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Genetic Structure of Tetraploid Italian and Westerwolds Ryegrasses (*Lolium* spp.) as Revealed by Enzyme and ISSR Polymorphism

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Abstract: The genetic variation of Italian ryegrass (*Lolium multiflorum* Lam.) and westerwolds ryegrass (*L. westerwoldicum* Breakw.) was investigated in eight tetraploid cultivars originated from Poland. The 337 individual seedlings, cultivated in uniform greenhouse conditions, were tested with horizontal gel electrophoresis according to five enzyme systems: diaforase (DIA), superoxide dismutase (SOD), glutamate oxalacetate transaminase (GOT), peroxidise (PX) and phosphogluco isomerise (PGI), which were used to array allelic diversity at five polymorphic loci. The estimation of genetic diversity in 64 plants of the same cultivars was studied using inter simple sequence repeats (ISSRs) as molecular marker. The genetic variation described by ISSR suggests that the polymorphism detected appears to be poorly informative at the taxonomic level. For statistical analysis, unweighted pair group method with arithmetic mean (UPGMA) based dendrograms and minimum spanning trees were constructed using Nei's distance and Jaccard's similarity coefficient.

Key words: *Lolium* spp., tetraploid cultivars, genetic structure, enzyme electrophoresis, inter simple sequence repeats markers.

1. Introduction

Italian ryegrass (*Lolium multiflorum* Lam.) is a fodder grass. This species, known for its good taste (due to high sugar content), is also valued as fodder in the form of hay and silage, so it is grown intensively in Asia, America, Europe and New Zealand.

In recent years, scientists have searched for markers to describe its genetic variation with the use of many techniques, such as enzyme electrophoresis and DNA sequencing. In genetic research on populations of *L. multiflorum*, mostly randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) markers have been used so far [1-4]. In this study, inter simple sequence repeats (ISSR) markers have been chosen instead, as this method has most of the benefits of SSR and AFLP, and it proves to be a good method for genetic diversity detection in other monocot crops, e.g., rice [5-7], rye [8], triticale [9, 10] and barley [11], as well as grasses of the genus *Bromus* [12]. ISSR segregates mostly as dominant marker following simple Mendelian inheritance and also enables distinction between homozygotes and heterozygotes.

Westerwolds ryegrass (*L. westerwoldicum*, syn. *L. multiflorum* ssp. *westerwoldicum*) is also an annual fodder grass, originating from the Westerwolde region in the Netherlands. This species is highly valued by breeders, so its local cultivars have been developed in many countries, including Poland. The variation of its Polish cultivars has been described in respect of morphological and chemical characters [13], but no genetic studies have been done.

In spite of intensive application of molecular methods, enzymatic studies of *Lolium* species, including *L. multiflorum* are still relevant [14]. They are used both in considerations of difference between

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species [15] and in tracing of the mechanisms of their inheritance [16].

As the genetic variation of *L. westerwoldicum* has not been investigated till now, this study was aimed to find some markers for comparison of the two species in question.

2. Materials and Methods

2.1 Plant Materials

Seedlings of three Polish cultivars of *L. westerwoldicum*, i.e., Kaja (KA), Koga (KO), Telga (TE), and five cultivars of *L. multiflorum*, i.e., Atos (A), Gaza (GA), Gisel (G), Turtetra (TU) and cultivar 604 (604), were grown from seeds in the same greenhouse conditions. The seeds were procured from breeding companies: Małopolska Plant Breeding HBP Ltd. (Kraków), Bartrążek Plant Breeding Ltd. (Olsztyn) and Danko Plant Breeding Ltd. (Szelejewo).

2.2 Electrophoretic Separation of Enzymes

Individual plants, at least 30 of each cultivar: KA (N = 58), KO (N = 30), TE (N = 30), A (N = 60), GA (N = 30), G (N = 31), TU (N = 66) and 604 (N = 32), were studied in respect of five enzymatic systems: diaphorase (DIA), superoxide dismutase (SOD), glutamic oxaloacetic transaminase (GOT), peroxidase (PX) and phosphogluco isomerase (PGI). Two leaves from individual plants were homogenised in double-distilled water for DIA, SOD, GOT and PX. Plants prepared for the PGI enzyme system were homogenized in 0.12 M Tris-HCl, pH 7.5. Electrophoresis was conducted in 11% starch gel (Sigma) prepared on the basis of lithium-boric buffer system, pH 8.1. Band patterns were visualised by a method previously used [14]. Electrophoretically detected phenotypes were used to calculate the genetic parameters: observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient (F) and polymorphic index (P_g). The genetic distances between cultivar populations were calculated on the basis of allele frequency according to Nei [17] and illustrated by minimum spanning trees and dendrograms.

2.3 Molecular Analysis

DNA was isolated from 64 plants (eight plants of each cultivar) by using Genomic Mini AX plant kit (A&A Biotechnology) according to the manufacturer's instructions. In polymerase chain reaction (PCR), Fermentas reagents were used. Amplifications were conducted in 25 μL of reaction volume containing 100 ng of DNA, and polymerase buffer was composed of 1.5 mM $MgCl_2$, 1 mM NTP, 0.25 mM of primer and 1.4 units of Taq polymerase. The sequences of primers were taken from Stepansky et al. [18]. PCR amplification was performed by 2720 thermal cycler (Applied Biosystems). Amplification products were separated in 1.5% agarose gel with ethidium bromide. The gels were visualised using the ImageMaster® VDS (Pharmacia Biotech) and Liscap Capture ver. 1.0 software. For analysis of band patterns, GelScan ver. 1.43 (Kucharczyk TE) software was used. Each plant subjected to ISSR analysis was regarded as an operational taxonomic unit (OTU). The 302 ISSR bands for 64 OTUs were encoded as a binary data matrix (with "1" indicating the presence and "0" the absence). The data were used to calculate the matrix of Jaccard's similarity coefficients and to generate a dendrogram by unweighted pair group method with arithmetic mean (UPGMA), using NTSYS ver. 2.11a software [19].

3. Results and Discussion

3.1 DIA (E.C.1.6.4.3)

All the populations were polymorphic in respect of three alleles of a single locus. The minimum spanning tree based on frequencies of alleles (allozymes, Fig. 1) indicates high similarity of cultivars TU, GA and KO, and of A and TE. Particularly noteworthy is cultivar KA, which is the most distant genetically from the others. P_g values ranged from 0.22 to 0.65 (see Table 1 for DIA and other enzyme systems).

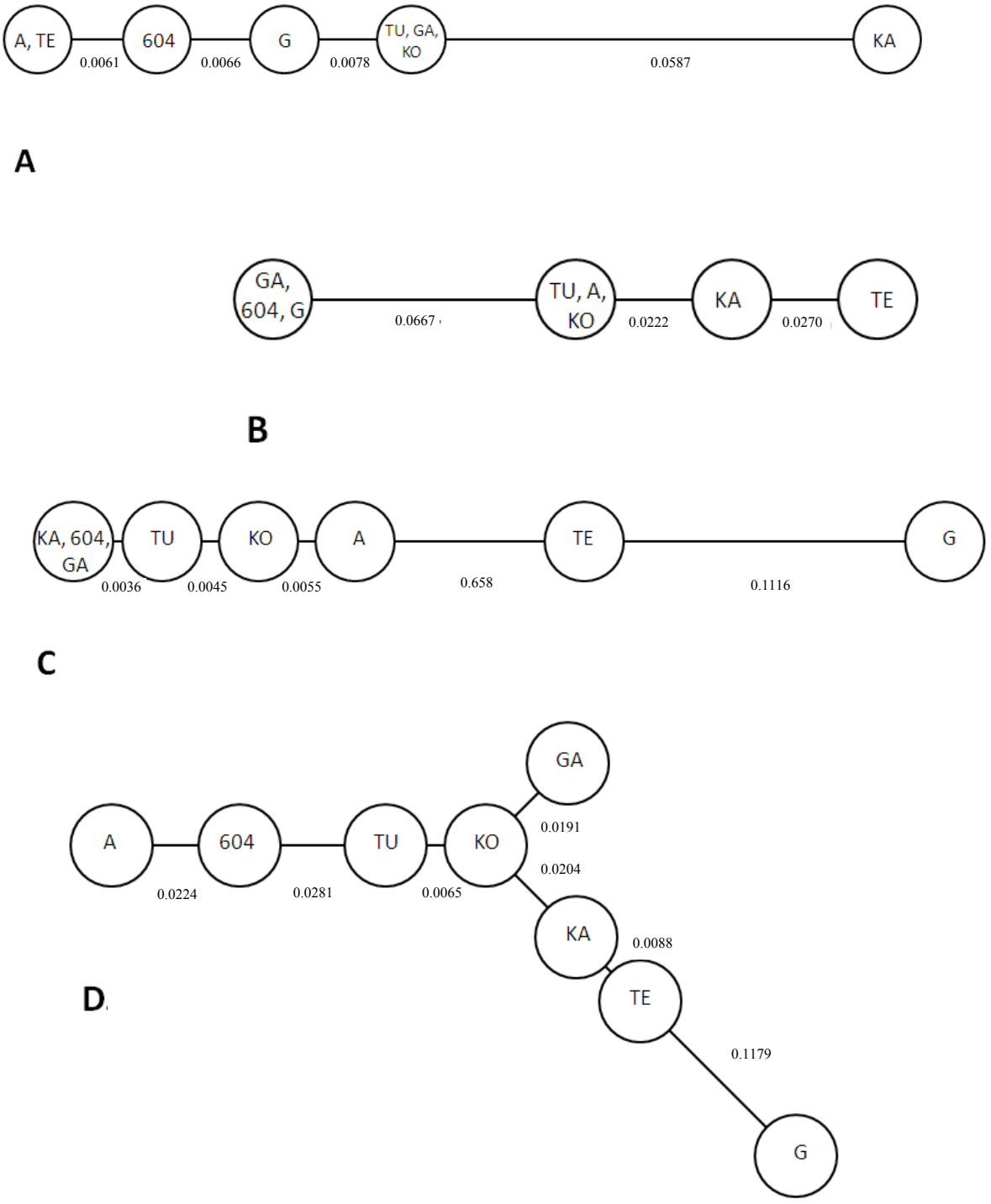

Fig. 1 The minimum spanning trees of genetic distances constructed for the four enzyme systems (A—DIA, B—GOT, C—SOD, D—PX) on the basis of gene frequency in eight cultivars.

Table 1 Genetic parameters for the eight *L. multiflorum* cultivars.

Locus	Cultivars	H_e	H_o	F	P_g
DIA D	604	0.5547	1.0000	-0.8028	0.2188
DIA D	A	0.4922	0.8750	-0.7778	0.2188
DIA D	G	0.5000	0.7500	-0.5000	0.4063
DIA D	GA	0.4922	0.8750	-0.7778	0.2188
DIA D	KA	0.3750	0.5000	-0.3333	0.5000
DIA D	KO	0.4922	0.8750	-0.7778	0.2188
DIA D	TE	0.4922	0.3750	0.2381	0.6563
DIA D	TU	0.4922	0.7500	-0.7778	0.2188
GOT G	604	0.2188	0.2500	-0.1429	0.3750
GOT G	A	0.1172	0.1250	-0.0667	0.2188
GOT G	G	0.2188	0.2500	-0.1429	0.3750
GOT G	GA	0.2188	0.2500	-0.1429	0.3750
GOT G	KA	0.2188	0.2500	-0.1429	0.3750
GOT G	KO	0.1172	0.1250	-0.0667	0.2188
GOT G	TE	0.3047	0.3750	-0.2308	0.4688
GOT G	TU	0.1172	0.1250	-0.0667	0.2188
SOD S	604	0.2188	0.2500	-0.1429	0.3750
SOD S	A	0.4297	0.6250	-0.4545	0.4688
SOD S	G	0.3750	0.2500	0.3333	0.5313
SOD S	GA	0.2188	0.2500	-0.1429	0.3750
SOD S	KA	0.2188	0.2500	-0.1429	0.3750
SOD S	KO	0.3750	0.5000	-0.3333	0.5000
SOD S	TE	0.5000	1.0000	-1.0000	0.0000
SOD S	TU	0.3047	0.3700	-0.2308	0.4688
PX X	604	0.4766	0.6250	-0.3115	0.6563
PX X	A	0.5313	0.7500	-0.4118	0.6250
PX X	G	0.6641	1.0000	-0.5059	0.6563
PX X	GA	0.5703	0.6250	-0.0959	0.6563
PX X	KA	0.5547	0.6250	-0.1268	0.6563
PX X	KO	0.4688	0.5000	-0.0667	0.5938
PX X	TE	0.5547	0.8750	-0.5775	0.4063
PX X	TU	0.4297	0.3750	-0.1263	0.5938
PGI P	604	0.7656	1.0000	-0.3061	0.7500
PGI P	A	0.8359	1.0000	-0.1963	0.8125
PGI P	G	0.8125	0.8750	-0.0769	0.7813
PGI P	GA	0.7813	0.8750	-0.1200	0.8438
PGI P	KA	0.7578	0.8750	-0.1546	0.7813
PGI P	KO	0.8203	1.0000	-0.2190	0.7813
PGI P	TE	0.7578	0.8750	-0.1546	0.6875
PGI P	TU	0.7969	1.0000	-0.2549	0.8125

3.2 SOD (E.C.2.6.12.1)

The minimum spanning tree based on frequencies of two alleles of a single locus (Fig. 2) showed that cultivar G is the most distinct, characterized by the highest P_g = 0.53. Three cultivars (KA, 604 and GA) do not differ in polymorphic index P_g = 0.37.

3.3 GOT (E.C.2.6.1.1)

As shown in the minimum spanning tree based on frequencies of three alleles of a single locus (Fig. 3), cultivars GA, 604 and G are the most similar to one another and distant genetically from the other populations. A similar group is composed of cultivars

TU, A and KO, which do not differ in polymorphic index P_g = 0.22. Overall, the polymorphism of populations was relatively low, as P_g values ranged from 0.22 to 0.47 for cultivar TE.

3.4 PX (E.C.1.11.1.7)

The minimum spanning tree based on frequencies of two alleles of a single locus (Fig. 1D) showed the distinctness of cultivar G. The similarity of cultivars TU and KO is noticeable, like in the previous enzymatic systems. P_g values varied from 0.40 to 0.66.

Lolium plants contain PXs of anodal migration [20]. Less frequently, peroxidises of cathodal migration are detected in grasses [21]. Taking into account that PXs are contained mainly in cell walls, such a location results in their activity at early stages of defence reactions of the plant to pathogen attacks. Besides, PXs have proven to provide useful markers for analysing intra-specific differentiation, as well as differences between species [21, 22]. In this study, PXs were used to detect differences between cultivars, which were not detected in the other four enzymatic systems. Only in respect of PXs for the three cultivars of *L. westerwoldicum* (KA, KO and TE), close genetic similarity was found (Fig. 1D).

3.5 PGI (E.C. 5.3.1.9)

All the tetraploid cultivars used in this study show a high polymorphism of four alleles of one locus. Generally, the PGI dimer is a highly polymorphic enzyme system and although it is the most expensive to work with, it should be recommended for broader investigations, especially in *Lolium* species [22]. Estimation of genetic diversity was shown in Table 1. The populations differ in heterozygosity level. P_g values ranged from 0.69 to 0.84. Genetic differences among cultivars are illustrated by a dendrogram (Fig. 2). Cultivar G is the most distant from others, which forms two subgroups. One of them is composed of two cultivars of *L. westerwoldicum* (KA and KO), while cultivars of *L. multiflorum* (604 and A) are accompanied by *L. westerwoldicum* TE. Examples of histograms generated for *L. multiflorum* GA and *L. westerwoldicum* (TE) were shown in Fig. 3.

ISSR-PCR was conducted with seven primers, but for analysis of the results, only those with the most readable band patterns (ISSR 01, ISSR 04, ISSR 05, ISSR 06 and ISSR 07) were used. The primers generated 2-12 products of various lengths, depending on the individual and primer. The total number of products of various lengths was as follows: 52 for

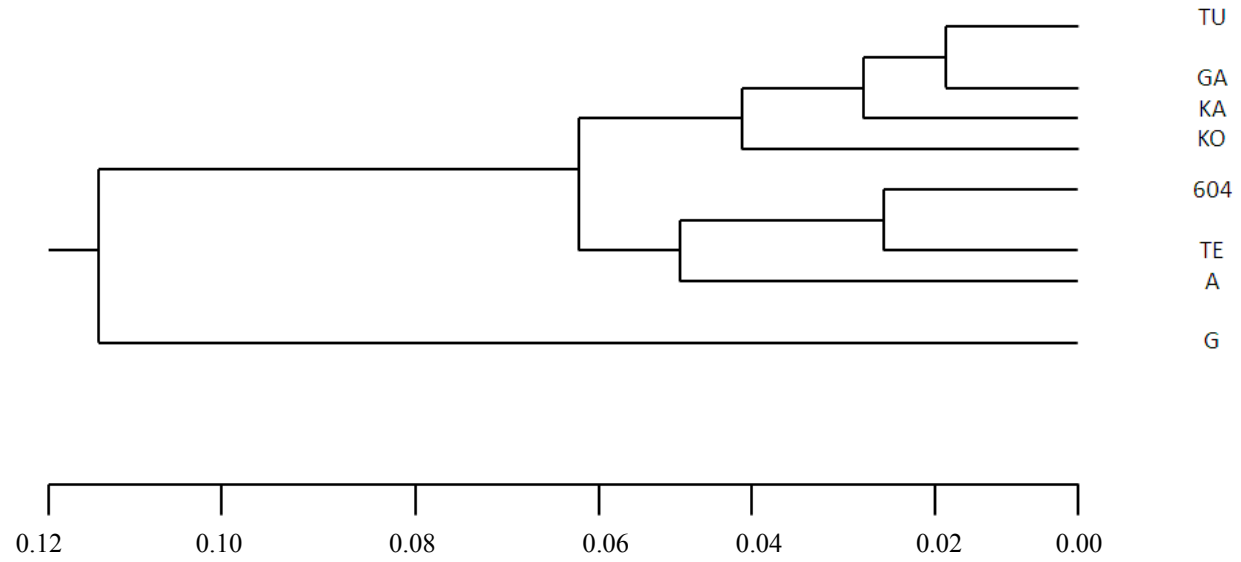

Fig. 2 PGI dendrogram constructed on the basis of gene frequency.

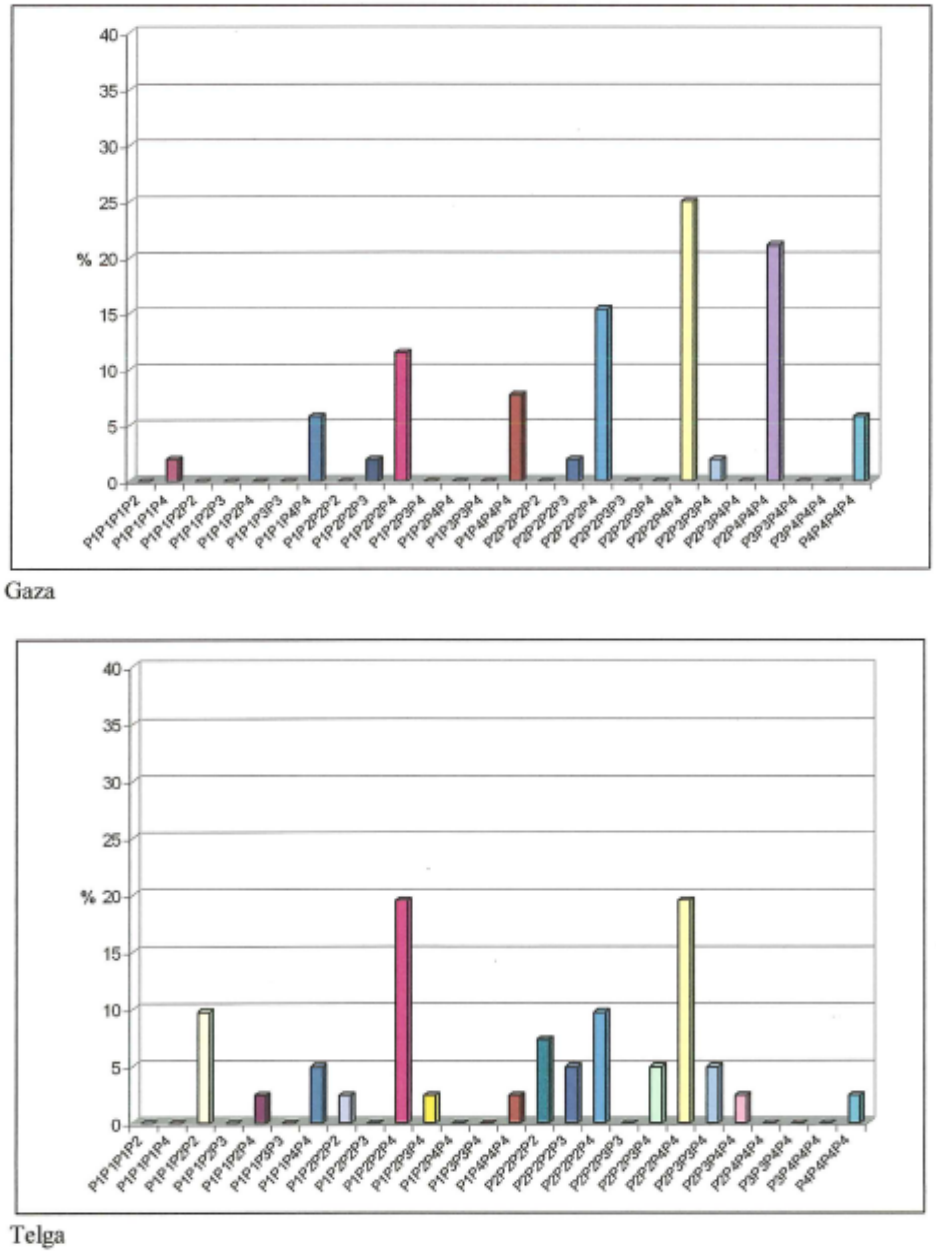

Fig. 3 Histograms of the PGI phenotypes generated for *L. multiflorum* (GA) and *L. westerwoldicum* (TE).

ISSR 01, 50 for ISSR 02, 55 for ISSR 04, 38 for ISSR 06 and 52 for ISSR 07. Electrophoretic separation of PCR products was shown in Fig. 4. The studied cultivars are clearly polymorphic at the DNA level. In the analysed material, no specific products (markers) were found for individual cultivars. The dendrogram (Fig. 5) generated for 64 individuals suggests that three groups of samples can be distinguished. The first group is created by KA and G, the second by cultivar 604 with TU and the third one is initiated by GA, TE, A and KO. The most marginal populations are occupied by KA and KO. It is noteworthy that two cultivars of *L. westerwoldicum*, KO and TE are included to the same group.

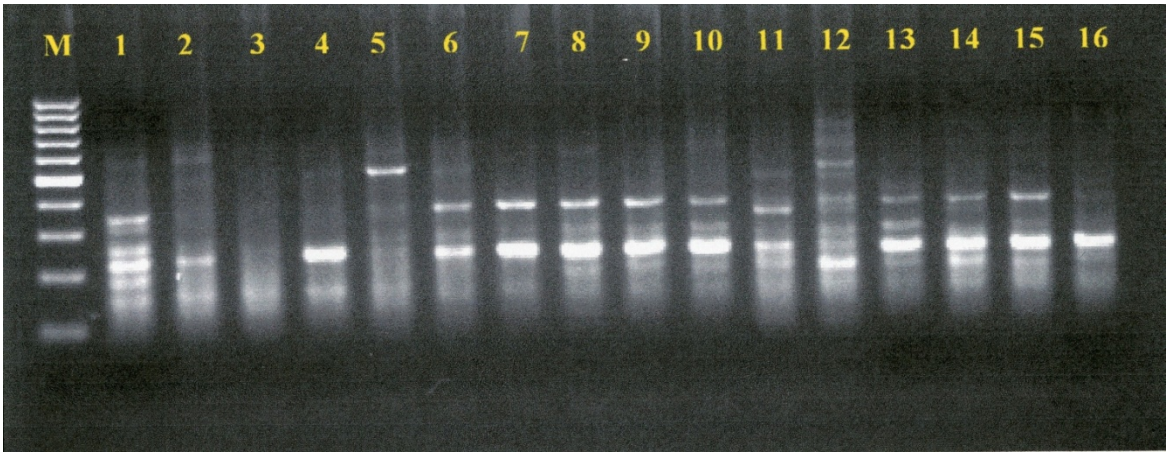

Fig. 4 Electrophoretic patterns of PCR—amplified ISSRs on 1.5% agarose gels.
M—100 pz markers, starter ISSR 04 $(ATG)_6$; 1-6: cultivar KA; 9-16: cultivar G.

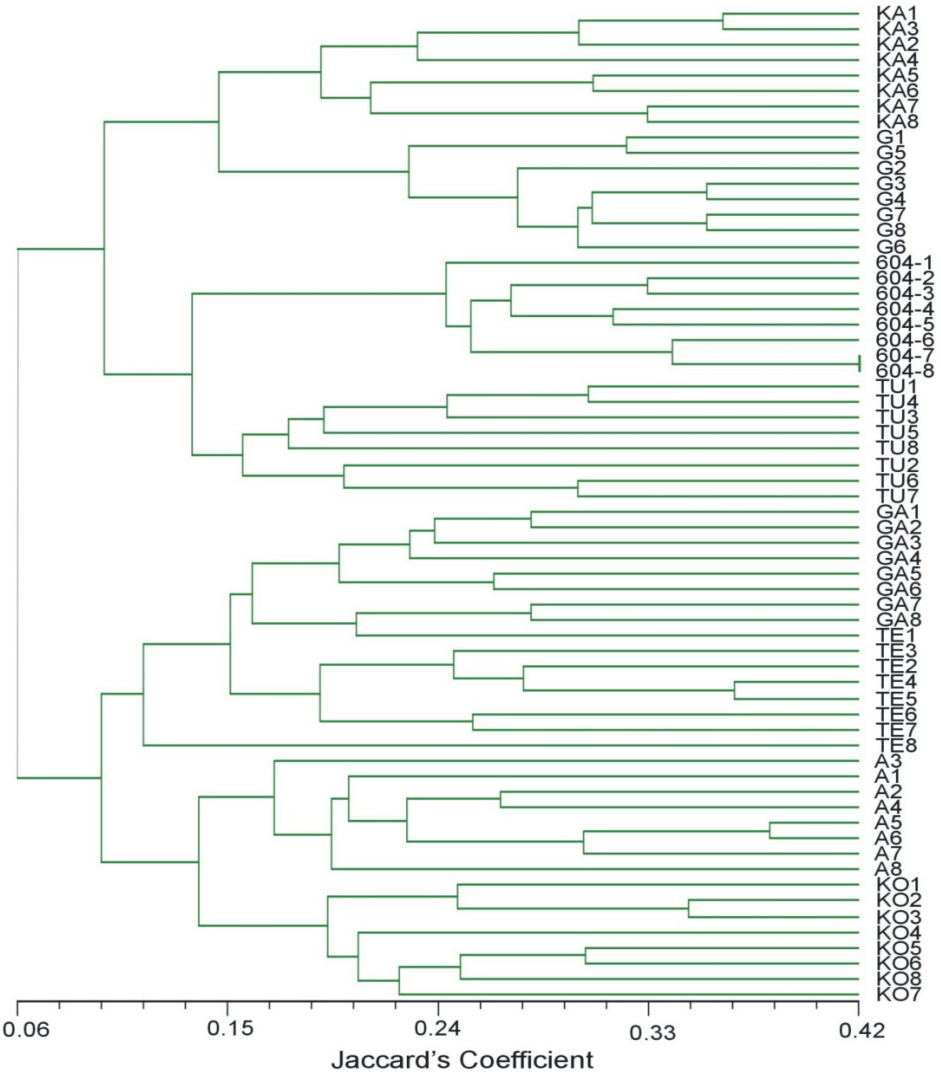

Fig. 5 Dendrogram from cluster analysis (UPGMA) based on Jaccard's genetic distances constructed for 64 OTUs.

4. Conclusions

All the compared Polish cultivars of *L. multiflorum* show genetic polymorphism. Out of the five studied enzyme systems, the most polymorphic ones were PGI and PX. This is interesting because both enzymes are involved in processes of plant resistance, so the knowledge of their genetic variation can be of practical importance. Broadening of research on those enzymes may provide new information on variation of the species.

In this study, ISSR-PCR proved to be useful for analysing associations between *Lolium* cultivars, although no markers specific to any cultivar were identified. In total, 401 plants were analysed simultaneously with the use of biochemical and molecular markers.

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