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ISSR analysis of chosen *Gleditsia* accessions obtained from Polish collections

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Abstract: Species of *Gleditsia* show considerable morphological variability that makes them difficult to distinguish using either vegetative or floral characters. Honeylocusts, especially the thornless cultivars, are popular ornamental, shade, street, attractive landscape trees. In this study the ISSR technique was used to evaluate the range of genetic variability between seven genotypes of *Gleditsia* cultivated in Polish dendrological collections [*Gleditsia caspica* Desf., *Gleditsia japonica* Miq., *Gleditsia japonica* Miq. var. *korainensis* (= *G. korainensis* Nakai), *Gleditsia triacanthos* L., *Gleditsia triacanthos* L. (bulk), *Gleditsia triacanthos* f. *inermis* (L.) Zabel (bulk). Forty ISSR primers were tested and 18 were selected for their ability to produce clear and reproducible patterns of multiple bands. A total of 177 loci of 260–2600 bp were amplified, of which 89 (50%) were polymorphic, 14 (8%) monomorphic and 74 (42%) were accession-specific. Accession-specific ISSR loci were obtained for all of the seven accessions tested. A dendrogram generated using the UPGMA, based on a similarity measure of total character difference, showed that the *Gleditsia* accessions were clustered into two main groups ('a' and 'b'). The first grup – 'a' – included: *Gleditsia triacanthos* L., *Gleditsia triacanthos* L. (bulk) and *Gleditsia triacanthos* f. *inermis* (L.) Zabel (similarity 0.61–0.75), the second – 'b' – included 2 species: *Gleditsia japonica* and *Gleditsia japonica* var. *korainensis* (similarity 0.43). Analysis of the phylogenetic similarity dendrogram has shown wide range of diversity between studied accessions. The clustering pattern obtained in our experiment was in agreement with the data based on morphological, allozyme and ITS analysis.

Additional key words: *Gleditsia*, genetic variability, fingerprinting, ISSR

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Introduction

The *Gleditsia* genus (locust trees), belonging to the *Caesalpinioideae* subfamily of the legume (pea) family *Fabaceae* (=*Leguminosae*), encompasses approximately twelve (14) species with natural stands in North America (2), South America (1), northern India (1), along the Caspian Sea in Azerbaijan and Iran (1) and several species in east and southeast Asia (Schnabel et al. 2003). These are trees (rarely shrubs) with characteristic twice-pinnate or pinnately compound leaves, thorns, polygamous dioecious flowers with radial symmetry (rarely encountered in this family) and fruits in the form of pods.

The North American honey locust (*Gleditsia triacanthos* L.), its thornless form [*Gleditsia triacanthos* f. *inermis* (L.) Zabel] as well as a few thornless cultivars are cultivated throughout Poland, but they are not very common (Seneta 1996, Czekalski and Danielewicz 1997).

Under the conditions existing in Poland, this tree goes through the full cycle of vegetative and generative development ending with the formation of seeds capable of germination (Czekalski and Danielewicz

1997, Kubus 1999). Other *Gleditsia* species, i.e., Caspian locust (*Gleditsia caspica* Desf.), Japanese honey locust (*Gleditsia japonica* Miq.) and Korean honey locust (*Gleditsia japonica* Miq. var. *korainensis*), are rarely cultivated in Poland, mostly in botanical gardens and arboreta, and no reference to their blossoming and fruit bearing can be found in the literature.

The morphological features of *Gleditsia* genus taxa are strongly diversified (Rehder 1960, Krüssmann 1960). However, unequivocal determination of the species affiliation of trees that have not undergone generative development is difficult, as in the case of the locust trees (*Gleditsia sp.*) described by Bojarczuk and Zieliński (1980), which have a characteristic columnar growth habit. Instability of sex expression is observed in honey locust (Michener 1986, Kubus 1999), and approximately 60% of seedlings of thornless forms (*G. triacanthos* f. *inermis*) do not develop the thorns characteristic of the species if they are propagated vegetatively (Fowells 1965, Blair 1990).

In this study, an attempt was made to determine the scope of genotypic variability within selected specimens of the *Gleditsia* genus from various regions of the world that are cultivated in Poland (excluding cultivars). The ISSR technique described by Zietkiewicz et al. (1994) was used. This technique amplifies DNA sequences located between tandem repetitive microsatellite sequences.

These sequences, or more precisely the variability within them and their occurrence in introns, exons, centromeres, and telomeres (Condit et al. 1991), have become in recent years the objects of a number of studies and were used to define the genetic variation and phylogenetic relationships between accessions of Douglas-fir of various origins (Tsumura et al. 1996), eucaplyptus germplasm (Van der Nest et al. 2000), tea (Mondal 2002), *Cicer* germplasm (Iruela et al. 2002, Rajesh et al. 2002, Sudupak 2004), *Morus* species (Vijayan and Chatterju 2003), lilacs of various morphologies and phenotypes (Rzepka-Plevneš et al. 2006) or maples from the Przelewice Arboretum collection (Rzepka-Plevneš et al. 2007).

In the present study, ISSR was used to determine variability, fingerprints and phylogenetic dependencies between locust tree taxa from Polish dendrological collections. Furthermore, attempts were made to determine the genetic relationship between the specimen described by Bojarczuk and Zieliński (1980) and the remaining genotypes in question and to compare the honey locust and its thornless form (*G. triacanthos* f. *inermis*) on the basis of descriptive genotyping of bulk samples of their DNA. The presented research represents a preliminary attempt to address these topics.

Materials and methods

Plant material representing seven locust trees was obtained from various Polish collections, including: *Gleditsia caspica* Desf., *Gleditsia japonica* Miq., *Gleditsia japonica* Miq. var. *korainensis* (= *G. korainensis* Nakai), *Gleditsia triacanthos* L. – pure species obtained from Arboretum of Polish Academy of Science in Kórnik (central part of Poland), *Gleditsia triacanthos* L. (bulk), *Gleditsia triacanthos* f. *inermis* (L.) Zabel (bulk) and *Gleditsia sp*. In the experiment in question, bulk DNA samples were formed by mixing equal amounts of DNA isolated separately from young leaves of five trees identified as *Gleditsia triacanthos* L. and four trees identified as *Gleditsia triacanthos* f. *inermis* (L.) Zabel growing in various stands in the city of Szczecin (north-west Poland). Morphological characteristics and origin of the aforementioned accessions were listed in the Table 1.

Table 1. Origin of the plant's material

Accesion	Origin	Place of groving/appearance	The age of tree [years]	Parameters: high [m]/circuit of strump [cm] at 1.3 m high
Gleditsia caspica Desf.	the south coasts of Caspian Sea Erewan Kanaker, Botanical Garden, Armenia	Arboretum of WULS-SGGW in Rogów near Łódź (central Poland)	48	11.5/43
Gleditsia japonica Miq.	Japan Kasukabe, Kanagawa Botanical Garden	Arboretum of WULS-SGGW in Rogów near Łódź (central Poland)	32	2.8/5.5 the shrub – with no central trunk
Gleditsia japonica Miq. var. *korainensis* (= *G. korainensis* Nakai)	North Korea Pyong-Yang H.B. Botanical Garden	Arboretum of WULS-SGGW in Rogów near Łódź (central Poland)	37	2/6 the shrub – with no central trunk
Gleditsia triacanthos L.	Canada, Toronto (the natural position)	Arboretum in Glinna near Szczecin (northwest Poland)	16	4.2/17
Gleditsia triacanthos f. *inermis* L.(Zabel)	USA (the provenience of the seeds is unknown)	Since 1926 in Botanical Garden – Szczecin (northwest Poland)	about 85	17/213
Gleditsia sp.	(the provenience of the seeds is unknown)	Arboretum of PAS in Kórnik near Poznań (central Poland)	about 75	13/124

WULS – Warsaw University of Life Science (SGGW)
PAS – Polish Academy of Science

The total genomic DNA from about 200 mg of fresh leaf material was extracted using the *Genomic Mini AX Plant* kit (A&A Biotechnology). 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, 1 out of 40 different 0.2 μM primers (UBC – University of British Columbia primer sets), 0.2 mM of each dNTP (Fermentas), 1.0 units of *Taq* DNA polymerase (Fermentas) and 100 ng template genomic DNA. The contaminating RNA was removed by digestion with RNase A (20 mg · cm^{-3}). DNA was amplified using a Mastecycler 5333 (Eppendorf) thermal cycler using the following programme: initial denaturation at 94°C for 7 min, 40 cycles of 30s at 94°C, 50 s at annealing temperature, 2 min at 72°C, and 7min at 72°C for a final extension. ISSR products were mixed with 6× Orange Loading Dye Solution and analysed by electrophoresis on a 2% agarose Basic LE (Prona) with ethidium bromide (0.5 mg · cm^{-3}). O'RangeRuler 200bp DNA Ladder (Fermentas) was used as a size marker (3000 – 200 bp). For ISSR data analysis (GelCapture – MiniBis Pro – Bio Imaging Systems – USA), the relative mobility position of all bands present in each analyzed *Gleditsia* accession was calculated and transformed in a data matrix in which the character '1' means the presence of a specific band and '0' represents its absence (Gelquant/MiniBis Pro – Bio Imaging Systems – USA). The Nei and Li (1979) algorithm contained in the TREECON computer package software was used to calculate the genetic distances between the *Gleditsia* accessions (Van de Peer and De Wachter 1994). Rooted phenograms were constructed by the UPGMA (unweighted pair group with arithmetic mean) method, and the robustness of the tree topology was assessed by 2,000 bootstrap resamplings (Felsenstein 1985).

Results

A set of 40 microsatellite primers was used in this study. They differed from one another by the sequence of the repeated motif and the number of anchoring nucleotides at their 3' end. Among the 40 primers used, distinct amplicons were generated in reactions with 18 primers. A total of 177 loci were amplified (469 amplicons), among which 14 (8%) turned out to be monomorphic, 89 (50%) polymorphic, and 74 (42%) were defined as accession-specific (Table 2, Fig. 1).

Table 2. ISSR primers used to characterize 7 accessions of *Gleditsia,* the number of total, monomorphic, polymorphic and specific fragments generated by ISSR technique

Primer no.	Primer sequence 5'–3'	Fragment size range (bp)	Number of amplified loci				*G. caspica*	*G. japonica*	*G. japonica* var. *koraiensis*	*G. triacanthos typicum* (bulk)	*G. triacanthos typicum*	*G. triacanthos* var. *inermis* (bulk)	*G. sp.*	Generated amplicons
			Total	Monomorphic	Polymorphic	Accession-specific								
802	$(GA)_8T$	470–1550	**13**	2	8	3	4	5	5	10	9	7	6	**46**
810	$(GA)_8T$	390–1700	**14**	2	7	5	2	6	6	8	8	2	8	**40**
811	$(CA)_8AT$	350–1560	**18**	1	6	11	5	7	5	5	4	4	5	**35**
819	$(GT)_8A$	510–1500	**11**	0	7	4	0	5	5	5	5	6	3	**29**
820	$(GA)_8YC$	460–1350	**9**	1	3	5	2	6	2	3	2	5	1	**21**
821	$(GA)_8YC$	450–1460	**11**	0	4	7	1	7	2	4	4	3	2	**23**
823	$(TC)_8C$	700–2600	**11**	1	3	3	2	2	2	3	1	3	2	**15**
824	$(TC)_8G$	680–1640	**7**	1	0	0	2	5	4	2	1	2	3	**19**
825	$(AC)_8T$	1540	**1**	0	9	5	1	1	1	1	1	1	1	**7**
830	$(TG)_8G$	650–2150	**14**	1	4	4	2	4	3	3	3	4	6	**25**
833	$(GT)_8YC$	430–1720	**9**	0	10	4	3	6	2	4	4	5	4	**28**
837	$(AC)_8YG$	260–1740	**14**	2	0	6	4	4	6	6	6	8	5	**39**
839	$(AG)_8T$	370–1600	**8**	1	9	1	6	3	3	2	2	2	2	**20**
840	$(GA)_8GT$	380–1350	**11**	0	5	5	3	4	5	6	6	7	4	**35**
841	$(GA)_8AT$	400–1470	**10**	0	3	0	2	4	3	3	4	5	3	**24**
859	$(GT)_8YC$	950–1070	**3**	0	3	1	1	2	2	2	2	2	1	**12**
863	$(GAA)_6T$	340–1370	**4**	1	6	2	1	0	2	3	3	3	2	**14**
873	$(CACA)_4$	750–1960	**9**	1	2	8	4	7	7	5	5	5	4	**37**
	Total		177	**14**	89	74	**45**	**78**	65	75	70	74	62	**469**
	Mean		**10**	**1**	**5**	**4**	3	**4**	**4**	4	4	4	3	26

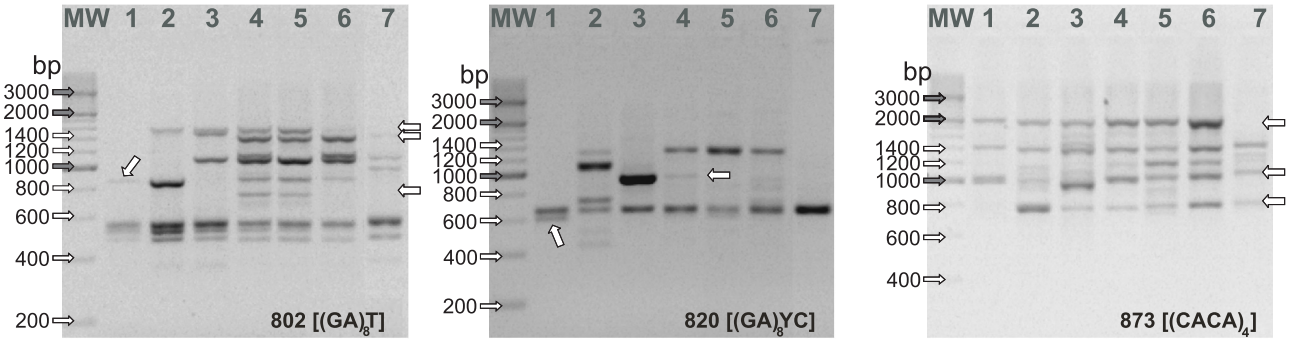

Fig. 1. Electrophoregrams of ISSR products amplified on DNA templates of seven accessions of *Gleditsia*. White arrows mark chosen ISSR polymorphic and accession-specific product

MW – Molecular Weight, 1 – *G. caspica*, 2 – *G. japonica*, 3 – *G. japonica* var. *korainensis*, 4 – *G. triacanthos typicum* (bulk), 5 – *G. triacanthos typicum*, 6 – *G. triacanthos* var. *inermis* (bulk), 7 – *G. sp.*

In general, approximately 10 loci, with lengths ranging from 260 (primer 837) to 2600 bp (primer 823) – Table 2, were amplified per reaction with one primer in the experiment.

The greatest number (18) was amplified in reactions with primer 811, and the smallest – one locus – was amplified with primer 825 (Table 2). The number of amplicons generated from the individual accessions of *Gleditsia* sp. is presented in Table 2.

In ISSR reactions with primers 802, 810 and 839, two monomorphic loci were amplified, and one locus was amplified in reactions with primers 811, 820, 821, 824, 825, 833, 840 and 873. No monomorphic loci were amplified in reactions with the remaining primers (Table 2).

The largest number of polymorphic loci (10 and 9) was amplified with primers 837, 830 and 840, and the lowest number of polymorphic loci (2 and 3) was amplified with primers 821, 820, 824, 859 and 863. Polymorphic loci were not found in the genetic profiles of the studied accessions in reactions with primers 825 and 839 (Table 2).

Analysis of electrophoregrams revealed that the *Gleditsia* sp. accessions studied in this experiment are characterised by a relatively high degree of variability. From each of them a number of accession-specific loci were amplified. These formed unique genetic profiles that distinguished each accession from the others (Table 3).

Only one locus was amplified with primers 840 and 863. No loci were amplified in reactions with primers 825 and 859 (Table 2 and 3, Fig. 1).

Twelve loci were amplified from *G. caspica* in reactions with eight primers. From *G. japonica*, a total of 20 loci were amplified in reactions with twelve primers. Fifteen genotype-specific loci were amplified from *G. japonica* var. *korainensis* in reactions with eleven primers. From two bulk DNA samples isolated from honey locust *G. triacanthos* and its thornless form *G. triacanthos* f. *inermis*, 75 and 74 ISSR products were generated, respectively. Seventy ISSR products were generated from the form used as a pure species (*G. triacanthos*) – Table 2, Fig. 1. Genotype-specific loci were generated from each of the three aforementioned genotypes (Table 3). They were found to be genetically diverse. Thus, six genotype-specific loci were amplified from *G. triacanthos* (bulk), three were amplified from *G. triacanthos* f. *inermis* (bulk), and two were amplified from *G. triacanthos* (pure species) in reactions with four, three and two primers, respectively (Table 3).

Genotype-specific loci in both *G. triacanthos* (bulk) and the pure species (*G. triacanthos*) were found in reactions with only one of the selected primers – 821 [$(GA)_8$YC]. From the bulk sample of *G. triacanthos*, specific loci of 1460 and 820 bp in lenght were amplified. From the pure species (reference) genotype, a specific locus of 870 bp was amplified (data not

Table 3. ISSR accessions-specific products amplified for 7 accessions of *Gleditsia*

Accession	Primers and generated products
G. caspica	$811_{1150, 780}$, 820_{620}, 823_{760}, 830_{2050}, 833_{1720}, 837_{440},$839_{1600, 950, 730, 550}$, 841_{400}
G. japonica	802_{850}, $810_{1700, 840}$, $811_{810, 350}$, $819_{1300, 670}$, $820_{1140, 540, 460}$, $821_{1090, 520, 450}$, 824_{980}, 830_{750},$833_{1530, 950}$ 837_{700}, $839_{370,}$ 841_{1120}
G. japonica var. *korainensis*	$811_{650, 420}$, $819_{1400, 510}$, $821_{900, 720}$, 823_{970}, 830_{1070}, 833_{1300}, $837_{370, 330}$, $839_{600,}$ 841_{690}, 863_{340}, 873_{1580}
G. triacanthos typicum (bulk)	$811_{850, 540}$, $821_{1460, 820}$, 823_{2600}, 830_{1420}
G. triacanthos typicum	821_{870}, 823_{1030}
G. triacanthos var. *inermis* (bulk)	820_{1140}, 823_{1000}, 841_{1060}
G. sp.	$802_{1450,1000}$, $810_{1420, 960, 910}$, $811_{1370, 460, 370}$, $823_{870, 700}$, $824_{1130, 860}$, 830_{2150}, 840_{380}, 841_{750}, 873_{1320}

Table 4. Values of genetic similarity between investigated accessions of *Gleditsia*

		1	2	3	4	5	6	7
1	*G. caspica* Desf.	1						
2	*G. japonica* Miq.	0.32	1					
3	*G. japonica* Miq. var. *korainensis* (=*G. korainensis* Nakai)	0.35	0.43	1				
4	*G. triacanthos typicum* L. (bulk)	0.26	0.30	0.42	1			
5	*G. triacanthos typicum* L.	0.28	0.31	0.40	**0.75**	1		
6	*G. triacanthos* var. *inermis* (Zabel) (bulk)	0.25	0.32	0.36	0.64	0.61	1	
7	*G. sp.*	**0.17**	0.26	0.26	0.31	0.34	0.37	1

shown). A relatively high degree of variability was found to exist between these genotypes, allowing them to be distinguished by reactions with just one primer. Similar dependencies were observed in electrophoregrams of ISSR products generated in reactions with primers 802, 823 and 873 (Fig. 1).

Analysis of the results confirmed the genetic separateness of *Gleditsia* sp. in relation to the remaining tested genotypes. Sixteen genotype-specific loci were amplified from *Gleditsia* sp. The most numerous (three loci each with lengths of 1420, 960, and 910 bp and 370, 460, and 730 bp, respectively) genotype-specific loci were amplified in reactions with primers 810 and 811. The remaining loci and the primers they were amplified by are presented in Table 3. This table also contains descriptions of all amplified loci for each of the tested genotypes.

Analysis of the dendrogram of phylogenetic similarity revealed that the similarity ranges from 0.17 (between *G. caspica* and *Gleditsia* sp.) to 0.75 [between *G. triacanthos* (pure species) and *G. triacanthos* (bulk)] – Table 4. The tested genotypes formed two distinct similarity groups, 'a' and 'b' (Table 4, Fig. 2).

The first group includes: *G. triacanthos* (pure species), *G. triacanthos* (bulk) and their thornless form – *G. triacanthos* f. *inermis* (bulk). Genetic similarity within this group ranges from 0.61 to 0.75 (Table 4). Two accessions comprise group 'b': *G. japonica* and *G. japonica* var. *korainensis*. The similarity between them was determined to be 0.43 (Table 4).

G. caspica and *Gleditsia* sp. were found to be significantly different from them. Analysis of the topology of the phylogenetic tree showed that *G. caspica* is grouped outside the aforementioned similarity groups, and that it is most similar to *G. japonica* (0.32) and least similar to *Gleditsia* sp. (0.17) – Table 4, Fig. 2. A similar relationship was found between *Gleditsia sp.* and the remaining accessions tested. It must be stated, however, that it was the most genetically related to the bulk DNA sample consisting of *G. triacanthos* f. *inermis* genotypes and the least related to the aforementioned *G. caspica* (Table 4, Fig. 2).

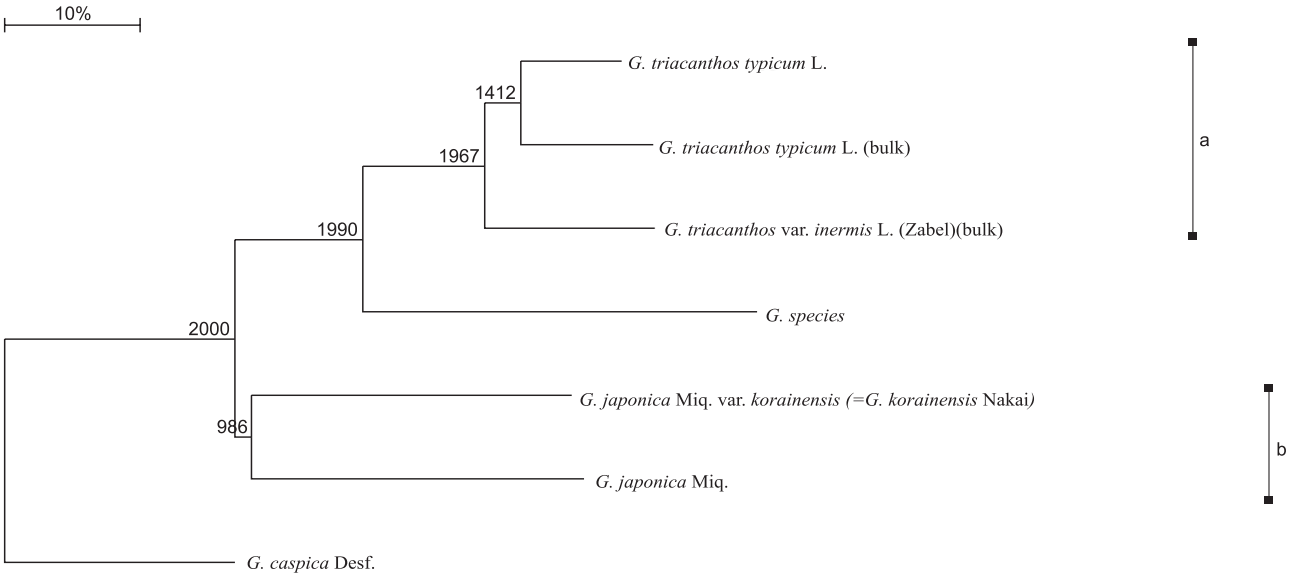

Fig. 2. Dendrogram of seven accessions of *Gleditsia* based on ISSR markers clustered by UPGMA technique. Numbers above branches indicate bootstrap values calculated using 2,000 replications

'a', 'b' – group of simililarity

Discussion

Nucleic acid sequences are a valuable source of data for phylogenetic research. As they provide a comprehensive approach to systematics and taxonomy (Irulea et al. 2002, Mondal 2002, Vijayan and Chatterju 2003, Sudupak 2004). Many plant species are characterised by the natural variability of alleles of a vast number of loci. Researchers can analyse a plant's genotype in each developmentall stage, which is especially important for the investigation of both phylogenetic similarities and differences between organisms (Wolko et al. 1999).

In this study the ISSR technique was used to evaluate the range of variability between seven genotypes of *Gleditsia* cultivated in Polish dendrological collections. The results of the analyses validate the opinions of numerous authors quoted in the present article on the utility of this method for this kind of research. The results confirm numerous reports that have already been published indicating an exceptionally wide range of variability within the *Gleditsia* genus (Schnabel and Hamrick 1990). In addition, the results presented here confirm the authors' opinions, which are the subject of ongoing discussion, about the close phylogenetic relationship between the two Asian species *G. caspica* and *G. japonica* (Paclt 1982, Schnabel and Hamrick 1990, Schnabel et al. 2003).

A relatively high degree of genotypic variability between *G. caspica, G. japonica* and *G. japonica* var. *korainensis* was demonstrated in this study. On the whole, a significantly smaller number of amplicons was amplified from *G. caspica* (only 45), while 78 and 65 amplicons were amplified from *G. japonica* and *G. japonica* var. *korainensis*, respectively. In numerous ISSR reactions, no ISSR products whatsoever were obtained from *G. caspica*, while visible and distinct amplicons were amplified from DNA templates from the remaining accessions. Based on these results, we can confirm the genetic separateness of *G. caspica* and *G. japonica,* as well as the genetic similarity of the botanical variety of *G. japonica* var. *korainensis* to *G. japonica*, for which a number of common and also polymorphic ISSR loci were amplified in our experiment.

Schnabel et al. (2003) proved on the basis of their own research that *G. caspica* is different from thirteen other species of the *Gleditsia* genus at the species and population levels. According to Schnabel and Wendel (1998), this is caused by its geographic isolation (South and South-East Asia).

Paclt (1982) considers *G. caspica* to be a subspecies of *G. japonica*, while Schnabel and Wendel (1998) and Schnabel et al. (2003) regard *G. caspica* and *G. japonica* as sister taxa, suggesting that *G. caspica* is a derivate of *G. japonica*. The latter interpretation was confirmed by the results of research based on the description of allozyme markers of two populations consisting of 75 individuals (Girkan Nature Reserve, Azarbaijan) and 30 adult individuals (Astara, Azarbaijan) – Schnabel and Krutovskii (2004), as well as by the results of research on phylogenetic relationships in *Gleditsia* conducted by Schnabel et al. (2003) using ITS sequences.

Differentiation of five and four bulk DNA samples of *G. triacanthos* and its thornless form (f. *inermis*) by means of the ISSR technique was another issue examined in this study. As has been shown by numerous tests and observations conducted over the last one hundred years (Arnold Arboretum of Harvard University – Michener 1986), a relatively wide range of variability is observed within this species, and the possibility of reversion of the thorniness feature is particularly interesting here (Michener 1986).

Fowells (1965) and Blair (1990) stated that the *G. triacanthos* species are characterised by significant genetic variability, and considering its wide distribution, northern and southern ecotypes were distinguished. The defenceless type of locust tree [*Gleditsia triacanthos* f. *inermis* (L.) Zabel] is also encountered in natural stands, and on the border of their distribution ranges, *G. triacanthos* and *G. aquatica* cross to produce fertile hybrids, *Gleditsia* ×*texana* Sarg.

Tumiłowicz (2005) pointed to considerable morphological differences between locust trees from North Korea (from the botanical garden in Pyong-Yang and from natural stands) and those obtained from seeds acquired in Kanagawa, Japan. Trees from Japan have significantly smaller pinnately compound leaves and leaflets whose number is also smaller. Japanese honey locusts are trees, but they have the form of shrubs (with no central trunk). This type of growth was caused by the fact that the trees became severely frostbitten at a young age.

In the present study, the formation of bulk DNA samples isolated from five *G. triacanthos* and four *G. triacanthos* f. *inermis* specimens growing in the city of Szczecin (Poland) and their comparison to the pure species (*G. triacanthos*) was an intentional procedure. The thorniness feature in *G. triacanthos* depends on numerous environmental factors, and the analysis of bulk samples from specimens of *Gleditsia triacanthos* and *G. triacanthos* f. *inermis* that had been under long-standing observation (Kubus 1999) was aimed at demonstrating their variability and potential differences between the features of thorniness and thornlessness at the bulk sample level. Only comparison of bulk samples allows the degree of variability within individual samples to be determined and makes it possible to identify potential loci differentiating the samples.

The analysis of the genetic profiles of the bulked samples revealed that they differed with respect to the number of polymorphic loci. Analysis of the phylogenetic tree showed that the three genotypes tested in the experiment, *G. triacanthos* (bulk), *G.*

triacanthos f. *inermis* (bulk) and the pure species *G. triacanthos*, were grouped in the same cluster, and the greatest phylogenetic similarity (0.75) was discovered between *G. triacanthos* (bulk) and the pure species *G. triacanthos*. This fact confirms the existence of variability within *G. triacanthos*, which is evidenced by the amplification of accession-specific loci. Thus two specific ISSR loci (821_{870} and 823_{1030}) were amplified from the *G. triacanthos* pure species standard, and six specific loci were amplified from the bulk sample prepared from G. *triacanthos,* out of which two were amplified with the $821_{1460, 820}$ primers and four with primers 811, 823 and 830. *G. triacanthos* (bulk) differed from *G. triacanthos* f. *inermis* (bulk) by two polymorphic loci amplified in a reaction with just one primer (823). A 823_{2600} locus was amplified from *G. triacanthos* (pure species), while a 823_{1000} locus was obtained from its thornless form. More differences of this type were found, and it can only be supposed that phenotypic and genotypic analyses of progeny obtained by crossing carefully selected thorny and thornless genotypes would be very interesting.

While describing a locust tree growing in the Kórnik Arboretum (Poland) exhibiting a characteristic columnar growth habit of the crown, Bojarczuk and Zieliński (1980) found it difficult to unequivocally determine its species affiliation. The authors excluded the possibility of it being a *Gleditsia triacanthos* L. honey locust. They pointed to the similarity of its vegetative features to Japanese honey locust or to an unidentified hybrid of this species. The results obtained with the ISSR technique demonstrated that this genotype exhibits the highest similarity (0.37) to the bulk DNA sample obtained from the thornless forms (*G. triacanthos* f. *inermis*) and the lowest similarity (0.17) to *G. caspica*. However, it was difficult to determine unequivocally which genotype *Gleditsia sp.* was most similar to by analysing the dendrogram of phylogenetic similarity because its phylogenetic similarity to pure species of *G. triacanthos* and to *G. triacanthos* (bulk) was 0.34 and 0.31, respectively, and its similarity to *G. japonica*, *G. japonica* var. *korainensis* and *G. caspica*, was 0.26, 0.26 and 0.17, respectively.

These results support the opinion of Bojarczuk and Zieliński (1980) that it is difficult to unequivocally determine the affiliation of the aforementioned form. Sixteen genotype-specific loci were amplified from *Gleditsia sp.* in this experiment. This is a testament to its genetic distinctness from the remaining analysed accessions.

In conclusion, it should be emphasised that the ISSR technique is a simple and repetitive method that is easy to apply, which is in accordance with the opinion of numerous authors quoted in the present study. Its application in this study made it possible to obtain results whose interpretation fulfilled the study's goals.

When comparing these results with the results obtained by other authors who used the same method to study genotypes belonging to the legume family (*Leguminosae*), it should be emphasised that the results presented here meet the standards presented in the relevant literature with respect to the number of the primers used, the number of primers that generate distinct genetic profiles, the number and the length of amplified loci, and the frequencies of monomorphic, polymorphic, and accession-specific loci (Zietkiewicz et al. 1994, Tsumura et al. 1996; van der Nest 2000, Iruela et al. 2002, Mondal 2002; Rajesh et al. 2002, Vijayan and Chatterju 2003, Sudupak 2004; Rzepka-Plevneš et al. 2006 and 2007).

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