Biochemical Systematics and Ecology 62 (2015) 20–24

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Genetic relationships among Eurasian *Puccinellia distans* genotypes

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ARTICLE INFO

Article history:
Received 13 April 2015
Received in revised form 22 July 2015
Accepted 26 July 2015
Available online 3 August 2015

Keywords:
Sequence related amplified polymorphism markers
SRAP
Weeping alkali grass

ABSTRACT

Puccinellia distans (Jacq.) Parl. is a common grass species found throughout the world. It can grow in arid and saline environments as well as under toxic boron concentrations. In this work we performed sequence related amplified polymorphism (SRAP) marker analysis on 20 wild *P. distans* genotypes to understand the genetic relationships among different genotypes and subspecies. We tested 119 SRAP primer pairs and found that 43 were polymorphic. The molecular data were then analyzed to determine the genetic relationships and population structure of the genotypes. We were able to trace the origin of genotypes that were carried to distant locations or gathered for research purposes. We also found that geographical distance between genotypes was not an important determinant of genetic relationships as even distantly located *Puccinellia* genotypes were closely related. As *P. distans* is known to be tolerant to salinity stress and toxic mineral concentrations, the findings of this work can be used as a starting point for selection of genotypes that should be tested under such conditions.

1. Introduction

Puccinellia distans (Jacq.) Parl., more commonly known as weeping alkali grass, is a perennial grass species originating from Eurasia (Hitchcock, 1971; Tarasoff et al., 2007). *P. distans* has been dispersed throughout the world via different methods including wind, traffic flow (Scott and Davison, 1985;Šera, 2010) and potentially even in mud stuck to cars (Schmidt, 1989). Currently, *P. distans* and its subspecies are most frequently found in saline meadows, alkaline waterside sand and gravel, along roads, in oases, and near springs (Grubov, 2001). In previous studies, weeping alkali grass was shown to have good tolerance to salt, drought and other abiotic stress conditions (Hughes et al., 1975). In addition, it is a hyperaccumulator of boron and can grow in areas with boron levels that are toxic to other species (Stiles et al., 2010). For this reason, *P. distans* is a good model for phytoremediation of soil in boron polluted areas and has been investigated for such use in previous studies (Aydın and Çakır, 2009; Stiles et al., 2011).

The study of genetic diversity is important because it helps in understanding the amount of variability present in individuals and populations. Such variability can tell us about an organism's past and help to determine its vulnerability to future environmental conditions. Molecular markers are often used to study diversity and to determine genetic relationships among accessions/genotypes within a species in different locations. Sequence related amplified polymorphism markers (SRAPs) are simple and reliable molecular markers that can be used for DNA fingerprinting with a moderate throughput ratio

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http://dx.doi.org/10.1016/j.bse.2015.07.031
0305-1978/

(Li and Quiros, 2001). SRAP markers are especially suited to studies of genetic diversity because they target random coding sequences within the genome. Such sequences tend to mutate less frequently than non-coding regions and provide a moderate number of markers (Li and Quiros, 2001). These properties of SRAP markers are important since the genotypes used in this work are not inbred. They are wild genotypes which may have several different alleles that can be used to determine their genetic relationships.

In this work we used SRAP markers to analyze the genetic relationships between *P. distans* genotypes collected from different countries as well as different locations within the same country. Although the phylogenetic relationships among *Puccinellia* species and other grasses were examined by Choo et al. (1994), this is the first systematic work using genotypes of *P. distans* from different locations of the world. We performed our work with 20 genotypes of *P. distans*, 17 of which were collected from nature in mid-Eurasia and three used for research purposes in the USA.

2. Materials and methods

2.1. Plant material

A total of 20 *P. distans* genotypes from different locations were used in this work (Table 1). Nineteen of these varieties were provided by the Germplasm Resource and Information Network, United States Department of Agriculture (GRIN, USDA), Washington DC and one was provided by Mehmet Babaoglu, Department of Field Crops, Faculty of Agriculture, Selcuk University, Konya Turkey. Plants were germinated in potting soil and grown for two weeks until they were approximately 10 cm long. Fresh leaves were immediately used for DNA extraction.

2.2. DNA extraction

DNA was extracted as bulk from leaves of approximately 20 plants of the each accession following a modified CTAB extraction method (Doyle and Doyle, 1990). After extraction, DNA concentration was determined by spectrophotometric measurement. Concentration of the samples was adjusted to 20 ng/μl. All genetic materials were stored at −20 °C.

2.3. Molecular marker analysis

SRAP analysis was performed with 7 forward and 17 reverse primers. A total of 119 forward and reverse primer combinations were tested using ME1 to ME7 and EM1 to EM17, respectively (Table 2). PCR mix consisted of 2 μl of 10X PCR Buffer, 0.01 mM F and R primers, 25 mM $MgCl_2$, 20 ng/μl Genomic DNA, 0.75 μl of 40 mM dNTP mix, 1 U Taq polymerase; 8.25 μl H_2O to a total of 20 μl. PCR amplification was performed in two stages. The first stage started with 5 min at 94 °C, 1 min at 94 °C, 1 min at 35 °C, 1 min at 72 °C for 5 cycles. The second stage continued with 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C, for 35 cycles finishing with 10 min at 72 °C. PCR products were then separated on 1% agarose gels and bands were observed in BioRad Gel Doc XR imaging system.

Table 1
Puccinellia distans genotypes from different locations in mid-Eurasia used in this study.

No	Accession	Species name	Origin	Source
1	Eskişehir	*Puccinellia distans* (Jacq.) Parl. ssp. *distans*	Turkey	Selcuk University
2	PI311722	*P. distans* ssp. *borealis*	Turkey	GRIN
3	PI316340	*P. distans* ssp. *borealis*	Turkey	GRIN
4	PI384942	*P. distans*	Iran	GRIN
5	PI381010	*P. distans*	Iran	GRIN
6	PI381009	*P. distans*	Iran	GRIN
7	PI381008	*P. distans*	Iran	GRIN
8	PI229458	*P. distans* ssp. *sevangensis*	Iran	GRIN
9	PI230249	*P. distans*	Iran	GRIN
10	PI230250	*P. distans*	Iran	GRIN
11	PI221955	*P. distans*	Afghanistan	GRIN
12	PI251220	*P. distans*	Afghanistan	GRIN
13	PI578856	*P. distans* FULTS strain	USA	GRIN
14	PI600922	*P. distans* FULTS strain	USA	GRIN
15	PI443386	*P. distans*	USA	GRIN
16	PI502581	*P. distans*	Former Soviet Union	GRIN
17	PI502580	*P. distans*	Russian Federation	GRIN
18	PI502582	*P. distans*	Russian Federation	GRIN
19	PI440628	*P. distans*	Kazakhstan	GRIN
20	PI251164	*P. distans* ssp. *limosa*	Former Serbia and Montenegro	GRIN

Table 2
Forward and reverse primers used in SRAP analysis.

Primer	Sequence
ME1 (Forward)	5′TGAGTCCAAACCGGATA3′
ME2 (Forward)	5′TGAGTCCAAACCGGAGC3′
ME3 (Forward)	5′TGAGTCCAAACCGGAAT3′
ME4 (Forward)	5′TGAGTCCAAACCGGACC3′
ME5 (Forward)	5′TGAGTCCAAACCGGAAG3′
ME6 (Forward)	5′TGAGTCCAAACCGGTAG3′
ME7 (Forward)	5′TGAGTCCAAACCGGTTG3′
EM1 (Reverse)	5′GACTGCGTACGAATTAAT3′
EM2 (Reverse)	5′GACTGCGTACGAATTTGC3′
EM3 (Reverse)	5′GACTGCGTACGAATTGAC3′
EM4 (Reverse)	5′GACTGCGTACGAATTTGA3′
EM5 (Reverse)	5′GACTGCGTACGAATTAAC3′
EM6 (Reverse)	5′GACTGCGTACGAATTGCA3′
EM7 (Reverse)	5′GACTGCGTACGAATTATG3′
EM8 (Reverse)	5′GACTGCGTACGAATTAGC3′
EM9 (Reverse)	5′GACTGCGTACGAATTACG3′
EM10 (Reverse)	5′GACTGCGTACGAATTTAG3′
EM11 (Reverse)	5′GACTGCGTACGAATTTCG3′
EM12 (Reverse)	5′GACTGCGTACGAATTGTC3′
EM13 (Reverse)	5′GACTGCGTACGAATTGGT3′
EM14 (Reverse)	5′GACTGCGTACGAATTCAG3′
EM15 (Reverse)	5′GACTGCGTACGAATTCGT3′
EM16 (Reverse)	5′GACTGCGTACGAATTCGG3′
EM17 (Reverse)	5′GACTGCGTACGAATTCCA3′

2.4. Data analysis

Agarose gel electrophoresis results were interpreted and polymorphic bands were determined for each primer pair. A scoring matrix was constructed with a score of 1 used for the presence of a specific band and 0 for the absence of that band. The matrix was processed in the DARwin5 (Perrier and Jacquemoud-Collet, 2006) program into a dissimilarity matrix using the Jaccard algorithm. The dissimilarity matrix was then used to reconstruct a tree with the unweighted neighbor joining method and the fit of the matrix to the tree was calculated with the fit criterion built in the program. Data were also subjected to a second analysis program, Structure (Pritchard et al., 2000). This program was used to calculate subpopulation (K) structure using K values of 1–10 with 20 iterations.

3. Results

In this work, seven forward primers (ME1 to ME7) and 17 reverse primers (EM1 to EM 17) were assayed on 20 *P. distans* genotypes. Of these 119 primer pair combinations, 43 (36.1%) were polymorphic. The polymorphic primer pairs provided 219 polymorphic bands which were scored for further analysis.

The dissimilarity matrix computed from the marker data gave a minimum value of 0.07 between genotypes PI502581 (16) and PI443386 (15) indicating that these genotypes were highly similar. The maximum dissimilarity between genotypes was 0.84 and was calculated between PI230249 (9) and Eskişehir (1) meaning that these genotypes are very different from each other. The dissimilarity analysis gave an overall average score of 0.65.

Using the dissimilarity matrix, an unweighted neighbor-joining tree was drawn (Fig. 1) with a very strong correlation between the distance matrix and the neighbor-joining dendrogram ($r = 0.99$). The genotypes fell into two main clusters. Cluster 1 had 11 genotypes and three subclusters: A, B and C. Cluster 1 mainly consisted of genotypes located in the eastern part of mid-Eurasia. This cluster had an average dissimilarity index of 0.65 with minimum and maximum values of 0.07 and 0.82, respectively. Cluster 2 had seven genotypes that were mainly collected in the western part of mid-Eurasia except for PI440628 (19) which was from Kazakhstan. In this cluster, the average dissimilarity index was 0.53 with minimum and maximum values of 0.31 and 0.66, respectively. Lastly cluster 3, containing genotypes PI316340 (3) and PI311722 (2) was an outgroup of the other two clusters as expected because these two genotypes are a subspecies of *P. distans* Parl. ssp. *borealis* (Holmberg) W.E. Hughes. The analysis performed with Structure data program supported the defined clusters however, five genotypes: PI384942 (4), PI230249 (9), PI578856 (13), PI600922 (14), and PI440628 (19) were found to be admixed (data not shown).

4. Discussion

Although *P. distans*, does not have any economic value, it possesses unique properties for abiotic stress tolerance and hyperaccumulation of toxic boron (Hughes et al., 1975; Stiles et al., 2010). The accessions we studied were collected from

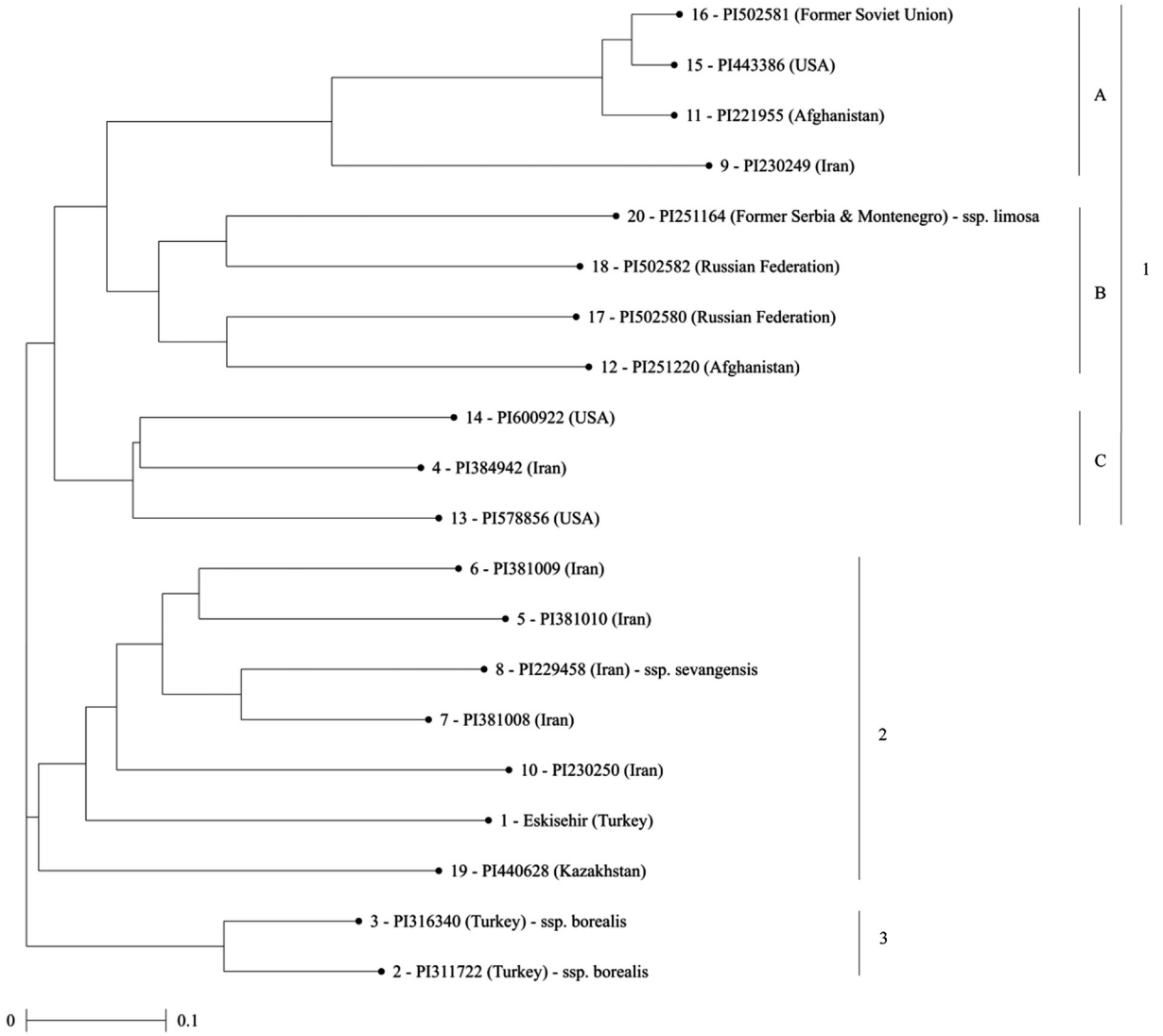

Fig. 1. Unweighted neighbor joining tree of *P. distans* genotypes. The tree contains three clusters: 1, 2 and 3. Cluster 1 contains 3 subclusters A, B and C. Cluster 3 is an outgroup.

various locations and environments and can be used to shed light on the relationship between the genotypes and their abiotic stress resistance phenotypes. Previously, Choo et al. (1994) performed work with 20 different *Puccinellia* accessions, three of which were used in this work, and constructed a phylogenetic tree. They obtained PI251164 (20), PI311722 (2) and PI384942 (4) from the Plant Introduction Station not as subspecies of *P. distans*, but as different species: *P. limosa* (20) and *P. poecilantha* (2 and 4). However, PI251164 (20) was received as *P. distans* (Jacq.) Parl. ssp. *limosa* (Schur) Soó and Jáv, from GRIN in this work and clustered with other *P. distans* accessions (Fig. 1) while PI311722 (2) was identified as subspecies *borealis* and PI384942 (4) simply as *P. distans*. In agreement with their re-classification by GRIN, PI311722 and PI251164 were found in different clusters. PI311722 (2) clustered as outgroup with another ssp. *borealis* accession, PI316340 (3) while PI384942 (4) was placed in subcluster C with other *P. distans* accessions. This organization shows a more distant relationship between these two accessions than that shown by Choo et al. (1994).

Because *P. distans* is not native to the Americas, it is assumed that PI578856 (13), PI600922 (14) in and PI443386 (15) were donated to the USA from other countries for research purposes and their original location is unknown. Genotypes PI578856 (13), PI600922 (14) were renamed FULTS and described in a turfgrass seed guide (Sports Turf Research Institute, 1981). According to our results, the origin of these registered landraces may be Iran because they are closely related to accession PI384942 (4). Interestingly, the two FULTS genotypes were only 54% similar indicating that they were selected from genetically distinct material. The other US genotype, PI443386 (15) probably originated from Russia as it was only slightly (0.06) dissimilar from PI502581.

Genotypes from Iran clustered with other Middle Eastern genotypes from Turkey and Kazakhstan. Because genotype PI229458 (8) is known to be *P. distans* Parl. ssp. *sevangensis* (Grossh.) Tzvelev, it is possible that the closely related genotypes from Iran, especially PI381008, may also be ssp *sevangensis*. Also located in this cluster is a genotype from Eskisehir, Turkey. This genotype is a boron hyperaccumulator and is able to accumulate 3.30 mg of boron per g of dry leaf tissue when grown in modified half strength Hoagland solution with 500 mg B/L (Stiles et al., 2010). Thus, it is possible that other genotypes in cluster 2 may be able to hyperaccumulate boron. Genotypes PI381008 (7) and PI381010 (5) are especially interesting and should be tested for such properties because they were collected near a salt flat and an alkali spot, respectively.

The outgroup cluster consisted of two genotypes of *P. distans* ssp. borealis from Kakliç Turkey. These two genotypes, accessions PI316340 (3) and PI311722 (2), showed low dissimilarity, a value of 0.20, indicating that they are closely related. This was expected as they were collected from the same location. The other two subspecies of *P. distans*, ssp. *limosa* and ssp. *sevangensis*, did not fall into the outgroup but, instead were clustered with *P. distans* genotypes. PI251164 (20) ssp. *limosa* was found in cluster 1, as already indicated, and PI229458 (8) ssp. *sevangensis* was found in cluster 2. Thus, our results, only showed a clear genetic distinction between ssp. *borealis* and the rest of the *P. distans* material.

While it is often expected that more closely located genotypes will be more closely related, we found that some distantly located genotypes were genetically similar. This seeming discrepancy may be explained by the anemochorous mode of seed dispersal of *P. distans* as well as the dispersal of seeds by motor vehicles between long distances as explained by Schmidt (1989). To further support this hypothesis, more samples from countries in the region should be gathered and added to the analysis. If enough genotypes are added, we may be able to see the pattern of dispersal and possibly find the point of origin of *P. distans* species. In establishing genetic relationships between *P. distans* accessions, this work may shed light on their physiological properties since certain genotypes of *P. distans* are known to be tolerant to drought, salt and boron abiotic stress (Hughes et al., 1975; Stiles et al., 2010). Thus, this work may be used as a starting point for selection of *P. distans* genotypes that should be tested for toxic mineral hyperaccumulation and abiotic stress tolerance.

Acknowledgments

This research was supported by the Scientific and Technological Research Council of Turkey (113Z930). We also would like to thank Mehmet Babaoğlu for providing the *P. distans* seeds from Eskişehir, Turkey.

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