Volume 18(4), 135- 140, 2014
JOURNAL of Horticulture, Forestry and Biotechnology
www.journal-hfb.usab-tm.ro

Evaluation of genetic diversity of some *Phalaris sp.* using microsatellites

Bloju O.[1*], Boldura Oana-Maria[2], Samfira I.[1], Popescu Sorina[1]

[1]University of Agricultural Sciences and Veterinary Medicine of Banat " King Mihai I of Romania " Timisoara, Faculty of Horticulture and Forestry; [2]University of Medicine and Pharmacy ,,Victor Babes", Timisoara, Faculty of Medicine

*Corresponding author. Email: bloju.octavian@gmail.com

Abstract In this experiment we took young vegetative samples from 12 varieties of Phalaris that were cultivated in USAMVBT didactical and experimental field having the main goal of genotyping this valuable biologic material. A number of 15 randomly chosen ISSR molecular markers were used in the preliminary screening experiment. From this set, four primers were further used, based on their qualities as resulted from the screening. Based on preliminary data a dendrogram of genetic similarities among Phalaris varieties was constructed. The varieties grouped in two main, distinct clusters and the polycross clone as part of the second cluster tended to separate from the others varieties.

Key words

Phalaris spp.; genetic diversity; ISSR molecular markers; genotyping

Phalaris is a type of grass, belonging to Plantae kingdom, Angiosperms, Monocots, Commelinids, Poales order, Poaceae family, Pooideae subfamily and genus Phalaris. 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 (8). Some species contain alkaloids (DMT), it has a potential in bioenergy, it tolerates wet soils, semy flooding and moderate saline soil which makes her grow in large quantitys, there are very competitive making them hard to eliminate. Life history traits and demographic history, including human-mediated introduction and dispersal, can influence the molecular signatures of diversity and differentiation. Self-incompatible, wind-pollinated plants, such as *P. arundinacea*, tend to have higher within-population diversity and lower levels of differentiation among populations (3, 4). The Phalaris plant, has the propriety to clean the soil content, absorbing polluted minerals 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. It is a winter active grass with short rhizomes, perennial plant with a great resistance and weak if affected by steam rust (4, 7, 9). The average temperature is 10ºC (10).

Description tussock-forming grass up to 2 m high that spreads by short rhizomes and seed bluish-green foliage, leaves 15-40 cm, broad (4-15 mm), flat and hairless – young leaves are rolled in bud ligule 3-5 mm long and membranous, transparent to white and rounded at the tip leaf sheath bluish-green, hairless, round in cross-section – when cut through at the base a pink sap exudes that is useful for identifying seedlings inflorescence is a dense compact cylindrical panicle 50-150 mm long and 10-15 mm in diameter (4, 8, 9, 10).

Neutral genetic markers have become an important tool for examining demographic histories of introduced species. Inter simple sequence repeats (ISSRs) are dominant markers located between microsatellite sites in the genome ISSRs are often highly polymorphic at the species level, require no prior sequence information, and are more reproducible than RAPD markers ISSR markers or microsatellites, name given by Litt and Lutty, there are multilocus probe that create patterns banding making oligonucleotide fingerprints. A microsatellite has a monomer length of 1 to 6 bp long monomer sequence that is repetable, SSRs or STRs mean short tandem repeats/simple sequence repeats (1, 2, 5, 6).

Materials 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 labbeled 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)

Young leaves were collected for each variety. In case of collections, were more individuals were at disposal leaves were collected, bulked in order to obtain a genetically homogenous and representative sample.

.

Microsatellites molecular markers were chosen for the genetic diversity assessment. For the initial screening experiment a set of fifteen ISSR primers (Table 2) were used

Table 2

Randomised primers used in initial screening (ISSR)

ISSR primer code	Sequence 5'...3'
A3	GACAGACAGACAGACAGACA
A7	AGAGAGAGAGAGAGAGAGAGT
A 10	CTCTCTCTCTCTCTCTCTCTT
A 12	GAGAGAGAGAGACC
A 13	GTGTGTGTGTGTCC
A 17	GTGGTGGTGGC
A 21	CACACACACACAAC
UBC 808	CACACACACACACAG
UBC810	AGAGAGAGAGAGAGAGC
UBC811	GAGAGAGAGAGAGAGAT
UBC834	AGAGAGAGAGAGAGAGYT
UBC873	GACAGACAGACAGAC A
UBC880	GGAGAGGAGAGGAGA
UBC884	HBHAGAGAGAGAGAGAG
UBC886	VDVCTCTCTCTCTCTCT

Single letter abbreviations for mixed-base positions: Y = (C, T), R = (A, G), B = (non A), D = (non C), V = (non T), H = (non G).

DNA was isolated and purified from approximately 50 mg of leaf fresh tissue using CTAB method (ISO 21571, 2005).

The quality and quantity of DNA was assessed by specrophotometry method (*NanoDrop 8000, Thermo Scientific*). In all cases, amplification reactions were carried out in volumes of 25 µl containing 75 ng of DNA template. The composition of amplification mixture 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.) and the 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 48°C-55°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 **(5).**

The resulting PCR products were run on 1.8 % agarose gels in TAE buffer at room temperature at a constant voltage of 100 V for 90 minutes. The PCR products were visualized and photographed under UV light (PhotoDocumentation System, UVP, England). The obtained data were analysed with VisionWorksLC software.

The dendrogram was assessed from a set of variables by using DendroUPGMA program (**8**). 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 15 ISSR primers were chosen for an initial screening in order to determine the most polymorphic primers that could be used further in the experiment. As template 5 µl of each DNA sample were collected and bulked together. For this screening experiment, a Gradient-PCR program, with annealing temperatures ranging from 48°C- 55°C was used. The DNA fingerprint obtained served to select primers of best quality in terms of amplified sequences number and well defined PCR product (**Figure 1**).

Based on gel image, four ISSR primers were selected to proceed with the genotyping experiment (Table 3).

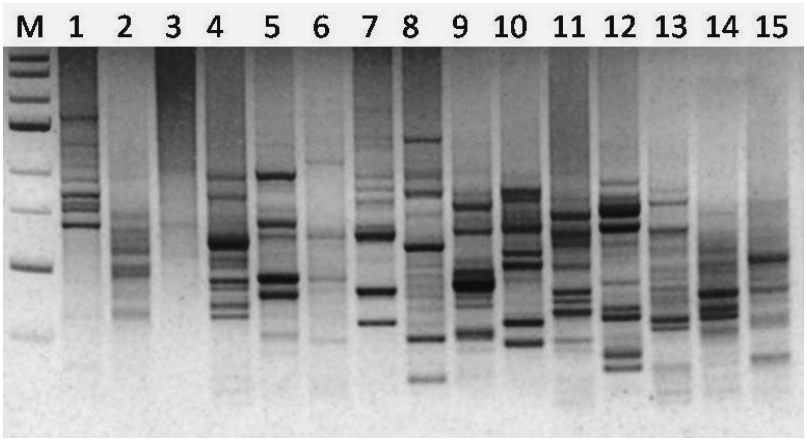

Fig. 1 PCR based screening with 15 ISSR primers: 1 – primer A3, 2-primer A7, 3- primer A10 ,4-primer A12 , 5-primer A13, 6-primer A17 ,7-primer A21 ,8- primer UBC 818, 9 - primer UBC 810, 10 - primer UBC 811 , 11- primer UBC 834, 12- primer UBC 873, 13. primer UBC 880, 14. primer UBC 884, 15. primer UBC 886, M- Molecular weight marker - GeneRuler Express DNA Ladder

Table 3

ISSR primers used in this study and data collected from 12 Phalaris genotypes

Primer	**sequence**	Fragment size range	Fraction polymorphic fragments
UBC 810	GAGAGAGAGAGAGAGAT	**1150-155**	**16/21**
UBC 811	GAGAGAGAGAGAGAGAC	**1020-260**	**13/19**
UBC 834	AGAGAGAGAGAGAGAGYT	**1090-160**	**16/22**
UBC 873	GACAGACAGACAGACA	**1720-250**	**23/24**
			68/86 (79%)

Primer UBC 810 proved to be highly polimorphic, yielding a total number of 21bands from which 16 were polymorphic (Figure 2). The most obvious differences can be noticed in the area of heavy molecular weights amplicons.

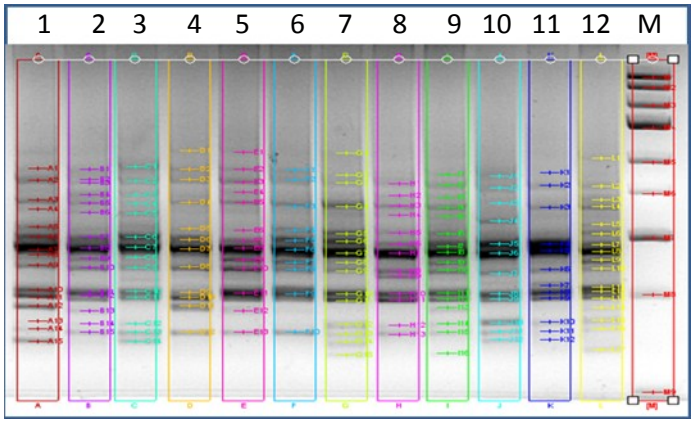

Fig. 2 DNA fingerprint obtained by using primer UBC 810: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier,3- P. arundinacea var. tardy ,4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,7-P. spp. Polycross bulk ,8- P. arundinacea var. Brandenbrug early ,9- 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 UBC 811, the DNA fingerprint consisted of 19 amplicons, ranging from 1020 bp to 260 bp, from those a number of 13 being polymorphic (Figure 3).

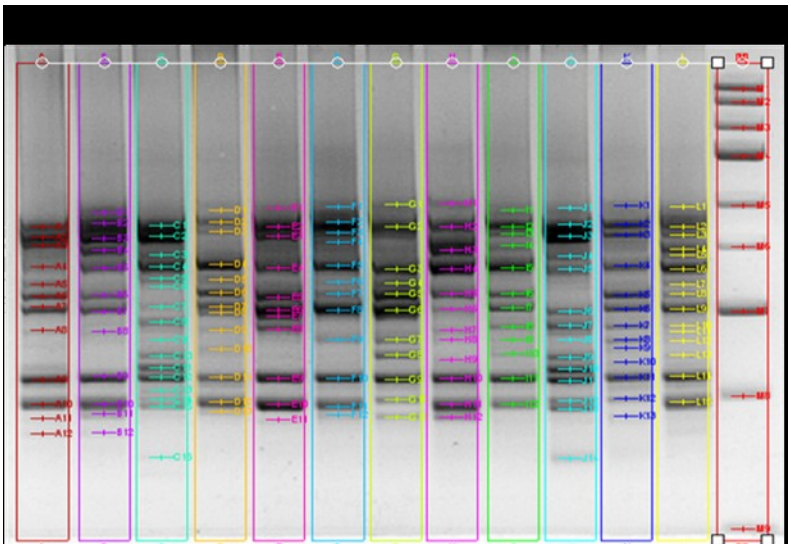

Fig. 3 DNA fingerprint obtained by using primer UBC 811: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier,3- P. arundinacea var. tardy ,4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,7-P. spp. Polycross bulk ,8- P. arundinacea var. Brandenbrug early ,9- 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.

Different from the previous fingerprint the most polymorphic fragments can be noticed in the medium sized amplified DNA fragments.

Analyzing the image of PCR amplification using primer UBC 834, it can be noticed that the obtained amplicons are in number of 22, from which 16 are polymorphic. The amplified fragments are ranging from 1090 to160 (Figure 4). Even if the primer is the less polymorphic comparing with the others three primers used in this experiment, the polymorphism can be scored for all amplicons in the fingerprint.

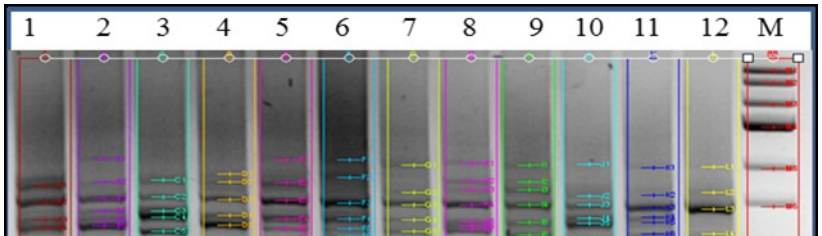

Fig. 4: DNA fingerprint obtained by using primer UBC 834: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier,3- P. arundinacea var. tardy ,4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,7-P. spp. Polycross bulk ,8- P. arundinacea var. Brandenbrug early ,9- 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.

For the primer UBC 873, the number of amplified fragments was 24, 23 of those were polymorphic especially in the case of individuals 6 and 8 (Figure 5). The amplicons size ranged from 1720 to 250, the polymorphism can be noticed for the large amplified fragments but also for small ones.

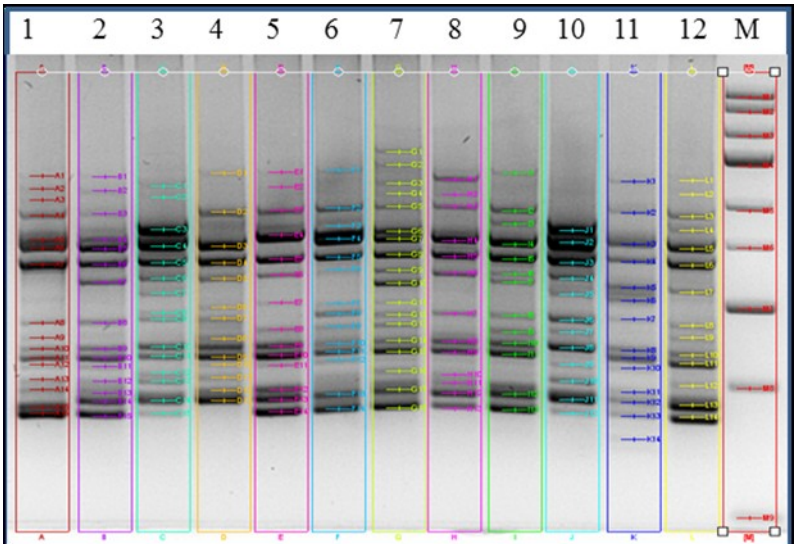

Fig. 5 DNA fingerprint obtained by using primer UBC 834: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier,3- P. arundinacea var. tardy ,4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,7-P. spp. Polycross bulk ,8- P. arundinacea var. Brandenbrug early ,9- 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.

Based on data that resulted from the analyses of PCR results, by using the gels analysis software, a binary matrix for each individual was set. A number of 85 were scored in this experiment and the scores were used to asses an intermediary UPGMA dendrogram (Figure 6).

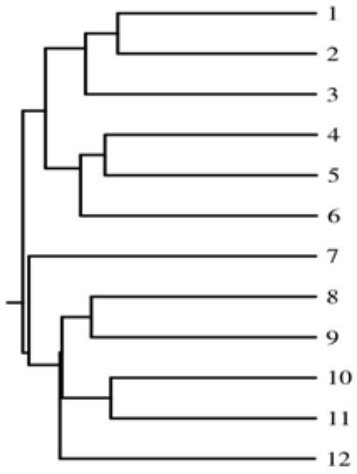

Fig. 6 Dendrogram of genetic distance of *Phalaris ssp*.created by DendroUPGMA program using data from four ISSR molecular markers: 1 –Phalaris pycta, 2-P. arundinacea var.Pemier, 3- P. arundinacea var. tardy , 4-P. arundinacea var. early , 5-P. arundinacea var. Premier (locally breaded), 6-P. canariensis ,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).

Analyzing the dendrogram it can be said that the genotypes are grouped in two large clusters. One contains genotypes 1, 2, 3, 4, 5, 6 and a second one containing the genotypes 7, 8, 9, 10, 11, 12. Considering the varieties used in this study, the obtained dendrogram is probably not the very accurate result due to the reduced number of primers used in the experiment. However, some concordance with the genetic identity of Phalaris varieties tend to conturate. For example in the second cluster, variety 7, a polycross clone, could not be joined with another variety most probably due to the diversity of genetic material that participated in construction of the genotype.

Conclusions

The study presented in this paper is a part of a complete project of characterizing a collection of Phalaris spp. genotypes. Even if the genotyping study is just at beginning some conclusions can be enounced:

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

2.Based on bibliographic references and data obtained from the presented experiment, ISSR molecular markers are very suitable to be used in the attempt to genotype varieties belonging to Phalaris genus.

3.The four ISSR primers used in this preliminary study are highly polymorphic and therefore recommended to be further used.

4.The polycross clone (variety 7) tend to separate from the varieties that donated the genetic material meaning that this could be a variety composed of very diverse set of genes.

5.It seems that the genetic diversity inside the genus Phalaris is not very high, meaning that a higher number of molecular markers need to be further scored.

References

1.Ramel, C., 1997, Mini- and Microsatellites. Environmental Health Perspectives, 5, 781-789

2.Nybom Hilde, Weising K. and Rotter B., 2014, DNA fingerprinting in botany: past, present, future, Investigative Genetics 5: 1-35

3.Michael F. Nelson,Neil O. Anderson,Michael D. Casler.Andrew R. Jakubowski, 2013, Population genetic structure of N. American and European Phalaris arundinaceae L. as inferred from inter-simple sequence repeat markers, Publication from USDA – ARS /UXL Faculty

4.Moore, G, Sanford, P & Wiley, T., 2006, Perennial pastures for Western Australia, Department of Agriculture and Food Western Australia, Bulletin 4690

5.Pharmawati M., Guijun Y., Patrik M. Finnegan, 2005, Molecular Variation and Fingerprinting of Leucadendron Cultivars (Proteaceae) by ISSR Markers, Annals of Botany 95: 1163–1170.

6.Swati P. Joshi, Prabhakar K. Ranjekar and Vidya S. Gupta, 2008, Molecular markers in plant genome analysis, Plant molecular biology group, division of biochemical sciences, National Chemical Laboratory, Pune, India.

7.http://en.wikipedia.org/wiki/Phalaris_%28grass%29

8.http://genomes.urv.cat/UPGMA/index.php

9.http://www.dpi.nsw.gov.au/agriculture/pastures/pastures-and-rangelands/species-varieties/a-z/phalaris

10.http://www.eeob.iastate.edu/research/iowagrasses/speciespages/PhalaArund/PhalaArund.html