APPLICABILITY OF ScoT (START CODON TARGETED) MARKERS IN EVALUATION OF *THYMUS* GENETICALLY VARIABILITY

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Abstract: *Identifying plant species and assessing variability, is often difficult if only morphological traits are analyzed. This statement is also valid in the case of thyme. For this reason, it is necessary to expand research in the field of biochemical and DNA analysis. The aim of this paper was to investigate the possibilities of using ScoT markers to assess diversity in different wild thymus genotypes. Therefore, 13 spontaneously ecotypes of Thymus collected from the western part of Romania were analyzed with 4 ScoT (Start Codon Targeted) markers (Scot 11, Scot 14, Scot 35 and Scot 36), compared to a cultivated genotype. The plants were collected from their natural habitat and the DNA was extracted from fresh leaves, based on CTAB method. ScoT primers were used because they combine the advantages of random amplification, which gives them a general character with the evaluation of coding chromosomal areas. Thus, 117 alleles were amplified, with an average of 29.25 alleles/primer. All bands were polymorphic and the analysis of the variance showed an average PIC (polymorphism information content) of 0.370 and an average polymorphic index (PI) of 10.57, which places the analyzed markers in the category of highly polymorphic, with increased discrimination power. Therefore, it has been shown that the assessment of variability and the establishment of similarity indices in Thymus using ScoT markers is possible and can be the basis for complex molecular genetic analysis. The four used primers generated complex DNA fingerprints, in which all bands were polymorphic, therefore no band was common to all analyzed ecotypes. The data obtained allowed the elaboration of a dendrogram that groups the analyzed ecotypes according to the similarity index. It has been shown that the assessment of variability and the establishment of similarity indices in Thymus using ScoT markers is possible and can be the basis of complex molecular genetic analysis.*

Keywords*: Thymus, ScoT markers, variability, dendrogram*

INTRODUCTION

Since the 1990s, the use of DNA markers in plant molecular genetic research has developed significantly (GUPTA *et al.*, 1999). The two directions of research were oriented towards the evaluation of genetic diversity in different plant species and the elaboration of genetic maps, which were the basis for finding linked markers to certain agronomically important traits or even identifying new genes (COLLARD *et al*., 2005).

Markers based on the PCR (Polymerase Chain Reaction) technique with a random amplification have generally been used to assess diversity, considering that they can generate a large number of polymorphic bands. In chronological order we can mention the RAPD (Random Amplified Polymorphic DNA) markers which have a short sequence, generally 10 nucleotides and random bind in the genome (WILLIAMS *et al*., 1990), the ISSR (Inter Simple Sequence Repeats) markers, which are annealed in microsatellite regions, amplifying the portion of DNA between them (BLAIR *et al*., 1999) or AFLP markers (Amplified Polymorphic DNA) based on restriction enzyme sectioning, followed by binding of adapters and amplification with specific primers (VOS *et al.*, 1995).

The unprecedented development of DNA sequencing techniques has allowed the introduction of new categories of markers, which are general, with possibilities for use in a large number of species, but which do not amplify random regions of the genome, but rather coding areas close to the genes.

Therefore, a marker category was used in this paper, developed in the late 2000s, and validated in 2009 (COLLARD *et al*., 2009). They are based on a chromosomal region around the start codon of each gene, which is a highly conserved area. A single primer is used, as for the other markers giving random amplification, whose design has been made to bind in the regions bordering the start codons of some neighboring genes, located on antiparallel chains. Thus, this marker category combines the benefits of random-amplification markers, which can be used in different species with the assessment of the coding regions of the genome.

The aim of this paper was to evaluate the using of the ScoT markers for variability assessing in different species of wild thyme. This was based on the fact that this category of markers has been used successfully over time for different plant species such as rice (BERTRAND *et al*., 2009), grapes (GUO *et al*, 2012), cicer (AMIRMORADI, 2012), sugarcane (QUE *et al*., 2014), wheat (HAMIDI, 2014) durum wheat (ETMINAN *et al*., 2016), palm (SABOORI *et al*., 2020). Tests have been performed even on species of the genus *Thymus* that have been promising (ALQAHTANI *et al.*, 2020)

MATERIAL AND METHODS

For the evaluation 13 genotypes of wild spontaneous thyme (1-8 and 10-14) and a cultivated specie were analyzed (9). The plants were collected from their natural habitat and the DNA was extracted from fresh leaves, based on CTAB method (DOYLE and DOYLE, 1987). For amplification four ScoT primers were used, with the following sequences: ScoT 11 – 5', AAGCAA TGGCTACCACCA3', ScoT 14 – 5'ACGACATGGCGACCACGC3', ScoT 35 - 5'CAT GGCTACCACCGGCCC 3' and ScoT 36 - 5' GCAACAATGGCTACCACC3'. The amplification program was as usual and the annealing temperature was 54 ºC (COLLARD et al. 2005). The amplification products were separated by 1.8% agarose gel electrophoresis and the bands were visualized in UV light, in ethidium bromide presence. The processing of the experimental data was done by variance analyzing the t test and ANOVA (CIULCA, 2006).

RESULTS AND DISCUSSIONS

The assessment of the genetic polymorphism of *Thymus* ecotypes was carried out using four dominant primers Scot , i.e.: Scot 11, Scot 14, Scot 35 and Scot 36 to see the ability to discriminate against *Thymus* ecotypes.

First, the marker ScoT 11 was analyzed. 29 fragments with different lengths were amplified, between 200 and 2200 bp, all being polymorphic, ie each ecotype had a specific DNA fingerprint, without any band common to all. Next, the other markers were analyzed: Scot 14, which generated a number of 27 fragments, with dimensions between 200 and 1500bp., ScoT 35, with a number of fragments of 27, between 200 and 1200 and ScoT 36 with 34 fragments with lengths of 150-1500 bp (Fig 1).

The total number of bands generated by the respective primers was 117, all of which were bands/primer was 29.25, with limits ranging from 27 to ScoT 14 and 34 to ScoT 36, respectively (Table 1).

Table 1

Analysis of the distribution of bands generated by ScoT primers for *Thymus* ecotypes

Parameter	Valoare
Number of primers	4
Total number of bands	117
Number of bands / primer	29,25
Number of polymorphic bands	117
Polymorphism rate (%)	100
Analysis efficiency index	29,25
Polymorphism / primer	0,351±0,018
Marker index	10,571+1,033

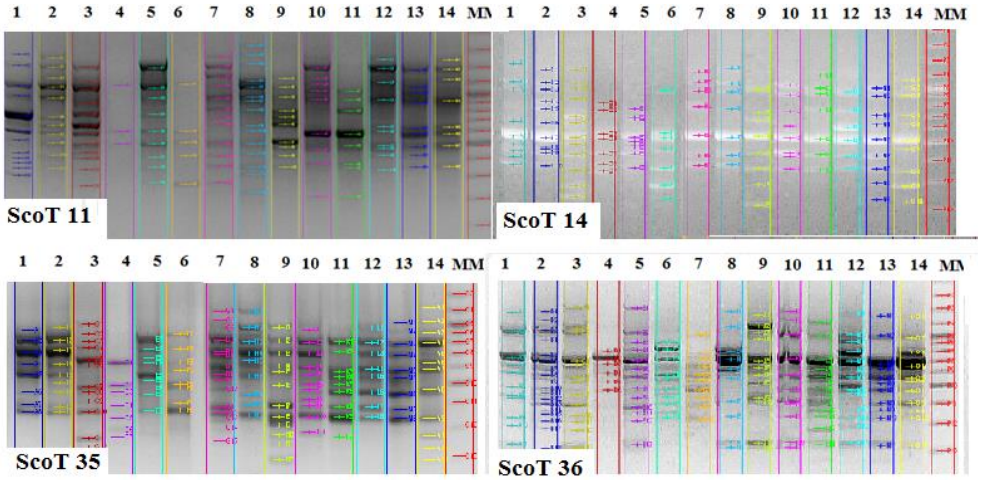

Figure 1. The DNA fingerprints generated by the ScoT primers (TNR 9, normal, center)

The total polymorphism generated by a certain primer (PIC) and its discriminatory power, showed values between 0.314 for ScoT35 and 0.400 for ScoT36, with an average of 0.35. The discrimination index (PI), which emphasize the efficiency of a certain primer in detecting polymorphism, had values between 9,122 for the ScoT14 primer and 13,602 for the ScoT36 primer, which had the highest capacity to generate polymorphic bands in the analyzed ecotypes (Table 2).

Table 2

Polymorphism rate for the *Thymus* ecotypes using ScoT primers

Nr. crt.	Primer	The nucleotide sequence nucleotidelor	Bands number		Polimor-phism (%)	PIC $\bar{x} \pm s_{\bar{x}}$	PI
			Total	Polimorph			
1	ScoT 11	AAGCAATGGCTACCACCA	29	29	100	0,350±0,021	10,143
2	ScoT 14	ACGACATGGCGACCACGC	27	27	100	0,338±0,027	9,122
3	ScoT 35	CATGGCTACCACCGGCCC	27	27	100	0,314±0,029	9,418
4	ScoT 36	GCAACAATGGCTACCACC	34	34	100	0,400±0,016	13,602

Considering the bands generated by the Scot 11 primer, it was observed that all 29 bands amplified by this primer are polymorphic, with frequencies between 7.1 and 78.6%.

Based on the similarity matrix, it was found that the interpopulation diversity presented values from 10.34% between ecotypes 10 and 2; 4 and 6; 12 and 14; up to 65.52% between ecotypes 7 and 10.

Scot14 primer had a polymorphism rate of 100%, given that all 27 bands were polymorphic, with frequencies between 7.1 and 78.60%. Half of the bands showed a high polymorphism of over 0.4, while the lowest value of the polymorphic capacity was 0.133 recorded in four bands.

The high polymorphic capacity of this primer was also observed from the matrix of genetic similarity, according to which the highest genetic differentiations (59.26%) were registered between ecotypes 2 and 13, or between 2 and 10 and 3 and 12 respectively (55.56%). The highest similarity for the alleles of this primer was observed between ecotypes 13 and 14 (88.89%), 7 and 8 (85.18%), respectively 2 and 3 (81.45%).

Considering the bands generated by the ScoT35 primer, based on a maximum level of polymorphism, the 27 bands showed frequencies between 7.1 and 85.7%.

Taking in account the slightly higher allelic similarity (63.78%) compared to the other ScoT primers, it was found that the interpopulation similarity registered values from 39.29% between ecotypes 5 and 9, respectively 42.86% between ecotypes 2 and 14, up to 89.29% between ecotypes 1 and 12, or 8 and 12.

Given the bands generated by the ScoT 36 primer, it was observed that all 34 bands amplified by this primer are polymorphic, with frequencies between 7.1 and 71.4%.

Based on the similarity matrix, it was found that interpopulation diversity showed values from 58.72% between ecotypes 1 and 13 to 79.41% between ecotypes 7 and 11. Regarding the analysis of variance for the studied *Thymus* ecotypes in terms of the amplified bands of the ScoT36 primer, high variance values were recorded for ecotypes 4, 11 and 12. Reduced variability in band distribution was observed for ecotypes 4 and 10. Ecotypes 2 and 3 have the greatest contribution to the diversity within the first cluster, respectively the population 10 in the second cluster, while the allele frequency for that primer in the population 7 has a lesser influence on the diversity between the ecotypes in the last cluster.

Regarding the values presented in Table 3, it was observed the existence of very close and statistically assured relation between the total diversity expressed through ScoT primers and the individual contributions of each of these primers.

Table 3

Values of the correlation coefficients established between the DNA fingerprints generated by ScoT primers

Primer	ScoT11	ScoT14	ScoT35	ScoT36	ScoT
ScoT11	1	0.165 *p=0,119*	-0,017 *p=0,867*	0,187 *p=0,076*	0,621*** *p=0,001*
ScoT14		1	0,052 *p=0,626*	0,273** *p=0,009*	0,627*** *p=0,001*
ScoT35			1	-0,081 *p=0,445*	0.409*** *p=0,001*
ScoT36				1	0,611*** *p=0,001*
ScoT					

As such, these primers can be used effectively to assess polymorphism and establish genetic diversity among different ecotypes of *Thymus*. It was also found that the analyzed ecotypes have similar genetic structures for the alleles of Scot14 and ScoT36 primers.

The high polymorphic capacity of the four primers was also observed from the matrix of genetic similarity according to which the highest genetic differentiations (50%) were registered between ecotypes 1 and 14, respectively 49.15% between ecotypes 3 and 10, or 47.46% between ecotypes 7 and 10. The highest similarity for the alleles of this primer was observed between ecotypes 13 and 12 (76.74%), 13 and 14 (74.58%), respectively 10 and 11 (72.88%).

Depending on the genetic similarity for the 117 ScoT alleles, the ecotypes were classified hierarchically into four main clusters, among which there is an average diversity of approximately 62% (Fig. 2).

The first group consists of 66.95% genetically similar ecotypes 1 and 2 to which are added the ecotypes 3 and 5 which show a genetic differentiation of 22.2% and about 4 % compared to 1 and 2 group

The second group consists of ecotypes 4 and 6 with a genetic similarity of about 72%.

Ecotypes 10 and 11 which have a common fund of 72.88% ScoT alleles, together with ecotypes 12 and 13 and the ecotype 14, form a third group at the level of which the average diversity is about 28%.

The last cluster includes the ecotypes 7 and 8, along with the cultivated population of *Thymus* (9), which has a common fund of about 67% ScoT alleles.

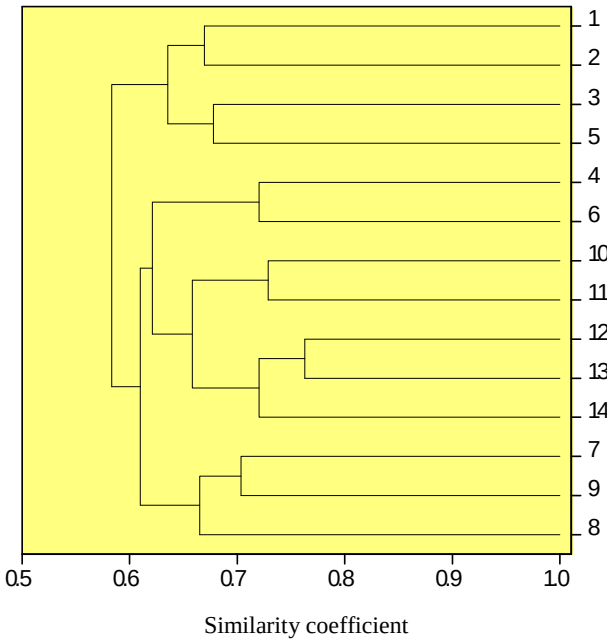

Figure 2. The dendrogram generated by ScoT primers amplification for the analyzed *Thymus* genotypes based on ANOVA analysis

Regarding the studied ecotypes (Table 4), a notable contribution to the total variability regarding the spectrum of the different amplified fragments of ScoT primers was observed in the case of the population 7, which is highlighted by a different allelic structure. The lowest values of variance were recorded in ecotypes 10 and 6. The highest variability of polymorphic bands within the first cluster was recorded in population 3 and population 6 for the second cluster, respectively. Population 10 shows a high influence on the diversity at the level of the third cluster.

Table 4

Variance analysis for *Thymus* ecotypes based on ScoT primers analysis

Genotype	Between groups		Inside the group		F test
	SP	GL	SP	GL	
1	3,420	1	23,335	116	17,00**
2	8,774	1	19,701	116	51,66**
3	2,535	1	25,940	116	11,34**
4	1,738	1	17,965	116	11,23**
5	3,413	1	22,349	116	17,72**
6	0,863	1	20,493	116	4,88*
7	11,246	1	17,229	116	75,72**
8	3,915	1	22,195	116	20,46**
9	3,179	1	22,932	116	16,08**
10	0,650	1	26,401	116	2,85
11	6,919	1	18,844	116	42,59**
12	4,720	1	20,297	116	26,98**
13	2,729	1	23,034	116	13,74**
14	1,279	1	25,161	116	5,90*

CONCLUSIONS

The evaluation of the ScoT marker have shown that their use to assess variability in wild thymus is possible. The four used primers generated complex DNA fingerprints, in which all bands were polymorphic, therefore no band was common to all analyzed ecotypes. A high number of fragments (117) was amplified with an average of 29.25 alleles / primer. The analysis of the variance showed an average PIC of 0.370 and an average polymorphic index (PI) of 10.57, which places the analyzed markers in the category of highly polymorphic ones, with high discrimination power. The data obtained allowed the elaboration of a dendrogram that groups the analyzed ecotypes according to the similarity index. Therefore, it has been shown that the assessment of variability and the establishment of similarity indices in *Thymus* using ScoT markers is possible and can be the basis of complex molecular genetic analysis.

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