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Full Length Research Paper

Assessment of genetic relatedness of the two *Amaranthus retroflexus* populations by protein and random amplified polymorphic DNA (RAPD) markers

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Two populations of *Amaranthus retroflexus* with different morphology were collected from field of the Maize Research Institute Zemun Polje, Serbia. Random amplified polymorphic DNA (RAPD) and seed protein analysis were performed to study the genetic differences in two grain *Amaranthus* populations. The studied populations have different protein and DNA profile. A total of 171 DNA fragments were generated by 31 RAPD primers, with an average of 5.5 fragments per primer. Of these, 61.4% fragments were polymorphic among the two populations. 18 protein fraction were obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The populations differed in the four protein fractions of different molecular weight. The seed protein electrophoresis and RAPD markers are useful for genetic determination of *A. retroflexus* populations and identification of biotypes with atypical morphology.

Key words: *Amaranthus retroflexus*, biotypes, molecular markers, proteins.

INTRODUCTION

The genus *Amaranthus,* with about 70 species, is characterized with a high degree of morphological diversity and a wide spectrum of adaptability to different ecological conditions. The amaranth gene pool involve a diverse group of wild relatives and weedy species, a group of cultivated grain species, and individual landrace populations collected from unique localities. The weed species *A. retroflexus* L., commonly referred as pigweeds, is one of the most widespread and frequent weeds of arable land worldwide as well as in Serbia (Stanojevic et al., 1996; Vrbnicanin et al., 2008). Populations of species *A.retroflexus* share many features, as a tendency to mutation and hybridization, and they demostrate different reaction to acetolactate synthase inhibitors that could be related to a evolution of resistance in their population (Ferguson et al., 2001).

Correct genotype identification is important to evaluate the genetic diversity of local *Amaranthus*, and for efficient weed control, but is often difficult because of similar morphological characteristics among species and variation within species, so misidentification is common (Horak et al., 1994; Wax, 1995). According to Wetzel et al. (1999) 12 of 92 *Amaranthus* accessions that had been collected and identified by weed scientists were misidentified. Ahrens et al. (1981) found that 13 of 14 accessions that were identified as redroot pigweed were actually smooth pigweed (*Amaranthus hybridus* L.) or Powell amaranth (*Amaranthus powellii* S. Wats.). Electrophoresis analysis of seed proteins proved to be useful for distinguishing species and cultivars of *Amaranthus*, for describing similarity between species and for estimation of its outcrossing rate (Zheleznov et al., 1997; Drzewiecki, 2001; Juan et al., 2007). For more accurate study of genetic diversity and phylogenetic relationships between *Amaranthus* species, different molecular markers including random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and amplified fragment length polymorphism (ALFP) have been used (Xu and Sun 2001; Wassom and Tranel 2005; Lee et al.,

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2008; Ray et al., 2008, Popa et al., 2010). The RAPD is a prefferd method for identification of genotypes because it is relatively inexpensive, utilizes arbitrary primers, and randomly samples a potentially large number of loci in a less complex pattern than other polymerase reaction (PCR) based markers (Hadrys et al., 1992; Williams et al.,1993, Das et al., 2005). Transue et al. (1994) and Chan and Sun (1997) used RAPD markers for the study of evolutionary relationships among grain amaranths and their wild relatives. Popa et al. (2010) studied genetic diversity and phylogenetic relationship among six species of *Amaranthus* by RAPD markers and showed that there is slightly intra and inter species polymorphism.

The *A. retroflexus* green plants with pronounced stem hairiness are widespread and frequently occurred weed in maize fields in Serbia as well as in field of Maize Research Institute, Belgrade. Recently, the plants of *A. retroflexus* with green plant with pronounced red pigment admixture and with sparse stem hairs were new appeared genotypes in part of same field of Maize Research Institute, Belgrade. The objective of this study was to evaluate the level of genetic differences between two populations of *A. retroflexus* as well as usefulnes of seed protein electrophoresis and RAPD markers for distinquishing plants with atypical morphology.

MATERIALS AND METHODS

Two *A. retroflexus* populations, population 1 (green plant with pronounced red pigment admixture and with sparse stem hairs) and population 2 (green plant with pronounced stem hairiness) were collected from field of the Maize Reserch Institute Zemun Polje. The limited number of plant of population 1 was the reason why we collected only 12 plants from each population.

DNA was extracted from leaf tissue of randomly chosen five individual plants as well as from pooled samples of all collected plants (12) of each populations by the method of Rogers and Bendich (1985). Approximately 250 mg young leaf tissue was frozen in liquid nitrogen, and ground to fine powder in a pre-cooled mortar. The ground tissue was suspended in extraction buffer (100 mM Tris-Cl pH 8.0, 1.4 m NaCl, 20 mM EDTA, 2%CTAB, and 1% PVP) and incubated 30 min at 65°C with occasional mixing by inversion, followed by chloroform extraction. After precipitation with CTAB precipitation puffer, dried samples were dissolved in TE puffer. The quality and concentration of the DNA was evaluated by viewing samples in agarose gels and by spectrophotometer analysis of 260 and 280 nm light absorption.

An analysis of five randomly choosen individual plants of each populations was conducted with the nine selected RAPD primers. For futher analysis PCR amplification of bulked DNA from all 12 plants, was tested on 40 10-mers arbitrary RAPD primers (Genosys Biotechnologies, Operon Technologies) in two rounds of amplification by the modified method of Williams et al. (1990). The list of RAPD primers is shown in Table 1. RAPD reactions were done in a volume of 25 µl containing 2.5 mM $MgCl_2$, 100 µl dNTPs, 0.2 µl of decamer primers, 2.5 U of Taq Polymerase (Fermentas, Canada), and 10 ng of template DNA. Amplifications were carried out in a PTC-100 Thermocycler (MJ Research, Waltham, MA) with the following program: an initial denaturation step at 94°C for 2 min followed by 45 cycles at 94°C for 30 s, annealing at 40°C for 1 min, and extension at 72°C for 1 min and a final cycle at 72°C for 7 min. The amplified products were separated by electrophoresis in 1.5% agarose in a 1×TBE buffer (Tris-borate 89 mM and EDTA 0.5 M pH 8.0), visualized after ethidium bromide staining and photographed under UV light. The Generuler 1kb DNA Ladder (Fermentas, Canada) was used as a standard molecular weight marker.

The band of equal molecular weight and mobility generated by the same RAPD primer were considered to be individual loci. Only consistely reproducible, well resolved fragments in the size range of ~250 to 3000 bp were scored. The presence or absence of each individual band was recorded for each line of the gel representing different plant sample.

Proteins were extracted from seeds of two populations according to Wang et al. (1994). Isolated proteins along with a PAGE ruler unstained protein ladder (Fermentas, Canada) were analyzed through SDS-PAGE following discontinuous method of electrophoresis (Laemmli, 1970). The gel concentration was 10%. After electrophoresis, gel was stained with coomassie brilliant blue for about 20 to 30 min and then destained in 5% methanol and 20% acetic acid until the color of the background disappeared and the electrophoretic bands were clearly visible. The differences in number, pattern and intensity of protein fractions were determined by direct observation of gels and photographs.

RESULTS

In the study of the 40 primers tested, an initial screening resulted in selection of 31 decamer primers that produced clear and reproducible RAPD profiles. RAPD assays of each population were performed at least two times each, with only reproducible, amplified fragments being scored. Initially screening of the randomly chosen five plants of both populations with the nine primers showed an identical or very similar profile of amplification products within individuals of same population (Figure 1). As observed, intrapopulation polymorphism was quite low for further analysis; we used group samples (bulk DNA) per population.

The RAPD data in the present study indicated that no two primers revealed identical profiles in the analyzed populations (Figure 2). RAPD primers yielded a total of 171 fragments, ranging from 250 to 3000 bp in size of which 105 fragments (61.4%) were polymorphic. The total number of polymorphic loci detected varied between primers. The primer GEN 2-80-4 gave the highest number of fragments (13), while the minimum number of fragments in the two analysed populations (2) was obtained with OPB05, OPB09, and GEN1-70-3 primers. One primer, Gen 2-80-3, was monomorphic. The average number of polymorphic fragments per primer among the two populations was 5.5. The percent polymorphism of primers ranged from 0% (GEN 2-80-3) to 88.8% (OPB01), the average was 56.7%. In OPB-01, eight bands out of nine were noted as polymorphic bands, whereas in GEN 4-70-5, six out of seven bands were polymorphic. Certain amplified bands appeared to be common to both populations, whereas others were present in one population, but absent in the other. Primers Gen-2-80-1 and OPB09, after confirmation, could be used as markers to differentiate population of *A. retroflexus* plants with normal morphology from

Table 1. List of primers along with their percent of polymorphism.

Primer	Sequence	Total number of fragment	Number of polmorphic fragment	Polymorphism (%)
GEN 4-70-10	CGCAGACCTC	8	5	62.5
GEN 2-80-4	GCAGCTCCGG	13	8	61,5
GEN 4-70-9	CCGGGGTTAC	10	7	70.0
GEN 4-7-5	CATGTCCGCC	7	6	85.7
GEN 2-80-8	GGCCACAGCG	9	6	66.6
GEN 1-70-5	TAGATCCGCG	4	2	50.0
GEN 1-80-9	GCACGGTGGG	5	4	80.0
GEN 2-80-1	GCAGCAGCCG	4	1	25.0
GEN 4-70-3	CTGTCGGCTC	10	4	40.0
GEN 2-80-10	CGCGAACGGC	5	3	60.0
GEN 1-70-3	ACGGTGCCTG	2	1	50.0
GEN 1-70-9/1	TGCAGCACCG	4	2	50.0
GEN 4-70-2	GGACCGACTG	5	4	80.0
GEN 2-80-3	ACCCGTCCCC	1	0	0.0
GEN 4-70-8	GAGAGGGAGG	6	3	50.0
GEN 4-70-7	CTATCGCCGC	5	4	80.0
GEN 1-80-4	CGCCCGATCC	4	2	50.0
GEN 1-70-1	CATCCCGAAC	0	0	0.0
GEN 4-70-4*	GGACCGCTAG	0	0	0.0
GEN 1-70-9/2*	GGACTCCACG	0	0	0.0
GEN 2-80-7|*	GCAGGTCGCG	0	0	0.0
GEN 2-80-5*	CGAGACGGGC	0	0	0.0
GEN 2-80-6*	ACCGCCTCCC	0	0	0.0
GEN 4-70-1*	GCCCCTCTTG	0	0	0.0
GEN 1-70-4*	CGCATTCCGC	0	0	0.0
OPB01	GTTTCGCTCC	9	8	88.8
OPB06	TGCTCTGCCC	3	2	66.6
OPB09	TGGGGGACTC	2	1	50.0
OPB 02*	TGATCCCTGG	0	0	0.0
OPB19	ACCCCCGAAG	3	2	66.6
OPB03	CATCCCCCTG	5	3	60.0
OPB05	TGCGCCCTTC	2	1	50.0
OPB11	GTAGACCCGT	5	3	60.0
OPB18	CCACAGCAGT	7	3	42.8
OPB04	GGACTGGAGT	3	2	66.6
OPB07	GGTGACGCAG	8	5	62.5
OPB12	CCTTGACGCA	7	4	57.1
OPB13	TTCCCCCGCT	4	1	25.0
OPB17	AGGGAACGAG	6	3	50.0
OPB20	GGACCCTTAC	5	4	80.0

*no clear and reproducible fragments

population with atypical morphology.Seed protein profiling of two *A. retroflexus* populations showed distinct polymorphism in electrophoretic banding patterns and led to detection of a total of 18 polypeptide bands of which 14 were polymorphic (Figure 3). The molecular weights of peptides ranged from 20 to 200 kDa with the presence or absence of particular band. The 22% variation in seed protein fraction was observed between the two popu-

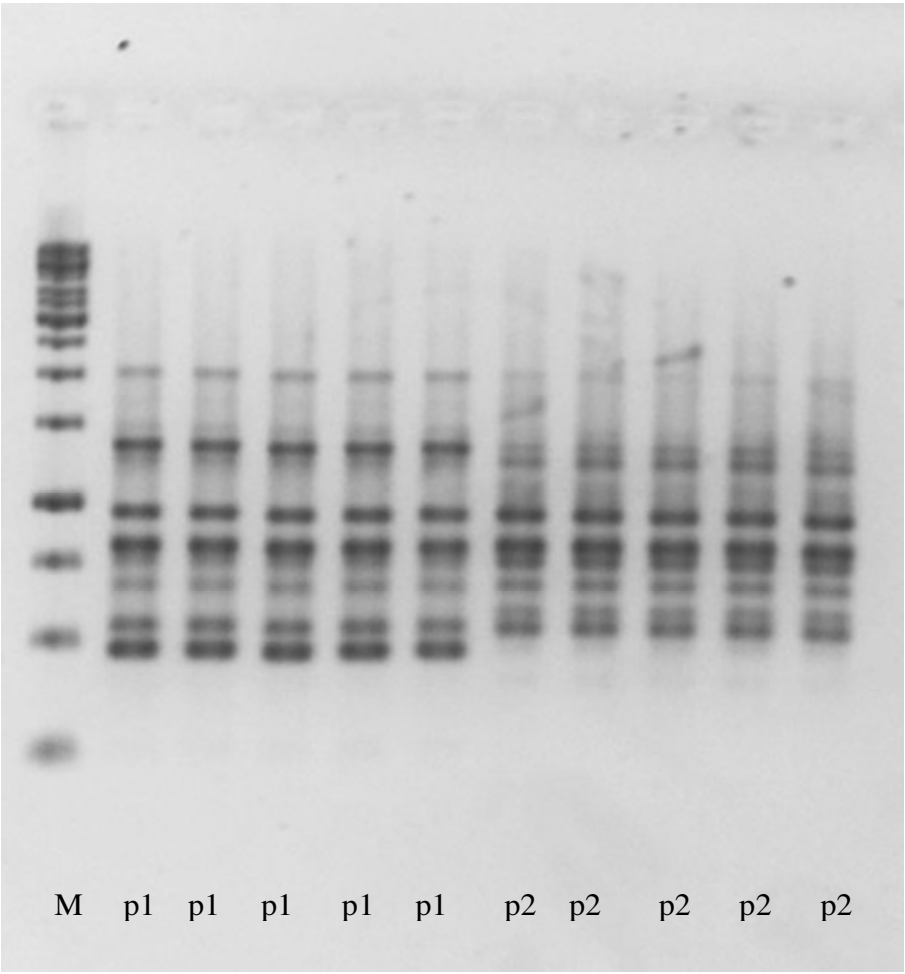

Figure 1. RAPD-agarose gel image of five individual plants per population with GEN 4-70-3.

lations. Population 1 had four more bands of different molecular weight compared with population 2. Seed protein profiling using SDS-PAGE has the potential to make a distinction between plants with atypical morphology from plants with normal one.

DISCUSSION

Weedy species of the genus *Amaranthus* have increased in frequency and severity over the past years. Identification of these weeds is difficult because of similar morphological characteristics among species and variation within species. In Serbia, *A. retroflexus* is an invading and economically harmful weed species. In the Maize Research Institute field, two biotypes of *A. retroflexus* is presented; population 1 having stem covered in sparse hairs and population 2 with notable dense stem hairs. The results of previous study of morphological traits of those two populations (Vrbnicanin et al., 2009) indicate that apart from differences in hairiness, they differ also in the anatomy of stem (stem diameter, epidermis thickness, cortex diameter, collenchymas thickness, and central cylinder diameter) as well as leaf anatomy (mesophyll thickness). According to same authors, such differences between populations provide basis for better understanding of plant reaction in terms of herbicide uptake and translocation that are potentially connected to an evolution of resistance to herbicides in the locality Zemun Polje, Serbia. To aid in the identification of morphologically atypical genotypes, the seed proteins was analyzed by SDS PAGE electrophoresis. Seed proteins, as genetic markers, are relatively frequently applied in the study of plant genomes for distinguishing species and for describing similarity between species, but there are relatively few studies on *Amaranthus* (Gudu and Gupta, 1988; Gorinstein et al., 1991; Zheleznov et al., 1997; Drzewiecki, 2001; Juan et al., 2007). The protein patterns obtained from the two populations consisted of 18 fraction of which 78% was polymorphic. Some polypeptides, presented in both analyzed populations, showed “conservativeness”, whereas some protein fractions were variable between populations. By protein patterns, population 1 with

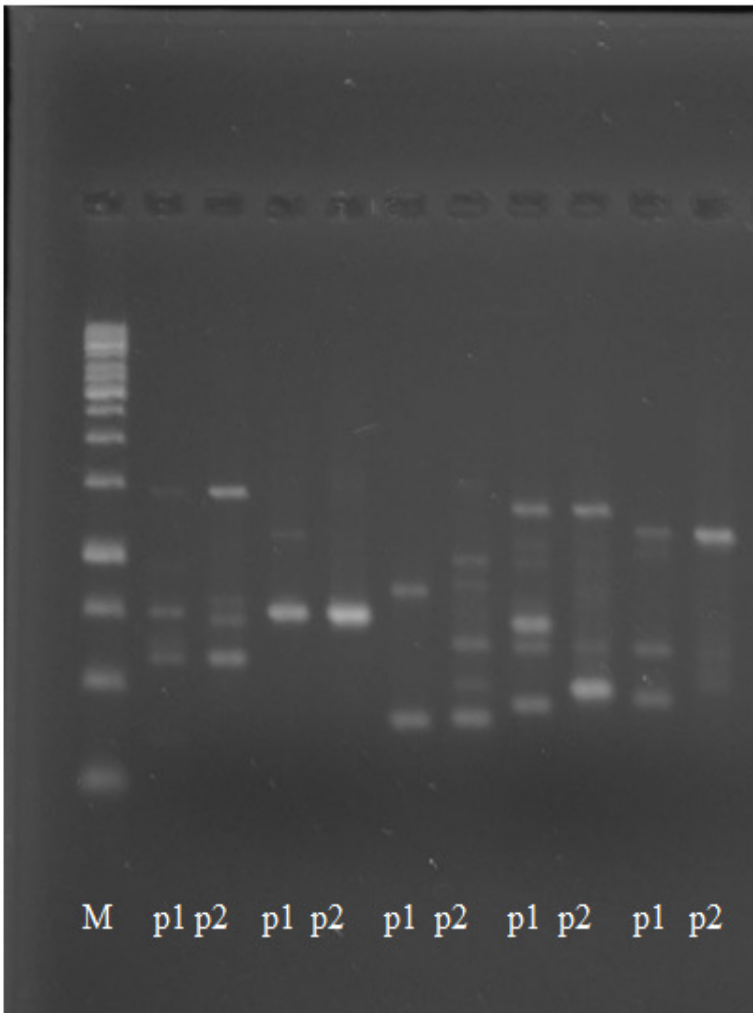

Figure 2. Polymorphic RAPD-agarose gel image of the two populations (p1, p2) obtained with primer OPB03, OPB05, OPB11,OPB18 and GEN 1-70-5.

atypical morphology clearly differs from the other population. Our results are in agreement with literature data that *Amaranthus* seed protein is characterized by essential molecular heterogeneity. According to Zheleznov et al. (1997), electrophoretic analysis of storage proteins is a very useful method of describing phylogenetic relationships between the ten species of *Amaranthus*, while Gorinstein et al. (1991) obtained very slight differences of proteins between four *Amaranthus* species.

The use of RAPD assay to identify genetic variation is preferred over the morphological and biochemical markers since these are completely devoid of the effects of environment and the stage of the experimental material, thus making them highly reliable. Analysis with nine RAPD markers of individual plants of both popualtions revealed a realtively low polymorphism. It is possible that the primers we used amplified mostly the conserved part of genome so they show low variation. So, for futher analysis, we used bulk DNA sample per population. This approach had positive results in the study of populations of a great number of plant species (Transue et al., 1994; Roman et al., 2003). 31 decamer primers selected for RAPD profiling of two populations produced 105 (61.4%) polymorphic and 66 monomorphic banding sites. The level of polymorphism is comparable with the results for other *Amaranthus* species (Mandal and Das, 2002; Popo et al, 2010). Our results indicate that RAPD markers could be useful for verifying the identity of genotypes with ambiguous or a typical morphology.

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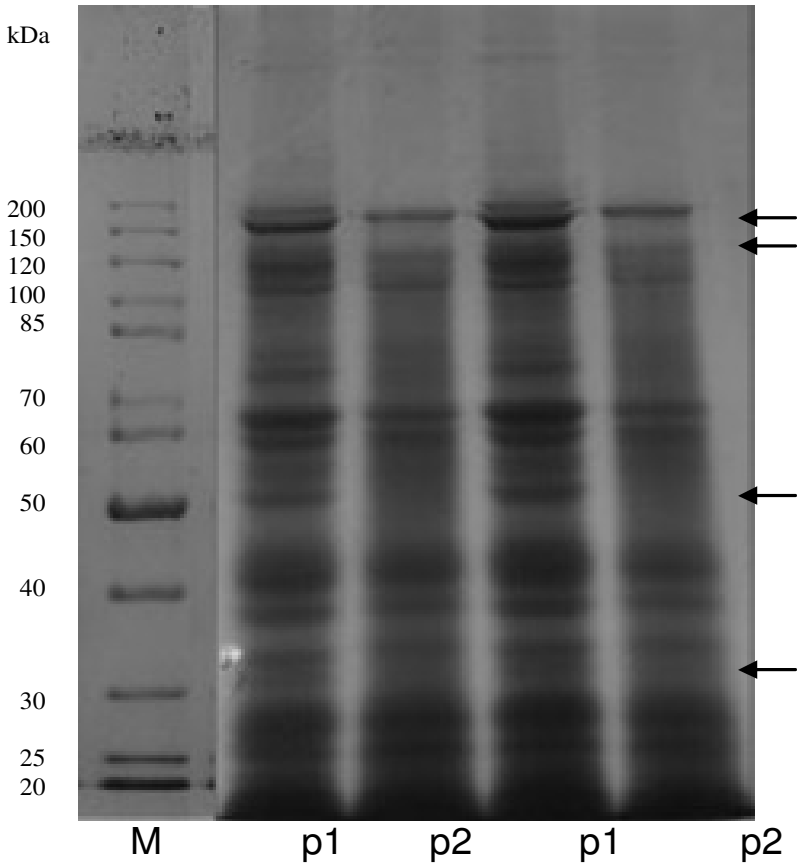

Figure 3. SDS PAGE electrophoresis of seed from the two populations.

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