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rDNA Variability Assessed in PCR Reactions of Selected Accessions of *Acer*

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Abstract

The region of rDNA (SSU, 5.8S, LSU and ITS1, ITS2 spacer sequences) of fourteen *Acer* accessions was tested in order to determine the possibility to identify genotypic variability within a rDNA sequence. The tests were performed using the PCR technique and a set of combination of several pairs of 'universal' primers designed to amplify rDNA sequences. 'Universal' primers were selected in such a way as to make the rDNA regions flanking possible in cases where a smaller or larger scope of variability was expected. Thus, within SSU or LSU sequences, monomorphic PCR products were amplified for the tested *Acer* accessions. Within the ITS sequence, a greater scope of variability was found. This was manifested by the presence of several additional PCR products in the genetic profiles of the tested *Acer* accessions. The range of their length relative to the ITS region or ITS1, ITS2 or 5.8S corresponded separately to that described in the literature. The analysis of the topology of the constructed tree revealed the presence of two similar groups, 'a' and 'b'. Cultivated varieties and one botanical variety of the Japanese maple were included respectively in these groups and the genetic similarity between the analyzed accessions ranged from 63 to 98%.

Keywords: *Acer*, additional products, Japanese maple, PCR, rDNA, 'universal' primers

Introduction

Maple (*Acer* L.-*Sapindaceae*) is a genus to which numerous species of trees and shrubs are included, which occur naturally throughout the northern hemisphere. At present, approximately 200 maple species are known, half of which occurs in China. Within this genus, polyploid species occur and the number of cultivated species derived from various genotypes of *Acer* in terms of ploidy amounts to over 600 (van Gelderen *et al.,* 1994; Hasebe *et al.,* 1998).

The *Acer* genus is difficult to classify taxonomically (Delendick, 1990), although such work has already been done for its individual sections (Hasebe *et al.,* 1998; Grimm *et al.,* 2007). The analysis of variability within genes encoding rRNA subunits (rDNA), that include ITS (internal transcribed spacers), IGS (intergenic sparcer) and sequences of rRNA genes: SSU (small subunit), 5.8S and LSU (large subunit) is one of many ways of determining the phylogenetic relationships between accessions (Pillay and Kenny, 1996; Smolik *et al.,* 2009; Poczai and Hyvönen, 2010). Variation in the rDNA repeats has been useful in addressing questions in population genetics (Capossela *et al.,* 1992). It is also considered a useful genetic marker for breeding applications (Poczai and Hyvönen, 2010).

One of the remarkable properties of nrDNA (including ITS) genes is that their paralogs within individuals are quite homogenous, resulting from concerted evolution (Nagylaki, 1984). nrDNA paralogs will, however, display polymorphisms in individuals where concerted evolution is incomplete, for example in cases where hybridization is involved (Álvarez and Wendel, 2003) or where concerted evolution cannot act between paralogs effectively when they are dispersed on non-homologous chromosomes in the genome (Hillis and Dixon, 1991).

Research on determination of phylogenetic relationships between taxons is focused mainly on amplification of the ITS region and sequencing of the obtained product. The bioinformatic analysis of the obtained sequences enables to draw conclusions about phylogenetic relationships between subjects. In polyploids the variability within rDNA sequences is high. Using 'universal' primers designed for this purpose allows to amplify several such sequences in a reaction with only one primers pair (multicopy character of amplicons). Thereby a difficulty of selecting an amplicon representative of rDNA sequences for sequencing and for further analyses is encountered, and a question arises whether selecting a specific amplicon for sequencing, and discarding others, results in too considerable error of results of the analyses.

Thus the aim of the present study was to demonstrate the possibility of amplification of this type of products in fourteen different *Acer* accessions using various combinations of 'universal' primers pairs designed for the amplification of different rDNA regions. The primers were used in different combinations in order to describe polymorphism in the places of their hybridization and of the target sequences. In addition, the range of variability within rDNA sequences, including the possibility to generate polymorphic or specific amplicons and the possibility of using those informative products, was determined.

Material and methods

Fourteen *Acer* accessions (*A. palmatum* var. *heptalobum, A. palmatum* Thunb., *A. palmatum* 'Elegans', *A. palmatum* 'Dissectum', *A. palmatum* 'Dissectum Ornatum', *A. palmatum* 'Dissectum Atropurpureum', *A. palmatum* 'Garnet', *A. palmatum* 'Nicholsonii' *A. palmatum* 'Sanguineum', A. *palmatum* f. *atropurpureum*, *A. japonicum* 'Aconitifolium', *A. shirasavanum* 'Aureum', hybrid-*A. palmatum* Thunb. × *A. palmatum* 'Elegans', *A. palmatum* f. *atropurpureum* and *A. diabolicum*) part of the collection of the Dendrological Garden in Przelewice (Poland), constituted the research material.

DNA preparation

Total DNA was extracted from *Acer* fresh leaves. Plant material was ground in liquid nitrogen with a mortar and pestle and incubated at 37^{0}C with Protease K. The process for total DNA extraction was described in Genomic Mini AX Plant protocol (A&A Biotechnology Gdynia-Poland). The extracted DNA pellet was resuspended in water (Sigma-Aldrich). RNaseA (Sigma-Aldrich) was added (2 µl) to the DNA samples to eliminate RNA contaminations by incubation at 37^{0}C for 5 min. Genomic DNA was quantified (GeneQuant DNA/RNA Calculator-Pharmacia LKB) to obtain 50 ng DNA pro 1µl of DNA template.

Oligonucleotides for PCR amplifications

The analysis encompassed nuclear rDNA, including the following sequences: SSU, ITS1; 5.8S; ITS2 and LSU are presented in Fig. 1. The figure also includes names, approximate locations and directions of the hybridization of primers used for PCR reactions.

The origin of primers, sequences and their authors are presented in Tab. 1.

Tab. 1. Universal primers used in this study

rDNA regions amplified	Primer name	Author(s)
SSU	NS1, NS2, NS4, NS3	White *et al.* (1990)
ITS1	NS5, ITS2, NS6, ITS5, ITS1	White *et al.* (1990)
	5.8S,	Vilgalys and Hester (1990)
	SR6R	Vilgalys*
	ITS1_F	Gardes and Bruns (1993)
ITS2	ITS3, ITS4	White *et al.* (1990)
	LR1, LR2	Vilgalys and Hester (1990)
	ITS4B	Gardes and Bruns (1993)
LSU	LR0R	Vilgalys*
	LR7, LR3R	Vilgalys and Hester (1990)

*Vilgalys unpublished [http://www.botany.duke.edu/fungi/mycolab]

PCR amplification of rDNA

PCR mixtures (25 µl) contained: 2.5µl 10× PCR buffer with $(NH_4)_2SO_4$, 2mM dNTPs, 25 mM $MgCl_2$ (Fermentas MBI), 2.5 µM of each primer, 1.0 units of *Taq* DNA polymerase (Fermentas MBI) and 100 ng of template DNA. DNA was amplified using a Mastercycler 5333 (Eppendorf) thermocycler and using the following program: initial denaturation at 94°C for 7 min, 40 cycles of 30 s at 94°C, annealing temperature, 2 min at 72°C and 7 min at 72°C for final extension. The annealing temperature was usually adjusted according to the T_m of the primers being used in the reaction (Tab. 1). Amplification reactions were performed according to the touchdown PCR protocol by raising the temperature of the first single cycles by 6°C above the annealing temperature.

Electrophoresis and data analysis

PCR products were mixed with 6× Loading Dye Solution and were analysed by electrophoresis on a 2% agarose (Basica LE-Prona) in 1× TBE buffer. PCR products were stained with ethidium bromide, visualized (MiniBIS Pro-

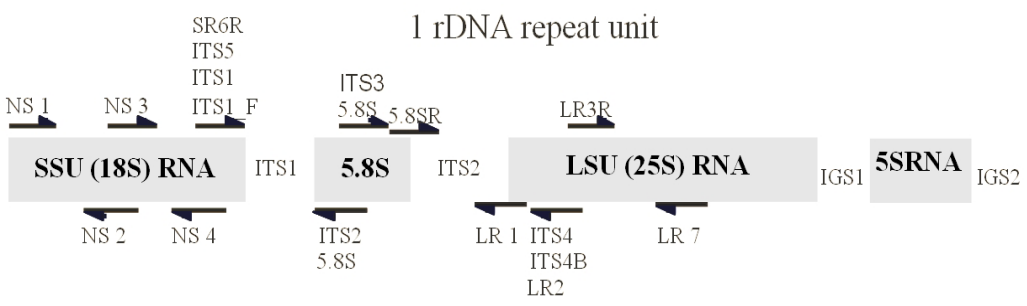


Fig. 1. rDNA unit. Arrows indicate approximate positions of primers used to generate amplicons in different rDNA regions. Primer names, sequences and their authors were listed in Tab. 1

DNT Bio-Imagining System-USA) and scored for band present or absence. O'RangeRuler 200bp DNA Ladder and O'RangeRuler™ 100+500bp DNA Ladder (Fermentas MBI) were used as a size markers. The rDNA fingerprints generated by each primer pairs selected (Tab. 1), were used to compare relatedness of *Acer* accessions. Presence (1) or absence (0) of each amplicon was scored for each accession. Only stable amplicons were scored. The similarity coefficient between accessions compared was determined by Jaccard's coefficient (S_{IJ}) according to the formula $S_{IJ} = a(n\text{-}d)$ where a is the number of matched 1's, n is the total sample size, and d is the total number of matched 0's. Dendrograms were produced by cluster analysis of the similarity coefficients using UPGMA (Nei and Li, 1979). The strength of the internal branches from the resulting tree was tested by TREECON bootstrap analysis application using 2000 replications (Van de Peer and de Wachter, 1994; Felsenstein, 1985).

Results

To determine genetic variability between fourteen *Acer* accessions, a combination of 28 'universal' primers pairs were used (Fig. 1). Amplification products were obtained in the reactions of 16. In total, 34 products were generated in the performed amplifications out of which 23 (68%) were polymorphic, 10 (29%) monomorphic and 1 product (3%) was a genotype-specific for *A. palmatum* f. *atropurpureum*. The length of the amplicons ranged from ~230 to ~1100bp (Tab. 2).

The largest number of polymorphic products were generated if the ITS5+ITS4 and SR6R+5.8S primers combination were used and their length ranged from ~230 to ~700 bp. The accession-specific product was amplified in a reaction with the NS3+NS4 (430bp) primers, designed for the SSU subunit. In the experiment 10 monomorphic reaction products were generated for the fourteen analyzed accessions of *Acer*. These were obtained by the amplification of appropriate regions encoding the small (SSU) and the large (LSU) RNA subunit in reaction with combination of 10 appropriately complementary primers (Tab. 2).

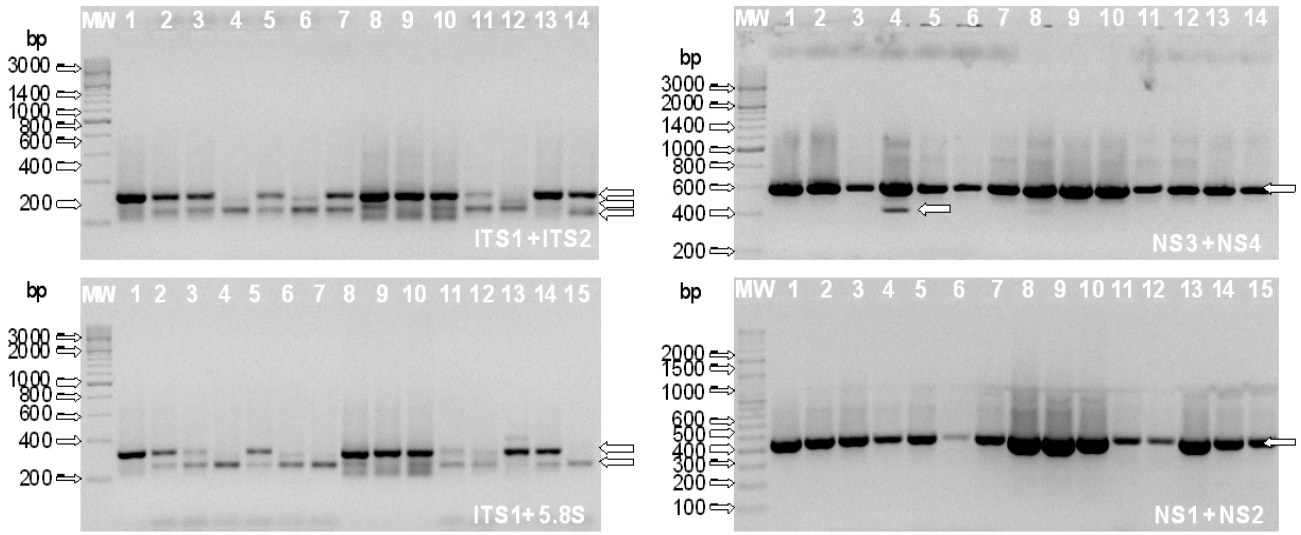
SSU

Three pairs of primers were used to amplify sequences encoding the SSU subunit. In reactions with each of the primers pairs, monomorphic products were generated (1100, 570 and 590bp, respectively). Although, in the reaction with the NS3+NS4 primers, one specific product (430bp) for *A. palmatum* f. *atropurpureum* was generated (Tab. 2, Fig. 2).

ITS1

Tab. 2. Characteristics of generated amplicons with different combinations of 'universal' primers obtained for accessions of *Acer*

rDNA region	Primers No.	Fragment size range (bp)	*A. p.* 'Dissectum'	*A. p.* 'Dissectum Atropurpureum'	*A. p.* 'Dissectum Ornatum'	*A. p.* f. *atropurpureum*	*A. p.* 'Garnet'	*A. p.* var *heptalobum*	*A. p.* 'Nicholsonii'	*A. diabolicum*	*A. japonicum* 'Aconitifolium'	*A. shirasawanum* 'Aureum'	*A. p.* 'Elegans'	*A. p.* Thunb.	*A. p.* 'Sanguineum'	*A. p.* Thunb. × *A. p.* 'Elegans'	Total	Monomorphic	Polymorphic	Genotype-Specific
SSU	NS1 + NS4	1100	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	1	0	0
	NS1 + NS2	570	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	1	0	0
	NS3 + NS4	600-430	1	1	1	2	1	1	1	1	1	1	1	1	1	1	15	1	0	1
ITS1	ITS1 + 5.8S	440-260	1	2	2	2	2	2	2	3	2	3	2	2	2	2	29	1	2	0
	NS5 + 5.8S	930	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	1	0	0
	ITS1-F + ITS2	300-260	1	1	1	1	1	1	1	2	2	2	1	1	1	1	17	1	1	0
	SR6R + 5.8S	380-240	2	3	2	3	2	2	2	1	3	1	3	4	3	3	34	0	4	0
	ITS1 + ITS2	320-230	2	2	2	1	2	2	2	3	2	3	2	1	1	2	27	0	1	0
ITS2	5.8SR + ITS4	470-330	3	3	3	2	3	2	2	2	1	2	2	1	1	2	29	1	2	0
	5.8SR + ITS4B	510-490	1	1	1	0	1	1	1	2	2	2	1	1	1	1	16	0	2	0
	ITS3 + ITS4B	540-480	1	1	1	1	1	1	1	2	1	2	1	1	1	1	16	0	3	0
	ITS3 + LR7	340-250	1	2	1	2	1	1	1	2	2	2	1	1	2	2	21	1	2	0
	5.8SR + LR1	420-350	2	2	0	1	1	1	2	2	2	2	0	2	2	1	20	0	2	0
ITS	ITS5 + ITS4	700-440	0	3	2	3	1	2	4	3	3	3	0	2	3	3	32	0	4	0
	ITS1-F + ITS4	640	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	1	0	0
LSU	LR3R + LR7	800	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	1	0	0
		Total	20	26	21	23	21	21	24	28	26	28	19	22	23	24	326	10	23	1



MW-Molecular Weight, 1-*A. palmatum* 'Dissectum', 2-*A. p.* 'Dissectum Atropurpureum', 3-*A. p.* 'Dissectum Ornatum', 4-*A. p.* f. *atropurpureum*, 5-*A. p.* 'Garnet', 6-*A. p.* var. *heptalobum*, 7-*A. p.* 'Nicholsonii', 8-*A. diabolicum*, 9-*A. japonicum* 'Aconitifolium', 10-*A. shirasavanum* 'Aureum', 11-*A. p.* 'Elegans', 12-*A. p.* Thunb., 13-*A. p.* 'Sanguineum', 14-hybrid-*A. p.* Thunb. × *A. p.* 'Elegans', 15-human sample

Fig. 2. PCR amplicons profiles generated in amplifications with selected primers pairs designed for investigation of rDNA variability. White arrows mark selected monomorphic or polymorphic amplicons

Five pairs of 'universal' primers, hybridized appropriately on the SSU and 5.8S subunit regions, were used for the amplification of ITS1 sequences (Fig. 1). As a result of the amplifications conducted with the NS5+5.8S primers, a monomorphic product (930bp) was generated for the fourteen *Acer* accessions. In the reaction with the ITS1+5.8S and ITS1_F+ITS2 primers combinations, both polymorphic and monomorphic products were generated. In a reaction with the ITS1+5.8S, one monomorphic product (440bp) and two polymorphic products (260 and 320bp) were amplified, whereas amplification with primer combination: ITS1_F+ITS2 primers yielded one monomorphic (300bp) and one polymorphic PCR product (260bp). The latter was present only in three out of the fourteen analyzed *Acer* accessions. In the PCR reaction with SR6R+5.8S and ITS1+ITS2 primer combinations, polymorphic products with lengths ranging from 230 to 380bp and 230 to 320bp, respectively, were amplified.

ITS2

A combination of five pairs of 'universal' primers were used for the amplification of ITS2 sequences. Polymorphic products were mostly amplified in reactions with the listed primers (Tab. 2). Their lengths ranged from 250 to 540 bp. Only two monomorphic amplicons were identified in electrophoregrams of the analyzed maple accessions when pairs of primers were used: ITS3+LR7 (270 bp) and 5.8SR+ITS4 (420 bp) (Fig. 2).

ITS1–5.8S–ITS2

Primers specific to the SSU rRNA sequences and primers specific to the LSU rRNA sequence were used in different combinations for the amplification of the ITS1–5.8S–ITS2 fragment. These primers were used in two combinations: ITS5+LR4 and ITS1_F+ITS4. One monomorphic product (640bp) was amplified in the reaction with the ITS1_F+ITS4, while two polymorphic products were amplified with the ITS5+ITS4 pair of primers with lengths of 440 and 700 bp.

LSU

One pair of primers (LR3R+LR7) was used for the amplification of genes encoding the LSU subunit. As the result one monomorphic product (800bp) was generated in a PCR reaction for all accessions of *Acer* (Fig. 2).

Phylogenetic tree

A dendrogram of phylogenetic similarity was drawn for the genetic profiles of the fourteen *Acer* accessions (Fig. 3). *A. diabolicum* was the species to which the drawn tree was rooted, as it was the only species belonging to the *Lithocarpa* section. The analysis of the topology of the obtained dendrogram revealed that the *Acer* accessions showed a similarity to one another within a range of 63% to 98%. Two similar groups were found. They were marked with letters 'a' and 'b' in Fig. 3. Four cultivated varieties of the Japanese maple were classified in the first group-'a': 'Elegans', 'Dissectum', 'Garnet', 'Dissectum Ornatum'

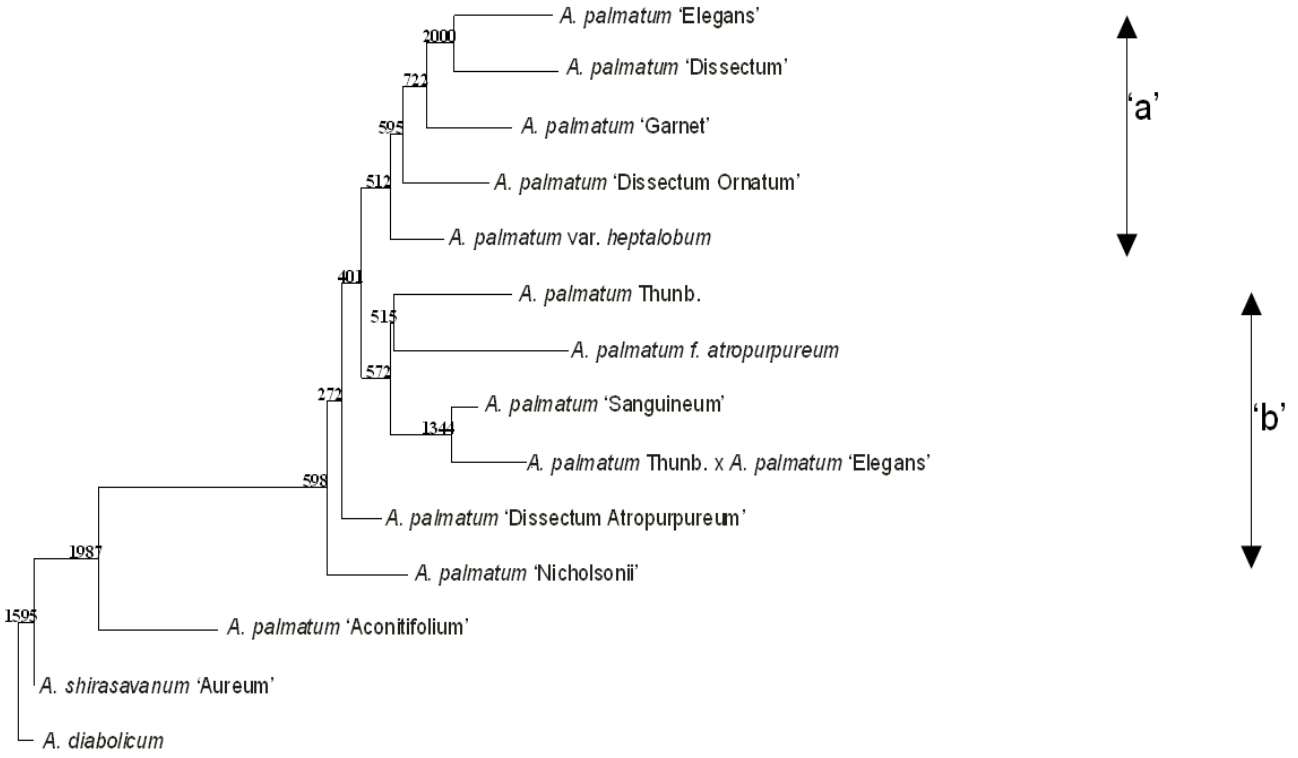


Fig. 3. UPGMA dendrogram based on similarity index, representing genetic relationships among *Acer* accessions analysed by PCR method. Numbers indicate the values of bootstraps in which the branch was observed in 2,000 replications

together with one botanical variety, the *heptalobum*. The other group 'b' contained three cultivated varieties of the Japanese maple: 'Sanguineum', 'Nicholsonii', 'Dissectum Atropurpureum', a cross-breed of *A. palmatum* Thunb. × *A. palmatum* 'Elegans', f. *atropurpureum* and *A. palmatum* Thunb. Apart from the aforementioned *Acer* accessions, *A. shirasavanum* 'Aureum' and the *A. p.* 'Aconitifolium' cultivar were classified.

Discussion

Within the *Acer* genus, research was initially based mainly on the analysis of phenotypic features and prove insufficient to systemize it in a reliable way (van Gelderen *et al.,* 1994; Suh *et al.,* 2000; Grimm *et al.,* 2006; Rzepka-Plevneš *et al.,* 2007).

In some excellent studies by many authors presenting research on phylogenetic relationships in selected species, including: *Viburnum* (Donoghue *et al*., 2004), *Acer* (Grimm *et al.,* 2006; 2007), *Malus* (Feng *et al*., 2007) or *Fraxinus* (Wallander, 2008), the authors do not mention the possibility of amplification of additional products (the most frequently within ITS sequences) in the reactions with primers used in the research (the most frequently primers flanking the ITS region). However, they state that the obtained PCR products, cloned into appropriate vectors, are often available in the number of 10 clones (maximum 10 different clones) for one subject of study. In the description of one's results, the authors state that the length of the cloned ITS products often varies by several bp. Thus Feng *et al*. (2007) estimated the ITS length for *Malus* at 590-595 bp, and Donoghue *et al*. (2004)-at 607-619 bp for *Viburnum*.

Results of our research were presented possibility of amplification of additional PCR products after using different 'universal' primers, similarly as it was demonstrated in the other below-mentioned studies. The fact that polymorphic fragments of nrDNA occurred in the analyzed *Acer* accessions depend on its genomic characteristics (ploidy). The amplification of nuclear rDNA sequences with the use of different combinations of 'universal' primers, limited to these sequences, demonstrated a relatively high range of variability for the *Acer* genus. Using different combinations of primers pairs complementary to the same rDNA regions was intentional (Fig. 1). It was proved that the selection of a given pair of primers used for the amplification of the same rDNA region is not without importance. As a result of the conducted research, distinct amplification products were obtained in reactions of 16 among 28 various pairs of 'universal' primers combinations. They were initially selected in various combinations, to obtain the possibility of the hybridizing of the target nrDNA within and outside the ITS1 and ITS2 sequences.

The application of the touchdown profile was aimed at eliminating the possibility of the appearance of artefacts after the PCR reaction, although formation may not be excluded (Cronn *et al.,* 2002). In order to control the con-

ditions of the conducted reactions, human genomic DNA template was added to the amplified rDNA samples. As a result, products polymorphic in length were amplified in the same reaction conditions for *Acer* accessions, while only one product was amplified for the added sample. We can corroborate the information contained in the study by Hillis and Dixon (1991) on the high genetic similarity occurring between various genera, including the *Homo sapiens* genus.

Thus, an analysis of the scope of variability within SSU and LSU sequences showed that, depending on whether the N series or LR series pairs of primers were used (White *et al.,* 1990) predominantly monomorphic products of PCR reaction were obtained. In terms of their length, the results of our experiment corresponded to those presented by Ki and Han (2005). These authors determined that the length of SSU sequences of *Alexandrinum tamarense* amounted to 18 bp and the length of LSU sequences was 3393 bp.

The amplification of ITS sequences, including ITS1 or ITS2, showed that the lengths of the generated amplicons for the tested ITS1 (440-260 bp) or ITS2 (540-250 bp) regions generally corresponded to the total length of those amplified for ITS1+5.8S+ITS2. Sequences amplified in reactions with primers flanking the ITS5+ITS4 and ITS1_F+ITS4 fragments were characterized by lengths ranging from 700 to 440 bp.

Similar lengths of tested amplicons (470-690 bp) were described for a germplasm collection of *Beta* (Yulong *et al.,* 1998). These authors used a methodological approach similar to the one presented in this study by amplifying the region of genomic nrDNA of 13 genotypes from the *Beta* idioplasm. They were used ITS2, ITS4 and ITS5 primers designed by Baldwin *et al.* (1992). Amplicons generated were sequenced. By comparing the obtained sequences, they found that the variability between the *Beta* accessions was large. After designing 15 specific primers for the following sections: *Beta* (BETA3), *Procumbentes* (PROC) and *Corollinae* plus *Nanae* (CORO2), they demonstrated a possibility of differentiating the tested genotypes after using combination of an ITS4 and each of the primer designed for the differentiation of genotypes from the aforementioned sections. The authors paid particular attention to the role of PCR reaction conditions (including the annealing temperature parameter) for the correct amplification and the reliability of obtained results.

Yasui and Ohnishi (1998) amplified a ~700 bp long fragment while analyzing ITS sequences in 20 *Fagopyrum* species from China. Similarly to the results obtained in the present study, these authors demonstrated the possibility of amplifying additional products, e.g. in tetraploid form of *F. cymosum* after a reaction with primers designed by White *et al.* (1990).

Suh *et al.* (2000) determined the length of the 5.8S RNA sequence as amounting to 164 bp and the variable ITS1 and ITS2 sequences were 220-242 and 215-250 bp long, which is appropriate for the 28 species of the *Acer* genus and *Dipteronia* species of the *Acer* genus.

The analysis of the phylogenetic tree based on the genetic profiles obtained showed that the similarity between the analyzed *Acer* accessions ranged from 63% to 98%. The *Acer* accessions analyzed in the experiment belong to the *Palmata* section, hence the interpretation of the results of their mutual phylogenetic connections seems easier. However, the inclusion of *A. dibolicum* in the analyses was intentional. This accession belonged to the *Lithocarpa* section and the drawn dendrogram was rooted from it. It should also be noted that *A. shirasavanum* 'Aureum' was grouped outside clades, in which cultivated and botanical varieties were classified. It should be emphasized that the dendrogram constructed on the basis of the genetic profiles obtained for the *Acer* successions does not present the actual phylogenetic relationships between them. It only reflects the relationships resulting from the presence of additional amplicons in the genetic profiles, which are not always taken into account in the cloning sequencing and therefore in the interpretation of results.

Conclusions

We succeeded in showing these PCR products were additionally amplified in more polymorphic regions, thus confirming the opinions and results obtained by other authors concerning their possible occurrence, i.e. the existence of rDNA polymorphic gene copies which have not been completely homogenized by concerted evolution.

References

Álvarez I, Wendel JF (2003). Ribosomal ITS sequences and plant phylogenetic inference. Mol Phylogenet Evol 29:417-434.

Baldwin BG (1992). Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: An example from the *Compositae*. Mol Phyl Evol 1:3-16.

Capossela A, Silander JA Jr, Jansen RK, Bergen B, Talbot DR (1992). Nuclear ribosomal DNA variation among ramets and genets of white clover. Evol 46:1240-1247.

Cronn RC, Small RL, Haselkorn T, Wendel JF (2002). Rapid Diversification of the Cotton Genus (*Gossypium*: *Malvaceae*) Revealed by Analysis of Sixteen Nuclear and Chloroplast Genes. Am J Bot 89(4):707-725.

Delendick TJA (1990). Survey of foliar flavonoids in the *Aceraceae*. Mem. New York Bot Gard 54:1-129.

Donoghue MJ., Baldwin BG., Li J, Winkworth RC (2004). *Viburnum* phylogeny based on chloroplast *trnk* intron and nuclear ribosomal ITS DNA sequences. Sys Bot 29(1):188-198.

Felsenstein J (1985). Confidence limits on phylogenesis: An approach using the bootstrap. Evol 39:783-791.

Feng TT, Zhou ZQ, Tang JM, Cheng MH, Zhou SL (2007).

ITS sequence support the hybrid origin of *Malus toringoides* Hughes. Can J Bot 85:659-666.

Gardes M, Bruns TD (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol 2:113-118.

Grimm GW, Denk T, Hemleben V (2007). Evolutionary history and systematics of *Acer* section *Acer*-a case study of low-level phylogenetics. Pl Syst Evol 267:215-253.

Grimm GW, Renner SS, Stamatakis A, Hemleben VA (2006). Nuclear Ribosomal DNA Phylogeny of *Acer* Inferred with Maximum Likelihood, Splits Graphs, and Motif Analysis of 606 Sequences. Evol Bioinfo Online 2:279-294.

Hasebe M, Ando T, Iwatsuki K (1998). Intrageneric relationships of maple trees based on the chloroplast DNA Restriction Fragment Length Polymorphisms. J Plant Res 111:441-451.

Hillis DM, Dixon MT (1991). Ribosomal DNA: Molecular evolution and phylogenetic inference. Q Rev Biol 66:411–453.

Ki JS, Han MS (2005). Molecular Analysis of Complete SSU to LSU rDNA Sequence in the Harmful Dinoflagellate *Alexandrium tamarense* (Korean Isolate, HY970328M). Ocean Sci J 40(3):43-54.

Nagylaki T (1984). The evolution of multigene families under intrachromosomal gene conversion. Genet 106:529-548.

Nei M, Li WH (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269-5273.

Pillay M, Kenny ST (1996). Structure and inheritance of ribosomal DNA variants in cultivated and wild hop, *Humulus lupulus L.* Theor Appl Genet 93:333-340.

Poczai P, Hyvönen J (2010). Nuclear ribosomal spacer regions in plant phylogenetics: problems and prospects. Mol Biol Rep 37:1897-1912.

Rzepka-Plevneš D, Smolik M, Drzewiecka K (2007). Genetic variation of some botanical and cultivar varieties of *Acer* sp. JFAE 5(3-4):481-485.

Smolik M, Jadczak D, Korzeniewska S (2009). Assessment of morphological and genetic variability in some *Thymus* accessions using molecular markers. Not Bot Hort Agrobot Cluj 37(2):234-240.

Suh Y, Heo K, Park Ch (2000). Phylogenetic relationship of Maples (*Acer* L; *Aceraceae*) implied by nuclear ribosomal ITS sequences. J Plant Res 113:193-202.

Van de Peer Y, De Wachter R (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comp App Biosci 10:569-570.

Van Gelderen DM, De Jong PC, Oterdoom HJ (1994). Maples of the World. Timber Press, Portland.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J of Bact 172:4238-4246.

Wallander E (2008). Systematics of *Fraxinus* (*Oleaceae*) and evolution of dioecy. Plant Syst Evol 273:25-49.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. In: Innis M, Gelfand D, Sninsky J, White T (Eds.) PCR protocols: A guide to methods and application. Academic Press, San Diego.

Yasui Y, Ohnishi O (1998). Phylogenetic relationships among *Fagopyrum* species revealed by the nucleotide sequences of the ITS region of the nuclear rRNA gene. Genus Genet Syst 73:201-210.

Yulong S, Newbury HJ, Ford-Lloy BV (1998). Identification of taxa in the genus *Beta* using ITS1 sequence information. Plant Mol Biol Reprt 16:147-155.