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Evaluation of genetic diversity of some *Phalaris sp.* Using minisatellites

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Abstract The experiment was conducted in order to see the genotypic expression of 12 varieties of Phalaris spp. in order to determine their degree of relatedness and genetic diversity. The samples become from USAMVBT didactical and experimental field were Phalaris is cultivated and breaded. 7 DAMD molecular markers randomly chosen were tested. After the preliminary screening, four primers were further used according to their polymorphic ability. The data collected from those probes made possible the development of a Dendrogram of genetic similarities among those 12 probes. In this paper is described the first attempt to genotype Phalaris spp. Using minisatellites molecular markers (DAMD).

Key words

Phalaris spp, genetic diversity, DAMD molecular markers, Dendrogram

Phalaris is a perennial grass which grows mainly in late autumn, winter and spring. Better suited to moderate to high fertility soils. Sensitive to acid soils. Tolerates wet soils, flooding, and moderately saline soils. Very persistent with appropriate management (8).

All varieties can cause phalaris poisoning. Rotational grazing preferred, especially for semi erect and erect types.There are between 15 up to 22 species some species are very toxic containing gramine that cause serious brain damage and it may cause death. The Phalaris manage to clean very well soil by absorbing polluted compounds from soil making from them a source of nutrition or souring those in biomass, this plant has a potential , a very big one because it is a medium to tall plant with big biomass , it may be used as bioenergy , food for animals not forgetting that some compounds like those alkaloids may harm (7, 8). Phalaris as a species is relatively intolerant of soil acidity especially where soil aluminium is high and phosphorus levels are low. Some varieties have been developed for improved tolerance of acidic soil conditions (7, 10, 5). In marginal situations use of these varieties may improve long term productivity and persistence. Genetic monitoring is the use of molecular markers to identify individuals, species or populations, or to quantify changes in population genetic metrics (such as effective population size, genetic diversity and population size) over time. Genetic monitoring can thus be used to detect changes in species abundance and/or diversity, and has become an important tool in both conservation and livestock management. The types of molecular markers used to monitor populations are most commonly mitochondrial, minisatellites or microsatellites or, single-nucleotide polymorphisms (SNPs), while earlier studies also used allozyme data. Species gene diversity is also recognized as an important biodiversity metric for implementation of the Convention on Biological Diversity(3,4,9). Minisatellites are tandemly repeated DNA regions of genomes, many of which showed high levels of allelic length variation due to differences in the number of repeated units. These loci are highly informative genetic markers that have been used extensively in many areas of genetics. Heath et al. (1993) reported a technique, called *directed amplification of minisatellite region DNA* (DAMD) to direct the PCR mediated amplification of minisatellite DNA region. It has been speculated that when a portion of a minisatellite DNA array is involved in an inversion, a single primer makes PCR is possible for the amplification of minisatellite core region (1, 2 , 6).

Matherial and Methods

The biological material was represented by 12 varieties of Phalaris specie, collected from the experimental field of B.U.A.S.V.M. and labeled according to Table 1.

Table 1

Phalaris species and varieties used in this study

No.	Specie	Variety
1	Phalaris pycta	
2	Phalaris arundinacea	Premier (field cultivated collection)
3	Phalaris arundinacea	tardy
4	Phalaris arundinacea	early
5	Phalaris arundinacea	Premier (locally breaded)
6	Phalaris canariensis	
7	Phalaris spp.	Polycross bulk
8	Phalaris arundinacea	Brandenbrug early
9	Phalaris arundinacea	Romanian clones (2011)
10	Phalaris arundinacea	Brandenbrug tardy
11	Phalaris arundinacea	Polycross collection
12	Phalaris arundinacea	Premier (vegetative pot cultivated collection)

The young leaves from the variety of Phalaris were collected and bulked in order to obtain a DNA sample that is genetically homogenous and representative.

For the genetic diversity assessment study minisatellietes molecular markers (DAMD) were chosen. For the initial screening experiment a set of 7 primers (Table 2) were used.

Table 2

DAMD primers used in initial screening

DAMD primer code	Sequence 5'...3'
URP 2F	GTGTGCGATCAGTTGCTGGG
URP 2R	CCCAGCAACTGATCGCACAC
URF 9F	ATGTGTGCGATCAGTTGCTG
URP 6R	GGCAAGCTGGTGGGAGGTAC
33.6	GGAGGTGGGCA
M13	GGCAGGATTGAAGC
14C2	GAGGGTGGCGGCTCT

DNA was extracted and purified from 100 mg of leaf fresh tissue using CTAB method (ISO 21571, 2005).
The quality and quantity of DNA was assessed by using the specrophotometric method (*NanoDrop 8000, Thermo Scientific*). Amplification reactions were carried out in volumes of 25 µl containing having 75 ng of DNA as template. The composition of master mix was carried out following the producer instructions for GoTaq Green Master Mix (*Promega*, USA). PCR was performed on a DNA Engine Peltier Thermal Cycler (MJ Research, U.S.A.) PCR program consisted of an initial denaturing step for 5 min at 94°C, followed by 45 cycles of denaturation at 95°C for 45 sec, annealing at 53°C- 56°C for 45 sec and extension at 72°C for 2 min, with a final step at 72°C for 5 min., according to literature data (**3).**

The resulting PCR products were run on 1.7 % agarose gels in TAE buffer at room temperature at a constant voltage of 90 V for 90 minutes.

The PCR products were visualized and photographed under UV light (PhotoDocumentation System, UVP, England). The obtained data were analyzed with VisionWorksLC software. The dendrogram was assessed from a set of variables by using DendroUPGMA program. The program calculates a similarity matrix and transforms similarity coefficients into distances and makes a clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

Results and Discussions

A set of 7 DAMD primers were chosen for an initial screening. Since there is no literature data recordings to state that minisatellites can be used in studies for determining the genetic diversity of Phalaris spp. This screening offered the possibility of selecting polymorphic primers suitable to be used further in the experiment. A bulked sample consisting of 5 µl of each

Phalaris variety DNA was used as template. For this screening experiment, a Gradient-PCR program, with annealing temperatures ranging from 57°C- 53°C was used. Based on the obtained screening result, 4 primers were selected to be used in the first step of the experiment (**Table 3**).

However, it can be noticed that all the primers yielded DNA fingerprint of good quality in terms of amplified sequences number and well defined PCR product (Figure 1), making them a valuable choice for further studies.

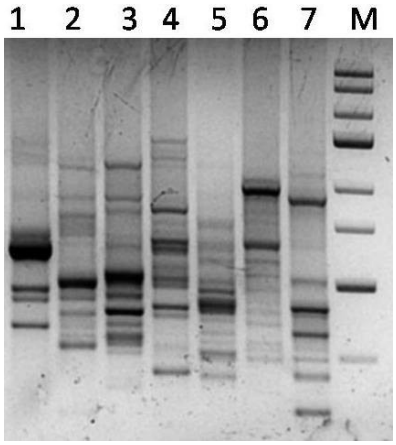

Fig. 1: PCR based screening with 7 DAMD primers: 1 – primer URP 2F, 2-primer URP 2R, 3- primer URP 9F ,4-primer URP 6R , 5-primer 33.6, 6-primer M13 ,7-primer 14 C2 , M- Molecular weight marker - GeneRuler Express DNA Ladder.

Table 3

DAMD primers used FOR genotyping study and data collected from 12 Phalaris genotypes

Primer	**sequence**	Fragment size range	Fraction polymorphic fragments
URP 2R	CCCAGCAACTGATCGCACAC	**2575 - 170**	**22/22**
URF 9F	ATGTGTGCGATCAGTTGCTG	**2010 - 150**	**21/21**
URP 6R	GGCAAGCTGGTGGGAGGTAC	**1525 - 335**	**17/17**
33.6	GGAGGTGGGCA	**1330-270**	**21/21**
			81/81 (100%)

Primer URP 2R proved to be highly polymorphic, yielding a total number of 22 bands from which 22 were polymorphic (Figure 2). It can be noticed that for P. pycta there is no record in the area of small molecular weight. For this primer, the most obvious differences can be noticed in the area of heavy molecular weights amplicons.

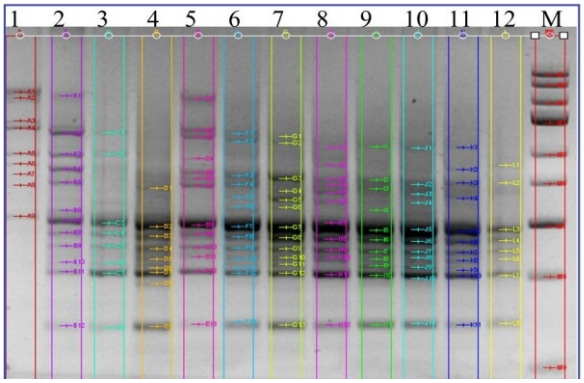

Fig. 2: DNA fingerprint obtained by using primer URP 2R: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier,3- P. arundinacea var. tardy ,4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,7-P. spp. Polycross bulk ,8- P. arundinacea var. Brandenbrug early ,9- P. arundinacea var. Romanian clones (2011) , 10-P. arundinacea var. Brandenbrug tardy ,11- P. arundinacea var. Polycross collection , 12- P. arundinacea var. Premier (vegetative pot cultivated collection) , M- Molecular weight marker - GeneRuler Express DNA Ladder.

Analyzing the image of PCR amplification using primer URF 9F, it can be noticed that the obtained amplicons are in number of 21, all of them being polymorphic. The amplified fragments are ranging from 2010 to150 (Figure 3). For this primer the most obvious differences can be noticed in the area of medium and small molecular weight amplified fragments.

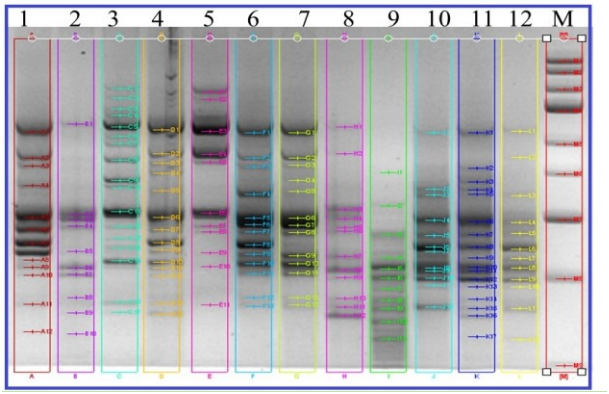

Fig. 3: DNA fingerprint obtained by using primer UBC 834: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier,3- P. arundinacea var. tardy ,4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,7-P. spp. Polycross bulk ,8- P. arundinacea var. Brandenbrug early , 9- P. arundinacea var. Romanian clones (2011) , 10-P. arundinacea var. Brandenbrug tardy ,11- P. arundinacea var. Polycross collection , 12- P. arundinacea var. Premier (vegetative pot cultivated collection) , M- Molecular weight marker - GeneRuler Express DNA Ladder.

In case of primer URP 6R, the DNA fingerprint consisted of 17 amplicons, ranging from 1525 bp to 335 bp and as previous all were polymorphic (Figure 4). The most polymorphic fragments can be noticed in the medium sized amplicons area.

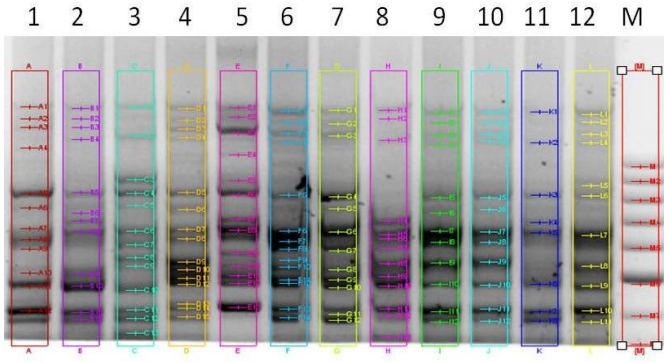

Fig. 4: DNA fingerprint obtained by using primer URP 6R: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier,3- P. arundinacea var. tardy ,4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,7-P. spp. Polycross bulk ,8- P. arundinacea var. Brandenbrug early ,9- P. arundinacea var. Romanian clones (2011) , 10-P. arundinacea var. Brandenbrug tardy ,11- P. arundinacea var. Polycross collection , 12- P. arundinacea var. Premier (vegetative pot cultivated collection) , M- Molecular weight marker - GeneRuler Express DNA Ladder.

For the primer 33.6, the number of amplified fragments was 21 and all were polymorphic especially in the case of individuals 3 and 5 (P. arundinacea var. tardy and P. arundinacea var. Premium respectively) (Figure 5). The amplicons size ranged from 1330 to 270, the polymorphism can be noticed for the large amplified fragments.

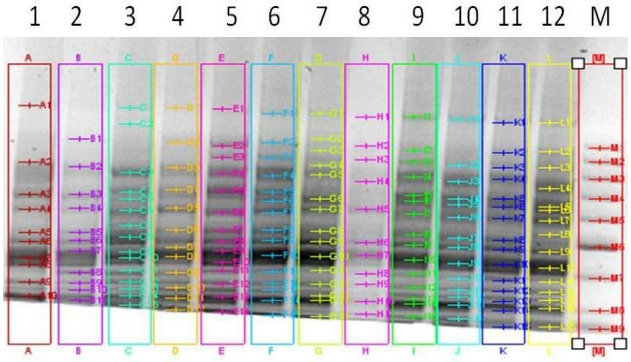

Fig. 5: DNA fingerprint obtained by using primer 33.6: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier,3- P. arundinacea var. tardy , 4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis, 7-P. spp. Polycross bulk , 8- P. arundinacea var. Brandenbrug early ,9- P. arundinacea var. Romanian clones (2011) , 10- P. arundinacea var. Brandenbrug tardy ,11- P. arundinacea var. Polycross collection , 12- P. arundinacea var. Premier (vegetative pot cultivated collection) , M- Molecular weight marker - GeneRuler Express DNA Ladder.

The data that resulted from the analyses of PCR results, as were given by the analysis software, a binary matrix for each individual was developed. A number of 81 were scored in this DAMD experiment and those scores were used to construct an intermediary UPGMA dendrogram (Figure 6).

Analyzing the dendrogram it can be said that among the genotypes there is a large genetic diversity scored with DAMD molecular markers. It is impossible to group the genotypes in large clusters. The variety 1, P. pycta is clearly distinct from the others, as it could be predicted from the raw data. All others genotypes were split in two clusters from wich very distinct are genotypes 3 and 5. Considering the varieties used in this study, the obtained dendrogram is probably not a very accurate result due to the reduced number of primers used in the experiment. Since the primers used were entirely polymorphic, there is a need of adding other molecular markers to the experiment in order to predict the genetic similarity of Phalaris varieties. For example in the large group, variety 7, a polycross clone, is joined with variety 6 – P. canariensis and most probably at the end of the study it will be clear in which percent the last one participated in construction of the polycross genotype.

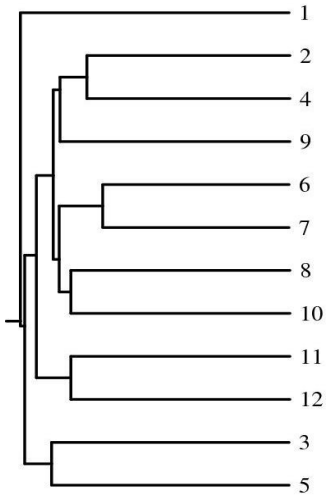

Fig. 6: Dendrogram of genetic distance of *Phalaris ssp*.created by DendroUPGMA program using data from four DAMD molecular markers: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier, 3- P. arundinacea var. tardy ,4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,7-P. spp. Polycross bulk , 8-P. arundinacea var. Brandenbrug early, 9- P. arundinacea var. Romanian clones (2011) , 10- P. arundinacea var. Brandenbrug tardy , 11- P. arundinacea var. Polycross collection , 12- P. arundinacea var. Premier (vegetative pot cultivated collection).

Conclusions

This study is a part of a complete project of characterizing a collection of Phalaris spp. genotypes. The novelty brought in this paper is the use of DAMD primers, being the first report of such experiment. Even if the genotyping study is not finished some conclusions can be enounced:

A good quality of Phalaris spp. DNA was obtained using CTAB extraction method, given been the fact that the genus is problematic, containing a high amount of alkaloids.

1.Even if there are no references in the specific literature, the data obtained from the presented experiment state that DAMD molecular markers are very suitable to be used in the attempt to genotype varieties belonging to Phalaris genus.

2.The four DAMD primers used in this preliminary study are entirely polymorphic and therefore recommended to be further used in similar experiments.

3.The polycross clone (variety 7) is found to be joined with P.canariensis, probably meaning that some percent of genetic material of the specie was participating in the construction of this polycross clone.

4.The DAMD experiment revealed a high genetic diversity inside the genus Phalaris, meaning that a higher number of molecular markers need to be further scored in order to develop an accurate conclusion.

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