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RFLP ANALYSIS OF MITOCHONDRIAL DNA IN THE GENUS *SECALE*

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RFLP analysis of mitochondrial DNA was carried out with eight restriction enzymes *Bam*HI, *Eco*RI, *Hae*III, *Hind*III, *Msp*I, *Pst*I, *Sal*I and *Xho*I, from which nine mitochondrial gene probes (*atp6*, *atp9*, *atp1*, *cox1*, *nad3*, *nad6*, *nad9*, *pol-r*, *orf25*) were hybridized, by means of digestion products, for seven species of the genus *Secale*. RFLP *Eco*RI/*pol-r* specific markers were determined for all the species of rye. To estimate the relationships among species, genetic pairwise similarities between them were estimated and a UPGMA dendrogram was constructed. The analysis separated the species into two groups. The first comprises the pair *Secale sylvestre* Host and *S. cereale* subsp. *segetale* Zhuk., exhibiting the greatest genetic similarity, that is, closest relationships. The second group is composed of *S. strictum*/Presl/Presl, *S. strictum*/Presl/Presl subsp. *kuprijanovii*/Grossh./Hammer, *S. strictum*/Presl/Presl subsp. *africanum*/Stapf/Hammer, *Secale cereale* L. and *S. vavilovii* Grossh., with one clear subgroup comprising *Secale strictum*/Presl/Presl and *S. strictum*/Presl/Presl subsp. *kuprijanovii*/Grossh./Hammer. The latter two species showed the highest genetic similarity to each other and relatively high genetic similarity to the remaining species in the group.

Key words: *Secale*, rye, mitochondrial DNA, RFLP, UPGMA, genetic similarity.

INTRODUCTION

The genus *Secale* is a small but agriculturally significant taxon. It belongs to the *Triticeae* tribe and includes perennial and annual species. Depending on the criteria used to define the species, the genus *Secale* embraces cultivated rye and from 3 to 15 species and subspecies (Roshevitz, 1947; Hammer et al., 1987; Frederiksen and Petersen, 1998). This wide range of taxa results from the lack of agreement in interpreting taxonomic studies based on morphological, crossing, cytogenetic and molecular research. Most rye taxons are allogamous, and preserving the original material is very difficult. All species in the genus *Secale* have 2n = 14 chromosomes (Petersen, 1991; Frederiksen and Petersen, 1998). Analysis of the karyotypes of particular species of *Secale* revealed that they are highly similar. They differ only in the number and extent of translocations (Riley, 1955; Khush and Stebbins, 1961; Khush, 1962; Kranz, 1963, 1973; Singh and Robbelen, 1977). Comparisons of karyotypes, however, have not produced a conclusive taxonomic division of the genus *Secale*. One of the first phylogenetic analyses of the genus *Secale* was based on RFLP of the plastid genome (Petersen and Doebley, 1993). In that analysis the only well differentiated species was *Secale sylvestre*. *Secale cereale* and *S. strictum* specimens were intermingled in the phylogenetic tree and *S. strictum* subsp. *africanum* was not differentiated from other forms of *S. strictum*. The second analysis was based on the characteristics of the ITS rDNA sequence (DeBustos and Jouve, 2002). Sequence analysis of internal intron 1 (ITS–1) in the 18S–5,8S–26SrDNA showed the presence of two different sequence motifs in three taxa: *S. sylvestre* Host, *S. strictum* subsp. *kuprijanovii* Grossh., and *S. strictum* subsp. *africanum* Stapf./Hammer. *Secale sylvestre* proved to have the greatest number of sequence differences and was the most distant taxonomic unit. The latest classification (Hammer et al., 1987; Hammer, 1990) divides the genus *Secale* into four species: annual and autogamous *S. sylvestre* Host and *Secale vavilovii* Grossh., annual allogamous *S. cereale*, and the perennial *S. strictum* Presl/Presl (syn. montanum).

Phylogenetic studies in animals are frequently based on analysis of the mitochondrial genome; this is very rarely done in plants (Brown et al., 1979;

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TABLE 1. Names, sequences, product length and origin of primers used in probe creation

Gene	Sequence 5' – 3'	PCR product (bp)	Origin, Gene Bank Accession no
atp1	ATPA-1: GCGGATTTCTCCCTTAAAAT ATPA-2: CGAGTAAGGGGCAACCTTTT	1603	X99020 *S. cereale*
atp6	ATP6-1: AGCGAGCAGAGCTGAAAAAG ATP6-2: AGAATCTTCGGCTCCTCGTT	1222	M24084 *Triticum aestivum*
atp9	ATP9-1: AACAGCGAAGGAAAAACGTG ATP9-2: GCCCCATCTATGGAACAAGA	369	X99020 *S. cereale*
cox1	COXI-1: GATAGGTGCACCTGACATGG COXI-2: CATAATGGAAATGTGCAACC	940	U93504 *T. monococcum*
nad3	NAD3-1: AGAGAACGAAGTGGGCTTTG NAD3-2: CCCCCTTTGCCCTTTCTA	404	*Lupinus luteus* +
nad6	NAD6-1:TTTGGGAGCAGATCTTTCAA NAD6-2: GCCCGCCTATAAATCCTTTC	740	*Lupinus luteus* +
nad9	NAD9-1: ATTGGAAGAGAAGAAGCGGAACT NAD9-2: AGCATTTCCTATTGATTTGTGCC	400	D50099 *Oryza sativa*
pol-r	POL-R-1: CCAGCCGAAAGAAAGCATAG POL-R-2: GCCCGCTATCCATCCTAACT	943	X74133 *S. cereale*
orf-25	ORF-25-1: CTTTCAAAAGTGAGCGAGCA ORF-25-2: GGATCCGCTCTTCTGTTAGC	680	AB022060 *Triticum timopheevi*

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Clayton, 1982; Hiesel et al., 1994; Abe et al., 1999; Isshiki et al., 2003; Muller et al., 2003; van Droogenbroeck et al., 2004; Ballard and Rand, 2005). Phylogenetic studies of the genus *Secale* have never used the mitochondrial genome. They have always been based on analysis of nuclear and chloroplast genomes (Bedbrook et al., 1980; Jones and Flavel, 1982; Cuadrado and Schwarzacher, 1998; Fuchs et al., 1998; Cuadrado and Jouve, 1995, 1997, 2002). Thus there is a gap in the literature on phylogenetic studies in *Secale*. In order to fill this gap we attempted to define relationships in the genus *Secale* on the basis of RFLP mtDNA analysis. This paper presents the results of restriction analysis of total DNA with the use of eight restriction enzymes and Southern's hybridization of nine mitochondrial gene probes with the obtained restriction fragments in seven taxa from the genus *Secale*.

MATERIALS AND METHODS

PLANT MATERIAL

RFLP analysis of mitochondrial DNA was carried out on seven forms of *Secale*: *S. cereale* L. cv. Dańkowskie Zielonkawe, introd. no. 1595, *S. cereale* subsp. *segetale* Zhuk. introd. no. 1782/94/INN, *S. strictum*/Presl/Presl introd. no. 1785/94/INN, *S. strictum* /Presl/Presl subsp. *africanum*/Stapf/ Hammer introd. no. 6043, *S. strictum*/Presl/Presl subsp. *kuprijanovii*/Grossh./ Hammer introd. no. 2705, *S. vavilovii* Grossh. introd. no. 1783/94/INN and *Secale sylvestre* Host introd. no. 6047. The seeds were obtained from the Botanical Garden of the Polish Academy of Sciences in Warsaw.

DNA EXTRACTION

DNA was extracted from fresh, 5–6-day-old etiolated seedlings (~300 seedlings/species) using the DNEasy PLANT MINI KIT (Qiagen, Germany). After extraction, 1–2 μg/μl DNA was obtained and this was concentrated by precipitation in 96% ethyl alcohol with ammonium acetate, washed with 70% ethanol and diluted in 30 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA) (acc. Sambrook et al., 1989). DNA integrity and quality was evaluated by electrophoresis on 0.8% agarose gels. DNA concentration was determined with a Gene Quant RNA/DNA Calculator (Pharmacia LKB, USA).

PROBE DESIGN AND LABELING

Probes composed of the mitochondrial genes *atp6, atp9, atp1, cox1, nad3, nad6, nad9, pol-r* and *orf25* were made in PCR reactions with reagents (MBI Fermentas, Lithuania). Primers for these mitochondrial genes were constructed using the Primer3 program. The primer sequences are shown in Table 1. Following the manufacturer's instructions, 100 ng DNA of each probe was labeled by a random prim-

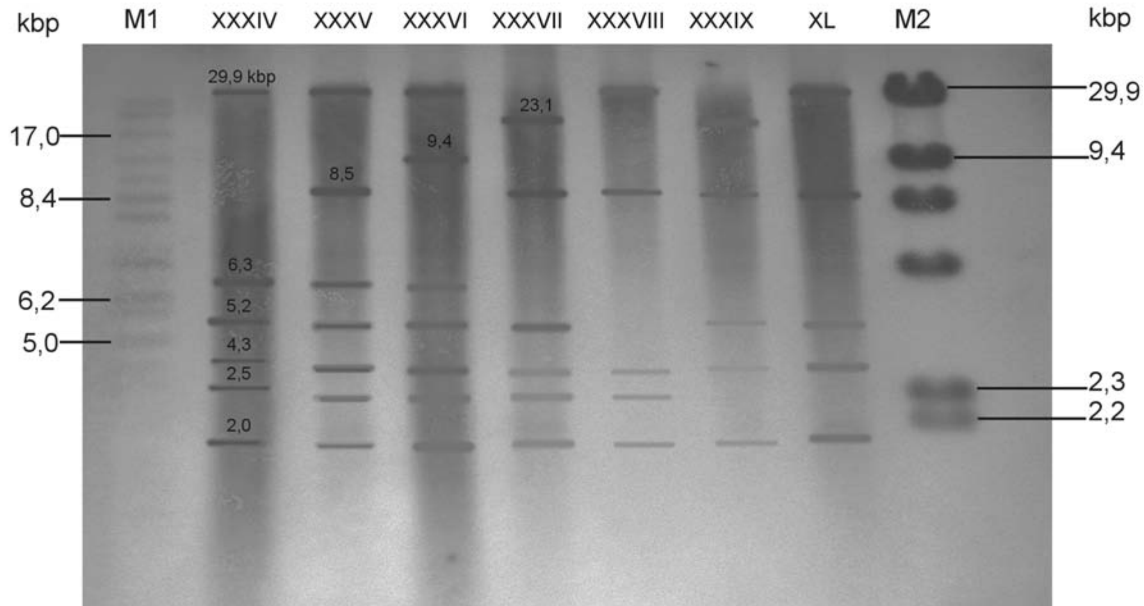

Fig. 1. Posthybridization patterns of *Eco*RI/*pol-r* in seven studied rye species; M1 – Lambda DNA/HindIII (MBI Fermentas); M2 – DNA MarkerII for Genomic DNA Analysis (MBI Fermentas); XXXIV – *S. strictum* subsp. *africanum*; XXXV – *S. strictum*; XXXVI – *S. strictum* subsp. *kuprijanovii*; XXXVII – *S. cereale* subsp. *segetale*; XXXVIII – *S. cereale*, XXXIX – S. *sylvestre*, XL – *S. vavilovii*.

ing method using reagents from the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany). The solutions of the labeled probes were stored at –20°C.

RFLP ANALYSIS

For digestion, 10 μg of total DNA was extracted from each species of rye and subjected to the action of eight restriction enzymes: *Bam*HI, *Eco*RI, *Hae*III, *Hind*III, *Msp*I, *Pst*I, *Sal*I and *Xho*I (MBI Fermentas, Lithuania). The reactions were conducted according to the method given by Maniatis et al. (1982). Digestion products were separated electrophoretically on 0.7% agarose gels with 1 × TBE buffer, run at 15 V for ~18 h, and then transferred to a nylon membrane (Roche, Germany).

SOUTHERN HYBRIDIZATION

The labeled probes with restriction products were hybridized with the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) according to the manufacturer's instructions.

DATA ANALYSIS

To estimate the relationships among the rye species, genetic pairwise similarities were estimated with the formula by Nei and Li (1979). The fraction of restriction fragments common to each pair of species (F) was calculated as follows:

$$F = 2Mxy/(Mx + My)$$

where Mxy is the number of bands shared by two species x and y, and Mx and My are the total number of bands resolved in species x and y. Distance values were calculated as 1-F. Cluster analysis was performed on the basis of the matrix of genetic similarity. The unweighted pair-group method with arithmetic averages (UPGMA) was used for clustering by the method of Nei and Kumar (2000), using GenStat5 (1993) software.

RESULTS

The results of Southern hybridization of eight restriction enzyme digests of total DNA to nine mitochondrial gene probes are shown in Table 2. For the seven studied rye taxa, 49 haplotype patterns emerged, labeled I to XLIX. Analysis of these haplotypes showed the occurrence of patterns I, II, III, V and VI in all of the studied species. Patterns IV-XXVII appeared in from two to six species of rye. The remaining 22 patterns were unique, appearing only once in a given taxon. Among them were patterns XXXIV–XL, formed by combinations between restriction fragments of *Eco*RI and *pol*-r probes observed in all rye species (Fig. 1, Tab. 2). These were regarded as RFLP mtDNA markers to identify the rye species. Other enzymes such as *Bam*HI, *Hind*III, *Msp*I, *Xho*I and *Sal*I cmbined with different probes also yielded unique haplotypes which were

TABLE 2. Restriction Fragment Length Polymorphism and haplotypes of mitochondrial DNA in seven studied rye species (*Secale cereale*)

Enzyme	Probe	Size (kbp)	Species						
			S. strictum subsp. *africanum*	*S. strictum*	*S .strictum* subsp. *kuprijanovii*	*S. cereale* subsp. *segetale*	*S. cereale*	*S. sylvestre*	*S. vavilovii*
*Bam*HI	*atp1*	29,9	+	+	+	-	+	+	+
		9,4	-	-	-	-	+	-	-
		8,4	+	-	+	-	+	+	+
		6,3	-	-	-	-	+	-	-
		5,2	+	-	-	-	-	+	+
		2,5	-	-	-	-	+	-	-
		haplotype	XVII	I	IX		XXVIII	XVII	XVII
	atp6	29,9	+	+	+	-	+	+	+
		6,5	+	+	+	+	+	+	+
		2,5	+	+	+	-	+	+	+
		haplotype	VIII	VIII	VIII	XVIII	VIII	VIII	VIII
	atp9	29,9	+	+	+	+	+	+	+
		17	+	+	+	+	+	-	-
		haplotype	III	III	III	III	III	I	I
	cox1	29,9	+	-	-	-	-	-	+
		23,1	+	+	+	-	-	-	+
		9,4	+	-	+	-	+	-	-
		haplotype	XXII	II	XXIII		VII		IV
	nad9	29,9	-	-	+	-	-	-	+
		23,1	+	+	+	+	+	-	+
		17	+	+	+	+	+	+	+
		9,4	+	+	+	+	+	+	+
		haplotype	XII	XII	XXIV	XII	XII	XXIX	XXIV
	orf25	9,4	+	-	+	-	+	-	-
		haplotype	VII		VII		VII		
	pol-r	29,9	-	+	-	-	+	-	+
		23,1	-	+	+	+	+	+	+
		haplotype		IV	II	II	IV	II	IV
*Eco*RI	*atp1*	29,9	+	+	+	+	+	+	+
		6,3	-	+	+	-	+	+	-
		haplotype	I	XIII	XIII	I	XIII	XIII	I
	atp9	29,9	-	+	+	+	+	-	+
		23,1	+	-	-	-	-	+	-
		17	-	+	+	+	+	-	+
		8,4	+	+	+	-	-	+	+
		6,5	-	-	-	-	-	-	-
		4,3	+	+	+	-	-	-	+
		haplotype	XXX	XIX	XIX	III	III	XXXI	XIX
	cox1	29,9	+	+	+	+	+	+	+
		23,1	-	-	+	-	-	-	+
		17	-	-	-	-	+	-	-
		8,4	-	+	+	-	+	-	-
		haplotype	I	IX	XXXII	I	XI	I	IV
	nad3	23,1	+	+	+	+	+	+	+
		17	-	+	+	-	+	+	+
		6,5	+	+	+	-	+	-	-
		haplotype	XXXIII	XX	XX	II	XX	V	V

TABLE 2. *(continued)*

*Eco*RI	*nad9*	29,9	+	+	+	+	+	+	+
		9,4	-	+	+	-	+	+	+
		haplotype	I	VI	VI	I	VI	VI	VI
	orf25	29,9	-	+	+	+	-	+	+
		23,1	+	+	+	-	+	-	-
		haplotype	II	IV	IV	I	II	I	I
	pol-r	29,9	+	+	+	-	+	-	+
		23,1	-	-	-	+	-	+	-
		9,4	-	-	+	-	-	-	-
		8,5	-	+	-	+	+	+	+
		6,3	+	+	+	-	-	-	-
		5,2	+	+	+	+	-	+	+
		4,3	+	+	+	+	+	+	+
		2,5	+	+	+	+	+	-	-
		2	+	+	+	+	+	+	+
		haplotype	XXXIV	XXXV	XXXVI	XXXVII	XXXVIII	XXXIX	XL
*Hae*III	*atp9*	2,5	-	+	+	-	-	-	-
		2,3	-	+	+	-	-	-	-
		2	-	+	+	-	-	-	-
		haplotype		XXVI	XXVI				
	nad9	23,1	-	+	+	-	-	+	+
		haplotype		II	II			II	II
*Hind*III	*atp1*	29,9	+	+	+	-	+	-	+
		17	-	+	-	-	-	-	-
		6,5	+	+	+	-	+	+	+
		haplotype	X	XXVII	X		X	XVIII	X
	atp6	29,9	-	-	+	-	-	-	-
		haplotype			I				
	nad3	29,9	+	+	+	+	+	+	+
		6,5	-	-	-	-	+	-	-
		haplotype	I	I	I	I	X	I	I
	nad6	29,9	+	+	+	+	+	+	+
		4,3	-	-	+	-	-	-	-
		haplotype	I	I	XLI	I	I	I	I
	nad9	29,9	+	+	+	+	+	-	+
		17	+	+	+	-	-	-	+
		9,4	-	+	+	+	+	-	-
		6,5	-	+	+	-	+	+	+
		haplotype	III	XXV	XXV	VI	XXI	XVIII	XXVII
	orf25	29,9	+	+	+	+	+	+	+
		23,1	-	-	+	-	-	-	-
		9,4	-	-	+	-	-	-	-
		8,4	-	+	+	-	-	+	+
		haplotype	I	IX	XLII	I	I	IX	IX
	pol-r	29,9	+	+	+	+	+	+	-
		23,1	-	-	-	-	-	-	+
		17	-	+	+	+	+	+	-
		8,4	-	+	-	+	+	+	-
		haplotype	I	XI	III	XI	XI	XI	II

TABLE 2. *(continued)*

*Msp*I	*atp1*	29,9	-	-	-	-	-	+	-
		23,1	+	-	-	-	-	-	-
		9,4	+	-	-	-	+	+	-
		haplotype	XXIII				VII	VI	
	atp6	29,9	+	+	-	+	+	+	-
		23,1	+	+	+	+	+	+	+
		17	+	-	-	-	+	+	-
		6,5	+	+	-	-	+	+	-
		haplotype	XIV	XLIII	II	IV	XIV	XIV	II
	atp9	29,9	-	+	+	-	+	-	+
		23,1	+	+	+	+	+	+	+
		17	+	+	+	+	+	+	+
		haplotype	V	XV	XV	V	XV	V	XV
	cox1	17	-	-	-	-	-	+	-
		8,4	+	-	-	-	-	+	-
		6,3	+	-	-	-	-	-	-
		haplotype	XLIV					XLV	
	nad9	23,1	-	-	-	+	+	+	+
		17	-	-	-	-	+	+	-
		6,5	-	-	-	-	-	+	-
		6,3	-	-	-	-	-	+	-
		haplotype				II	V	XLVI	II
*Pst*I	*atp1*	29,9	-	-	-	-	+	-	-
		23,1	-	-	+	+	+	+	+
		17	-	-	-	-	-	-	+
		haplotype			II	II	IV	II	V
	atp6	29,9	-	-	+	+	+	+	+
		haplotype			I	I	I	I	I
	atp9	29,9	-	-	-	-	+	-	-
		23,1	-	-	+	+	-	+	+
		haplotype			II	II	I	II	II
	cox1	29,9	-	-	-	-	+	-	-
		23,1	-	-	+	+	+	+	+
		17	-	-	-	-	-	-	+
		9,4	-	-	-	-	+	-	-
		haplotype			II	II	XXII	II	V
	nad9	29,9	-	-	-	-	+	-	-
		23,1	-	-	+	+	+	+	+
		17	-	-	-	-	-	-	+
		haplotype			II	II	IV	II	V
	orf25	29,9	+	+	+	+	+	+	+
		17	-	-	+	+	+	-	+
		haplotype	I	I	III	III	III	I	III
	pol-r	29,9	+	+	+	-	+	+	+
		17	+	+	+	-	+	+	+
		haplotype	III	III	III		III	III	III

TABLE 2. *(continued)*

*Sal*I	*atp1*	29,9	+	+	+	+	+	+	-
		23,1	+	+	+	-	-	-	-
		17	-	-	-	-	-	+	-
		haplotype	IV	IV	IV	I	I	III	
	atp6	29,9	+	+	+	-	+		+
		9,4	+	+	+	+	+	+	+
		6,5	-	+	+	-	-	-	-
		haplotype	VI	XXI	XXI	VII	VI	VII	VI
	atp9	29,9	+	+	+	+	+	+	+
		23,1	-	-	+	-	-	-	-
		haplotype	I	I	IV	I	I	I	I
	cox1	29,9	+	+	+	+	+	+	+
		17	-	-	+	-	-	-	-
		haplotype	I	I	IV	I	I	I	I
	nad3	29,9	+	+	+	+	-	+	+
		23,1	-	-	+	-	-	-	+
		haplotype	I	I	IV	I		I	IV
	nad6	29,9	+	+	+	+	+	+	+
		23,1	-	-	-	-	+	-	-
		17	-	+	+	-	-	-	-
		haplotype	I	III	III	I	IV	I	I
	nad9	29,9	+	+	+	+	+	+	+
		23,1	-	-	+	-	-	-	-
		17	-	-	+	-	-	-	-
		9,4	-	-	+	-	-	-	-
		6,5	-	-	+	-	-	-	-
		haplotype	I	I	XLVII	I	I	I	I
	orf25	29,9	+	+	+	+	+	+	+
		17	-	-	+	-	-	-	-
		haplotype	I	I	III	I	I	I	I
	pol-r	29,9	+	+	+	+	+	+	+
		23,1	-	-	+	-	-	-	-
		17	-	-	+	-	-	-	-
		6,5	-	-	+	-	-	-	-
		haplotype	I	I	XIV	I	I	I	I
*Xho*I	*atp1*	29,9	-	-	-	-	+	-	-
		23,1	+	+	+	+	+	+	+
		haplotype	II	II	II	II	IV	II	II
	atp6	29,9	+	+	+	+	+	+	+
		17	-	-	-	-	+	-	-
		haplotype	I	I	I	I	III	I	I
	cox1	29,9	+	+	+	+	+	-	+
		17	-	+	+	+	+	+	-
		5,2	-	+	+	+	+	+	+
		haplotype	I	XVI	XVI	XVI	XVI	XLVIII	XLIX
	nad9	23,1	+	+	+	+	+	+	+
		17	-	+	+	-	+	-	-
		haplotype	II	V	V	II	V	II	II
	orf25	29,9	+	+	+	+	+	+	+
		17	-	-	-	-	+	-	-
		haplotype	I	I	I	I	III	I	I

TABLE 3. Coefficients of genetic similarity of the studied rye (*Secale cereale*) species

Similarity	Species					
	S. strictum subsp. *africanum*	*S. strictum*	*S. strictum* subsp. *kuprijanovii*	*S. cereale* subsp. *segetale*	*S. cereale*	*S. sylvestre*
S. strictum	0.7517					
S. strictum subsp. *kuprijanovii*	0.6588	0.8108				
S. cereale subsp. *segetale*	0.6179	0.6957	0.6415			
S. cereale	0.6709	0.7514	0.7010	0.6939		
S. sylvestre	0.6714	0.6968	0.6250	0.7287	0.6951	
S. vavilovii	0.6901	0.7389	0.7303	0.7023	0.6867	0.7162

treated as RFLP markers for particular taxa. Different numbers of RFLP markers were defined for certain *Secale* species (Tab. 2).

On the basis of the obtained haplotype mtDNA patterns found by RFLP, genetic pairwise similarities between studied species were estimated with Nei and Li's (1979) formula (Tab. 3). Using the matrix of genetic similarity, cluster analysis was performed and a UPGMA dendrogram was constructed (Fig. 2). Cluster analysis of the genetic similarity coefficients clearly separated two groups of species. The first includes *S. cereale* subsp. *segetale* and *S. sylvestre*, and the second group comprises the rest of the analyzed species. The second group has a subgroup of most closely related species: *S. strictum* and *S. strictum* subsp. *kuprijanovii*. The next-highest genetic similarity was between those species and *S. vavilovii*, *S. cereale* and *S. strictum* subsp. *africanum*.

DISCUSSION

In the genus *Secale*, phylogenetic studies have not fully explained the relationships between its forms. Phylogenetic analyses based on 2, 3 or 4 intron sequences of alcohol dehydrogenase *1-Adh*1 placed subspecies of *Secale strictum* in a monophyletic group on the basis of identical sequences. On the other hand, two subspecies of *S. cereale* were not confined to monophyletic groups, and the position of S. *sylvestre* was uncertain (Petersen et al., 2004). *Secale* studies based on RFLP analysis of the plastid genome placed *Secale sylvestre* as a sister group to the remaining taxa of rye, but did not confirm *Secale cereale* and *Secale strictum* as monophyletic groups (Petersen and Doebley, 1993). On the basis of the characteristics of ITS rDNA sequences, DeBustos and Jouve (2002) found no sequence differences within the subspecies of *S. cereale*, explained by fast homogenization of different members of the multigene systems related to speciation (concerted evolution). In the *S. strictum* complex they presented sequence differences within and between taxa, most visible between *S. strictum* subsp. *africanum* and the remaining taxa. *Secale sylvestre* was separated from the remaining taxons by the greatest sequence differentiation. *Secale vavilovii* was characterized by sequence similarity to subspecies of the *S. strictum* and *S. cereale* groups. Our results are somewhat similar to those. RFLP mtDNA of seven species of rye showed two groups of rye taxa: one group of two species with the highest genetic similarity, *S. sylvestre* and *S. cereale* subsp. *segetale*, separated from a large group including *S. strictum*, *S. strictum* subsp. *kuprijanovii*, *S. vavilovii*, *S. cereale* and *S. strictum* subsp. *africanum*. We named the latter the *strictum* group. Rye taxa in it exhibited high genetic similarity. Among them, *S. strictum* and *S. strictum* subsp. *kuprijanovii* formed a subgroup of the highest genetic similarity, the most closely related species of rye. RFLP analyses revealed variation among the rye species, particularly visible in the *Eco*RI/*pol-r* combination. Different patterns of this combination occurred in all the analyzed species and were the RFLP marker for them.

A problem in RFLP analysis involves the different types of recombinations occurring between simple and inverted repeats of nucleotide sequences of mtDNA, which are common in the mitochondrial genome of plants. Recombinations between repeated sequences are a source of variability in plant mitochondrial genomes (Palmer and Shields, 1984; Lonsdale et al., 1988; Coulthart et al., 1990, 1994; Andre et al., 1992; Bendich, 1993). The size of the mitochondrial genome of *Secale* cereale was put at 410 kbp, and its buoyant density at 1,705 g/ml (Tudzynski et al., 1986). In the literature there are data on the organization of a repeat family of recombination unit 18S/5S in rye, confirming its polymorphism and its similarity to the organization of this unit in wheat and showing the common origin of rye and wheat (Falkonet et al., 1984; Coulthart et al.,

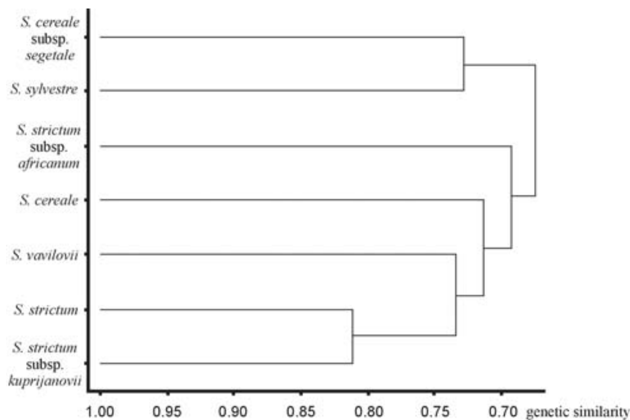

Fig. 2. UPGMA dendrogram describing genetic similarity of the examined rye species as found by mtDNA RFLP (Nei and Kumar, 2000)

1993). RFLP and Southern hybridization studies on repeat unit 18S/5S in different species of *Secale*: *dighoricun*, *segetale*, *vavilovii*, *montanum* and *cereale* showed different genome rearrangements that caused differentiation in the number of loci (one or three), not accompanied by changes in the number of gene copies. It was concluded that *Secale montanum* probably is an ancestral species for *S. cereale* and that the remaining species are Kodiak-type hybrids (Coulthart et al., 1994). In our studies we found unique patterns of hybridization for the *Eco*RI/*pol-r* combination in all the analyzed forms of rye. We considered them to be RFLP markers specific to the studied species. They proved to be the only RFLP markers for *S. cereale* subsp. *segetale*; only two other species, *S. strictum* and *S. cereale*, had two specific RFLP markers – including one with *Eco*RI/*pol-r*. Gene *pol-r* is an open reading frame which codes a protein showing significant homology to DNA-B polymerase, coded by linear plasmid S_1 from maize, and high homology to plasmid pC_1K_1 from *Claviceps purpurea*. The product of *pol-r* occurs in small quantities; probably it is subject to degradation as in the case of petunia. That is why it is regarded as evolutionarily young, probably introduced into the mtDNA of rye as the result of recombination with free plasmids (pS_1 – maize and pC_1K_1) (Dohmen and Tudzynski, 1994). The *Eco*RI/*pol-r* combination occurred in different hybridization patterns in the analyzed species of rye, showing the organizational changeability of this gene. This in turn indicated the organizational differentiation of mitochondrial genomes in the studied species of rye. The unique band *Bam*HI/*atp1* occurred in *S. cereale*, and *Msp*I/*atp6* in *S. strictum*. The calculated genetic similarity between the species of rye was very high between *S. sylvestre* and *S. cereale* subsp. *segetale* as well as between *S. strictum* and *S. strictum* subsp. *kuprijanoviii*. After *S. strictum* subsp. *kuprijanovii*, *S. cereale* is the next most genetically similar to *S. strictum*. The RFLP mtDNA data presented here accord with the suggestion that *S. sylvestre* was the first species to separate from others in the course of evolution, and partially in agreement with the statement that *S. vavilovii*, *S. strictum* subsp. *africanum*, *S. strictum* and *S. strictum* subsp. *kuprijanovii* are evolutionarily the youngest (Hammer et al., 1987).

Our observations also partly coincide with the results of Cuadrado and Jouve (2002). On the basis of the distribution of SSR, 5SrRNA markers and three repeat sequences of rye, they showed that *S. sylvestre* separated from *S. strictum*, creating a branch which evolved after amplification of the family of 610 bp repeats. Next, *S. strictum* subsp. *africanum* separated itself before amplification of 480 bp repeats and interstitiality of 120 bp repeats. They also confirmed that *S. vavilovii* and *S. cereale* are of common origin but separated and evolved independently. These last two studies considered traits encoded by nuclear genes, while our investigations focused on mitochondrial genes. Differences in the data obtained may be expected to result from the disparity of approaches, as nuclear gene trees may not correspond to mitochondrial phylogeny.

Rye tends to generate annual races from perennial plants, and vice versa. Its tendency towards annuality made it easier for rye weed to develop and bear seed simultaneously with annual wheat and annual barley, and thus to proliferate in fields with these crops (Nowiński, 1970). Moreover, self-pollination ensured the multiplication and territorial expansion of rye. The enormous ability of rye to generate various forms – annual, perennial, autogamous, allogamous, and of differentiated morphology – is one reason why it is so difficult to classify this crop. Our work is the first attempt at RFLP analysis based on the mitochondrial genome. It is generally known that the mitochondrial genome of plants is extremely changeable. It dynamically organizes coding and noncoding sequences, and cumulates mutations. These characteristics, along with presumably uniparental inheritance, make it excellent material for phylogenetic studies. *S. cereale*, characterized by bipaternal transmission of mitochondria, is an exception among flowering plants (Mogensen and Rusche, 2000). This also hinders recognition and classification of the forms of rye within the genus *Secale*.

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