INTRASPECIFIC GENETIC VARIABILITY OF *HYSSOPUS OFFICINALIS* L.

ANA MUTU[1], STELIANA CLAPCO[1*], RODICA MARTEA[1], ANGELA PORT[1], ELVIRA GILLE[2], MARIA DUCA[1]

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Abstract: Random amplified polymorphic DNA (RAPD) was used to investigate the intraspecific variability among *Hyssopus officinalis* L. collected from three different populations in Romania and Republic of Moldova. Twenty seven tested primers generated 169 amplification products, with an average of 8,0 bands per primer. A total of 120 bands were polymorphic. Analysis within individual populations showed that population from Piatra Neamt (Romania) had a relatively higher RAPD polymorphism (46,3%) than the Secuieni (Romania) and Botanical Garden (Chisinau, Moldova) populations (34,4 and 36,0%). Cluster analysis indicated a classification of the genotypes into three main groups, corresponding with tested populations.

INTRODUCTION

The genus *Hyssopus* (Family: *Lamiaceae*) comprises about 10-12 species of aromatic perennial herbs or subshrubs, natively spread from the east Mediterranean to central Asia. Due to the high content of essential oils (0,3-1,0%), one of the most economically important species among *Hyssopus* is *H. officinalis* L. (Hissop), commonly used in medicine as a carminative, antispasmodic, antiseptic, antitussive, diaphoretic, expectorant, sedative, stomachic, tonic and vasodilator, in the food industry as a flavor, condiment or supplement for sauces. Furthermore, hyssop oil possesses antimicrobial properties, which are essential in such industries as canning, beverage production and cosmetics (Fathiazad *et al*, 2011; Kizil *et al*, 2010).

H. officinalis L. is a perennial herb, mainly cultivated, but also found in the wild flora, characterized by a significant heterogeneity, various subspecies, varieties and forms having been identified, such as: ssp. *canescens* BriQ; ssp. *montanus* BriQ.; ssp. *aristatus* BriQ. (Syn. *H. officinalis* ssp. *pilifer*) (Dzamic *et al*, 2013); ssp. *officinalis* (Syn. *H. officinalis* ssp. *angustifolius* (M. Bieb.) Benth. (Ozer *et al*, 2005); *H. officinalis* ssp. *officinalis* var. *decumbens (Jord. & Fourr.) Briq.*) (Mazzanti *et al*, 1998), with different variations – var. *vulgaris* Benth., var. *decussata* Pers. (Syn. var. *latifolius* Fisch. ex Benth.) and, depending of the color of the corolla, *cyaneus, ruber* and *albus* forms (Chalchat *et al*, 2001). The high intraspecific diversity of hyssop could be observed for morphological (leaf, inflorescence, flower color, form and position) and, especially, for biochemical traits (quantitative and qualitative composition of essential oils). A remarkable phytochemical polymorphism within some chemotypes and subspecies is reported by several studies, but there is no report on the differences among individuals at the DNA level (Goncearuc and Balmuş, 2013; Kizil *et al*, 2010; Varga *et al*, 1998).

Intraspecific diversity is an important component of adaptive evolution, determining the ability of plants to survive in more diverse habitats, to colonize habitats of wide ecological amplitude and to respond to different environmental changes (Albert *et al,* 2011). Biodiversity at the species level is usually measured by genetic diversity, which refers to the variety of alleles found in a species and it is reflected in the differences between individuals across many traits, from DNA sequence and biochemical traits (protein structure or isoenzyme properties) to physiological and morphological properties (ex. flower color or plant form). The mechanisms causing changes to the combination of alleles found in different populations and, ultimately, determining the heterogeneity of these populations are natural selection, genetic drift and gene flow (Frankham *et al,* 2002; Rao and Hodgkin, 2002).

Knowledge of genetic diversity and relationship among individuals, populations, plant varieties promotes the use of genetic variation in crop improvement, selection of superior genotypes for cultivation, provides materials for germplasm conservation and contributes to studying the evolutionary ecology of the populations (Debnath, 2009; Hughes *et al,* 2008).

Diversity can be detected using the various genetic markers, such as morphological, chromosomal, biochemical and molecular markers (Cruzan, 1998). Morphological traits are commonly used for a primary evaluation of polymorphism, species identification/delimitation and substantiation of taxonomic classification. This approach is complemented by more informative biochemical and molecular techniques. The most widely used biochemical markers, due to their efficiency in estimation of intra- and interspecific variation and their cost effectiveness, are allo- and isozymes. Thus, by isozyme profile analysis, the genetic diversity within and among the different populations of medicinal plants was estimated (Johnson, 2012; Jugran *et al,* 2011). Allozymes are particularly useful for determining genetic relationships between conspecific populations, closely related species, crops and their wild relatives (Hamrick and

Godt, 1997). They have been successfully applied to describe patterns of genetic variation both within and among forest tree populations and for estimating mating systems and gene flow (White *et al,* 2007).

Molecular markers directly linked to the genome and independent of environmental conditions, are the most effective for understanding the basis of diversity, for distinguishing individuals and estimating genetic relatedness (Vieira *et al,* 2003). Various types of molecular markers are utilized to evaluate DNA polymorphism (RFLP, AFLP, RAPD and SSR). A notable number of studies have highlighted the benefits (low cost, rapidity and simplicity of the procedure) of Random Amplified Polymorphic DNA markers in identification of genetic diversity, genetic maps construction, assisted selection of desirable traits, fingerprinting and population genetics studies (Timmerman and McCallum, 1993).

Therefore, the objective of this research was to characterize genetic diversity within and among hyssop populations, using RAPD markers.

MATERIALS AND METHODS

Nine individuals of *Hyssopus officinalis L.* with different flower colors (Fig. 1, A-C) were picked from three geographically distinct locations in the Republic of Moldova and Romania, as follows:

- *collection of Botanical Garden*, *Chisinau, Republic of Moldova* (marked in the text as BG);
- *collection of Biological Research Center Stejarul, Piatra-Neamt, Romania* (marked as PN);
- *collection of Agricultural Research Station*, *Secuieni, Romania* (marked as S);

A. **B.** **C.**

Figure 1 - *Hyssopus officinalis L.* with blue (A), pink (B) and white flowers (C)

Extraction of DNA from hyssop young leaves was carried out by use of CTAB (Murray and Thompson, 1980). PCR amplification was performed in the following mixture: 50-60 ng DNA, 200µM of each dNTP type, 1,0 U per reaction of Taq DNA-polymerase, 2,5 mM $MgCl_2$ and 0,4-0,6 µM of decamere primers (Tab. 1) in the Applied Biosystem GeneAmp PCR System 9700 (Singapore) thermocycler, programmed with the cycling profile: initial denaturation at 95°C for 5 min followed by 35 cycles: 95°C - 1 min, 34– 36°C – 1,0 min, 72°C - 1 min and a final extension at 72° C for 3 min.

Table 1 - Oligonucleotides used in RAPD - PCR analysis

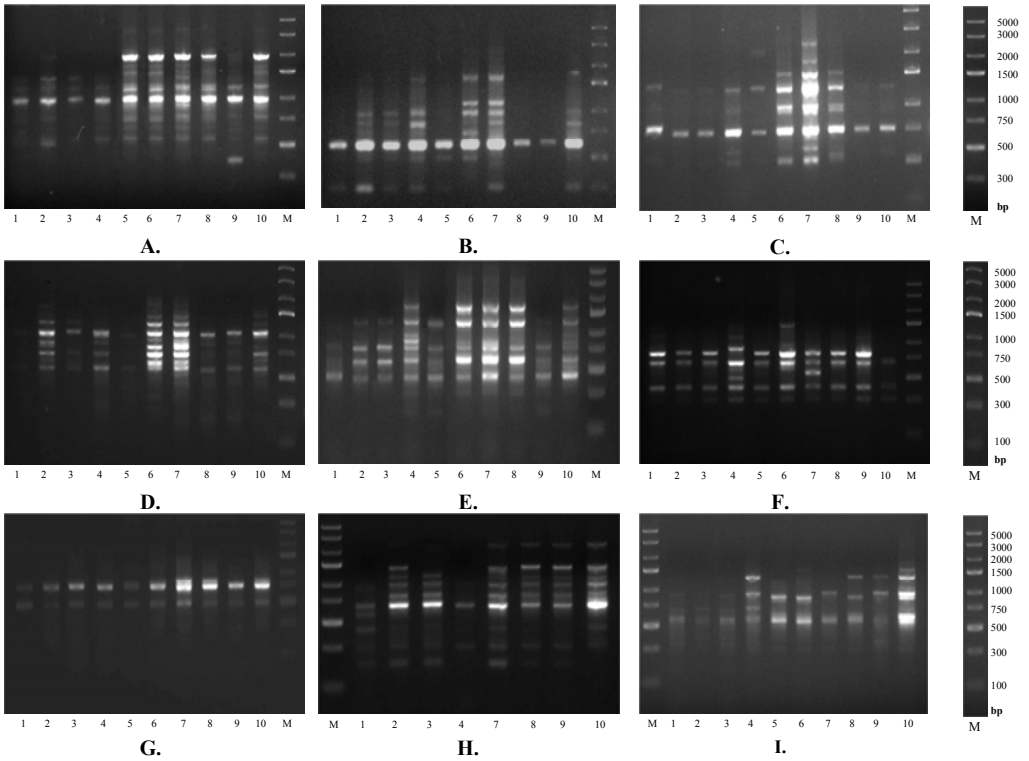
Primers	Primer Sequence	Annealing T (°C)	Primers	Primer Sequence	Annealing T (°C)
***OPA*$_{1}$**	5'-CAGGCCTTC-3'	30	***OPH*$_{15}$**	5'-AATGGCGCAG-3'	32
***OPA*$_{2}$**	5'-TGCCGAGCTG-3'	34	***OPI*$_{16}$**	5'-TCTCCGCCCT-3'	32
***OPA*$_{06}$**	5'-GGT CCC TGA C-3'	34	***OPJ*$_{O1}$**	5'-CCCGGCATAA-3'	32
***OPA*$_{9}$**	5'-GGGTAACGC C-3'	34	***OPU*$_{11}$**	5'-AGACCCAGAG-3'	32
***OPA*$_{11}$**	5'-CAATCGCCGT-3'	32	***OPK*$_{17}$**	5'-CCCAGCTGTG-3'	34
***OPA*$_{19}$**	5'-CAAACGTCG G-3'	32	***OPV*$_{09}$**	5'-TGTACCCGTC-3'	32
***OPB*$_{01}$**	5'-GTTTCGCTCC-3'	32	***OligoA*$_{1}$**	5'-GGTGCGGGAA-3'	32
***OPB*$_{03}$**	5'-CATCCCCCT G-3'	34	***OligoA*$_{2}$**	5'-AAG AGCCCGT-3'	32
***OPB*$_{10}$**	5'-CTGCTGGGAC-3'	34	***OligoA*$_{3}$**	5'-CCC GTCAGCA-3'	34
***OPB*$_{13}$**	5'-TTCCCCCGCT-3'	34	***UBC*$_{215}$**	5'-TCACACGTGC-3'	32
***OPG*$_{05}$**	5'-CTG AGACGG A-3'	32	***UBC*$_{250}$**	5'-CGACAGTCCC-3'	34
***OPG*$_{6}$**	5'-GTG CCTAACC-3'	32	***Oligo*$_{28}$**	5'-AGGTCACTGA-3'	30
***OPG*$_{10}$**	5'-AGG GCCGTCT-3'	32	***Oligo*$_{391}$**	5'-GCGAACCTCG-3'	34
***OPE*$_{17}$**	5'-CTACTG CCGT-3'	32			

The amplification products were separated by electrophoresis in agarose gel (1,5%). The gel was visualized in a UV transilluminator and photographed with gel documentation system DOC – PRINT-VX2.

DNA markers were scored as either present or absent in all samples used in the analysis. These data were used for the calculation of pairwise genetic distances between trees using the Jaccard coefficient similarity matrices. The data sets were analyzed using DendroUPGMA, a dendrogram construction utility (Garcia-Valve et al., 1999).

RESULTS AND DISCUSSIONS

The 27 tested RAPD primers generated distinct amplification bands with variable molecular weight, ranging from 200 to 2300 base pairs (bp). The number of bands for each primer varied significantly, the most bands (15) being generated by OPJ_{01}, followed by OPB_{10}, $OligoA_1$ and OPG_{10} with 11-12 bands (Fig. 4). The least number of amplicons (3) was identified by $Oligo_{28}$. The primers which showed no amplification (OPA_1, OPA_{06} and OPG_6) or generated ambiguous bands only for some of the individuals (OPU_{11}, OPI_{16} and OPB_{13}) were excluded. A total of 169 different DNA fragments were amplified, with an average of 8,0 bands per primer. The results indicate a considerable level of genetic diversity among the studied individuals, forty nine amplicons were monomorphic in all populations and 120 bands (71,0%) were polymorphic. Several examples of polymorphism detected with RAPD in hyssop are shown in Figure 2.



Genotypes collected from: Chisinau (Republic of Moldova) – 1-4; Piatra-Neamt – 5-7 and Secuieni (Romania) – 8-10.

Figure 2 - RAPD profile of *Hyssopus officinalis* L. generated by primer $OLIGOA_1$ (A), OPB_{O1} (B), OPG_{10} (C), OPK_{17} (D), OPA_9 (E), $OLIGO_{391}$ (F), $OLIGO_{28}$ (G), OPJ_{O1} (H) and OPG_{O5} (I)

The maximum number of polymorphic bands (12) belonged to primer OPJ_{01}, followed by OPA_9 and OPB_{03} with 8 polymorphic fragments. A majority of primers generated 6-7 polymorphic bands. The percentage of polymorphic loci was similar to those revealed by RAPD markers in different accessions of medicinal plant *Cassia occidentalis* (71,17%) (Arya *et al,* 2011), *Chlorophytum borivilianum* (67,49%) (Dwivedi and Sharma, 2011), *Changium smyrnioides* (69%) (Fu *et al,* 2003) and, lower in comparison to other plants such as *Terminalia bellirica* (90,0%) (Bharti and Vijaya, 2013), *Lonicera caerulea* (83,9%) (Naugžemys *et al*, 2007), *Campanula microdonta* Koidz. (94%) (Oiki *et al,* 2001).

It is notable that, twenty one of the identified fragments appeared to be genotype-specific and, thus, potentially useful as marker (Tabel 2). The highest number of specific DNA fragments was observed in genotypes collected from Piatra Neamt (13), seven of them being detected in individuals with white color of corolla, four and two – in genotypes with blue and, respectively, pink flowers.

Table 2 - The specific RAPD amplicons of the *H. officinalis* genotypes

Botanical Garden population		**Piatra Neamt population**		**Secuieni population**	
Genotypes	*Specific bands*	*Genotypes*	*Specific bands*	*Genotypes*	*Specific bands*
BG, blue	Oligo A_1^{500} Oligo A_2^{1400}	PN, blue	OPG_{10}^{2000}, OPJ_{01}^{1700}, OPJ_{01}^{2300}, OPB_{03}^{1600}	S, blue	Oligo A_1^{400} OPE_{17}^{400}
BG, pink	-	PN, pink	OPB_{10}^{600}, $Oligo_{391}^{1400}$	S, pink	OPV_{09}^{1750}
BG, white	$Oligo_{391}^{500}$	PN, white	OPA_{19}^{700}, OPA_{19}^{1400} OPB_{01}^{450}, OPG_{10}^{1750}, OPG_{10}^{2300}, UBC_{215}^{1100}, $Oligo_{391}^{550}$	S, white	OPA_2^{800} OPE_{17}^{450}

Primers OPG_{10} and $Oligo_{391}$ are the most informative for genotype discrimination, generating a maximal number of specific fragments (three fragments for each primer). Thus, OPG_{10} produced two amplicons with a variable size – 1750 and 2300 bp specific for plants with white corolla and one amplicon (2000 bp) specific for plants with blue flowers from Piatra Neamt. The second mentioned primer generated two bands (550 and 1400 bp) specific for genotypes from Piatra Neamt (white and pink corrola, respectively) and one characteristic for the genotype with white flowers collected from the Botanical Garden. No specific bands were revealed for the genotype with pink flowers from the Botanical Garden.

Analysis within individual populations showed a maximum number of scored bands (147) in the Piatra Neamt population and a minimum number (114) - in population belonging to the Botanical Garden, with an average of 77 monomorphic fragments (Figure 3). Population from Piatra Neamt had a relatively higher RAPD polymorphism (46,3%) than the Secuieni and Botanical Garden populations (34,4 and 36,0%).

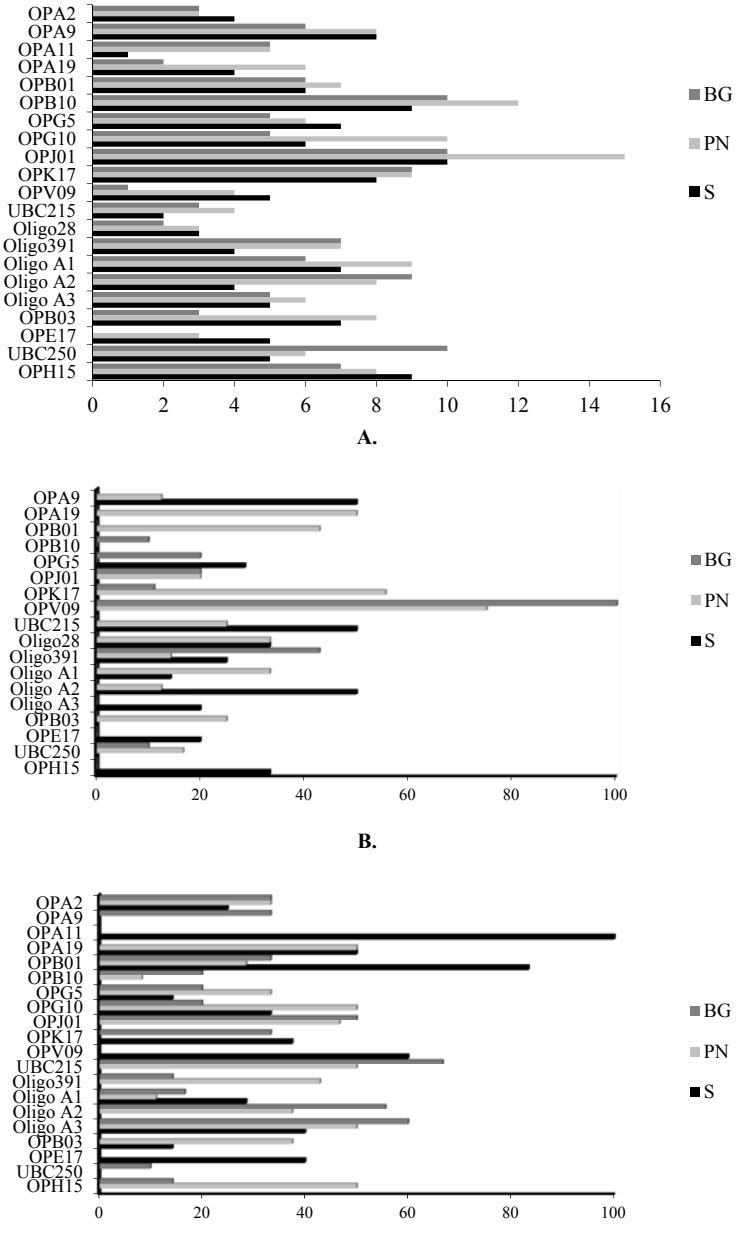
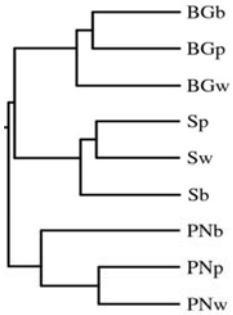


Figure 3 - Polymorphism revealed by RAPD primers within *Hyssopus officinalis* L. populations: total number of bands (A); polymorphic bands, % (B); specific bands, % (C).

It was found that the level of polymorphism among populations ranges from 24,3% to 40,2%, being lower comparative to those revealed within populations. Higher genetic variability

within rather than among populations was also found in bilberries from different Belgium habitats (Albert *et al*, 2004) as well as in a species of desert tree *Haloxylon ammodendron* (Sheng *et al*, 2005). Using RAPD markers and one morphological marker for investigation of population structure of a selfing annual plant species *Medicago truncatula* it was established that the intra-population variance component accounted for 55% of the total variance, while the inter-population variance component accounted for 45% (Bonin *et al*, 1996). W. Shi *et al*, 2008 found out that a slightly higher proportion of genetic variation resided within populations (58,0%) of the traditional Chinese medicinal plant *Coptis chinensis* and a relatively lower degree of genetic variation was noticed among these populations (42,0%) (Shi *et al*, 2008). Higher intra-population rather than inter-population genetic variability indicates minor gene flow. Additionally, four unique bands present in all individuals from one population and absent in all of the others were observed and could be used potentially as markers for population identification. Three of the revealed DNA fragments – OPB_{03}^{800}, OPE_{17}^{500}, OPE_{17}^{1750} were specific for the PN population and one – UBC_{250}^{250} was specific for genotypes collected from BG.

The genetic similarity matrix was calculated on the basis of Jaccard's algorithm for RAPD (Fig. 4). Cluster analysis indicated a distinct classification of the genotypes into three main groups, corresponding to the sampled populations.



	GBb	GBp	GBw	PNb	PNp	PNw	Sp	Sb	Sw
GBb	1	0,770	0,696	0,567	0,591	0,573	0,550	0,603	0,592
GBp		1	0,760	0,534	0,539	0,522	0,556	0,618	0,563
GBw			1	0,504	0,556	0,539	0,512	0,561	0,531
PNb				1	0,647	0,626	0,557	0,520	0,518
PNp					1	0,788	0,640	0,561	0,645
PNw						1	0,620	0,532	0,625
Sp							1	0,752	0,782
Sb								1	0,726
Sw									1

Figure 4 -
UPGMA dendrogram based on Jaccard's similarity coefficient between *Hyssopus officinalis* genotypes

The similarity values between hyssop genotypes ranged between 0,512 and 0,788. The maximum similarity was noticed between genotypes with white and pink corolla in populations from Piatra Neamt and Secuieni, genotypes with blue flowers being more distant. By contrast, in the BG population genotypes with blue and pink flowers are grouped together. The genetic similarity of the samples slightly correlated with their geographic location. Thus, the average similarity value between genotypes from Romanian populations was 0,580 while the similarity between the genotypes from Romania and Moldova constituted 0,556. However, due to low geographic distance between populations, no significant dissimilarities were found out.

A relationship between genetic diversity and geographical distribution has been reported for several species of medicinal and aromatic plants, such as *Hesperozygis ringens* Benth. (Fracaro and Echeverrigaray, 2006), *Ocimum* spp. (Vieira *et al*, 2003). In most of the cases, the genotype similarity is correlated with geographical distribution.

CONCLUSIONS

In this study, the RAPD technique was used to estimate genetic relationship within and among populations of *Hyssopus officinalis* L. A polymorphism between populations (24-40%) from different geographical areas (Romania and Republic of Moldova) was found. Analysis within individual populations showed that population from Piatra Neamt (Romania) had a relatively higher RAPD polymorphism (46,3%) than the Secuieni (Romania) and Botanical Garden (Chisinau, Moldova) populations (34,4 and 36,0%).

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[1] University of the Academy of Sciences of Moldova, University Center of Molecular Biology, Chisinau, Republic of Moldova
[2] National Institute of Research and Development for Biological Sciences, Biological Research Centre "Stejarul"

*stela.clapco@gmail.com