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Genetic similarity of chosen *Syringa* species determined by the ISSR-PCR technique

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Abstract: Inter-simple sequence repeat (ISSR) amplification was used to analyze polymorphism of microsatellite sequences in the lilacs genom and to evaluate genetic diversity among seven lilacs species (*Syringa* × *prestoniae* McKelvey., *S. reflexa* K.C. Schneid., *S. villosa* Vahl, *S.* × *chinensis* Willd., *S. meyeri* K.C.Schneid., *S. vulgaris* L. and *S. reticulata* (Blume) var. *amurensis* (Rupr.)). The plant material was originated from the collection of Dendrological Garden in Przelewice. A total of 30 primers, containing different simple sequence repeat motifs were tested for amplification. Out of the 30 primers only 13 gave interpretable banding patterns in all lilacs species. A total of 182 ISSR fragments were generated with 13 primers of which 109 (60%) were polymorphic and 57 (31.2%) species-specific. ISSR–PCR with genomic DNAs of the showed lilacs yielded DNA fragmets ranging form 2200 to 123 bp in size. Species-specific ISSR fragments were detected for each lilacs accessions. UPGMA cluster analysis was used to construct a dendrogram and to estimate the genetic distances between lilacs species. The ISSR-based phylogeny was generally consistent with *Syringa* taxonomy based on morphological and phenological evidence.

Additional key words: lilacs, Przelewice Dendrological Garden, genetic variability, ISSR

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Introduction

The lilacs comprise one of the most important collections of trees and bushes in the Przelewice Dendrological Garden where one may find 31 classification units, described as regards morphological and phenological traits, attributed to different species of this plant (Chylarecki et al. 1997). The sample collection of lilacs, existing in the mentioned garden, composes a valuable source of material for molecular phylogenetic studies, which were an important complement of their morphological and phenological characteristic. The present work describes the results of studies conducted on seven lilacs species from the subsection *Syringa* section *Villosae* (*S.* × *prestoniae* McKelvey., *S. reflexa* K.C.Schneid., *S. villosa* Vahl.), three from section *Syringa* (*S.* × *chinensis* Willd., *S. meyeri* K.C.Schneid., *S. vulgaris* L.) and the species *S. reticulata* (Blume) var. *amurensis* (Rupr) from the subsection *Ligustrina* (Jasnowska et al. 1999).

The characteristic of genetic relations between the compared species was obtained using the ISSR-PCR (inter simple sequence repeat – polymerase chain reaction) method, which is considered by many authors to be both precise and reliable. It combines the simplicity of analyses comparable with that of the RAPD method with the reliability or results obtained by the AFLP method (Readdy et al. 2002).

The ISSR-PCR method identifies the genetic differences between repeated motives of microsatellite sequences occurring within coding regions, both centromeric and telomeric which, in the opinion of

numerous authors, are highly polymorphic (Zietkiewicz et al. 1994).

The method discussed was used in studies on the genetic separateness of different forms of the eucalyptus (Van der Nest et al. 2000), the endemic bush *Tetracena mongolica* (Ge et al. 2003), *Anacardium occidentalle* from ten breeding centres of this plant in India (Archak et al. 2003), melon (Monforte et al. 2003), species of *Juniperus* of various origin (Adams et al. 2003) and many other species of wild and cultivated plants.

The present work aimed at determining by the ISSR-PCR technique the genetic similarity and phylogenetical relations between seven species of lilacs from the Przelewice Dendrological Garden.

Material and methods

The plant material was originated from the collection of the Przelewice Dendrological Garden. The material consisted of seven *Syringa* species from the subsection *Syringa* section *Villosae* (*S.* × *prestoniae* McKelvey., *S. reflexa* K.C.Schneid., *S. villosa* Vahl.), three from section *Syringa* (*S.* × *chinensis* Willd., *S. meyeri* K.C.Schneid., *S. vulgaris* L.) and the species *S. reticulata* (Blume) var. *amurensis* (Rupr.) from the subsection *Ligustrina* (Jasnowska et al. 1999). Out of the examined species, *S. reflexa* K.C. Schneid. and *S. villosa* Vahl were parental components of *S.* × *prestoniae* McKelvey, whereas *S.* × *chinensis* Willd. was obtained from the crossing of two species: *S. vulgaris* L. and *S. persica* L., the latter not included in the studies (Erhardt et al. 2002). Lilac is an poliploid species propagated exclusively by vegetative means. Replicate DNA extraction from the leaves of each of the seven species were used to assess the reproducibility of band patterns. Three leaves were sampled per species (from the three plant).

The total DNA from about 100 mg of fresh leaf material was extracted using the DNA PrepPlus kit (A&A Biotechnology – Poland). The contaminating RNA was removed by digestion with RNase A (10 mg · ml^{-1}). The concentration of the analysed templates was equalised so as to obtain 20 ng DNA per 1 μl of solution (GeneQuant DNA/RNA Calculator – Pharmacia LKB).

PCR mixtures (25 μl) contained: 1.5 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl pH 8.3, 0.1% Triton X-100, 0.2 μM primer, 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 – Germany) 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 (Table 1). The amplified products were mixed with 6× Orange Loading Dye Solution and were analyzed by electrophoresis on a 2% agarose gel, at a constant voltage of 80 V for 2 hours, using 1× TBE buffer at a room temperature. O'RangeRuler 200bp DNA Ladder (Ferments) was as a size marker (3000 – 200 bp). The negative controls with water replacing the template DNA were applied to monitor the contamination. The PCR products were visualized with ethidium bromide (0.5 mg · ml^{-1}) on a UV-21 transilluminator (Fotodyne). Gels were photographed (Polaroid DS-34). Only those bands that showed consistent amplification were considered for this study. The smeared and weak bands were excluded.

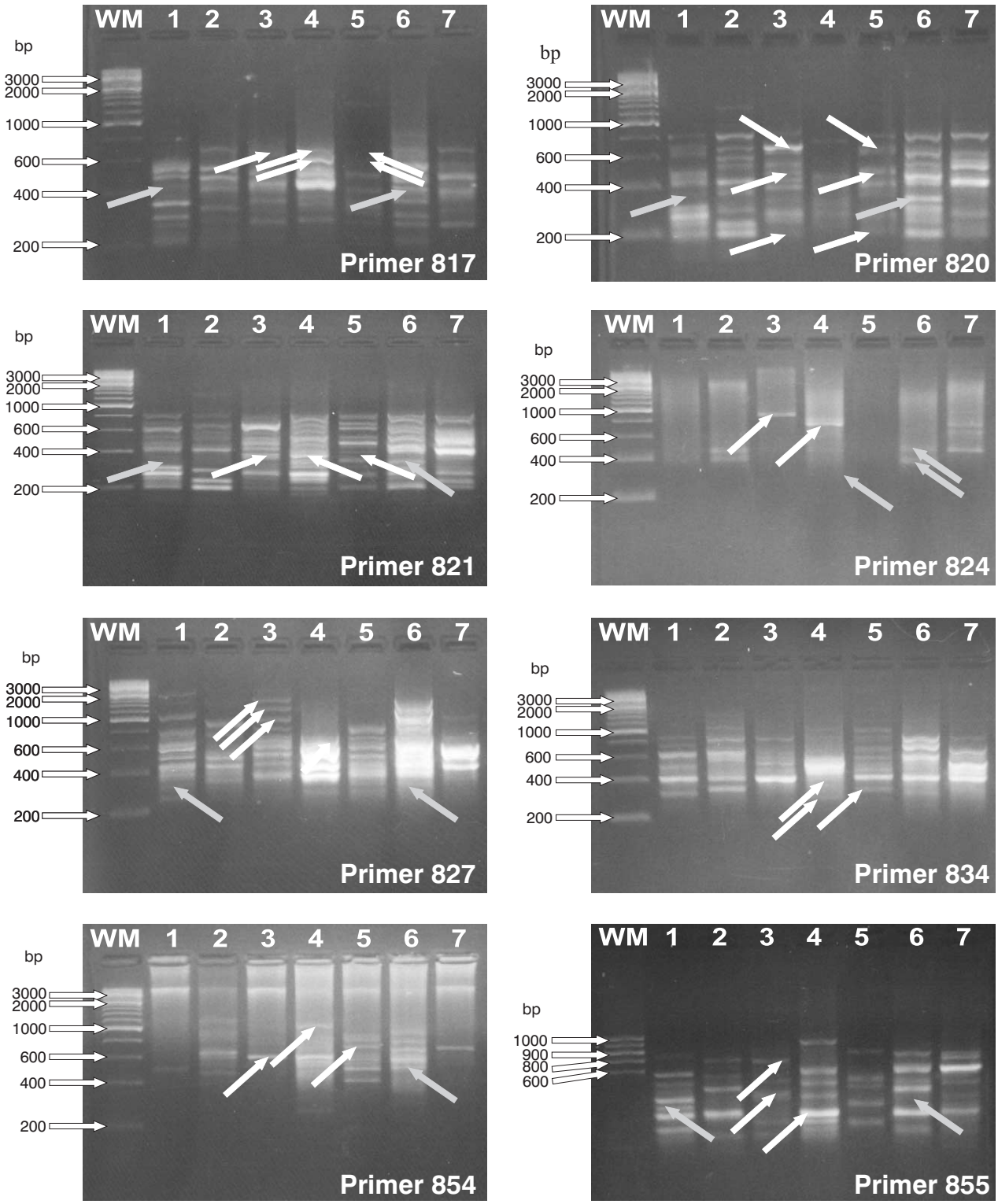
It was expected that each DNA band would represent a single locus. DNA fragments, detected not in all individual species spectra were considered as polymorphic. Each fragment that was amplified using ISSR primers, was coded in a binary form by '0' or '1' for absence or presence in each species, respectively. 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 (Nei and Li 1979) – dendrogram using software packages Diversity one 1.3 (Pharmacia LKB). Molecular weight of each bands was calculated using the same software packages.

Results

Thirty microsatellite ISSR primers were used in order to evaluate the genetic similarity of the compared lilac species (Table 1). Thirteen (817, 818, 820, 821, 824, 827, 832, 833, 834, 836, 854, 855 and 880) of the thirty primers generated scorable bands in both the low and high molecular weight regions for every assayed lilac species (Table 1, Fig. 1). Inter-SSR amplifi-

Table 1. ISSR primers used in the present study

Primer number	Sequence (5'–3')	Primer number	Sequence (5'–3')	Primer number	Sequence (5'–3')
817	$(AG)_8YG$	827	$(AC)_8GG$	854	$(TG)_8C$
818	$(CA)_8G$	832	$(AC)_8GA$	855	$(AG)_8YG$
820	$(GA)_8YC$	833	$(GT)_8YC$	880	$(GGAGA)_3GT$
821	$(GA)_8YC$	834	$(AG)_8GC$		
824	$(TC)_8G$	836	$(AC)_8YA$		



WM – molecular weight, *1. S.* × *chinensis, 2. S. meyeri, 3. S.* × *prestoniae, 4. S. villosa, 5. S. reflexa, 6. S. vulgaris, 7. S. reticulata* var. *amurensis*

Fig. 1. Electrophoregrams of ISSR product amplified on DNA templates of seven lilac species. White arrows mark ISSR polymorphic products

cation of the seven lilac species with those primers yielded a total of 182 fragments of which 109 (60%) were polymorphic, 16 (8.8%) were monomorphic and 57 (31.2%) were species-specific for all lilac (Table 2). On average one primer was amplified 14 *loci*. Their size ranged from 2200 bp (primer 827) to 123 bp (primer 880) (Table 2). The largest number (24) of ISSR products was generated with primer 820,

Table 2. ISSR primer sequences and results of experiments performed among accession of lilac species with ISSR markers

Primer	Primer sequence	Length of amplification products (bp)	Number of bands per primer	Monomorphic products	Polymorphic products	Species specific products	Total number of bands for all genotypes
817	$(AG)_8$YG	796–205	16	1	9	6	44
818	$(CA)_8$G	1060–184	15	4	9	2	64
820	$(GA)_8$YC	1295–150	24	2	13	9	58
821	$(GA)_8$YC	809–175	15	4	8	3	60
824	$(TC)_8$G	798–367	5	0	4	1	14
827	$(AC)_8$GG	2200–264	19	3	10	6	67
832	$(AC)_8$GA	1060–184	13	0	8	5	25
833	$(GT)_8$YC	1081–310	10	0	9	1	28
834	$(AG)_8$GC	1595–287	13	2	9	2	50
836	$(AC)_8$YA	1159–446	8	0	5	3	16
854	$(TG)_8$C	1104–254	11	0	8	3	35
855	$(AG)_8$YT	947–230	20	0	10	10	42
880	$(GGAGA)_3$GT	1171–123	13	0	7	6	28
		Total	182	16	109	57	531
		Mean	14	1.2	8.4	4.4	40.8
		Percentage		8.8	60.0	31.2	

whereas the smallest number (5) with primer 824 (Table 2). In total, ISSR-PCR with genomic DNAs of the showed lilacs gave 531 products of which the highest number of ISSR fragments was obtained for *S. vulgaris* (97 – data not shown), whereas the smallest (59 – data not shown) for *S.* × *chinensis* (Table 2). All of the thirteen oligonucleotides generated polymorphic and monomorphic markers (Table 3 and 4). The monomorphic products were amplified with oligonucleotides: 817, 818, 820, 821, 827 and 834 (Table 3).

Table 3. Monomorphic bands revealed through ISSR fingerprinting

Species	Primers and length of amplification products (bp)
S. × *chinensis*	$817_{[255]}$
S. meyeri	$818_{[533, 482, 334, 281]}$
S. × *prestoniae*	$820_{[774, 420]}$
S. villosa	$821_{[809, 492, 412, 200]}$
S. reflexa	$827_{[716, 594, 434]}$,
S. vulgaris	$834_{[796, 617]}$,
S. reticulata var. *amurensis*	

Structually, most of primers were dinucleotide repeats varying in size of the 3'-anchor. The first group of dinucleotide $(AG)_8$-motif primers (817, 834 i 855) gave 49, whereas the second (primers 827, 832 and 836 – $(AC)_8$-motif) gave 40 and the third group (820 and 821 – $(GA)_8$-motif) were generated 39 polymorphic, monomorphic and species-specific bands for all lilacs species, respectively (Table 2, 3 and 4).

The analysis of profiles of the ISSR products amplified for *S.* × *prestoniae* and its parental components (*S. reflexa* and *S. villosa*) shows the presence of both common products and products specific for the hybrid of (*S.* × *prestoniae*) and one of two parental forms: *S. reflexa* or *S. villosa* (data not shown). A similar relationship, confirming a hybrid character of both forms, was observed between *S.* × *chinensis* and *S. vulgaris*.

UPGMA analysis was carried out to classify the seven lilac species and to verify the ability of ISSR markers to reveal genetic similarity (Fig. 2). The phylogenetic tree revealed one separate group including *S. reticulata* var. *amurensis*, *S. vulgaris* and *S. villosa*. In this group the smallest divergence is observed between *S. vulgaris*, *S. reticulata* var. *amurensis* (69.7%).

Table 4. Species-specific bands revealed through ISSR fingerprinting

Species	Primers and length of amplification products (bp)
S. × *chinensis*	$821_{[581]}$, $827_{[2200, 388]}$, $855_{[404, 363, 250]}$
S. meyeri	$820_{[1295, 607, 360]}$, $827_{[845, 340]}$, $834_{[1595]}$, $854_{[954, 475, 254]}$, $855_{[574]}$
S. × *prestoniae*	$820_{[395]}$, $827_{[513]}$
S. villosa	$817_{[568, 377, 344]}$, $820_{[328]}$, $821_{[175]}$, $833_{[818]}$, $834_{[287]}$, $836_{[666, 576, 446]}$, $855_{[947]}$, $880_{[1171, 995, 268, 123]}$,
S. reflexa	$817_{[451]}$, $818_{[1060]}$, $820_{[647, 488, 374]}$, $855_{[540, 379, 289]}$
S. vulgaris	$817_{[796]}$, $820_{[342]}$, $821_{[326]}$, $827_{[882]}$, $832_{[1123]}$, $880_{[598]}$
S. reticulata var. amurensis	$817_{[490]}$, $818_{[255]}$, $824_{[592]}$, $832_{[782, 579, 519, 374]}$, $855_{[779, 436]}$, $880_{[628]}$

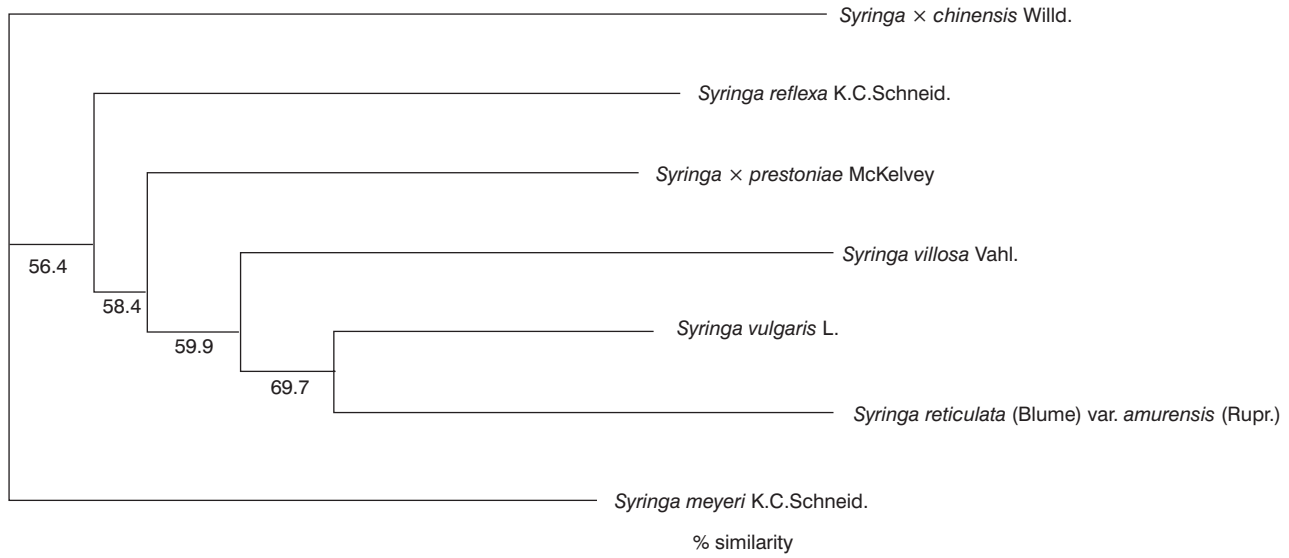


Fig. 2. UPGMA dendrograms representing genetic relationships among the seven lilac species analysed by ISSR markers

The other species are approximately equidistant from the group and from each other (Fig. 2).

It was observed that the phylogenetic similarity between the examined species varied from 38.5% (between *S. villosa* and *S.* × *chinensis*) to 69.7% (*S.* × *chinensis* and *S. reticulata* var. *amurensis*). The phylogenetic similarity between lilacs belonging to the section of *Villosae* i.e. *S.* × *prestonie* and its parental components (*S. reflexa* i *S. villosa*) amounted respectively to 58.5 and 59.4%, whereas the similarity between lilacs from the *Vulgaris* section, to which *S.* × *chinensis* and its parental components *S. vulgaris* belong, was 50.6%.

Discussion

In the past, the methods used in order to determine the phylogenetic relations between species of cultivated plants were based on morphological and phonological observations, the chromosome arrangement, etc. (Stutz 1972, Jasnowska et al. 1999). Currently, with increasing frequency, for studies of this type an analysis of genetic and gene polymorphism is applied, using DNA markers (Bhat et al. 1999, Cabrita et al. 2001, Korzun 2002).

The available literature indicates that for the differentiation of species and determination of mutual phylogenetic relations between them it is possible to use such markers as RAPD (Archak et al. 2003), AFLP (Cabrita et al. 2001) and ISSR-PCR (Ge and Sun 1999, Crowford et al. 2001, Herrera et al. 2002, Mondal 2002). Studies conducted by those authors changed the existing opinions on the origin and taxonomy affiliation of many species of cultivated plants and also of plants growing in natural conditions.

Crowford et al. (2001) used the ISSR technique to determine the intra-species differences between 89 *Lactoris fernandeziana* (*Lactoridaceae*) plants, growing on Masatierra island, part of the Juan Fernandez archipelago (Chile). During the course of the studies they was proved that only one of the examined plants showed a genotype typical for the *Lactoridaceae* family. The remaining plants differed in one or more *loci*. The observed differences did not depend on the origin. They were caused rather by disturbances in the build of the pollen and seeds, genetic drift, self-fertilization and mutations. The obtained results indicate that the evaluation of morphological, ecological and physiological traits does not always fully reflect the differentiation between individuals within one species or family.

Another example of this type of differences was brought by the studies conducted by Herrera et al. (2002) on the originality and authenticity of cultivated varieties of grape-vines (*Vitis vinifera*), in many countries of the world used for the production of wines. The plants were examined using the RAPD and ISSR-PCR techniques. The experimental material consisted of the following varieties: 'Cabernet Sauvignon', 'Cabernet Franc', 'Merlot' and 'Carmenere', obtained from various vineyards in Chile and France (model). The studies showed that cloned 'Merlot' varieties differed genetically from clones of the same variety cultivated in France. The differences were confirmed by both used techniques.

The low stage of molecular genetic similarity between varieties and their parents was demonstrated by Archak et al. (2003) for *Anacardium occidentale*. These authors stated, on the basis of a dendrogram, grouping the genetically most similar varieties of the nut, that four of them, though having a common parent, belong to different phylogenetic groups, while the two others, without a common parentage, constitute one phylogenetic group.

Also the studies reported by Skuza (2004) did not justify the division of ray into two sections, as performed by Khush (1962). The author observed, that the ancestor of the examined ray species is not, as expected, *Secale strrictum*, but probably *S. africanum*, a perennial, autogamous species. According to the author it was from this species that *S. strictum*, an allogamous perennial, originates.

The results presented here demonstrate a certain genetical diversity of the examined lilac species. However, the use of a small number of ISSR primers rendered it possible to characterise a series of polymorphic *loci*, including *loci* specific for the species, while an analysis of the phylogenetic dendrogram indicated genetic similarities between species which differed from those accepted in taxonomy.

In the presented studies the ISSR-PCR technique was applied for genetic differentiation of seven species of lilac. In the course of the amplification a series of both mono- and polymorphic products of the reaction were generated. Among them specific products were also isolated. A genetic similarity between two species: *S.* × *prestoniae*, *S.* × *chinensis* and their parental components, respectively: *S. reflexa*, *S. villosa* and *S. vulgaris* was confirmed as well. The presence of bands, common for the hybrid and its parental forms was shown on electroforegrams of the ISSR products. At the same time, systems were observed in which there were bands of a hybrid and of one of parental components on some elecroforegrams, whereas on others there were only bands specific for parental components, not occurring in hybrids.

The results of the studies shown in the present paper illustrate large genetic differentiation in lilacs. What is more, they are in agreement with the results of studies carried out by Kochieva et al. (2004) in relation to the number, the length of amplified products of reaction and the coefficient of genetic similarity. However, on the basis of the carried out studies it is difficult to draw a conclusion about phylogenetic relationships between the studied genotypes. These studies, as well as those reported by other authors, are always limited by the number of applied primers for it shows the accuracy with which the examination of a genome has been carried out and in many cases it is only of a screening character.

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