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Investigation of Genetic Structure in Mesomorphic Bluegrasses, Poa Section Stenopoa Dum, by Using ISSR Markers

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Abstract. A morphological and genetic analysis of five mesomorphic bluegrasses populations, Poa section Stenopoa, from a range of geographical locations were performed. We have established that all the populations have different level of the morphological and genetic diversity. A total of 79 DNA bands were obtained from six ISSR primers, including 61 polymorphic bands. Molecular data have shown no clear difference between morphologically homogeneous populations and hybrid populations *P. palustris* and *P. nemoralis*. This result may be due to the high genetic diversity of the populations studied.

INTRODUCTION

Stenopoa Dum. is one of the most important sections in the genus *Poa*. Different models of specification are implemented within this section. It can serve as a good model for the study of evolutionary processes. Due to its ecological plasticity, species of this section mastered a wide variety of habitats, playing an important role in the formation of phytocenoses. One of the most tricky and intricate complexes of this section are hybrids between *Poa palustris* and *Poa nemoralis*. N.N. Tsvelev [1] suggested that these species are the most ancient, and they are the ancestors of the other species of the section. The main distinguishing features of these species are following: the structure of the surface of the spikelet axis and the length of the ligule. *P. palustris* has a bare shaft and a long, tapering upwards, more than 2 mm ligule. It prefers wet and open space not like *P. nemoralis*, which has pubescent axis of the spikelet and the blunt ligule which is less than 1 mm in length. However, during Pleistocene migration these species were involved into hybridization processes [1], which lead to a mixing of these features. In Siberia region and North-East Europe *P. palustris* and *P. nemoralis* are appear as numerous of distinct, in varying degree, races and morphotypes of unclear taxonomic status. The study of P. Asherson [2] for Central Europe reported about seven varieties of *P. palustris* and more than 10 for *P. nemoralis*. Furthermore, G. Hegi [3] reported about 12 varieties and forms for these species. In Flora of Western Siberia P. Krylov allocated four varieties for *P. palustris* and six for *P. nemoralis* [4].

Using of molecular genetic data to determine the evolutionary relationships of taxa becomes more popular among modern taxonomists. However, the correct conducting of this kind of research is not possible without a good knowledge of the study group, its species composition, variability, distribution and ecological preferences [5].

One of the most common method is the ISSR (Inter Simple Sequence Repeats). Along with RAPD (Random Amplified Polymorphic DNA) [6], SSR (Simple Sequence Repeats) [7] and AFLP (Amplified Fragment Length Polymorphism) [8], ISSR allows analyzing genome polymorphism. The markers based on intermicrosatellite sequences have several advantages – easy handling, inexpensiveness and small amount of starting material required for genetic amplification as well as higher reproducibility and specificity in comparison with other methods [9–12]. It was established that all kinds of microsatellites (from mono to hexanucleotide repeats) are abundant in the non-coding regions of plants, animals and other eukaryotic organisms [13–15]. This explains the widespread use of ISSR technique for genome mapping, studying of the genetic structure of populations, plant

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passporting, as well as in phylogenetic analysis [16, 17]. ISSR primers consist of short tandem repeats of two to four base pair motifs, the total length of 15-24 nucleotides and one selective nucleotide at the 3' end [18].

In this article, we attempt to use the ISSR method to identify possible genetic polymorphism and differences between populations of *P. palustris* and *P. nemoralis*.

MATERIALS AND METHODS

We used herbarium collections from LE, MW, NS, NSK, SSBG and TK. In addition, we included our specimens from different areas of Siberia and Ural. Considering that virtually impossible to use only genetic methods for a large number of populations, we selected five populations *P. palustris* and *P. nemoralis* from different geographical ranges. Probably, some of our samples may be hybrids, because they have characteristics from both species (Table 1). The study was based on classical comparative morphology and the ISSR molecular method.

TABLE 1. The study populations

№	Species	Occurrence and habitat	Samples size for the morphological analysis	Samples size for the genetic analysis
1	*Poa palustris* × *Poa nemoralis*	Tomsk Oblast, Shegarskiy district, Ilovskiy reserved forest, grass-gramineous meadow, 28.6.2011	33	12
2	*Poa palustris* × *Poa nemoralis*	Sverdlovsk Oblast, an environs of Yekaterinburg, a pinewood on the periphery of a quarry, 6.7.2011	25	11
3	*Poa palustris*	Primorsky Krai, lozovskiy district, on a river alluvion, 20.08.2011	28	10
4	*Poa nemoralis*	Southeast outskirts of Magadan, an alder underbrush, 26.7.2011	25	11
5	*Poa palustris* × *Poa nemoralis*	Tomsk Oblast, Tomsk district, a floodplain of the Tom river, grass-gramineous meadow, 15.7.2011	27	12

Note: voucher specimens are stored in the TK.

Morphological analysis took into account the following characteristics: V1 – total plant height, V2 – the length from the base to the top node, V3 – the length from the top node to the base of the panicle, V4 – the length of the second top internode, V5 – the length of the top leaf blade, V6 – sheath length of the top leaf, V7 – the width of the upper leaf blade, V8 – the length of the ligule of the top leaf blade, V9 – panicle length, V10 – the width of the panicle, V11 – the number of branches in the lower tier of the panicle, V12 – the length of the largest branches of the panicles, V13 – the number of spikelets on the highest twig panicles, V14 – the number of flowers in a spikelet, V15 – spikelet length, V16 – the length of upper glume, V17 – the width of upper glume, V18 – the length of the lower glume, V19 – the width of the lower glume, V20 – length of lemmas, V21 – the width of the lemmas, V22 – the length of the lower segment of the axis of spikelets, V23 of – the structure of the surface of the spikelet axis, V24 – pubescence between lower glume veins, V25 – pubescence of callus, V25 – the shape of the stem at the bottom, V26 – color of the lower node. Statistical analysis was performed using the software package STATISTICA 10 [19] by principal component analysis (PCA) and factor analysis. Quantitative and qualitative characteristics were analyzed separately. The V8 feature was analyzed as both, quantitative and qualitative, taking encoding from 1 to 3 (samples with the ligula length less than or equal to 1 mm were coded as 1, samples with a length of the ligula 1 to 2 mm were coded as 2, and the specimens with the ligula length more than 2 mm were coded as 3).

The ISSR assay was performed to estimate genetic polymorphism. Optimization of a PCR protocol for the ISSR was made in accordance with recommendations [20]. Genomic DNA was isolated from dry samples according to the protocol of a commercial kit DiamondDNA Plant Kit D (DiamondDNA, Russia). Isolated DNA was dissolved in 50 μl of the TE buffer (DiamondDNA, Russia). The concentration and quality of isolated DNA was estimated using the spectrophotometric ratio of the light absorbance at wavelengths of 230, 260 and 280 nm using the spectrophotometer P330 (Implen, Germany).

A total of six primers (Medigen, Russia) were used for ISSR amplification. They are 17898A ($(CA)_6AC$), 17898B ($(CA)_6GT$), 17899B ($(CA)_6GG$), M2 ($(AC)_8YG$), UBC836 ($(AG)_8YA$) and UBC846 ($(CA)_8RT$). The optimal hybridization temperatures were 50 °C for primers 17898A, 17898B, 17899B, 51 °C for primer

UBC836, 54 °C for primer UBC846 and 55 °C for primer M2. For acceptable banding patterns is sufficient for at least 35 cycles of amplification for all six primers.

Thermo Scientific's reagents (USA) were used for polymerase chain reaction. The final reaction mixture volume was 15 µl, containing 1.5µl of 10X Taq Buffer (with 500 mM of KCl, 15 mM of $MgCl_2$, 100 mM of Tris-HCl and 0.8% Nonidet P40), 0.12 µl of dNTPs (25 mM), 0.6 µl of $MgCl_2$ (25 mM), 0.2 µl of Taq-polymerase (5 u/µl), 2 µl of ISSR primer (10 mM), 9.08 µl of dH_2O and 1.5 µl of DNA sample (15 ng).

PCR was performed in a programmable thermocycler Thermal Cycler S1000 (Bio-Rad, USA). The amplification conditions were as follows: initial DNA denaturation for 3 min at 95 °C, 35 cycles each comprising denaturation for 1 min at 95 °C, annealing for 45 sec at 50-54 °C (depend on a primer), extension for 1 min at 72 °C and final elongation for 10 min at 72 °C.

The PCR products were separated electrophoretically for 3 hours at a voltage of 70 V using a horizontal chamber The Sub-Cell Model 192 (Bio-Rad, USA) in 1.9% agarose gels (Helicon, Russia) with ethidium bromide. 1x TAE served as a buffer solution. Subsequent imaging was carried out using a gel documentation system Universal Hood II (Bio-Rad, USA).

The ISSR products were scored for the presence (1) and absence (0) of homologus DNA bands. ISSR band matrices were analyzed by using FAMD software. The final dendrogram was visualized using Fig Tree v1.4.2.

RESULTS AND DISCUSSION

Our preconceptual study of herbarium material and observation in nature has shown that for some samples from Siberia are impossible to determine the exact species of *Poa palustris* or *Poa nemoralis*. All this samples have the mixed morphological features, such as pubescence on spikelet, length and shape of ligula. In totality, we have not revealed the strong geographical connection for particular features and their combinations. Conversely, the samples with different mixed features were frequently mounted on one herbarium sheet and grew together in wild. It might be connected to a high intrapopulation morphological variation. We analyzed the quantitative and qualitative features of the five populations to measure intrapopulation variability. The quantitative data was performed by using the PCA and factor analysis. The figure 1 shows significant difference of the objects spread. It has indicated a different type of variation of the study populations.

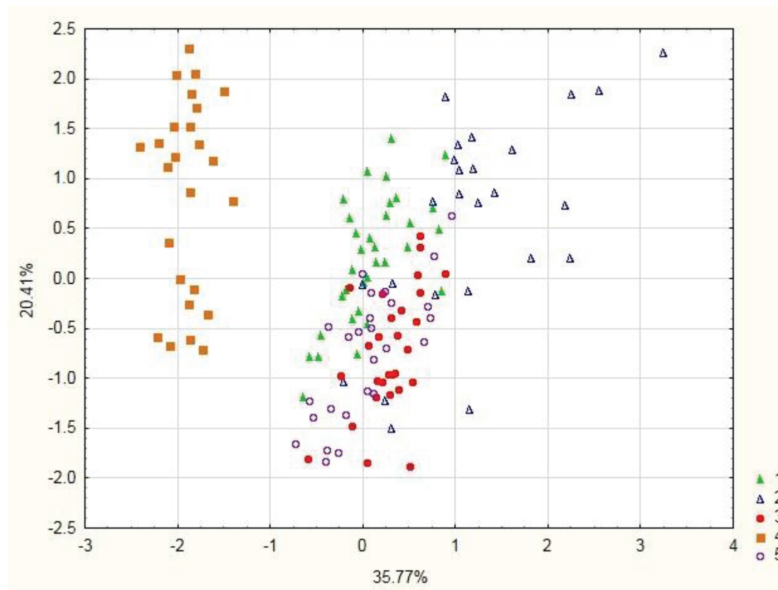

FIGURE 1. Projection of the samples from the populations 1–5 with the features V1–V22 by the PCA. X-axis is the first principal component (PC I), Y-axis is the second principal component (PC II).

The graph clearly showing isolation of the population 4 (Magadan). All the samples from that population morphologically correspond to the individuals of *P. nemoralis*. The samples from the population 3 (Primorsky Krai) are relatively compact and situate in the center of the main array. Those samples correspond to the individuals that morphologically similar with *P. palustris*. The rest of the populations, 1 (Tomsk region, Ilovskiy reserved forest), 2 (Yekaterinburg) and 5 (Tomsk region, floodplain of Tom river), which is reporting the highest number of forms that combine the characteristics of the both species are closer to the "core" of *P.*

palustris. The greatest scope of variation is noted in the population 2 (Yekaterinburg), which is also characterized by a certain isolation. Its main core is shifted up and to the right along the PC II from the third population. This distribution of the samples of the five populations by the quantitative variables are consistent with the opinion of N. N. Tsvelev [21], that *P. palustris* in Siberia almost completely replaces *P. nemoralis*. This can be explained by a higher polymorphism and plasticity of *P. palustris*.

Factor analysis has shown that the PC I mainly reflects the variability of reproductive features such as glumes and lemmas. The PC II reflects the vegetative features, which forms a common habit (table 2). The features, that reflecting the variability of the reproductive parts of the plants, are more conservative, than the features of the vegetative organs of the plants. The reproductive parts are less affected by the environment and most probably developed during some evolutionary events and fixed genetically.

TABLE 2. The correlation coefficient of the morphological characters with the axes I and II of the studied *P. palustris* and *P. nemoralis* populations.

No	Principal component I		№	Principal component II	
V15	Spikelet length	0.711	**V1**	Total plant height	0.819
V16	Length of upper glume	0.890	**V6**	Sheath length of the top leaf	0.825
V17	Width of upper glume	0.842	**V10**	Width of the panicle	0.832
V18	Length of the lower glume	0.916	**V12**	Length of the largest branches of the panicles	0.874
V20	Length of lemmas	0.915			
V21	Width of the lemmas	0.791			

*Note: coefficients less than 0.7 are not shown.

Then we built an UPGMA dendrogram on the basis of the individual states of the qualitative features, which differentiate *P. palustris* and *P. nemoralis*. As a result, the samples of the studied populations were divided into two unequal branches of the first order. One of them includes only population with the samples from the countryside of Yekaterinburg (2). The remaining populations that located on another branch, also divided into two branches of the second order. The samples of the populations (1, 3 and 5) are located on one of the branches. Its indicates a high similarity between the data samples. Some isolation of the samples from Magadan population (4) should be noted. Thus, the material proved to be very uneven in terms of the quality features. That may happen due to the influence of the complex conditions of existence.

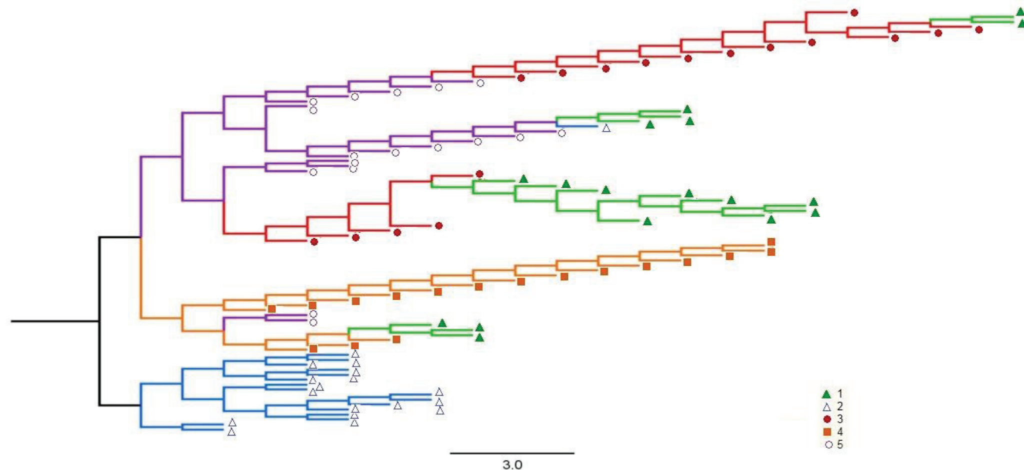

FIGURE 2. The UPGMA dendrogram of the five populations based on Euclidian distances between V8 and V23–V26 quality features.

The variation of morphological features can not be explained solely by the influence of different growth conditions, since every single feature within a species can only be changed insofar as this is possible within the

existing relations, which were formed in the process of adaptation and separation of the species [22–24]. On this basis, we can assume the heterogeneity of the populations at the genetic level.

To prove this idea we used ISSR fingerprinting. The number of bands varied from 10 (primer 17899B) to 17 (M2) with length from 230 bp to 1655 bp. The total number of bands from the six primers was 79, with the polymorphism level 77% (61 polymorphic bands).

The lowest polymorphism level (16%) was observed in intrapopulation from Magadan (3). These samples morphologically correspond to *P. nemoralis*. At potential hybrid populations from Tomsk oblast (1, 5) and Sverdlovsk Oblast (2) polymorphism levels were 22%, 23% and 47% respectively.

Primorsky Krai's population, which morphologically looks like *P. palustris*, was totally monomorphic by all the six primers (Fig. 3). We presume that this population has the apomixis mechanism of spreading.

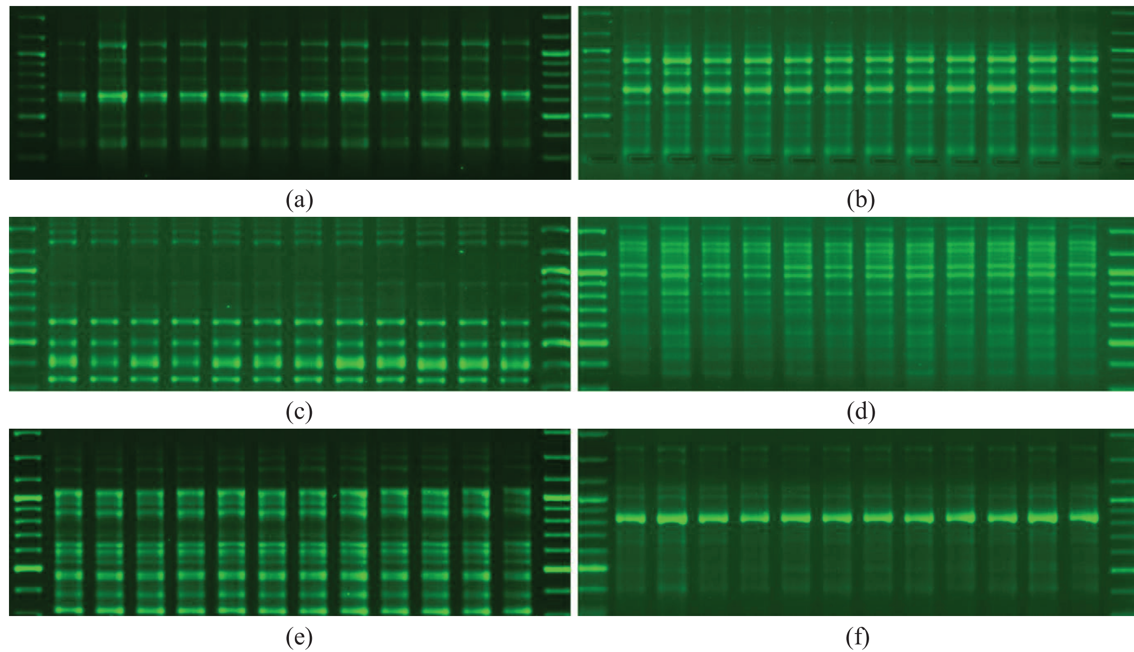

FIGURE 3. Gel electrophoresis image of Primorsky Krai's population. (a) primer 17898A, (b) primer M2, (c) primer 17898B, (d) primer UBC836 (e) primer 17899B, (f) primer UBC846.

We built a dendrogram using the neighbour-joining (NJ) algorithm in FAMD to identify possible phylogenetic distances between the samples (Fig. 4).

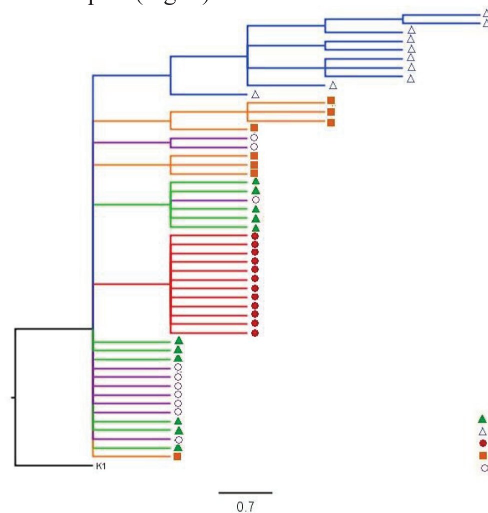

FIGURE 4. The NJ dendrogram of relationships among *P. palustris* and *P. nemoralis* populations (1–5) based on Nei's unbiased genetic distance. K1 – outgroup (*Poa compressa* L.).

The dendrogram illustrates a clear association into separated clades by the samples from Sverdlovsk Oblast (2) and Primorsky Krai (3). The samples from Tomsk oblast were again divided at random. Consequently, the populations from Tomsk oblast have hybrid nature. Unfortunately, we were not able to analyze genetic variation between *P. palustris* and *P. nemoralis*. Nevertheless, our data about genetic polymorphism of the populations *P. palustris* and *P. nemoralis* have been correlated with morphological analysis.

CONCLUSION

Study of the distribution that based on morphological characteristics and comparative morphological analysis of populations of *P. palustris* and *P. nemoralis* revealed a rather high variability of these features in the studied populations.

Such a distribution of Siberian plant materials by the morphological characteristics may indicate a hybrid nature of the samples that included in the analysis. The NJ-dendrogram that based on the results of ISSR-analysis excellently confirms this assumption and reveals a relatively high genetic diversity of the studied populations.

For more detailed results and determination of the taxonomic structure of the hybrid complex more detailed morphological, geographical and phylogeographic studies are required.

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