crops (Lamb et al., 2006). The main reasons being due to: a complex genetic structure (autotetraploidy, $2n = 4x = 32$), allogamy and tetrasomic inheritance in alfalfa, as well as plant architecture, hermaphroditic flowers, and meadow conditions (Scotti and Brummer, 2010). Alfalfa breeding programs are mainly based on recurrent phenotypic selection, with or without progeny testing, with an aim to accumulate desirable alleles at high frequency within a population (Li and Brummer 2012). Using phenotypic selection, there was great improvement in disease and pest resistances, winter tolerance and forage nutritive value, but increase of yield has been less successful (Veronesi et al., 2010).
In papers published around the end of the 20th century, alfalfa breeders reported that significant specific combining abilities existed in hybrids made by crossing divergent genotypes and populations of alfalfa (Riday and Brummer, 2002a; Segovia-Lerma et al., 2004). A proposal has been made to the start with the development of semihybrids, which are the product of crossing genetically divergent germplasm and identifying heterotic groups in the alfalfa gene pool (Brummer 1999; Riday and Brummer 2002b). The strategy for developing semihybrids based on making crosses among populations should enable the partial utilization of the heterosis effect and could help in improvement of yield per se in alfalfa (Brummer, 1999). In Europe, Rotili et al. (1999) suggested that heterotic effects could be partially utilized in alfalfa through the development of free hybrids from lines resulting from two to three generations of selfing. Recent studies support idea of the semihybrid breeding of this crop (Scotti and Brummer 2010, Katić et al., 2011, Milić et al., 2013). The concept involves: breeding more uniform alfalfa populations, identification of heterotic germplasm and the production of seed of the population hybrid (Milić et al., 2011).

Since severe inbreeding depression hinders development of inbred lines, all commercial cultivars are synthetic populations generated by crossing different numbers of selected genotypes (Rowe et al., 2011). Because of the tetraploid structure of the alfalfa genome, cross pollination and severe inbreeding depression, cultivars can exhibit different levels of genetic variation (Julier et al., 2000, Julier et al., 2003b, Flajoulot et al., 2005, Živković et al., 2012, Taski-Ajdukovic et al., 2012). Therefore, information about germplasm diversity and relationships within and among elite breeding material is of great importance for any efficient and successful alfalfa breeding program.

Although the molecular breeding approaches like marker assisted selection (MAS) could enhance efficiency of cultivar development in term of gain per unit cost and time (Wang et al., 2013), it has not been widely implemented in perennial forage breeding programs, mainly due to the fact that: a) they are small breeding programs with limited resources; b) there is a lack of genomic tools and infrastructure and c) ploidy level of main forage species such as alfalfa (Riday et al., 2011).

Different types of molecular markers have been used in alfalfa populations (Gherardi et al., 1998; Flajoulot et al., 2005; Vandemark et al., 2006; Noeparvar et al., 2008) and other Medicago germplasm sources (Carelli et al, 2009) for estimation of their relationships, variety and ecotype identification (Morales et al., 200; Pupilli et al., 2000), analysis of
population genetic structure (Falahati-Anbaran et al., 2009), construction of genetic linkage maps (Yu et al., 1993, Sledge et al., 2005), and identification of inter species hybrids (Milić et al., 2014).

Genetic diversity within and among alfalfa populations have been assessed by using various types of DNA markers: Random Amplified Polymorphic DNA (RAPD) (Crochemore et al., 1996; Skuza et al., 2013; Vyšniauskienė et al., 2013), Amplified Fragment Length Polymorphism (AFLP) (Barcaccia et al., 1999; Segovia-Lerma et al., 2003), Restriction Fragment Length Polymorphism (RFLP) (Kidwell et al., 1994), Simple Sequence Repeat (SSR) (Bagavathiannan et al., 2010; Sakiroğlu et al., 2010), Intersimple Sequence Repeat (ISSR) (Touil et al., 2008) and Sequence Related Amplified Polymorphisms (SRAP) (Ariss et al., 2007; Al-Faifi et al., 2013). Results of all this research lead to conclusions (i) that alfalfa populations, both wild and cultivated, are highly diverse and (ii) that most genetic variation in alfalfa resides within populations.

Among DNA markers, RAPD (Williams et al., 1990) is generally considered a fast, informative and inexpensive type of marker, which despite dominance and low reproducibility, allows analysis of the polymorphism in many individuals with good coverage of the entire genome (Melchinger et al., 1993).

Information about germplasm diversity and relationships within and among breeding material is crucial for alfalfa breeding programs. Therefore, the initial evaluation of the potential usefulness of RAPD markers as an inexpensive, quick and efficient tool for diversity screening, and possible application of MAS in alfalfa breeding programs was started at the Institute of Field and Vegetable Crops (Novi Sad, Serbia). The aims were to determine genetic variation and relationship among five varieties, potential parental populations, and their F₁ progenies by using RAPD markers (i) and to determine how understanding the genetic variation and population structure of the analyzed breeding material affects their application in alfalfa breeding (ii).

**Plant material**

For the study 30 tetraploid alfalfa populations were chosen, including 5 alfalfa parental populations of different geographic origin: NS Banat ZMS II (B), Gareh Yon Geh (G), Zuzana (Z), Pecy (P) and RSI 20 (R) (Table 1). From each variety, 10 individual samples were taken for DNA isolation and further RAPD analysis.
Table 1. Description of alfalfa parental populations

<table>
<thead>
<tr>
<th>Variety</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banat (NS Banat ZMS II)</td>
<td>An old variety developed at the Institute of Field and Vegetable Crops, Novi Sad, Serbia by individual selection from local populations (Pannonian type of alfalfa). Has rapid initial growth and fast regrowth after cutting. Plant height at early flowering is over 80 cm. Proportion of leaves in green forage yield is 450–500 g kg$^{-1}$. Green forage yield is about 80 t ha$^{-1}$, hay yield is 15–20 t ha$^{-1}$. Resistant to drought, low temperatures and frequent cuttings.</td>
</tr>
<tr>
<td>Ghareh (Ghareh Yon Geh)</td>
<td>Variety developed at the Institute Karaj in Iran, the center of alfalfa origin. Well adapted to environmental conditions of Serbia (resistant to drought and low temperature). It has tall, large plants that regrow fast after cutting (37.1 cm) and high hay yield (16–19 t ha$^{-1}$).</td>
</tr>
<tr>
<td>Zuzana</td>
<td>An old variety developed at the breeding station in Zelezice, Brno, Czech Republic, with good adaptation to environmental conditions of Serbia. Has good dry matter yield (14 t ha$^{-1}$), a larger number of shorter internodes (tolerance to lodging), slower regrowth after cutting (higher dormancy), and higher susceptibility to drought. Represents a transition between Pannonian and Western European type of varieties.</td>
</tr>
<tr>
<td>Pecy</td>
<td>An old French variety developed by company R2N. A typical variety of the Western European type. Has good resistance to lodging and main alfalfa diseases. Well able to withstand low temperatures but is susceptible to drought. Has an exceptional quality, with high proportion of leaves in yield (48–56%), and larger number (13) of short internodes (5.2 cm).</td>
</tr>
<tr>
<td>RSI 20</td>
<td>Breeding population from Spain. Early-maturing variety with high dry matter yield (17.9 t ha$^{-1}$) and excellent quality (crude protein content of 22.2%). Has low dormancy (fast regrowth after cutting −39.4 cm), tolerance to high temperatures and drought, but it is sensitive to cold. Because of smaller number (10) of long internodes (6.3 cm) it is susceptible to lodging.</td>
</tr>
</tbody>
</table>

Also were included 20 F$_1$ populations from the reciprocal diallel crosses and 5 S$_1$ populations (Bs, Gs, Zs, Ps, Rs) from self-pollination used in alfalfa breeding program at the Institute of Field and Vegetable Crops, Novi Sad. DNAs isolated from individual samples in parental populations were mixed into the bulk samples. In progeny populations, genomic DNA extraction was performed using the mixture of leaves collected from ten to twenty plants.
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per population. Total genomic DNA was isolated from leaves according to the protocol of Somma (2004).

**RAPD analysis**

In order to test amplification profiles for polymorphism, readability and reproducibility, sixty decamer primers from ROTH®GmbH kits X, Y and Z were initially tested (Nagl et al., 2010). For analysis of genetic variation and relationship in parental varieties, seventeen primers were used for further RAPD analysis (Table 2). For the analysis in progenies and bulked parental samples, additional three primers were used: X03, Y09 and Y18 (marked italic in Table 5).

Amplification was carried out in a 25 μl reaction volume containing 2.5 μl buffer; 0.2 mM L^{-1} of each dNTP; 0.5 μM L^{-1} of primer; 2 units of Taq polymerase (Thermo Scientific) and 30 ng of DNA, following the amplification profile: denaturation at 94°C for 4 min, followed by 40 cycles under 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, with final elongation on 72°C for 10 min. The amplified products were separated by electrophoresis on 1.5% agarose gels containing 0.005% ethidium bromide and visualized under UV light. As the molecular standards were used GeneRuler 100 bp plus and GeneRuler 1 kb DNA Ladder (Thermo Scientific).

**Data analysis**

Each amplified fragment was treated as binary unit character and scored “0” for absence and “1” for presence. In order to measure informativeness of the markers, the polymorphism information content (PIC) for each primer was calculated (Smih et al., 1997). Estimation of genetic variation within and among parental varieties, as well as within groups of populations (parental varieties, cross-pollinated progenies and self-pollinated progenies) was carried out by using the POPGENE software package (version 1.32; Molecular Biology and Biotechnology Center, University Alberta, Canada http://www.ualberta.ca/~fyeh/Pop32.exe) for calculation of the following parameters: number of polymorphic loci P (no) and their percentage P (%), effective number of alleles per loci, expected heterozygosity (He) based on allelic frequencies (Nei, 1987) and Shannon’s information index (I) of phenotypic diversity (Lewontin, 1972) based on marker frequencies. For estimation of variance components among and within the tested varieties and the groups of populations analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed, using the ARLEQUIN 3.11 software.
(Excoffier et al., 2005) Jaccard’s coefficient of similarity was used for grouping of the populations by unweighted pair group arithmetic mean (UPGMA) cluster method. Dendrograms were drawn using SAHN clustering method as available in NTSYSpc software package (version 2.11a, Department of Ecology and Evolution, State University of New York) and generated by using TREE display option. Robustness of the clustering pattern was tested by bootstrap analysis using Free Tree software (version 0.9.0.1.50, Faculty of Science, Charles University, Prague, http://ijs.sgmjournals.org/content/vol51/issue3/).

Results

Parental populations

Genetic diversity

Seventeen selected RAPD primers generated stable and reproducible bands in samples of investigated parental varieties (Figure 1). A total of 156 polymorphic bands were generated, ranging from 300 to 6,000 bp (Table 2), with average number of bands per primer of 10.6 and polymorphism information content (PIC) value of 0.278. The highest number of polymorphic bands was achieved with primer X17 (17 bands), while the most informative primer was Z17 with PIC value of 0.374.

Figure 1. RAPD profiles of individual samples from five alfalfa varieties generated by primer Y15: a) Banat, b) Ghareh, c) Zuzana, d) Pecy and e) RSI 20, 100bp – GeneRuler 100bp plus DNA ladder.
Table 2. Description of RAPD primers used for analysis of parental alfalfa varieties.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Max. no. of bands</th>
<th>Band size range (bp)</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>X07</td>
<td>GAGCGAGGCT</td>
<td>14</td>
<td>400-2,000</td>
<td>0.267</td>
</tr>
<tr>
<td>X09</td>
<td>GGTCCTGGTTG</td>
<td>12</td>
<td>600-3,000</td>
<td>0.286</td>
</tr>
<tr>
<td>X12</td>
<td>TCGCCAGCCA</td>
<td>13</td>
<td>400-2,000</td>
<td>0.174</td>
</tr>
<tr>
<td>X17</td>
<td>GACACGGACC</td>
<td>17</td>
<td>400-3,000</td>
<td>0.368</td>
</tr>
<tr>
<td>Y02</td>
<td>CATCGCCGCA</td>
<td>7</td>
<td>900-3,500</td>
<td>0.314</td>
</tr>
<tr>
<td>Y05</td>
<td>GGCTGCGACA</td>
<td>11</td>
<td>700-4,000</td>
<td>0.285</td>
</tr>
<tr>
<td>Y06</td>
<td>AAGGCTCACC</td>
<td>8</td>
<td>1,000-3,500</td>
<td>0.358</td>
</tr>
<tr>
<td>Y07</td>
<td>AGAGCCGCTCA</td>
<td>14</td>
<td>700-4,000</td>
<td>0.291</td>
</tr>
<tr>
<td>Y10</td>
<td>CAACAGTGGG</td>
<td>9</td>
<td>650-4,000</td>
<td>0.320</td>
</tr>
<tr>
<td>Y11</td>
<td>AGACGATGGG</td>
<td>13</td>
<td>300-2,500</td>
<td>0.308</td>
</tr>
<tr>
<td>Y13</td>
<td>GGCTCGCGGT</td>
<td>10</td>
<td>500-2,800</td>
<td>0.345</td>
</tr>
<tr>
<td>Y15</td>
<td>AGTCGCCCTT</td>
<td>9</td>
<td>350-3,000</td>
<td>0.361</td>
</tr>
<tr>
<td>Z01</td>
<td>TCTGTGCCAA</td>
<td>6</td>
<td>450-1,600</td>
<td>0.011</td>
</tr>
<tr>
<td>Z07</td>
<td>CCAGAGGAGC</td>
<td>11</td>
<td>600-3,000</td>
<td>0.158</td>
</tr>
<tr>
<td>Z12</td>
<td>TCAACGGGCAC</td>
<td>8</td>
<td>1,000-6,000</td>
<td>0.242</td>
</tr>
<tr>
<td>Z14</td>
<td>TCGAGGGTTC</td>
<td>8</td>
<td>900-3,500</td>
<td>0.256</td>
</tr>
<tr>
<td>Z17</td>
<td>CTTTCCACT</td>
<td>10</td>
<td>400-5,000</td>
<td>0.374</td>
</tr>
</tbody>
</table>

PIC - polymorphism information content

An estimate of genetic variation among and within alfalfa varieties based on RAPD markers is presented in Table 3. Although none of the scored loci was monomorphic in all varieties, they were usually uniform in some populations, while showing different levels of polymorphism in others. The number and percentage of polymorphic loci, as well as effective number of alleles were the highest in variety Zuzana, while the most uniform was variety RSI 20. The values of expected heterozygosity in the tested varieties ranged from 0.217 to 0.256, with average of 0.226 within varieties and overall value of 0.286 among varieties. As a measure of the degree of variation within population, the Shannon’s diversity index was the lowest in RSI 20 (I=0.318) and the highest in Zuzana (I=0.375). The mean value within varieties was 0.322 and total genetic diversity across populations was 0.426. Among these five varieties, variety Zuzana exhibited the highest level of variation (P=104, P(%)=66.67, Ne=1.453, He=0.256, I=0.375), whereas variety RSI 20 exhibited the lowest level of variation (P=87, P(%)=55.77, Ne=1.385, He=0.217, I=0.318).
Most of the genetic variability estimated by AMOVA was attributed to variation among individuals within varieties (88.39%) and only 11.61% was found between varieties (Table 4).

**Table 3.** Estimates of genetic variation in parental alfalfa varieties, using RAPD markers.

<table>
<thead>
<tr>
<th>Variety</th>
<th>P (no.)</th>
<th>P (%)</th>
<th>Ne</th>
<th>He</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banat</td>
<td>90</td>
<td>57.69</td>
<td>1.389 ± 0.400</td>
<td>0.220 ± 0.212</td>
<td>0.323 ± 0.300</td>
</tr>
<tr>
<td>Ghareh</td>
<td>88</td>
<td>56.41</td>
<td>1.387 ± 0.389</td>
<td>0.217 ± 0.209</td>
<td>0.319 ± 0.298</td>
</tr>
<tr>
<td>Zuzana</td>
<td>104</td>
<td>66.67</td>
<td>1.453 ± 0.399</td>
<td>0.256 ± 0.208</td>
<td>0.375 ± 0.292</td>
</tr>
<tr>
<td>Pecy</td>
<td>88</td>
<td>56.41</td>
<td>1.393 ± 0.407</td>
<td>0.220 ± 0.215</td>
<td>0.322 ± 0.305</td>
</tr>
<tr>
<td>RSI 20</td>
<td>87</td>
<td>55.77</td>
<td>1.385 ± 0.402</td>
<td>0.217 ± 0.213</td>
<td>0.318 ± 0.302</td>
</tr>
<tr>
<td>Mean</td>
<td>91.4</td>
<td>58.59</td>
<td>1.399 ± 0.399</td>
<td>0.226 ± 0.211</td>
<td>0.322 ± 0.299</td>
</tr>
<tr>
<td>Overall</td>
<td>129</td>
<td>82.69</td>
<td>1.498 ± 0.377</td>
<td>0.286 ± 0.189</td>
<td>0.426 ± 0.257</td>
</tr>
</tbody>
</table>


**Table 4.** Analysis of molecular variance (AMOVA) for parental alfalfa varieties.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Df</th>
<th>SSD</th>
<th>Variance components</th>
<th>Percentage variation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>4</td>
<td>143.464</td>
<td>2.03623</td>
<td>11.61</td>
<td>&lt;10⁻⁵</td>
</tr>
<tr>
<td>Within populations</td>
<td>45</td>
<td>697.666</td>
<td>15.5369</td>
<td>88.39</td>
<td>&lt;10⁻⁵</td>
</tr>
<tr>
<td>Total</td>
<td>841.130</td>
<td>17.53992</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Df – degrees of freedom, SSD – sum of squared deviations, P – probability of obtaining a more extreme component estimate by chance alone, estimated by computing 16,000 permutations

**Cluster analysis**

UPGMA dendrogram was drawn to visualize relationships among parental alfalfa varieties (Figure 2). It showed that Banat and Ghareh formed one subcluster with high level of similarity, while Pecy and Zuzana formed another. These four varieties formed a consistent group (94% of bootstraps), while variety RSI 20 was apart. The cophenetic correlation coefficient was high for dendrogram clustering populations, with value $r = 0.885$.

The dendrogram representing the relationship between individual plants (Figure 3) did not divide all genotypes into distinct groups resembling the analyzed alfalfa populations. Genotypes from Banat and Ghareh varieties
Figure 2. Dendrogram of parental alfalfa varieties based on unweighted pair group arithmetic mean method cluster analysis of Jaccard’s genetic similarity coefficients. Numbers represent bootstrap confidence limits for 1000 replicates.

Figure 3. Dendrogram of 50 individual samples based on unweighted pair group arithmetic mean method cluster analysis of Jaccard's similarity coefficient. (B1 - B10 - Banat, G1 - G10 - Ghareh, Z1 - Z10 - Zuzana, P1 - P10 - Peczy, R1 - R10 - RSI 20). Numbers represent bootstrap confidence limits for 1000 replicates.
were divided in two groups, while Zuzana was distributed along the dendrogram, showing large intrapopulation diversity. The genotypes of variety Pecy formed one cluster with two subclusters: first with eight Pecy genotypes and the second with two Pecy and one Zuzana genotype. RSI 20 genotypes formed distinct cluster. The cophenetic correlation coefficient for dendrogram of individuals was \( r = 0.657 \).

**Progenies**

**Genetic diversity**

Twenty selected primers generated 217 bands ranging in size from 300 to 6000 bp (Table 5), with an average number of bands per primer of 10.85. Primer Y10 produced the highest number of bands (17), while primer Z14 produced the lowest (4). The most informative primer was Z17 with PIC value of 0.374.

**Table 5.** Description of RAPD primers used for analysis of alfalfa progenies.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Max. no. bands</th>
<th>Band size range (bp)</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>X03</td>
<td>TGGCGCAGTG</td>
<td>13</td>
<td>800-5000</td>
<td>0.341</td>
</tr>
<tr>
<td>X07</td>
<td>GAGCGAGGCT</td>
<td>12</td>
<td>550-2000</td>
<td>0.233</td>
</tr>
<tr>
<td>X09</td>
<td>GGTCTGGTTG</td>
<td>11</td>
<td>650-2700</td>
<td>0.250</td>
</tr>
<tr>
<td>X12</td>
<td>TCGCCAGCCA</td>
<td>12</td>
<td>350-2000</td>
<td>0.097</td>
</tr>
<tr>
<td>X17</td>
<td>GACACGGACC</td>
<td>15</td>
<td>450-2000</td>
<td>0.270</td>
</tr>
<tr>
<td>Y02</td>
<td>CATCGCCGCA</td>
<td>11</td>
<td>630-2300</td>
<td>0.266</td>
</tr>
<tr>
<td>Y05</td>
<td>GGCTGCGACA</td>
<td>6</td>
<td>1800-4500</td>
<td>0.275</td>
</tr>
<tr>
<td>Y06</td>
<td>AAGGCTCACC</td>
<td>13</td>
<td>500-3750</td>
<td>0.259</td>
</tr>
<tr>
<td>Y07</td>
<td>AGAGCCGTCA</td>
<td>11</td>
<td>700-3500</td>
<td>0.206</td>
</tr>
<tr>
<td>Y09</td>
<td>AGCAGCGCAC</td>
<td>11</td>
<td>900-2900</td>
<td>0.215</td>
</tr>
<tr>
<td>Y10</td>
<td>CAAACGTGGG</td>
<td>17</td>
<td>600-4000</td>
<td>0.330</td>
</tr>
<tr>
<td>Y11</td>
<td>AGACGATGGG</td>
<td>9</td>
<td>300-1500</td>
<td>0.303</td>
</tr>
<tr>
<td>Y13</td>
<td>GGGTCTCGGT</td>
<td>9</td>
<td>1300-5000</td>
<td>0.285</td>
</tr>
<tr>
<td>Y15</td>
<td>AGTCGCCCTT</td>
<td>12</td>
<td>350-3000</td>
<td>0.272</td>
</tr>
<tr>
<td>Y18</td>
<td>GTGGAGTGCAG</td>
<td>12</td>
<td>350-4000</td>
<td>0.291</td>
</tr>
<tr>
<td>Y19</td>
<td>TGAGGGTCCCT</td>
<td>15</td>
<td>450-1700</td>
<td>0.249</td>
</tr>
<tr>
<td>Z07</td>
<td>CCAGGAGGAC</td>
<td>7</td>
<td>1450-3000</td>
<td>0.154</td>
</tr>
<tr>
<td>Z12</td>
<td>TCAACGGGAC</td>
<td>8</td>
<td>1100-6000</td>
<td>0.156</td>
</tr>
<tr>
<td>Z14</td>
<td>TCGAGGGTTC</td>
<td>4</td>
<td>900-4000</td>
<td>0.256</td>
</tr>
<tr>
<td>Z17</td>
<td>CCTTCCCACCT</td>
<td>9</td>
<td>1000-3300</td>
<td>0.374</td>
</tr>
<tr>
<td>Total</td>
<td>217</td>
<td>300-6000</td>
<td>0.246</td>
<td></td>
</tr>
</tbody>
</table>

PIC - polymorphism information content
Eleven new bands, absent in the parental populations, were detected in the progenies. Some of them occurred in only one progeny population, like band 2200 bp Y07 which was specific for the progeny population PxG; band 3900 bp Z12 was specific for the progeny population PxB (Figure 4) and band 800 bp X17 was specific for the progeny population GxB. Some bands were present in two progeny populations: band 1400 bp X09 was present in populations GxP and PxB, while 750 bp Y02 band was present in populations PxB and PxZ. The other bands were detected in three or more hybrid populations. Three bands (350 bp, 550 bp and 650 bp) amplified by X12 occurred in one progeny population coming from self-pollinated offspring Zs. The band 750 bp X17 was present only in parental population Ghareh, but not in progenies.

**Figure 4.** Representative RAPD profiles of primer Z12, showing the occurrence of the new band in population Pecy x Banat. L1 - GeneRuler 100 bp plus DNA ladder, L2 - 1 kb DNA Ladder.

Hybrids had the highest level of genetic variability, while parameters of genetic variability were similar in parental populations and self-pollinated progenies (Table 6). The overall percentage of polymorphic bands
was 74.65% of the total number of bands. Progeny populations coming from cross-pollination showed the highest percentage of polymorphic bands (68.20%) and the effective number of alleles \((Ne = 1.414 \pm 0.383)\), while progeny populations coming from self-pollination and parental populations had similar values. The value of expected heterozygosity \((He)\) in the parental populations was 0.178, in self-pollinated progeny 0.177 and in hybrid progeny 0.240. The Shannon’s diversity index \((I)\) in parental populations was 0.260, in self-pollinated progeny was 0.259 and in \(F_1\) progeny 0.357.

**Table 6.** Estimates of genetic variation in alfalfa populations, using RAPD markers.

<table>
<thead>
<tr>
<th>Variety</th>
<th>P (no.)</th>
<th>P (%)</th>
<th>Ne ± 0.386</th>
<th>He ± 0.209</th>
<th>I ± 0.300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parents</td>
<td>95</td>
<td>43.70</td>
<td>1.313</td>
<td>0.178</td>
<td>0.260</td>
</tr>
<tr>
<td>S1</td>
<td>96</td>
<td>44.24</td>
<td>1.307</td>
<td>0.177</td>
<td>0.259</td>
</tr>
<tr>
<td>F1</td>
<td>148</td>
<td>68.20</td>
<td>1.414</td>
<td>0.240</td>
<td>0.357</td>
</tr>
<tr>
<td>Mean</td>
<td>113</td>
<td>52.07</td>
<td>1.345</td>
<td>0.198</td>
<td>0.292</td>
</tr>
<tr>
<td>Overall</td>
<td>162</td>
<td>74.65</td>
<td>1.440</td>
<td>0.256</td>
<td>0.383</td>
</tr>
</tbody>
</table>

P (no) – number of polymorphic loci, P (%) – percentage of polymorphic loci, Ne – effective number of alleles, He – expected heterozygosity, I – Shannon’s information index, S1 – self-pollinated progeny, F1 – hybrid progeny.

**Table 7.** Analysis of molecular variance (AMOVA) for alfalfa populations.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Df</th>
<th>SS</th>
<th>Variance components</th>
<th>Percentage variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among groups</td>
<td>2</td>
<td>124.450</td>
<td>4.663</td>
<td>14.6%</td>
</tr>
<tr>
<td>Within groups</td>
<td>27</td>
<td>735.850</td>
<td>27.254</td>
<td>85.4%</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>860.300</td>
<td>31.917</td>
<td>100%</td>
</tr>
</tbody>
</table>

Df – degrees of freedom, SS – sum of square, Fixation index = 0.146, P <0.001

Most of the genetic variability (85.4%) estimated by AMOVA was attributed to variation within population groups, and only 14.6% was found between the tested groups (Table 7.).

**Cluster analysis**

A genetic distance based analysis was performed by calculating the Jaccard’s coefficient similarity. The distance ranged from 0.8344 (between populations RxZ and RxP) to 0.5823 (between populations Zuzana and Bs), indicating that there was a high amount of genetic diversity among the tested populations.

UPGMA dendrogram was drawn to visualize relationships among parental populations and their progenies (Figure 5). Hybrid progenies and
Figure 5. Dendrogram generated by unweighted pair group arithmetic mean method (UPMGA) cluster analysis showing relationships between 30 alfalfa populations based on RAPD profiling.

parental populations formed the first cluster, while self-pollinated progenies formed second cluster with high bootstrap values. In the first cluster, only hybrids with Zuzana as parental variety formed distinct group, as well as hybrids with parental variety RSI.

Discussion

The assessment of genetic diversity and structure of germplasm, as well as their relationship, is essential for the efficient organization and utilization of breeding material. Having that in mind, the aim of this research was to estimate genetic variation of populations already included in alfalfa breeding program and discuss how that knowledge might affect their further involvement.

Most alfalfa varieties are genetically broad-based, developed by crossing selected parents and improving their offspring through several generations. It is well known that inbreeding tetraploid alfalfa results in more substantial depression in vigor (yield) than might be expected based solely on the decrease in heterozygosity. The large variation may improve the adaptation to a wide range of environments, but the high level of genetic variation may
also slow down genetic progress by slowing the concentration of desirable alleles and limiting the purging of deleterious alleles (Veronesi et al., 2010).

Since the investigation was done on the valuable breeding material, the genetic diversity was estimated by using a larger number of RAPD primers than would usually be used in such investigation (Gherardi et al., 1998; Tucak et al., 2008), with the aim to generate as many polymorphic bands as possible. Genetic diversity in parental populations was high, which agrees with previous research on tetraploid alfalfa (Noeparvar et al., 2008; Mengoni et al., 2000). A very high level of genetic variation within varieties, detected by AMOVA, is in agreement with results of investigations done on diverse alfalfa populations (Flajoulot et al., 2005; Pupilli et al., 2000). This contrasts with results of Crochemore et al. (1996), who attributed 50% of the total variance to within-population genetic variability in landraces and varieties of distinctly different origins. The large genetic variation at the intrapopulation or variety levels can be explained by alfalfa alogamy, its autotetraploidity and sexual propagation. It can also be a reflection of differences in the amount and type of germplasm used for variety development (Tucak et al., 2008).

The cluster analysis of individuals in parental populations clearly illustrated population structure of the tested varieties. It showed the presence of subpopulations in Banat and Ghareh, but also illustrated that varieties Pecy and RSI 20 are more uniform, forming tight, clearly defined clusters. As the estimates of genetic diversity indicated, variety Zuzana showed very high level of variation (Nagl et al., 2011 a, b). The obtained results are in accordance with breeding history of the analyzed varieties: Zuzana is old, very adaptable synthetic variety developed by crossing large number of parents, Banat and Ghareh were developed mainly by collecting local germplasm with certain level of uniformity in yield and morphological traits, while Pecy and RSI 20 resulted from intensive breeding programs.

The use of investigated populations in the alfalfa breeding programs would, in our opinion, depend on their population structure and diversity. The population Zuzana, with high level of genetic variation, might be used as a source for selection of desirable germplasm and development of new varieties. Sub-populations in Banat and Ghareh could be used for development of new, unique and homogenous germplasm. The germplasm with higher level of uniformity, like Pecy and RSI 20 in this investigation, could be used as potential parents in semi-hybrid breeding program, for producing hybrid progenies.

The previous research in alfalfa showed that selection of very diverse parental genotypes according to their molecular markers distance has not been successful in predicting heterosis (Kidwell et al., 1999; Riday et al., 2003; Maureira et al., 2004). However, the populations selected for our
investigation, although with different geographic origin, were bred for
growing in similar environmental conditions. Therefore, we suggest that if
highly adapted, relatively uniform populations, with optimal coefficient of
similarity, are taken as the parental components, the chances of predicting
hybrid effect might increase.

In the analysis of bulk samples, some of the polymorphic bands were
detected only in the parental varieties, while some bands could be detected
only in progenies. This might indicate the presence of specific loci in the
genotypes studied, especially variety Pecy, since it was parental population
for the most hybrids where new bands were detected. It is well known that
RAPD variation resides in many different types of mutational events that
occur in the annealing site of the primer and between the two adjacent sites
responsible for the amplification (Mengoni et al., 2000). Therefore, the
presence or absence of RAPD bands may indicate the occurrence of genetic
changes in the genome of the hybrids, either through the loss or
rearrangement of some of their nucleotides (Farzaneh et al., 2010). Also,
detection of the band 750 bp, generated by primer X17, in the parental
variety Ghareh, indicates that some primers have potential to be used in
identification of alfalfa breeding material (Taški-Ajduković et al., 2014a, b).

Although variability of the tested populations was high, it could be
noticed that self-pollinated progenies and parental populations had similar
values of genetic variability parameters, which were smaller than in F₁
hybrids. One of the reasons for it might be a decrease of heterozygosity
levels and increased similarity within populations, due to self-pollination
(Rotilli et al., 1999). Furthermore, the parental populations are maintained
through open pollination, which enables crosses between similar and/or
closely related plants, thus reducing variability and heterozygosity.

The AMOVA revealed a higher distribution of genetic variation within
groups of populations (parental varieties, self-pollinated progenies, hybrids),
then between them. It is to be expected, since the individual RAPD analysis
of parental varieties revealed very high distribution of genetic variation
within them (Nagl et al., 2011).

Since the selfing of alfalfa not only reduced level of heterozygosity,
leading to higher similarity within population, but also increased the
differences between parental populations and their S₁ progenies, cluster
analysis grouped self-pollinated progenies in separate sub-cluster or group.
Additionally, cluster analysis placed the hybrid offspring where Žuzana, and
RSI 20 were maternal parents in separate groups (Taški-Ajduković et al.,
2013). In our opinion, this could be the consequence of maternal effect,
which was already confirmed in diallel crosses, on this breeding material, for
plant height and number of stems (Milic et al., 2011).
Although the bulked-sample DNA analysis with RAPD markers results in fewer bands compared with analysis of individual samples, it can be used in estimation of genetic relatedness among heterogeneous alfalfa cultivars (Chandra, 2007). The results of this study show that they can also be very efficient in evaluating the genetic relationships between closely related genetic material, i.e. parental populations and their offspring. Use of bulked DNA samples can reduce the number of individual analysis, and enable the screening of the larger number of populations, making the use of MAS in alfalfa breeding programs more cost-effective.

Acknowledgment

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References


63. Taski-Ajdukovic K., Nagl N., Milic D., Katic S. Genetic variation and relationships of tetraploid alfalfa parental populations and their progenies based on RAPD markers. 30th EUCARPIA Fodder Crops and Amenity Grasses Section Meeting, 12-16. May 2013, Vrnjacka Banja, Serbia, 86.


