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Assessment of Morphological and Genetic Variability in some *Thymus* Accessions Using Molecular Markers

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Abstract

The aim of the study was to determine the morphological and genetic variability in seven accessions of *Thymus*: *T. vulgaris*, *T. vilosus*, *T. praecox*, *T. serpyllum*, *T.* × *citriodorus*, *T. serpyllum* 'Aureum' and *T.* × *citriodorus* 'Silver Queen'. Morphological differences between the studied accessions were determined on the basis of morphometric measurements of some traits including: the length of the longest sprout, the width of pair leaves, length and width of leaves and essential oils content. DNA polymorphism was assessed based on the analysis in between region microsatellite sequences (ISSR-PCR) and InterGenic Spacers between copies of 5s RNA subunits genes (IGS-PCR). Amplicons were analyzed by electrophoresis on a 2% agarose gel. The 0/1 matrixes were used to calculate phylogenetic similarity and were employed to construct an UPGMA dendrograms. Morphometric studies showed that there were significant differences among *Thymus* accessions in all studding parameters. The ISSR reactions were carried out using 7 out of 20 microsatellite primers tested. Those amplified a total of 127 loci (309 amplicons). Specific DNA profiles were generated Only for *T. vulgaris* and *T. serpyllum*, which were different from other accessions, whereas in nested-PCR reactions (B and NSP primers) monomorphic products were amplified

Keywords: genetic variability, IGS, ISSRmorphology, *Thymus*

Introduction

The genus *Thymus* is exceptionally rich in species, and due to the diversity and plasticity of these plants, their geographical range is very wide. *Thymus* species differ with regard to their morphological features and metabolism, which influences their chemical constitution. Within individual species, particularly *T. vulgaris* and *T. serpyllum,* there are chemical variations that are characterized by different plant oil compositions, usually without any morphological differences. Typically, each plant has two predominant types of oil, phenols and terpenes are particularly characteristic of the *Thymus* genus, for example *T. serpyllum* contains about 50% phenols, while cymene, pinene, linalool, and borneol are only accompanying components.

The presence of other components and the proportions that these components occur in can undergo considerable variation depending on the plant's origin, variety, and environmental factors. Other species of thyme are of lesser importance; nevertheless, they are often used in cultivation as components for crossbreeding, and may considerably influence the qualitative composition of essential oils. Based on the genetic traits of the plant, the breeder tries to create new varieties, and increase desirable features while decreasing negative ones (Rumińska, 1983).

Increasingly, plant breeding has taken advantage of developments in molecular biology in order to genotype the species of interest in a way that considerably accelerates their selection. These types of approaches consist of choosing desired genotypes on the basis of molecular markers, or having prior knowledge of the genes that determine the formation of a particular trait in a plant (Pradeep *et al.,* 2002).

Genetic markers are easily recognized markers of important traits in cultivation, and have become an object of interest for many researchers. Identification of markers basically consists of determining the exact relationship between a specific DNA sequence and the examined trait, or a plant identity that is determined by particular genes. One method for identifying molecular markers is the ISSR technique, which is based on amplifying DNA regions that create gaps between recurrent blocks of specific DNA sequences of several nucleotides (Zietkiewicz *et al.,* 1994). It is also possible to use moderately variable sequences for this purpose, for example, transcribed sequences separating sequences of ribosomal genes or some mitochondrial genes (Thompson *et al.,* 1994).

An area that is often examined within ribosomal RNA genes is a sequence that encodes three subunits of rRNA, together with external and internal transcribed spacer sequences (Godwin ? 1997). Genes encoding the 5s rRNA (5s rDNA) subunit create a group of 200 – 900 bp tandemly arranged sequences. They are characterized by the presence of highly conserved sequences within coding sequences, and variability in both the length and quality of non-transcribed sequences (Moreno *et al,.*1998).

The aim of this study was to determine the extent of morphological and genetic variability, and the phylogenetic relationship within a subset of thyme accessions. We also wished to analyze the genotypes of these accessions using the ISSR method and through amplification and sequencing of the intergenic spacer (IGS) regions that separate copies of the 5s rRNA gene.

Materials and methods

The research material consisted of seven accessions of thyme: *Thymus vulgaris* L., *Thymus vilosus* L., *Thymus serpyllum* L., *Thymus serpyllum* L. 'Aureum', *Thymus praecox* L., *Thymus* × *citriodorus* L. and *Thymus* × *citriodorus* L. 'Silver Queen', from a collection at the Vegetable Faculty of the Agricultural University of Szczecin. The phenotypic variability of the studied *Thymus* accessions was determined in a field experiment established at the Vegetable Research Station in Dołuje. Genotypic variability within 5s RNA was determined using ISSR and IGS techniques at the Department of Horticultural Plant Breeding of West Pomeranian University of Technology in Szczecin.

Phenotypic variability

The research material consisted of herb cuttings collected from mother plants in April 2006. Twenty-four cuttings were taken from each species. The cuttings were rooted in multi-purpose soil in a seedling tray with 4 cm diameter cells. In May 2006, 10 plants from each species were planted in a field using a spacing of 30×40 cm. During their growth, the plants were fertilized twice with ammonium saltpetre (10 $g \cdot m^{-2}$). The plants were nurtured by loosening the soil between rows of plants, removing weeds and periodically watering the plants. Biometric measurements of the plants were made during the vegetation period (every 12-14 days), determining their height (cm), the width of a leaf pair (cm), the length and width of a leaf blade (cm). The herbs were then picked, sprouts were separated from leaves, and all plants parts were weighed and dried in a drying room. After the drying process, the herbs were weighed again, and the before- and after-drying weights were compared. Essential oils were extracted using a Deryng apparatus.

Genetic variability

The total DNA from about 100mg of fresh leaf material was extracted using the AandA Biotechnology kit (DNA PrepPlus). ISSR-PCR mixtures (25 μl) contained: 2.0 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl pH 8.3, 0.1% Triton X-100, 0.2 μM primer (for ISSR), 0.2 mM of each dNTP, 1.0 units of *Taq* DNA polymerase (Fermentas MBI) and 50 ng template genomic DNA. DNA was amplified using a Mastecycler (Eppendorf) thermal cycler and using the following program: initial denaturation at 94°C for 7 min, 40 cycles of 30 s at 94°C, 50 s at annealing temperature, 2 min at 72°C, and 7 min at 72°C for a final extension. The annealing temperature was usually adjusted according to the T_m of the primers being used in the reaction (Tab. 1).

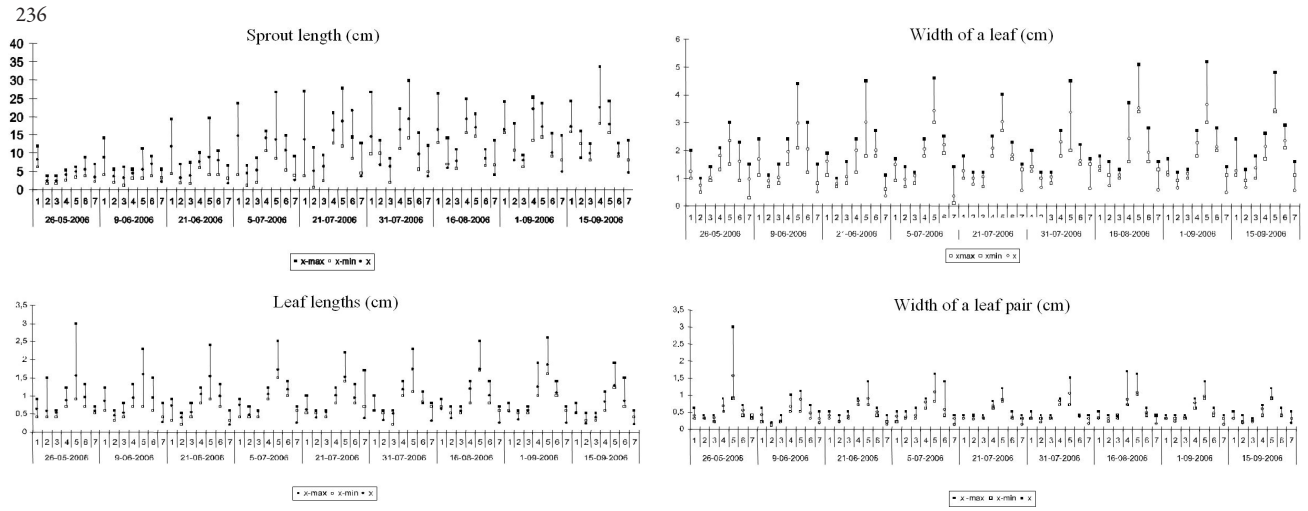
IGS-PCR

This DNA was then used as template for PCR amplification of 5S rRNA repeat based on three primers designed through Ko and Henry (1996) for cereals genomes (A: 5' TTT AGT GCT GGT ATG ATC GC 3'; B: 5' TGG GAA GTC CTC GTC TTG CA 3'; NeStedPrimer: 5' ACA CTC TTG CCA CCT TCA CGA 3'). DNAs were amplified for 35 cycles of 1 min at 94°C, 45 s at 55°C, 1 min at 72°C and one final cycle of 5 min at 72°C. The amplified products were analyzed by electrophoresis on a 2% agarose gel and visualized with ethidium bromide (0.5 $\mu g \cdot ml^{-1}$) on a UV-21 transilluminator (Fotodyne). The PCR products were photographed (Polaroid DS-34). Only those bands that showed consistent amplification were considered for this study. DNA fragments, detected not in all individual species spectra were considered as polymorphic. Each fragment that was amplified using ISSR primers, was coded in a binary form by '0' and '1' for absence or presence in each species, respectively. To infer phylogenetic relationships, the 0/1 matrix was used to calculate genetic similarity and then employed to construct an unweighted pair-group method with arithmetic means - UPGMA – dendrogram using software packages Diversity one 1.3 (Pharmacia LKB) (Nei and Li 1979, van der Peer *et al.,* 1994). The robustness of the tree topology was assessed by 1,000 bootstrap resamplings (Felsenstein 1985, Van de Peer and De Wachter 1994).

Results

Assessment of morphological variability

Biometric measurements were used to study the selected thyme accessions, and a wide range of morphological variety was observed. This variety was manifested in the sprout length, the width



1 – *T. vulgaris*, 2 – *T. vilosus*, 3 – *T. praecox*, 4 – *T. serpyllum*, 5 – *T.* × *citriodorus*, 6 – *T. serpyllum* 'Aureum' L., 7 – *T.* × *citriodorus* 'Silver Queen'

Fig. 1. Growth dynamics and average sprout length, width of a leaf pair, leaf lengths and widths, determined for selected accessions from the *Thymus* genus

and length of a leaf pair, and the width of individual leaves (Fig. 1.).

The hybrid *T.* × *citriodorus* plants were the largest of the *Thymus* accessions examined, and the *T. praecox* plants were the smallest (Fig. 1). These two accessions formed two extreme groups that had different average values for most of the traits analyzed. The *T.* × *citriodorus* 'Silver Queen' variety was considerably different from the form it was derived from regarding the traits analyzed in this study. The 'Silver Queen' is a short plant with small, narrow leaves (Fig. 1). The rest of the accessions had variable values for the traits analyzed, and their growth over the course of the study was exceptionally steady. Fig. 1 provides information regarding the individual values of each trait, and the rate of growth over the vegetation period. After the biometric measurements had been made, the herbs of the characterized *Thymus* genotypes were picked for drying and extraction of essential oils. It was observed that the contribution of the leaves and the sprouts to the mass of the plants varied. The largest contribution of the leaves to plant mass was observed in the smallest plants,

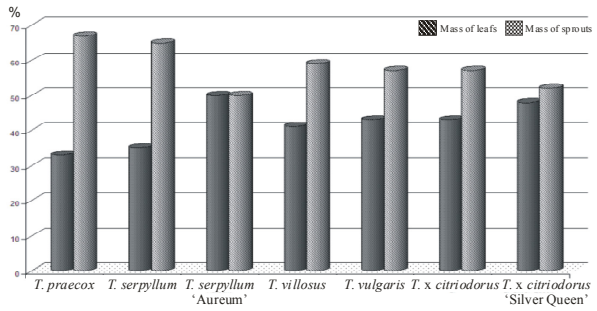


Fig. 2. The contribution of individual *Thymus* organs to plant mass after harvest

the *T.* × *citriodorus* 'Silver Queen' (48 %) and *T. serpyllum* 'Aureum' (50 %) (Fig. 2.).

After drying, the contributions of the leaves and the sprouts to the mass of the herb were similar to the contributions before drying. However, the mass of the air-dried leaves considerably decreased relative to the dried mass of the sprouts (Fig. 2.).

The largest amount of essential oil was observed in *T. vulgaris* (3.5%), and this was followed by *T. serpyllum (*2.5%) and *T.* × *citriodorus* (2%). In the rest of the studied thyme species, *T. serpyllum* 'Aureum' produced 1.5% essential oil, and *T. vilosus* and *T.* × *citriodorus* 'Silver Queen' produced ~1%. The smallest amount of essential oil was found in *T. praecox* (0.5%).

Assessment of genetic variability

Twenty microsatellite primers were used in the ISSR reactions. Clear reaction products were generated from reactions with seven of the primers (Tab. 1.).

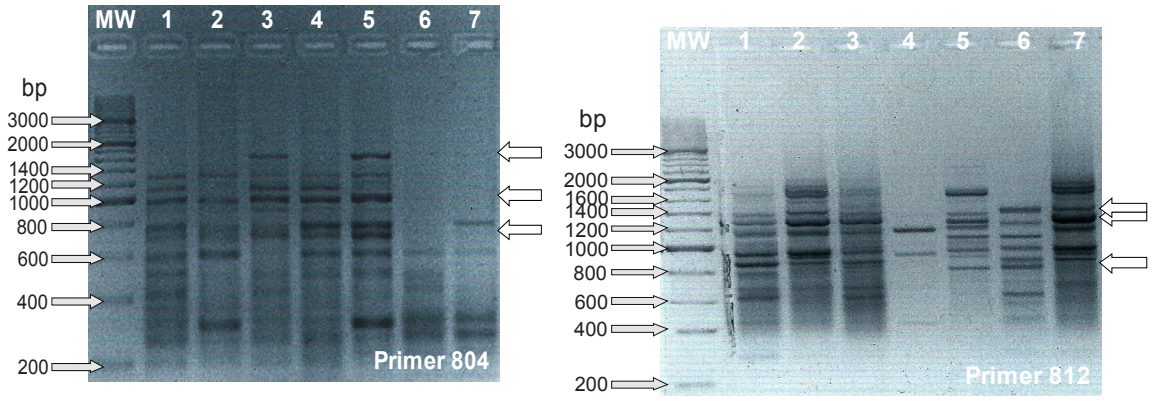
For the examined thyme accessions, 127 loci were amplified, generating 309 amplicons. The length of the amplicons varied from ~200 to ~3100 bp (Tab. 1, Fig. 3.).

A wide range of morphological variability was observed within the studied *Thymus* accessions. Interestingly, there were a large number of polymorphic loci (75 loci - 59%), and many of the amplified loci were specific to particular genotypes (49 loci - 39%). Two monomorphic loci were amplified (Tab. 1, 2).

Specific DNA profiles were generated for each of the studied *Thymus* genotypes, allowing them to be distinguished them from each other. Six specific loci were amplified for *T.* × *citriodorus* in reactions with three primers (813, 819 and 839), while two loci were amplified using two primers for the 'Silver Queen' variety. The

Tab. 1.Characteristics of ISSR's generated for seven accession of *Thymus*

Primer	Sequence 5' – 3'	Product length (bp)	Loci number				Amplicons number							Total
			Total	Polymorphic	Monomorphic	Accession-specifico	*T. vulgaris*	*T. vilosus*	*T. praecox*	*T. serpyllum*	*T. × citriodorus*	*T. serpyllum'Aureum'*	*T. × citriodorus* 'Silver Queen'	
804	$(AC)_8C$	1600 - 200	15	12	3	0	13	11	14	13	15	6	5	77
812	$(GA)_8C$	2000 - 420	17	15	0	2	12	13	10	3	9	8	14	69
813	$(GT)_8C$	2900 - 660	18	6	0	12	2	4	7	1	6	5	2	27
819	$(GT)_8A$	1760 - 410	20	10	0	10	8	5	9	0	9	7	1	39
824	$(TC)_8G$	2370 - 530	11	5	0	6	0	3	6	0	4	3	3	19
826	$(AC)_8C$	3100 - 970	18	12	0	5	7	4	6	0	6	8	3	33
839	$(TC)_8GT$	1620 - 340	29	15	0	14	10	7	7	3	10	8	0	45
		Σ	127	75	3	49	52	47	59	20	59	45	28	309
		Average	18	11	0	7	7	7	8	3	8	6	4	44



MW - Molecular Weight, 1 - *T. vulgaris*, 2 - *T. vilosus*, 3 - *T. praecox*, 4 - *T. serpyllum*, 5 - *T. × citriodorus*, 6 - *T. serpyllum 'Aureum'*, 7 - *T. × citriodorus* 'Silver Queen'

Fig. 3. ISSR-PCR fingerprints of seven *Thymus* species using the 3' anchored selecte primer. The selected bands marked with white arrows polymorphic

largest number of specific *loci* (15) was amplified in *T. praecox*. The smallest number of loci (2, in reactions with 1 primer) was amplified from *T. serpyllum* (Tab. 3). Genotypically-specific loci and the primers used to amplify them are shown for the *Thymus* genotypes in Tab. 2.

On the basis of the UPGMA cluster algorithm and the Nei and Li's coefficient (1979), a phylogenetic tree (Fig. 4) was drawn for the DNA profiles obtained using ISSR reactions. Two similarity groups, 'a' and 'b', were determined in the tree. Three species were included in group 'a': *T. serpyllum*, *T. vilosus* and *T. praecox*. *T. × citriodorus* 'Silver Queen' and *T. serpyllum* 'Aureum' were included in group 'b'. The other accessions were grouped outside of these two clusters (Fig. 4.).

DNA polymorphisms within IGS sequences

In the PCR reactions that were conducted with a specific pair of primers (A and B) for the *Thymus* accessions, three polymorphic reaction products of ~780, ~740 and ~430 bp, respectively, were amplified (Fig. 5.).

They were observed as unique DNA profiles that differentiated *T. vulgaris* and *T. serpyllum* from the other five *Thymus* accessions studied. A small range of variability was observed within the sequences that separated copies of the rDNA genes that encode the 5s rRNA subunit. Monomorphic PCR products that measured ~740 bp were generated for five of the *Thymus* accessions, and were gel-extracted and used as a template for a nested PCR with the B and NSP primers. After the reaction, the amplicons were separated by electrophoresis in an agarose gel (Fig. 5.). No differences were found within the complementary sequences for the NSP primer for the five *Thymus* accessions analyzed, and the monomorphic products that measured ~430 bp were amplified using PCR (Fig. 5).

Tab. 2. Accessions-specific products revealed through ISSR fingerprinting

Accession	Primer no., ISSR *loci* amplified (bp)z
T. vulgaris	813 [~1220], 819 [~520], 826 [~2330, ~2000, ~1300] 839 [~1590, ~1450, ~880, ~410, ~340]
T. vilosus	813 [~1720, ~660], 819 [~1760, 510] 839 [~770]
T. praecox	812 [~980], 813 [~2900, ~1880, ~1400], 819 [~1450, ~530, ~480], 824 [~2370, ~1770, ~1500, ~1200, ~530], 826 [~1450], 839 [~550, ~390]
T. serpyllum	813 [~2000, ~1380]
T. × *citriodorus*	813 [~1240, ~680], 819 [~1380, 470], 839 [~1620, ~1260]
T. serpyllum 'Aureum'	813 [~2240, ~1280], 819 [~740, 470], 826 [~1800], 839 [~1380, ~1160, ~730, ~660]
T. × *citriodorus* 'Silver Queen'	812 [~2000], 824 [~1410]

An analysis of a dendrogram of phylogenetic similarity showed that the biggest one (67%) was between *T. vulgaris* and *T. serpyllum.* The level of similarity between *T. serpyllum* and *T. praecox*, *T. vilosus*, *T. serpyllum* 'Aureum', *T.* × *citriodorus* and *T.* × *citriodorus* 'Silver Queen' reached

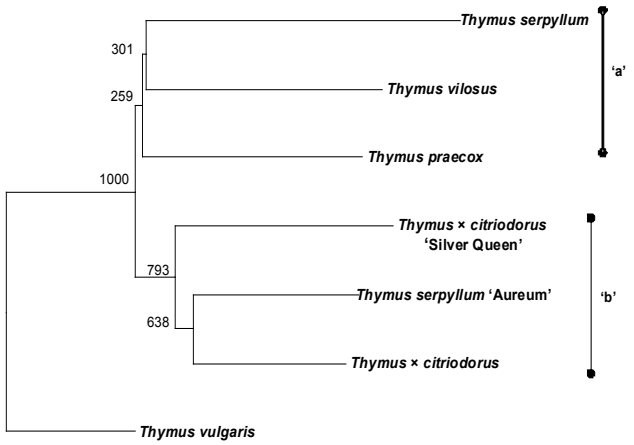


Fig. 4. Phylogenetic tree using the ISSR PCR products. Numbers above branches indicate bootstrap values calculated using 1,000 replications

33%, while the similarity of the accessions belonging to group 'a' reached 100% (Fig. 6).

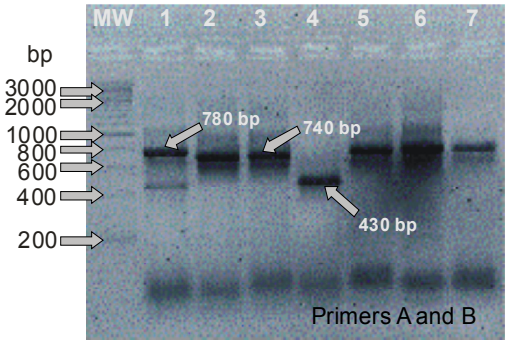


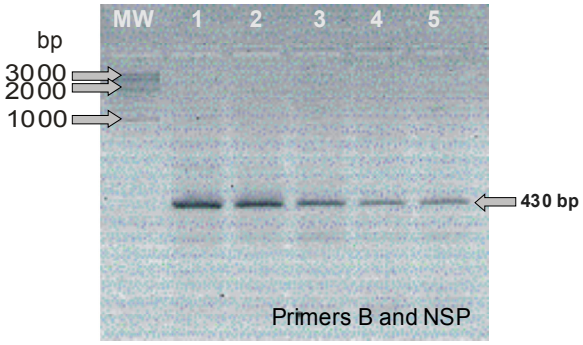
Fig. 5. MW - Molecular Weight, 1 - *T. vulgaris,* 2 - *T. vilosus*, 3 - *T. praecox*, 4 - *T. serpyllum*, 5 - *T.* × *citriodorus*, 6 - *T. serpyllum* 'Aureum', 7 - *T.* × *citriodorus* 'Silver Queen'

Discussion

Base materials play a very important role in plant breeding. They are components that are desirable to introduce into a hybrid, in order to later select the recombinants with the most valuable traits. A high base material quality generally guarantees successful breeding, and gives breeders a chance to quickly register new varieties with high agronomic values. Access to base materials is relatively easy in the *Thymus* genus, since members of this genus easily crossbreed, both within varieties and species, and produce numerous offspring.

Lakawiczius and Jaskonis (1968) found around 20 spontaneous hybrids surrounding Vilnius. These included *T. pulegioides* × *T. serpyllum*, while Chladek and Spurna (1964) found successful interspecies crossbreeds between *T. vulgaris* and *T. serpyllum,* as well as between *T. vulgaris* and *T. Marschallianus*.

The cultivation of *Thymus*, as well as of *Mentha*, *Sanguisorba* or *Nepeta* is orientated towards obtaining new varieties that contain large amounts of biologically active substances, particularly essential oils. Terpenes, linalool, caryophyllene, cineol, geraniol, borneol and citral are all found in the oil extracted from Thymus, and are of particular importance. There are many physiological varieties within *Thymus* genotypes, and these may cause problems



MW - Molecular Weight, 1 - *T. vilosus*, 3 - *T. praecox*, 4 - *T.* × *citriodorus*, 6 - *T. serpyllum* 'Aureum', 7 - *T.* × *citriodorus* 'Silver Queen'

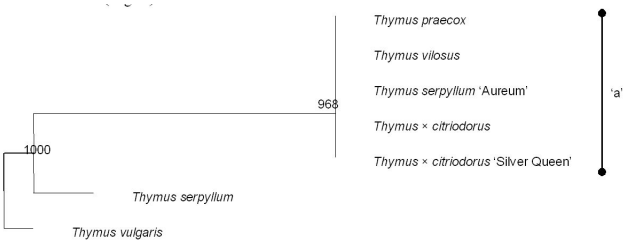


Fig.. 6. Phylogenetic tree using the IGS products. Numbers above branches indicate bootstrap values calculated using 1,000 replications

in cultivation. These varieties differ from one another solely in their essential oil content (Melchior and Kastner 1978; Rumińska 1983).

Schantz and Ivars (1964) observed a predominance of terpenes in thyme essential oil in a Finnish territory. Plants from the northern part of the country contained mainly linalool and linalool acetate (39 - 57 %), whereas plants from the south contained high levels of caryophyllene (19 - 27 %), cineol (18 - 28 %), geraniol, borneol and citral. Schratz *et al.* (1968) found a predominance of terpenes in most (83 %) of the specimens studied in Switzerland, while phenols were predominant in only some of the species.

T. vulgaris is cultivated in Europe and North America, and the main component of its raw material is oil. The oil content of *T. vulgaris* varies significantly depending on the origin of the plant, the variety and the weather conditions. In domestic material, the total oil content can range from 0.7 to 2%, or even more in some varieties. In the thyme accessions studied in this work, the total oil content ranged from 0.5 (*T. praecox*) to 3.5% (*T. vulgaris*).

Schratz *et al.* (1961) determined the essential oil contents of 325 thyme specimens (*Thymus vulgaris*), and found the content ranged from 0.8 to 3.4 %. Similar results (0.55% to 0.80 %) were obtained by Osińska and Węglarz (1999).

The occurrence of physiological varieties that are difficult to differentiate makes it impossible for a breeder to select appropriate components during breeding programm that allow varieties to be obtained that contain large quantities of the desired compounds without specialist training. The results of our studies may be of use to herb breeders, since we present the results of field experiments, which allowed us to determine a number of morphological parameters for the *Thymus* accessions studied. The experiments confirmed the existence of a wide range of morphological variability, which is evident in the type and length of sprouts, the size, shape and colour of leaves, the weight of the green parts, and the amount of essential oil. The results of our studies are consistent with information provided by Osińska and Węglarz (1999).

A wide range of variability was found both within microsatellite sequences and the IGS sequences separating numerous copies of 5s RNA genes. In addition to the morphological characteristics of the thyme species, these genotypes also provide valuable information for breeders of thyme species. The results of these studies, i.e. the average number of amplified loci in reactions with one primer, the length of amplicons and the percent of poly-, mono- and genotypically specific amplicons, are consistent with the results of studies by other authors, for example, *Thymus praecox* (Langergott *et al.,* 2006), grapevine (Moreno *et al.,* 1998), lemon (Fang and Roose 1997), mint (Smolik *et al.,* 2007) and burnet (Jadczak *et al.,* 2007).

The amplification of IGS sequences confirmed data that has been presented by other authors, regarding the wide range of variability within these sequences and their use for identifying varieties or species. The use of IGS sequences is not restricted to plants (Baldwin 1992). In PCR reactions with primers designed for to analyze IGS sequences (A and B, as well as B and NSP) in grains, polymorphic products were amplified that allowed the selected *Thymus* accessions to be differentiated. The results obtained confirmed that there was high degree of conservation of 5s RNA sequences and the variability in spacer sequences within accessions with different origins (Ko and Henry, 1994).

Conclusions

Our study indicated that PCR may be used as a technique to assess the degree of genetic variability within microsatellite sequences, as well as the spacer sequences that separate 5s RNA coding genes, in seven different *Thymus* accessions. A number of genotypically specific ISSR *loci* and polymorphic IGS sequences within 5s RNA were amplified using PCR. The combination of the information regarding morphological variability that was determined in field studies and genetic information may be of interest to breeders of this species, since it may allow them to apply the methods described in the study to select plants during cultivation. The information provided in this paper may also be of use in studies investigating the role of genetic background in the production of particular types of essential oils.

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