Genetic diversity of *Poa pratensis* L. depending on geographical origin and compared with genetic markers

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Background. *Poa pratensis* is one of the most common species of meadow grass in Europe. Most cultivars of the species found in Poland were originally derived from its ecotypes. We compared the effectiveness of the RAPD and ISSR methods in assessing the genetic diversity of the selected populations of *P. pratensis*. We examined whether these methods could be useful for detecting a possible link between the geographical origin of a given population and its assessed genetic variation. **Methods.** The molecular markers RAPD and ISSR were used and their efficiency compared using, inter alia, statistical multivariate methods (UPGMA and PCA). **Results.** The low value of Dice's coefficient (0.369) along with the significantly high percentage of polymorphic products indicates a substantial degree of genetic diversity among the studied populations. Our results found a correlation between the geographical origin of the studied populations and their genetic variations. For ISSR, which proved to be the more effective method in that respect, we selected primers with the greatest differentiating powers correlating to geographical origin. **Discussion.** The populations evaluated in this study were characterized by a high genetic diversity. This seems to confirm the hypothesis that ecotypes of *P. pratensis* originating from different regions of Central Europe with different terrain structures and habitat conditions can be a source of great genetic variability.

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15 ## Abstract

16 **Background.** *Poa pratensis* is one of the most common species of meadow grass in Europe.

17 Most cultivars of the species found in Poland were originally derived from its ecotypes. We

18 compared the effectiveness of the RAPD and ISSR methods in assessing the genetic diversity of

19 the selected populations of *P. pratensis*. We examined whether these methods could be useful for

20 detecting a possible link between the geographical origin of a given population and its assessed

21 genetic variation.

22 **Methods.** The molecular markers RAPD and ISSR were used and their efficiency compared

23 using, inter alia, statistical multivariate methods (UPGMA and PCA).

24 **Results**

25 The low value of Dice's coefficient (0.369) along with the significantly high percentage of

26 polymorphic products indicates a substantial degree of genetic diversity among the studied

27 populations. Our results found a correlation between the geographical origin of the studied

28 populations and their genetic variations. For ISSR, which proved to be the more effective method

29 in that respect, we selected primers with the greatest differentiating powers correlating to

30 geographical origin.

31 **Discussion**

32   The populations evaluated in this study were characterized by a high genetic diversity. This
33   seems to confirm the hypothesis that ecotypes of *P. pratensis* originating from different regions
34   of Central Europe with different terrain structures and habitat conditions can be a source of great
35   genetic variability.

Introduction

37   *Poa pratensis* L. is one of the most common species of meadow grass in Europe. It occurs
38   throughout the country and is highly valued as a grass cultivar that can be used for various
39   purposes (Mirek & Pięknoś-Mirkowa, 2007; Szenejko, 2014). Due to its high plasticity, high
40   spreading capability and significant expansiveness, *P. pratensis* is considered a cosmopolitan
41   species capable of occupying a wide range of different habitats. Its wide range of distribution and
42   the ease with which the species adapts to very different conditions has resulted in a great
43   diversity of ecotypes thriving in a miscellany of habitats. In the case of *P. pratensis,* the selection
44   of particular ecotypes became the basis for breeding work . In Poland, most cultivars of the
45   species were derived from natural grasslands, often from individual plants. Because apomixis is
46   the dominant manner of reproduction in *P. pratensis,* achieving high genetic variability within
47   individual populations is rather difficult (Müntzing, 1933; Muller, 1964; Felsenstein, 1974;
48   Pamilo et al., 1987; Huff & Bara, 1993). In addition to vegetative and sexual reproduction
49   processes, the plant can also reproduce asexually from seeds, which are formed through
50   apospory or diploid parthenogenesis and without any fertilization process (Mazzucato et al.,
51   1996; Albertini et al., 2001; Spillane et al., 2001; Albertini et al., 2005; Matzk et al., 2005). As a
52   result, offspring and parent plants may be genetically identical (Albertini et al., 2004; Carneiro et
53   al., 2006). This implies that the initial forms i.e. the different ecotypes and cultivars used for
54   breeding did not produce new combinations of genes. As a result, cultivars derived from such
55   initial material have similar or related genotypes. The results of our earlier research, which
56   concerned the evaluation of genetic variability in cultivars and ecotypes of *P. pratensis* native to
57   Central Europe using RAPD markers, confirmed the considerable genetic relatedness of those
58   cultivars and ecotypes (Szenejko et al., 2009; Szenejko & Rogalski, 2015). The populations
59   evaluated in those studies originated from Lower Silesia and Podlasie, regions of Poland with
60   different habitats and climatic conditions. The ecotypes analyzed in those previous studies
61   differed with respect to their phenotypic traits but showed little genetic variability, whereas the
62   populations in the present study were selected from habitats as diverse as possible, i.e. lowlands,

63   uplands and mountains. The ecotypes in this study originated from the western and southern

64   regions of Poland as well as from the Kujawy and Podlasie regions. It was assumed that long

65   distances between regions would affect habitat conditions, and that natural spatial barriers would

66   cause the greatest genotypic diversity among the research material. In addition, three cultivars

67   (including two of the oldest Polish cultivars of *P. pratensis*) were included in our research and

68   subjected to the same analyses. All the plant material was evaluated for genetic diversity using

69   two systems of markers based on DNA amplification (RAPD and ISSR). In the present study, the

70   effectiveness of the methods in assessing the genetic diversity of the selected forms of *P.*

71   *pratensis* was compared. Furthermore, we investigated whether these methods could be useful in

72   establishing a link between the geographical origin of a given population and their assessed

73   genetic variability. Primers with the greatest differentiating powers correlating with geographical

74   distance were selected for ISSR, the more effective method in that respect. Principal Component

75   Analysis (PCA) was used for this procedure, which was performed on the chosen values of DNA

76   amplification products obtained in the presence of those selected ISSR markers with the highest

77   genetic differentiating power with respect to the studied forms of *P. pratensis.*

Materials & Methods

79   **Plant material**

80   This assessment of the genetic diversity of 18 selected forms of *P. pratensis* was carried out in

81   2014. The study involved 3 cultivars and 15 ecotypes selected from locations as distant as

82   possible from each other. The seeds used in the study came from the national collection of the

83   Botanical Garden of the Institute of Plant Breeding and Acclimatization in Bydgoszcz (IHAR);

84   the caryopses used for establishing this collection were collected during field trips organized by

85   the staff of the Botanical Garden and originated from different geographical regions of Poland,

86   including 8 different voivodships (provinces) (Fig. 1, Table 1). The main criteria when

87   harvesting the caryopses were the diversity of plant material and variety of habitats. The seed

88   material was collected from wastelands and arable land (meadows and pastures), as well as from

89   environmentally valuable areas. The assessment of genetic diversity was performed for three

90   cultivars used for different purposes, including 'Eska 46', one of the oldest Polish fodder cultivar

91   and two lawn cultivars: 'Limousine', an apomictic uniclonal German cultivar, and 'Alicia', the

92   oldest Polish lawn cultivar.

93   **DNA isolation**

94   Genomic DNA was isolated from the plant tissue using magnetic beads (NOVABEADS Plant
95   DNA STANDARD Purification KIT), following the procedure for monocotyledons and the
96   manufacturer's instructions (Novazym, Poland). For each form of *P. pratensis* 100-150 mg of
97   material was taken from 80 random etiolated seedlings. The DNA was isolated in two
98   repetitions.

99   **RAPD method**

100   RAPD analysis, based on random amplification of polymorphic DNA, was performed according
101   to the modified method described by Williams et al. (1990). Sixty-nine primers were tested and 7
102   were chosen to evaluate RAPD polymorphism (Table 2). The amplification reactions were
103   performed in a T100™ Thermal Cycler (Bio-Rad Polska). The thermal profile proposed by
104   Rajasekar et al. (2005) was used: initial denaturation for 5 minutes at 95°C, then 94 °C – 1
105   minute, 37 °C – 90 seconds, and 72 °C – 1 minute in 45 cycles, with a final elongation for 7
106   minutes. The reactions were carried out on a total volume of 25 μl containing the following
107   ingredients: 1xbufor for PCR reaction with Mg^{2+} (Novazym, Poland), 1.5 mM $MgCl_2$, 0.2 mM
108   dNTPs, 1.0 μM primer, 1.25 U RedAllegro *Taq* Polymerase (Novazym, Poland) and 125 ng of
109   DNA.

110   **ISSR method**

111   ISSR analysis was performed following the modified method described by *Ziętkiewicz et al*.
112   (1994) and the experimental conditions optimized by Wang (2010). Having pretested 66
113   microsatellite primers, 13 of these (Oligo IBB PAN, Poland) were chosen to evaluate ISSR
114   polymorphism (Table 2). The reaction mixture, 25 μl in volume, contained: 1xbufor for the PCR
115   reaction with Mg^{2+} (Novazym, Poland), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.4 μM of primer 1 U
116   RedAllegro *Taq* Polymerase (Novazym, Poland) and 50 ng of DNA.

117   The amplification reactions were carried out in a T100™ Thermal Cycler (Bio-Rad Polska) for
118   40 cycles. The ISSR PCR reaction was as follows: initial denaturation at 94 °C for 4 minutes,
119   then 94 °C – 30 seconds, 52 °C – 45 seconds, 72 °C – 2 minutes, with a final elongation carried
120   out at 72˚C for 7 minutes.

121   **Data analysis**

122   The resulting RAPD and ISSR amplification products were separated on 2.0% agarose gel with
123   ethidium bromide (5 μg/ml; Sigma-Aldrich) in a TAE buffer for 4-5 hours. Visualization,
124   documentation and analysis of the results were carried out using Gel Doc™ XR+ and Quantity

125 One 4.6.5 software manufactured by Bio-Rad. The Polymorphism Information Content (PIC)

126 index for dominant primer systems was calculated for both RAPD and ISSR (Ghislain et al.,

127 1999). The Assay Efficiency Index (AEI) value was also calculated (Pejic et al., 1998). The

128 Genetic Similarity Index (Si) of the studied ecotypes and cultivars of *P. pratensis* was

129 determined in accordance with Dice's coefficient (Dice, 1945) following Nei and Li (1979). The

130 correlations between them were determined on the basis of genetic similarity matrices obtained

131 in the presence of the RAPD and ISSR markers by calculating Spearman's rank correlation

132 coefficient (r; $p < 0.05$); then the determined genetic similarity matrix was used to construct

133 UPGMA dendrograms in FreeTree and TreeView 1.6.6 software (Pavlíček et al., 1999; Hampl et

134 al., 2001). To analyze the quality of information from particular RAPD and ISSR primers,

135 Principal Component Analysis (PCA) was used. The information on the size of the selected

136 amplification products obtained in the presence of each primer was used as the initial data; then

137 the PCA assessment was carried out by reducing three-dimensional space to two dimensions.

138 The results of multiple analyses were compared to the PCA results. The PCA results obtained

139 using the initial data, in the form of information on the geographical location of the place of

140 origin of particular ecotypes, was used as the target matrix. These analyses were carried out

141 using the STATISTICA 10.0 PL statistical package.

142 **Results**

143 **RAPD analysis**

144 In the course of RAPD analysis, in total 246 amplification products were obtained for which

145 100% polymorphism was demonstrated (Table 3). The primers chosen to evaluate polymorphism

146 generated bands in a wide size range: from 149 bp to 2367 bp in the presence of primer C16. The

147 greatest number of amplicons (43) was obtained as the result of reactions with primers G19 and

148 M14 (Fig. 2). The primers used for the analysis initiated the synthesis of 79 specific

149 amplification products which differentiated the studied forms of *P. pratensis*. Of these, the

150 largest number of DNA fragments (16) which had a unique composition were obtained in

151 reaction with the M14 primer (Fig. 2). As many as 68 specific products were found for the

152 ecotypes, with the largest number of them in a population from Silesia (bes03 373). In the

153 presence of 6 primers, we were able to generate 15 products with a unique composition, ranging

154 in size from 149 bp (for C16) to 1478 bp (for M14). Unique DNA fragments of approximately

155 the same size were identified in amplification reactions with different primers. A significant

156 number of specific bands were also identified for two ecotypes, one from Podkarpacie (bie04

157 189) and the other originating from the borderland region of Lubuskie and Lower Silesian

158 voivodships (provinces) (lbs05 628) 11 and 10 bands respectively.

159 **ISSR analysis**

160 As the result of amplification reactions involving 13 microsatellite primers, a total of 514

161 products ranging in size from 303 bp (for 811) up to 3105 bp (for 808) were obtained (Table 3

162 and Fig. 3). In reactions with primers N01 and 840 a large number of bands were generated (51

163 and 50 respectively), whereas in the presence of primer 807 only 23 products (the smallest

164 number) were obtained. A high level of polymorphism was observed in the amplified DNA

165 fragments and in the presence of 8 primers only polymorphic products were obtained. We

166 identified a total of 124 specific amplification products in the ecotypes and cultivars of *P.*

167 *pratensis* analyzed in this study. The largest number (17) was obtained in reaction with primer

168 808, while in the presence of primer 807 only one product with a unique composition (649 bp)

169 for ecotype lbs05 170 was obtained (Fig. 3). In total, 95 such products were identified for the

170 ecotypes. The largest numbers were recorded for populations lbs05 170 (15 bands) and lbs05 628

171 (13 bands), and for ecotype pom06 175. Specific products of similar molecular size were

172 identified in the presence of different primers. A significant number of unique bands were also

173 identified for the lawn cultivars, including the German 'Limousine' (11).

174 **Evaluation of RAPD and ISSR polymorphisms**

175 Using RAPD and ISSR markers, a significant number of specific amplification products were

176 obtained. 98.6% of the bands obtained using ISSR were polymorphic, whereas those obtained in

177 RAPD reactions were 100% polymorphic (Table 3). A large number of products were obtained

178 using both methods in the presence of single primers (on average 30 products for each method)

179 (Table 4). For both RAPD and ISSR methods the polymorphism information content (PIC) was

180 calculated; the average PIC value for each method was almost identical and was 0.264 for RAPD

181 and 0.270 for ISSR. The value of the assay efficiency index, which indicates the average number

182 of polymorphic products identified in the presence of a single primer, was also calculated for

183 both RAPD and ISSR marker systems. The obtained AEI value was higher for ISSR markers.

184 Specific DNA fragments were obtained for almost all the studied populations (Tables 3 and 4);

185 RAPD primers were more effective in identifying them. In a reaction with a single RAPD

186 primer, we were able to identify as many as 11 unique bands. Dice's genetic similarity matrix

187 was determined for all the studied forms of *P. pratensis*. Independently of each other, the RAPD

188 and ISSR methods revealed little genetic relationship between the studied populations and

189 cultivars (Table 4). On average Dice's coefficient for RAPD was 0.371, while it was 0.367 for

190 ISSR markers. The greatest genetic relationship was demonstrated between two populations from

191 Podlaskie voivodship, pod02 215 and pod02 317 (0.733 for RAPD, 0.650 for ISSR and 0.678 for

192 RAPD+ISSR). The lowest values of Dice's coefficient for RAPD (0.154) were obtained for

193 populations lbs05 170 and bie04 189, while in the cases of ISSR and RAPD+ISSR the smallest

194 degree of genetic relatedness was found between ecotype lbs05 628 and the cultivar 'Alicia'. The

195 following ecotypes were found to be most genetically distant: bie04 189, lbs05 628, and lbs05

196 170. To highlight the compatibility of the results of the RAPD and ISSR methods, the correlation

197 between them was calculated. Using Spearman's coefficient (0.461), the statistical correlation

198 between the genetic similarity matrices obtained on the basis of electrophoretic images for

199 RAPD and ISSR was calculated (Fig. 4).

200 **Cluster analysis**

201 The applied measure of genetic similarity was used to construct UPGMA dendrograms (Fig. 5).

202 There was greater compatibility with respect to the clustered forms for the ISSR and

203 RAPD+ISSR dendrograms. Regardless of which method was used, two asymmetrical and

204 distinct major clusters were clearly distinguishable in each dendrogram. The majority of the

205 studied forms were assigned to the second cluster, within which two subclusters of similarity

206 were distinguishable 11 forms for RAPD and RAPD+ISSR and 12 forms for ISSR. In the ISSR

207 dendrogram, the first main cluster included two ecotypes from Kujawsko-Pomorskie (pom06

208 175) and from West Pomerania voivodships (zap00 240). The combined RAPD+ISSR analysis

209 also included ecotype dos01 405 from Lower Silesia. In the case of RAPD, the first main cluster

210 contained two other ecotypes (bie04 189 and ora03 178), while pom06 175 and zap00 240 were

211 assigned to the second main cluster and to both common subclusters (Fig. 5). Two ecotypes from

212 Lubuskie voivodship (lbs05 170 and lbs05 628) turned out to be genetically most distant. These

213 were not assigned to any pair or subcluster; this is visible in all the dendrograms (Fig. 5). These

214 populations also differed among themselves; this was confirmed by the value of Dice's similarity

215 index (0.224 for RAPD+ISSR). In the ISSR and RAPD+ISSR dendrograms, other genetically

216 different forms included an ecotype from Podkarpacie region (bie04 189), while in the RAPD

217 dendrogram, a form from Silesia (bes03 373) was genetically different. Among the cultivars,

218 'Eska 46' was the most distinctly different: in the RAPD and ISSR dendrograms, it was
219 connected loosely with the second cluster, while the image obtained for RAPD+ISSR depicted it
220 as an unpaired form. In each of the dendrograms the ecotypes from Podlasie and the lawn
221 cultivars 'Alicia' and 'Limousine' formed a common subcluster of forms. In the RAPD
222 dendrogram, the Silesian population bes03 354 joined them, whereas in the ISSR and
223 RAPD+ISSR dendrograms this form was included in the second subcluster together with other
224 ecotypes from Silesia and populations from Lesser Poland and Lower Silesia. In the ISSR
225 diagram, dos01 405 was placed with ecotypes from Lower Silesia, while in the RAPD diagram it
226 was only loosely connected with the second cluster and in the RAPD+ISSR diagram it was
227 included in a different cluster.

228 **Principal Component Analysis**

229 We examined the position of the ecotypes of *P. pratensis* in two-dimensional space using
230 principal component analysis (PCA), which was carried out on the geographical coordinates of
231 the habitats from which the surveyed populations had come. Figure 6A depicts this analysis,
232 which was used as a reference template for the selection of primers with the highest
233 differentiating power.   PCA was performed independently for RAPD and ISSR, using the
234 amplification products generated in the presence of individual primers. For each of the methods
235 the four starters were chosen which most clearly and coherently revealed the relationship
236 between the geographic location of the assessed ecotypes and their genetic variability.
237 Subsequently all possible combinations of the selected primers were analyzed for their main
238 components; this was done separately for RAPD, ISSR and (RAPD + ISSR). A total of 60 PCAs
239 were performed for the RAPD and ISSR primers, both for their combinations within one method
240 and also for both methods combined. For the final PCA, DNA amplification products were
241 chosen which had been obtained from the studied ecotypes and cultivars of *P. pratensis* in the
242 presence of ISSR microsatellites 807, 808, 834 and 840; these were the dinucleotide repeat
243 sequences $(AG)_n$ $(GA)_n$, anchored at the end of 3'. The studied populations were distributed on
244 both sides of the axis of coordinates and distinctly away from its center. They formed five
245 separate groups, which indicates their considerable diversity. Three populations from Podlasie
246 (13, 14, 15), and ecotypes from West Pomerania (2) and Kujawy regions (1) together with the
247 lawn cultivar 'Limousine' (17), formed two clearly distinguishable groups (Fig. 6). These were
248 distributed on the same side of the first axis (horizontal) and clearly differed from the remaining

249   forms of *P. pratensis,* which originated from regions in southern and western Poland. Another

250   group, which was located on the opposite side of the second axis, contained two ecotypes

251   (dos01 468 and lbs05 628 (4, 7)) which came from the borderlands of the Lower Silesian and

252   Lubuskie voivodships respectively. Populations from Silesia (10) and Lesser Poland (11) were

253   also contained in this group. On the same side of the horizontal axis we find another group

254   containing the fodder cultivar 'Eska 46' (18) and three ecotypes (3, 5, 9) from the Lubuskie,

255   Lower Silesian and Silesian voivodships respectively. In the course of the principal component

256   analysis, two forms of *P. pratensis* were identified (12 and 18 ('Alicia')), which were not

257   included in any of the groups. As with the UPGMA dendrograms, the population from

258   Podkarpacie (bie04 189) differed distinctly from the other ecotypes and constituted a distinct

259   genotype (Fig. 5 and 6).

260   **Discussion**

261   DNA markers have become a useful tool for breeding work and for evaluating the genetic

262   diversity of many different plant species, including the family *Poaceae*. Molecular analysis, in

263   conjunction with the characteristics of morphological traits, may be used to classify plants and

264   select the initial forms for creating new cultivars or breeding lines. In the case of *P. pratensis*,

265   this was confirmed by Shortell et al. (2009), Tamkoc & Arslan (2010), Raggi et al. (2015) and

266   Yuan et al. (2015). In this study two markers (RAPD and ISSR) based on DNA amplification

267   reactions were used to assess the genetic diversity of ecotypes and cultivars of *P. pratensis*.

268   These methods do not require any prior information on the analyzed DNA sequence. They do not

269   take a lot of time and are also relatively simple and therefore quite universal. Furthermore they

270   require only a small amount of DNA; this is why they are often employed in the genetic

271   evaluation of different plant species. They can be used either in combination (Nkongolo et al.,

272   2005; Alam et al., 2009; de Lima et al., 2011; Singh et al., 2011; Debajit et al., 2015) or

273   independently of each other (Al-Humaid & Motawei, 2004; Wang, 2010; Guo et al., 2009;

274   Bednarskaya et al. 2014). In grasses, RAPD and ISSR markers are most commonly used to

275   evaluate genetic variability within and between populations, and for molecular characterization

276   and identification of different species and their hybrids, cultivars and genotypes (Posselt et al.,

277   2006; Pivorienė et al., 2008; Al-Humaid et al., 2011; Motawei & Al-Ghumaiz, 2012; Madesis et

278   al., 2014; Yuan et al., 2014). In *P. pratensis* genetic analyses employing RAPD and ISSR focus

279   mainly on the identification of sexual and apomictic genotypes (Huff & Bara, 1993; Mazzucato

280   et al., 1995; Barcaccia et al., 1997, 1998; Stephens et al., 2006); the assessment of genetic

281   variability and identification of genotypes and cultivars (Lickfeldt et al., 2002; Ning et al., 2005;

282   Liang et al., 2009; Fard et al., 2012; Wang et al., 2012; Yuan et al., 2015); determining the

283   genetic relationships between different genotypes and species belonging to the genus *Poa* and

284   their hybrids (Johnson et al., 2002; Curley & Jung, 2004; Patterson et al., 2005; Goldman, 2008;

285   Goldman, 2013); and identification of pathogens responsible for fungal diseases in grasses

286   (Hsiang et al., 2000). Both marker systems can generate high resolution band patterns and high

287   levels of polymorphism as high as 90%. The presence of these markers resulted in a significant

288   number of amplification products in our study: 246 for RAPD and 514 for ISSR. However, ISSR

289   gives better reproducibility of results, which can be explained by its use of longer primers

290   (microsatellites) and higher annealing temperatures (Gostimsky et al., 2005; Reddy et al., 2000;

291   Fernandez et al., 2002; Vaillancourt et al., 2008). Similarly, in previous studies concerning the

292   assessment of the morphological and genetic variability of domestic ecotypes of *P. pratensis*

293   originating from the areas of Lower Silesia and Podlasie (Szenejko & Rogalski, 2015), RAPD

294   primers F05, G19 and M14 proved to be most useful in the evaluation of polymorphisms. The

295   highest number of DNA fragments were obtained in the presence of these primers. In the case

296   of ISSR markers, as in the studies of Bednarskaya et al. (2014), better results were obtained in the

297   presence of primers with dinucleotide repeat sequences $(AG)_2, (GA)_2$ and $(AC)_2,$ anchored at the

298   end of 3'. As suggested by Al-Humaid et al. (2004, 2011), dinucleotide and trinucleotide repeats

299   are abundant in genomes of fodder grasses and thus might constitute potential markers for

300   assessing their genetic diversity and identifying their fungal diseases using the ISSR method. In

301   grasses, $(CAC)_n$, $(CTC)_n$ and $(GA)_n$ and $(CA)_n$ are particularly abundant. In the genus *Poa*, ISSR

302   primers with dinucleotide $(AC)_n$ $(CA)_n$ $(GA)_n$ and $(AG)_n$ repeats turned out to be particularly

303   useful for the identification and evaluation of genetic relationships between various species,

304   including *P. angustifolia*, *P. trivialis*, *P. arachnifera*, *P. pratensis*, *P. compressa*, *P. ligularis*,

305   and *P. secunda* and their hybrids (Goldman, 2008; Arslan & Tamakoç, 2011). A high level of

306   polymorphism was observed in numerous amplification products generated in the course of our

307   analyses. The nature of ISSR, which targets regions especially rich in microsatellites, may also

308   explain the high level of polymorphism, since those regions are known to accumulate a large

309   number of mutations due to DNA polymerase slippage during replication and unequal crossing-

310   over (Kashi & King, 2006; da Costa et al., 2011). In the case of ISSR markers, this can lead to

311 longer dinucleotide repeat sequences and insertion into at least one of them. In this way new

312 variants of microsatellite sequences of different lengths appear, which enriches the collection of

313 alleles identified as polymorphic in a population. However, according to many researchers, in the

314 course of RAPD and ISSR analyses significant levels of polymorphism can be generated, and for

315 various forms and species belonging to the genus *Poa* this can exceed 90% (Liang et al., 2009;

316 Arslan & Tamkoç, 2011; Fard et al., 2012; Wang et al., 2012; Cichorz et al., 2014). In our

317 analyses, a slightly greater percentage of polymorphic DNA fragments was obtained in reaction

318 with RAPD primers. The data available in the literature indicates that the RAPD method is

319 somewhat superior due to its higher levels of generated polymorphism in the presence of various

320 molecular marker systems which is explained, inter alia, by their polyallelic nature (Alam et al.,

321 2009; Bhattacharya et al., 2010; Naik et al., 2010). A possible explanation for the difference in

322 resolution of RAPD and ISSR is that the two-marker techniques target different portions of the

323 genome (Debajit et al., 2015). Although a greater percentage of polymorphic DNA products was

324 identified in the presence of RAPD primers, it was microsatellites that we found to be more

325 effective in detecting them. This finding was confirmed by calculating the average efficiency

326 index (AEI), which was slightly higher for ISSR markers. The slight differences in the

327 assessment of RAPD and ISSR polymorphisms did not rule out the possibility of establishing a

328 correlation between them. Even though Spearman's coefficient was not very high (0.461), the

329 result was statistically very significant. Hence it can be concluded that the results presented in

330 this study obtained using RAPD and ISSR markers are comparable. Budak *et al*. (2004), in a

331 study examining the phylogenetic relationships between seed and vegetative biotypes of *Buchloe*

332 *dactyloides,* used various systems of molecular markers (ISSR, SSR, RAPD and SRAP), for

333 which they determined correlations. In the case of RAPD and ISSR, the Spearman's coefficient

334 (0.461) calculated by Budak was similar to ours, but their results turned out to be statistically

335 insignificant. Both RAPD and ISSR markers revealed a small genetic similarity among the

336 studied ecotypes and cultivars of *P. pratensis*. The average value of Dice's coefficient for both

337 methods combined (RAPD+ISSR) was low (0.369), which together with the high number of

338 polymorphic products may indicate substantial genetic differences between the studied

339 populations and their low level of relatedness. It can be speculated that natural spatial barriers

340 might have inhibited and minimized the transfer of genes among these populations; this would

341 certainly have contributed to their diversity. At the same time, we can surmise that there were

342   populations among the studied ecotypes in which apomixis was the main mode of reproduction.

343   The ecotypes originating from three voivodships (Podkarpackie, Lower Silesian and Lubuskie

344   (bie04 189, lbs05 628 and lbs05 170)), were determined to be those genetically most distant from

345   the other forms. These populations had the lowest similarity index and included the most

346   uncommon genotypes, for which the largest number of specific amplification products were

347   identified. In comparison to the other cultivars, 'Eska 46' was clearly different in terms of its

348   genetic structure, and, overall, few amplification products were generated for it. It should be

349   stressed that in previous studies aimed at evaluating RAPD polymorphism in different

350   populations of *P. pratensis,* such a high genetic diversity among ecotypes had never been found,

351   while the average Dice's coefficient was about 0.7 (Szenejko & Rogalski, 2015). Other authors

352   also reported significant genetic relatedness among the different genotypes and cultivars of *P.*

353   *pratensis* (Johnson et al., 2002; Ning et al., 2005; Tamkoc & Arslan, 2010; Fard et al., 2012).

354   UPGMA dendrograms based on genetic similarity matrices revealed discrepancies in the mode

355   of clustering of the studied forms of *P. pratensis*. Greater similarity in that respect was

356   demonstrated for the ISSR and RAPD+ISSR methods. This difference in the clustering process

357   between the RAPD and ISSR dendrograms was also demonstrated in research by Bhattacharya et

358   al. (2010), who were assessing the genetic diversity of *Cymbopogon winterianus* and by

359   Souframanien & Gopalakrishna (2004), who were studying genotypes of *Vigna mungo.* Similarly

360   to our results, these authors found greater clustering conformity for ISSR and RAPD+ISSR.

361   Looking at the distribution in individual UPGMA dendrograms, it appears that the ISSR

362   dendrogram depicts the relationship between the geographical origin of the ecotypes and their

363   genetic similarity in the clearest way. With the exception of populations bie04 189, lbs05 628

364   and lbs05 170, which differed from the others genetically, the remaining ecotypes were clustered

365   according to their geographical distribution. In the course of PCA, and depending on their

366   geographical origin, a similar tendency in the distribution of the studied populations in two-

367   dimensional space was observed. It was noted that ecotypes from the same voivodships

368   (provinces) or from regions located not far from one another were depicted similarly in two-

369   dimensional space as in the UPGMA dendrogram constructed for the ISSR process. Those

370   populations from eastern Poland and from West Pomerania and Kujawy clearly differed from the

371   ecotypes originating from the western and southern parts of the country and were located on the

372   opposite side of the horizontal axis of coordinates. They also differed from one another, which

373   can be seen in their arrangement on both sides of the vertical axis. Some discrepancies were
374   found between the UPGMA and PCA clusters of the studied forms, which were related, inter
375   alia, to the distribution of the cultivars. Only a modest relationship between genetic divergence
376   and geographical origin in *P. pratensis* was reported by several authors (Johnson et al., 2002;
377   Fard et al., 2012; Raggi et al., 2015). For comparison, Abbaszade et al. (2013), who were
378   studying the genetic diversity of *Lolium multiflorum*, and Rahmati et al. (2013), who were
379   studying *Festuca arundinacea,* demonstrated a greater result consistency between UPGMA and
380   PCA. However, neither of these research teams was able to demonstrate a correlation between
381   the genetic variability of the grass genotypes they analyzed and their geographical origin.

Conclusions

383   Our results suggest that both the systems of dominant RAPD and ISSR markers that were used in
384   our research can be used to assess the genetic variability of selected ecotypes and cultivars of *P.*
385   *pratensis.* However, given their better reproducibility of results, greater efficiency in detecting
386   polymorphic amplification products in the presence of a single primer and also their
387   differentiating properties (which make them capable of distinguishing studied forms with respect
388   to their genetic structure), ISSR markers seem to be a more useful and reliable tool for this kind
389   of analysis. The plant material evaluated in this study was characterized by a high genetic
390   variability, which seems to confirm the hypothesis that ecotypes of *P. pratensis* originating from
391   different parts of Central Europe, each with different terrain structures and habitat conditions,
392   can provide great genetic variability and a large collection of alleles. Microsatellites with
393   dinucleotide repeat sequences $(AG)_n$, $(GA)_n$ anchored at the end of 3' proved particularly useful
394   for assessing the genetic diversity of these ecotypes and determining their main directions of
395   variability. PCA helped to establish a correlation between the geographical origin of the studied
396   ecotypes and their genetic variation.

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399   Biology, University of Szczecin for sharing with us her invaluable advice and knowledge regarding the
400   development and interpretation of RAPD and ISSR results.

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611

612   **BIOSKETCH**

613   All the authors work at the Institute for Research on Biodiversity of the Faculty of Biology. Current
614   projects include the study of the ecology, biogeography and conservation genetics of different species of
615   plants and animals in Poland and beyond.

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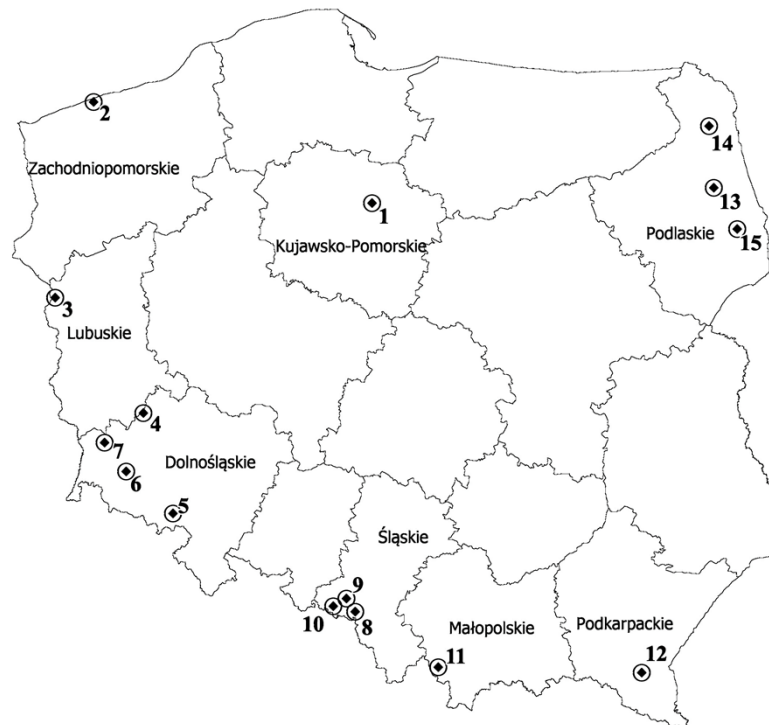
Figure 1:

 Map depicting the locations of the 15 populations of *P. pratensis* used in the study.
1 – pom06 175; 2 – zap00 240; 3 – lbs05 170; 4 – lbs05 628; 5 – dos01 333; 6 – dos01 405; 7 – dos01 468; 8 – bes03 373; 9 – bes03 379; 10 – bes03 35; 11 – ora03 178; 12 – bie04 189; 13 – pod02 215; 14 – pod02 172; 15 – pod02 317

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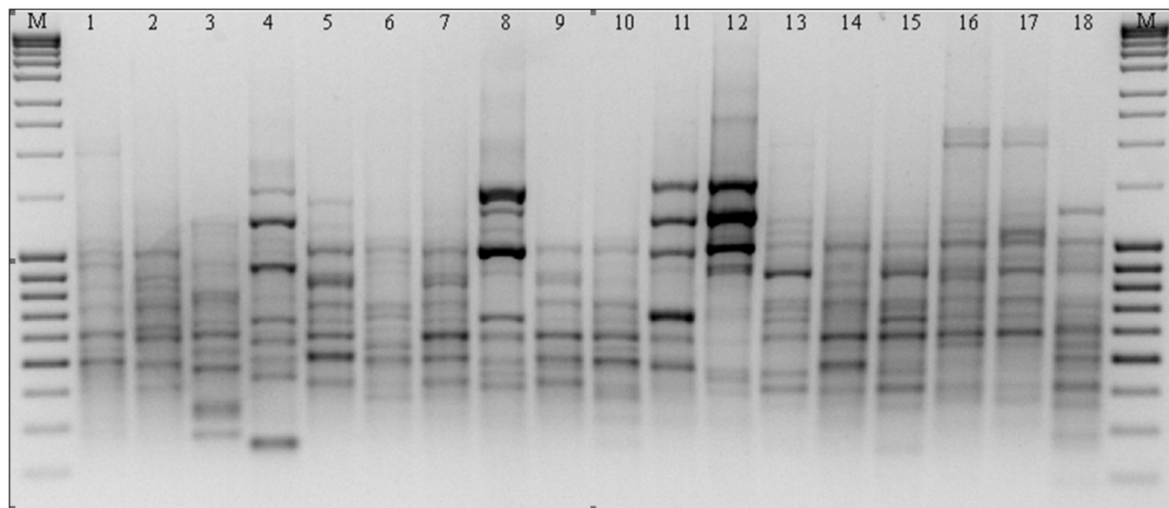
Figure 2:

RAPD patterns amplified with primer M14 for *P. pratensis*.

M – MassRuler™ Ladder Mix, ready-to-use (Thermo Scientific); 1 – pom06 175; 2 – zap00 240; 3 – lbs05 170; 4 – lbs05 628; 5 – dos01 333; 6 – dos01 405; 7 – dos01 468; 8 – bes03 373; 9 – bes03 379; 10 – bes03 354; 11 – ora03 178; 12 – bie04 189; 13 – pod02 215; 14 – pod02 172; 15 – pod02 317; 16 – 'Alicia'; 17 – 'Limousine'; 18 – 'Eska 46'

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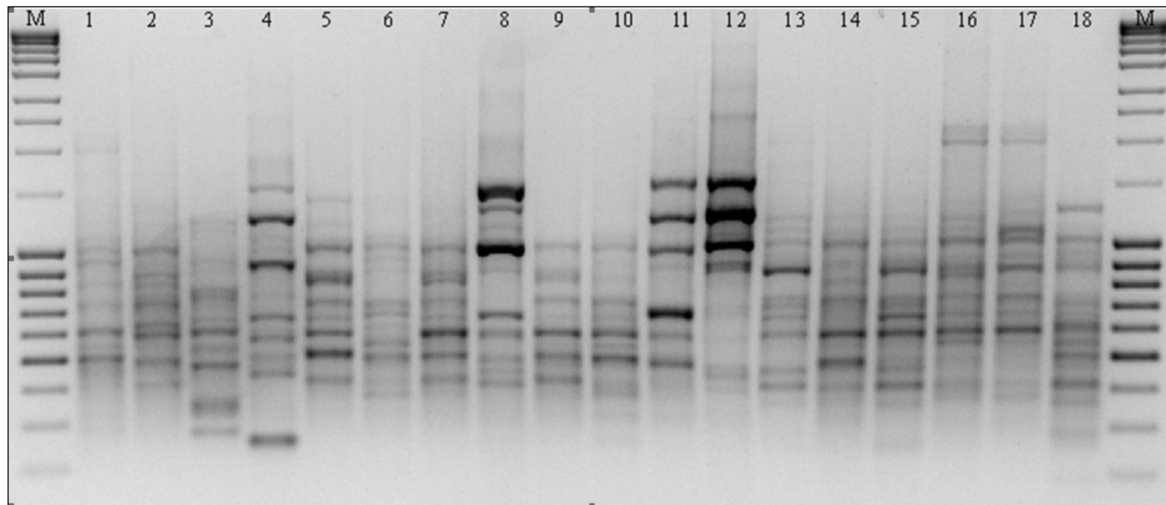
Figure 3:

ISSR patterns amplified with primer 808 for *P. pratensis*.

M – MassRuler™ Ladder Mix, ready-to-use (Thermo Scientific); 1 – pom06 175; 2 – zap00 240; 3 – lbs05 170; 4 – lbs05 628; 5 – dos01 333; 6 – dos01 405; 7 – dos01 468; 8 – bes03 373; 9 – bes03 379; 10 – bes03 354; 11 – ora03 178; 12 – bie04 189; 13 – pod02 215; 14 – pod02 172; 15 – pod02 317; 16 – 'Alicia'; 17 – 'Limousine'; 18 – 'Eska 46'

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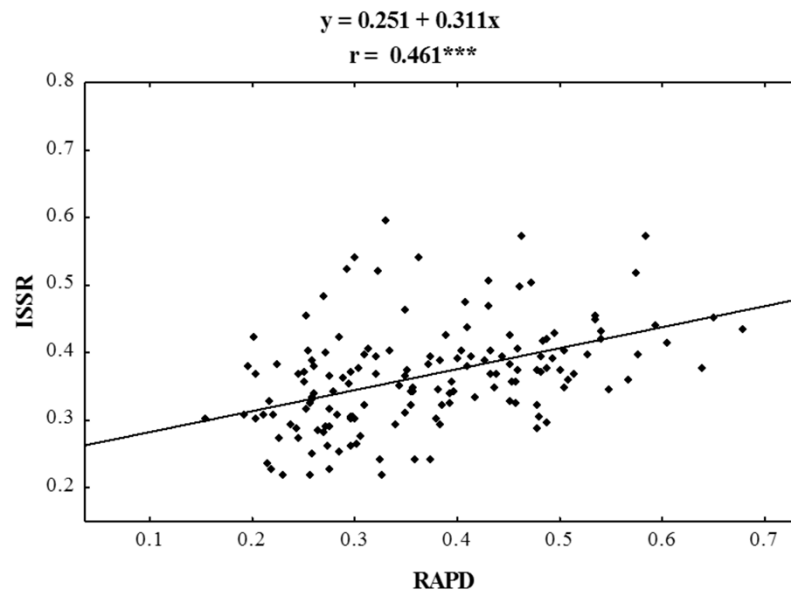
672   **Figure 4:**

673   **Correlation analysis of similarity matrices obtained using RAPD and ISSR markers in**
674   **ecotypes of *P. pratensis.***
675   The symbol *** indicates that the value is significant assuming P=0.001 as the level of
676   significance.

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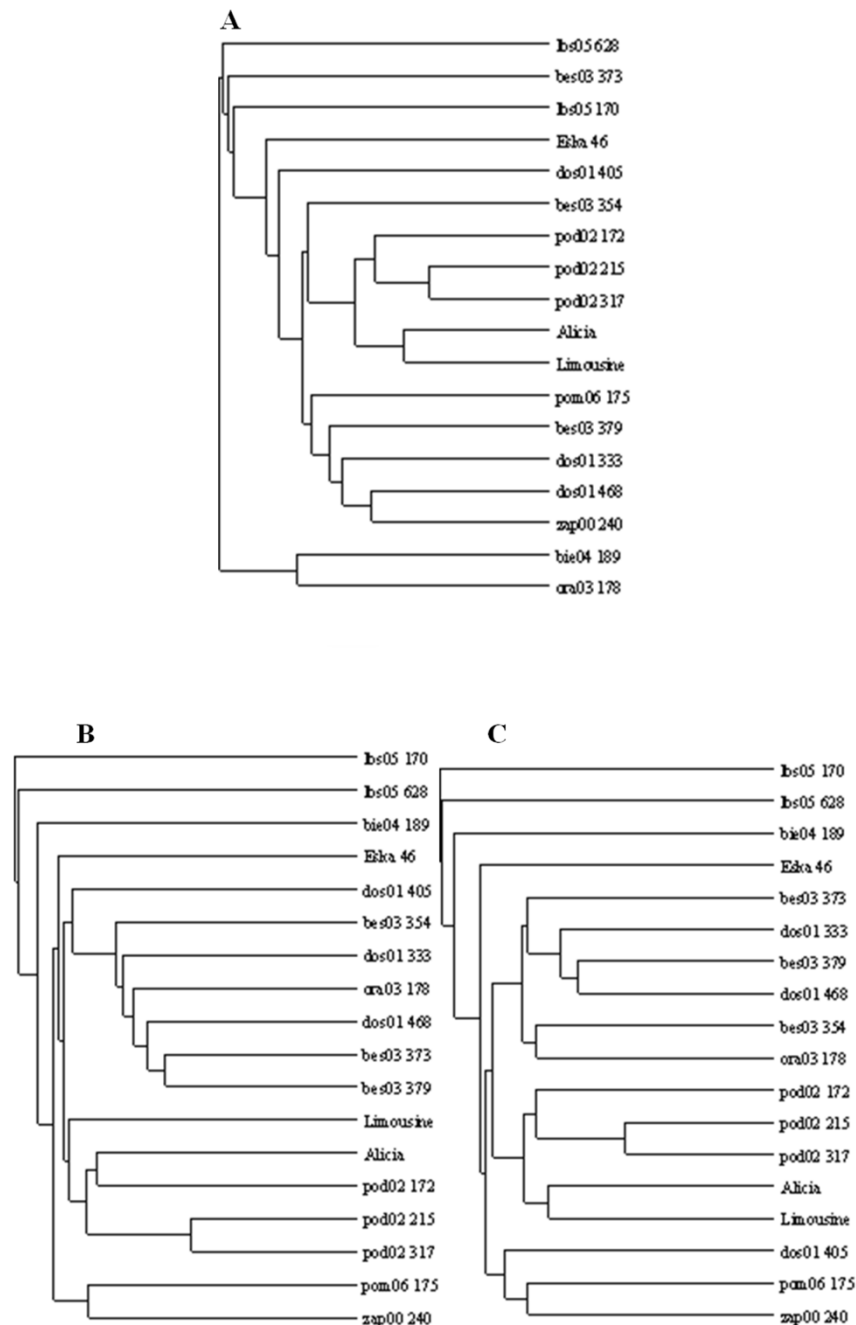
698 **Figure 5:**
699 **UPGMA dendrograms of genetic similarity of the studied ecotypes and cultivars of *P.***
700 ***pratensis* obtained on the basis of polymorphism markers constructed using the TreeView**
701 **1.6.6.**
702 A – RAPD, B – ISSR, C – (RAPD+ISRR)
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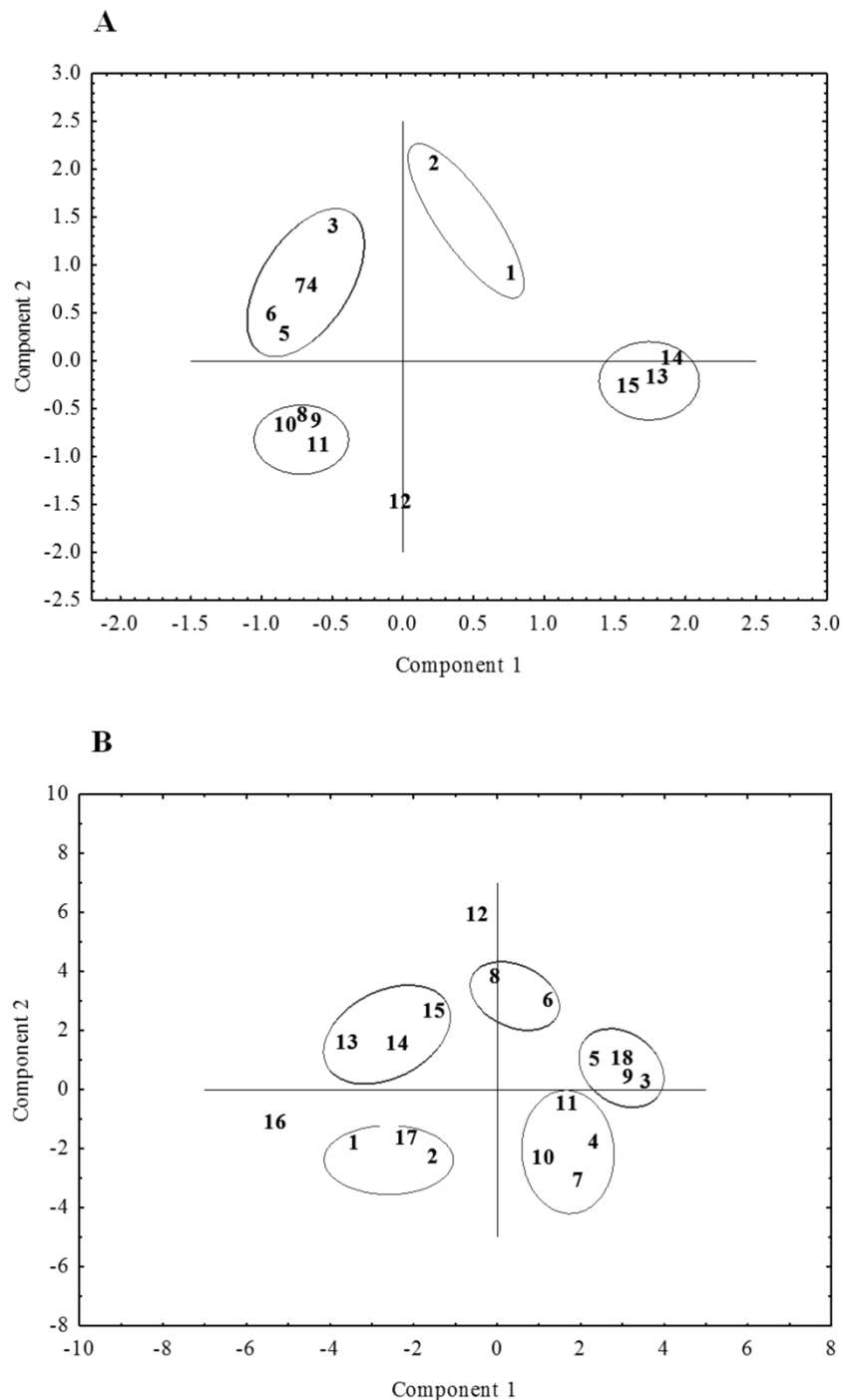
705 **Figure 6: The distribution of the studied ecotypes and cultivars of *P. pratensis* in two-**

706 **dimensional space (PCA).**

707 A – the result for the initial data in the form of geographic coordinates of their habitats.
708 B – the final result of PCA analysis using the size of the amplification products generated in the presence
709 of four ISSR primers: 807, 808, 834, 840, the closest to the geographical location of the selected
710 ecotypes: 1 – pom06 175; 2 – zap00 240; 3 – lbs05 170; 4 – lbs05 628; 5 – dos01 333; 6 – dos01 405; 7 –

711 dos01 468; 8 – bes03 373; 9 – bes03 379; 10 – bes03 354; 11 – ora03 178; 12 – bie04 189; 13 –
712 pod02 215; 14 – pod02 172; 15 – pod02 317; 16 – 'Alicia'; 17 – 'Limousine'; 18 – 'Eska 46'

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714 **Table 1:**
715 **Ecotypes of _P. pratensis_ analyzed in the study and the locations where their caryopses were**
716 **collected.**

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Ecotype	Town/Place	Latitude	Longitude	Habitat
pom06 175	Wielkie Stwolno	53°26'34.1"N	18°39'25.8"E	Vistula River oxbow lake
zap00 240	Łukęcin	54°02'58.4"N	14°52'03.7"E	beach
lbs05 170	Owczary	52°28'16.3"N	14°39'08.0"E	Ostnicowy nature reserve
lbs05 628	Przemków	51°32'50.6"N	15°46'33.4'E	meadow
dos01 333	Rybnica Leśna II	50°42'08.5"N	16°18'00.3"E	pasture
dos01 405	Dziwiszów - Góry Kaczawskie	50°57'39.4"N	15°49'23.6"E	meadow
dos01 468	Oleszna Podgórska	51°21'02.3"N	15°28'12.1"E	meadow
bes03 373	Ustroń - Wielka Czantoria	49°40'45.6"N	18°47'42.7"E	mountain meadow
bes03 379	Ustroń - Polana Goryczkowa	49°41'05.6"N	18°46'58.6"E	meadow pasture
bes03 354	Jaworzynka	49°31'20.2"N	18°51'31.2"E	meadow
ora03 178	Lipnica Wielka	49°27'05.3"N	19°37'40.6"E	meadow
bie04 189	Płonna	49°26'11.7"N	22°07'05.9"E	wasteland
pod02 215	Lipina	53°19'59.6"N	23°25'28.5"E	meadow/pasture
pod02 172	Walne	53°58'20.0"N	23°04'04.6"E	meadow
pod02 317	Pieńki	53°03'32.3"N	23°38'47.2"E	pasture

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738 **Table 2:**
739 **The sequences of primers used in the study.**
740 Random amplification of polymorphic DNA was held in the presence of 7 10-nucleotide primers
741 manufactured by Oligo IBB PAN, Poland. 13 microsatellite primers were used for the ISSR-PCR
742 reaction. Most of them were dinucleotides composed of 17 and 18 bp.
743

Primer RAPD	Sequence 5' - 3'	Primer ISSR	Sequence 5' - 3'
B10	CTGCTGGGAC	807	$(AG)_8T$
B17	AGGGAACGAG	808	$(AG)_8C$
C16	CACACTCCAG	810	$(GA)_8T$
F05	CCGAATTCCC	811	$(GA)_8C$
G19	GTCAGGGCAA	834	$(AG)_8YT$
M14	AGGGTCGTTC	840	$(GA)_8YT$
P08	ACATCGCCCA	841	$(GA)_8YC$
		857	$(AC)_8YG$
		888	$BDB(CA)_7$
		R07	$(AC)_8YT$
		M09	$(AC)_8YG$
		N01	$CA(GT)_8$
		TGA	$(TGA)_6A$

760 **Notes:**

761 **Y**: T or C; **B**: C, G or T; **D**: A, G or T

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779 **Table 3:**
780 **RAPD and ISSR primers used, total number of scored bands (NSB), number of**
781 **polymorphic bands (NPB), their unique bands (NUB) and percentage of polymorphism**
782 **bands (PPB) for each primer.**

Name of primer	Size range (bp)	NSB	NPB	NUB	PPB
RAPD					
B10	213-1867	35	35	11	100
B17	209-1483	32	32	7	100
C16	149-2367	33	33	15	100
F05	316-2366	34	34	11	100
G19	201-1902	43	43	9	100
M14	226-2243	43	43	16	100
P08	179-1173	26	26	10	100
Total	149-2367	246	246	79	-
ISSR					
807	341-1974	23	23	1	100
808	325-3105	43	43	17	100
810	338-2523	35	35	8	100
811	303-2748	40	40	10	100
834	404-2686	35	34	7	97
840	373-2967	50	50	8	100
841	373-2504	37	36	7	97
857	380-2577	46	45	7	98
888	501-2414	26	24	5	92
R07	576-2327	37	37	13	100
M09	353-2096	47	47	9	100

N01	404-2256	51	51	15	100
TGA	362-2209	44	43	17	98
Total	303-3105	514	508	124	-

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797 **Table 4:**
798 **Evaluation of RAPD and ISSR polymorphisms.**
799 The average Number of Scored Bands (NSB) including Number of Unique Bands (NUB)
800 obtained in the presence of a single primer, the value of Polymorphism Information Content
801 (PIC), Assay Efficiency Index (AEI), and Genetic Similarity (Si).
802

Parameter/Index	RAPD	ISSR	RAPD+ISSR
NSB	35.1	38.0	36.8
NUB	11.3	7.7	9.3
AEI	35.1	37.3	36.4
PIC	0.264	0.270	0.268
Si	0.371	0.367	0.369
(range)	(0.154-0.733)	(0.218-0.650)	(0.222-0.678)

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