



Phylogenetic relationships among different morphotypes of StY-genomic species *Elymus ciliaris* and *E. amurensis* (Poaceae) as a unified macroevolutional complex

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ABSTRACT

Microevolutionary relationships between the Far Eastern StY-genomic species *Elymus ciliaris* and *E. amurensis* were studied using a set of experimental methods. No relationship was found between the formal species affiliation of a particular accession and the component composition of protein spectra. The consensus neighbor-joining (NJ) dendrogram based on the variability of ISSR markers showed the features of differentiation among different morphotypes of the two species. Pubescence of leaf blades is not a marker of differences between the studied species. A level of crossability in *E. ciliaris* s. l. did not allow to study the inheritance of morphological traits. According to the results of sequencing of the nuclear gene *GBSS1*, the sequences of the St subgenome are the most informative in terms of microevolutionary differentiation. It is proposed to return to the early treatment by N.N. Tzvelev, where *E. ciliaris* and *E. amurensis* were considered as subspecies of *E. ciliaris* s. l.

Key words: *Elymus*, SDS-electrophoresis, *GBSS1*, ISSR, dendrogram, taxonomy

РЕЗЮМЕ

Агафонов А.В., Шабанова (Кобозева) Е.В., Емцева М.В., Асбаганов С.В., Дорогина О.В. Филогенетические взаимоотношения между различающимися морфотипами StY-геномных видов *Elymus ciliaris* и *E. amurensis* (Poaceae) как единого микроэволюционного комплекса. Изучались микроэволюционные отношения между дальневосточными StY-геномными видами *Elymus ciliaris* и *E. amurensis* с применением комплекса экспериментальных методов. По результатам анализа вариабельности белков эндосперма методом SDS-электрофореза не обнаружено связи между формальной видовой принадлежностью конкретного образца и компонентным составом белковых спектров. При анализе полиморфизма межмикросателлитных последовательностей ДНК (ISSR-маркеров) использовались 6 праймеров. Консенсусная дендрограмма, построенная методом ближайшего соседа (NJ) по данным изменчивости, показала особенности дифференциации ISSR-профилей у различающихся морфотипов среди двух видов. Опушение листовых пластин не является маркером различий между изучаемыми видами. Восемь идентифицированных морфотипов из разных ветвей дендрограммы были взяты в секвенирование ядерного гена *GBSS1*. Наиболее информативными в плане эволюционной дифференциации выступают последовательности субгенома St. Уровень скрепляемости у биотипов *E. ciliaris* s. l. не позволила изучить наследование морфологических признаков. На основе результатов предлагается вернуться к ранней обработке Н.Н. Цвелева, где *E. ciliaris* и *E. amurensis* рассматриваются как подвиды *E. ciliaris* s. l.

Ключевые слова: *Elymus*, SDS-электрофорез, *GBSS1*, ISSR, дендрограмма, таксономия

The genus *Elymus* L. belongs to wild perennial grasses of tribe Triticeae Dum. from the family Poaceae Barnh. It is widespread, rich in biodiversity and the largest genus in the tribe Triticeae by the current generalized treatments (Tzvelev 1991, Chen & Zhu 2006, Barkworth et al. 2007). Most species of the allopolyploid genus *Elymus* are facultative self-pollinators. The main reasons of taxonomists' discrepancies about the composition of taxa and the construction of genus system are the wide areas of most species, their frequent joint vegetation and the occurrence of hybridization and/or introgression events in populations, which received generalized term "the reticulate evolution" (Grant 1981, Mason-Gamer 2004).

Within the genus *Elymus* and the section *Goulardia* (Husnot) Tzvelev, the subsection *Ciliares* (Nevski) Tzvelev contains closely related species *Elymus ciliaris* (Trin.) Tzvelev and *E. amurensis* (Drob.) Czer. (Tzvelev 2008). These species are spread on the areas of Eastern and North-Eastern China, Korea and Japan (Bothmer et al. 2005). In Russia, they occur only within the southern Russian Far East. Their StY-genomic constitution was established cytogenetically by means of hybridization of specimens, referred to the *E. ciliaris* (Dewey 1984). The species *E. ciliaris* and *E. amurensis* grow jointly in many places of area and differ from each other by the small number of quantitative morphological traits. It allows taxonomists from different countries to

interpret freely enough the limits and taxonomical rank of two species.

Earlier, according to Probatova (1985), the most significant trait for differentiation of *E. ciliaris* and *E. amurensis* was the presence-absence of trichomes on lemmas and pilose leaf blades (LB), but in the publication by Tzvelev & Probatova (2010) these species differ only by pilose LB and length of anthers. These distinctions are fixed in the recent fundamental book “Grasses of Russia” (Tzvelev & Probatova 2019).

In China, where the main part of the taxa’s distribution area located, *E. ciliaris* is considered as the one species, which contains 6 varieties (Chen & Zhu 2006), including *E. ciliaris* var. *amurensis* (Drob.) S.L. Chen (Chen 1997), which was separated on the basis of hairiness of lemmas and LB.

Previously, we have studied the nomenclature and synonymy of *E. ciliaris* and *E. amurensis* and carried out a comparative morphological analysis, which questioned the clear separateness of these species (Kobozeva et al. 2011). Diagnostic traits of *E. ciliaris* and *E. amurensis* broadly varied, depending, besides other factors, on the growth conditions and developmental stage. In the sites with closed cover of *E. ciliaris* and *E. amurensis* species, along with typical species individuals, we have found the local micropopulations with deviating morphological characteristics. It can be an evidence of the active recombinational and introgressive processes in the natural growth conditions. We have concluded that *E. amurensis* most probably represents the complex of morphotypes of *E. ciliaris* s. l., which do not possess the characteristics of the separate species. Along with it, the lack of precise experimental data does not allow to formulate the suggestions on taxonomical structure of subsection *Ciliares* (Nevski) Tzvel., which includes these taxa. The origin of *E. amurensis* from isolated species *E. ciliaris* and *E. pendulinus* (Agafonov et al. 2016), which was supposed by Tzvelev & Probatova (2010), also wasn’t confirmed yet.

Earlier, Mason-Gamer with coauthors (Mason-Gamer 2001, 2004, 2007, 2008, 2013, Mason-Gamer et al. 1998, 2010) have shown high effectiveness of the use of sequencing of the low-copy nuclear gene *waxy* (granule-bound starch synthase 1, *GBSS1*) for the study of phylogenetic relationships between species of Triticeae tribe.

This work presents results of the study of *E. ciliaris* and *E. amurensis* differentiation within Russia using several approaches and methods: SDS-electrophoresis of the endosperm proteins, hybridological method, molecular genetical ISSR-analysis and sequencing of the nuclear gene *GBSS1*. The main purpose of this work was the construction of taxonomical model of the subsection *Ciliares*.

MATERIAL AND METHODS

To study the microevolutionary relationships between species from the Primorye Territory we mainly used our original collections (Tables 1, 2) and the accessions shown in Tables 3, 4.

Electrophoresis. The extraction of endosperm storage proteins from individual grains and electrophoresis in SDS-system were carried out according to Laemmli (1970) with modifications (Agafonov & Agafonova 1992). Grains of the *E. sibiricus* accession ALT-8401 were used as a standard, in which the polypeptide components of the electrophoretic patterns were previously calibrated using standard molecular weight markers. Scales of relative electrophoretic mobility (REM) were built for more precise identification of each component.

Analysis of the polymorphism of intermicrosatellite DNA sequences (ISSR). For the DNA probes sampling we used the dried green mass of young specimens, grown from the seeds of natural plants. DNA was isolated by Nucleospin Plant II kit (Macherey-Nagel, Germany) by using standard protocol with some modifications for the genus *Elymus*. ISSR-analysis was carried out with primers, which were successfully used previously for marking the *Elymus* species – (CA)₈GG, (AC)₈CG, (AC)₈YG, (CA)₆R, (CTC)₃GC, (CAC)₃GC (Medigen, Russia). PCR and assessment of polymorphism of ISSR markers were conducted according to the previously described procedure (Kobozeva et al. 2015).

Sequencing of the nuclear gene *GBSS1* (*waxy*) in selected biotypes of *E. ciliaris* and *E. amurensis* with different morphology. The set of accessions, included in the analysis of *GBSS1* sequences, comprised the most contrasting morphotypes of two species (Table 3). Sequences of other StY-genomic species and diploid carriers of genomes St (*Pseudoroegneria*) and H (*Hordeum*) were taken as a reference ones from the NCBI database (<http://www.ncbi.nlm.nih.gov/nucleo>) (Table. 4). Amplification, cloning and sequencing of *GBSS1* fragments from 9 through 14 exons were performed in accordance with recommendations of Kellogg (1992), Mason-Gamer et al. (1998) and with our modifications (Agafonov et al. 2019). Results of comparison of gene sequences were obtained. Dendrograms were constructed on the full gene fragments with exons and introns by using the TREECON ver. 1.3b software (Peer & Wachter 1994).

Hybridization. Intraspecific and interspecific hybridization was performed by using express-method, developed by us earlier (Agafonov 2004). Parental forms, which differed from each other by the number of morphological traits, were grown from the seeds of wild plants, collected in the

Table 1. Locations of the collection of populations of two species growing together by O.V. Dorogina, D.E. Nikonova (Gerus), E.V. Shabanova (Kobozeva) and A.V. Agafonov

| Population | Locations: Primorye Territory, Khasan District |
|-----------------------------------|---|
| Slavyanka (SLA) | Town Slavyanka, railway exclusion zones and meadow fragments (transect), alt. 3–10 m, from point N 42°52.659'N 131°23.327'E to point 42°51.852'N 131°23.145'E |
| Andreyevka (AND) | Town Andreyevka, boundary between the meadow slopes and roadsides of unpaved roads (transect), alt. 2–14 m, 42°38.010'N 131°8.272'E |
| Marine experimental station (MES) | Vicinity of Marine experimental station of the Pacific Institute of Bioorganic Chemistry (PIBOC) FEB RAS, meadow slopes and roadsides of forest paths and unpaved roads (transect), alt. 3–50 m, 42°37.326'N 131° 8.257'E |

different points of their distribution area. Hybridity of F_1 plants was determined by the morphological markers of assumed hybrid in combinations, when parental plant had divergent dominant traits.

RESULTS AND DISCUSSION

Electrophoretic study of polymorphism and specificity of endosperm storage proteins

For evaluation of relationships between species *E. ciliaris* and *E. amurensis*, we carried out an analysis of SDS-electrophoretic spectra of endosperm storage proteins from several populations of southern Primorye Territory.

The polymorphism of spectra allows to identify distinct biotypes, analyze heterogeneity of populations and evaluate levels of relatedness (Agafonov et al. 2016). In general, populations of *E. ciliaris* and *E. amurensis* from southern Primorye Territory are polymorphic in component composition of spectra, which allows to study the levels of affinity between biotypes and populations. The greatest diversity in mixed micropopulations with morphotypes of *E. ciliaris* and *E. amurensis* was noted in the southern part of Khasansky District in Primorye Territory in vicinity of Slavyanka township and Andreyevka settlement, and in vicinity of the Marine Experimental Station PIBOC FEB RAS.

Table 2. Locations of the separate accessions of *Elymus ciliaris*, *E. amurensis* and morphologically deviating forms (MDF) from the Primorye Territory.

| Accession code | Locations and authors of collectings |
|--|---|
| <i>Elymus ciliaris</i> – <i>E. amurensis</i> | |
| ANI-8616 | Partizansk District, vicinity of Anisimovka, roadside, alt. 224 m, 43°10.338'N 132°46.094'E (A. Agafonov, O. Dorogina) |
| VLA-8629; 8630; VLA-0947; VOK-8631; 0711; VOK-0725; 1011 | Vicinity of Vladivostok, shrub in exclusion zone between the railway stations Sanatornaya and Okeanskaya, from alt. 8 m, 43°13.892'N 131°58.975'E to alt. 5 m, 43°14.010'N 132°0.190'E (A. Agafonov, O. Dorogina, M. Agafonova) |
| VBG-8635* VBG-0844* | Vladivostok, Botanical Garden–Institute FEB RAS, mixed forest at the top of the hill, alt. 159 m, 43°13.121'N 131°59.355'E (A. Agafonov, O. Dorogina, M. Agafonova) |
| RUS-0731 | Russky Island, vicinity of settlement Paris; shrub near the coast of the bay, alt. 5 m, 43°1.041'N 131°52.892'E (A. Agafonov, D. Gerus) |
| PRA-1260 | Khasan District, vicinity of Ryazanovka, territory of Marine biological station of Far Eastern Federal University, alt. 27 m, 42°47.582'N 131°15.147'E (A. Agafonov) |
| ZAR-0708 | Khasan District, vicinity of Zarubino, tall grass meadow near the bus station, alt. 8 m, 42°38.375'N 131°04.523'E (A. Agafonov, D. Gerus) |
| COR-8992 | Received from the Democratic People's Republic of Korea (DPRK) according to delectus exchange, morphologically verified as <i>E. ciliaris</i> |
| Population of MDF | |
| MES-1111 ^{MDF} | Khasan District, vicinity of MES PIBOC FEB RAS, meadow slope and roadside of forest unpaired road, transect from point alt. 88 m, 42°37.047'N 131°8.632'E to point 42°36.995'N 131°8.527'E (E. Kobozeva, A. Agafonov) |

* accession with pilose upper surface of LB that corresponds to *E. amurensis* according to diagnosis of Tzvelev & Probatova (2010);
^{MDF} morphologically deviating form, characterized by absolutely glabrous lemmas, the absence of cilia and by elongated paleae

Table 3. Locations of additional accessions of *E. ciliaris* and *E. amurensis*, taken for the sequencing of *GBSS1* gene.

| Accession code | Clones in author's catalogue | Accession numbers in NCBI | Location |
|----------------------------|--|----------------------------------|--|
| PNA-0906*# | amuPNA06_6-St amuPNA06_1-Y | MT326222 MT326214 | Vicinity of Nakhodka, mixed forest on the slopes of bald mountain, alt. 48 m, 42°50.485'N 132°54.613'E |
| PNA-0907* | cilPNA07_1-St cilPNA07_2-Y | MT326221 MT326213 | The same |
| SAD-1030 | cilSAD30_1-St cilSAD30_2-Y | MT326220 MT332692 | Vicinity of Vladivostok, Sadgorod township, rocky cape in the Amur Bay, alt. 1 m, 43°16.10'N 132°0.92'E |
| SLA-1162* | cilSLA62_3-St cilSLA62_2-Y | MT326225 MT326216 | Khasan District, Slavyanka, railway exclusion zone near the coast of Slavyansky bay, alt. 3 m, 42°52.052'N 131°23.002'E |
| SLA-1161*# | amuSLA61_a-St amuSLA61_h-St amuSLA61_1-Y | MT326223 MT326224 MT326215 | The same |
| MES-1112 | cilMES12_5-St cilMES12_1-Y | MT326226 MT326219 | Khasan District, vicinity of MES PIBOC FEB RAS, downhill to the Peschanaya bay, alt. 6 m, 42°36.795'N 131°8.488'E |
| MES-1107*# | amuMES07_4-St amuMES07_2-Y | MT326228 MT326217 | Khasan District, vicinity of MES PIBOC FEB RAS, meadow on the border of forest fragment, alt. 21 m, 42°37.478'N 131°07.889'E |
| MES-1111_26 ^{MDF} | amuMES11_d-St amuMES11_3-Y | MT326227 MT326218 | Khasan District, vicinity of MES PIBOC FEB RAS, forest road below the ridge, alt. 30–85 m, 42°37.031'N 131°8.623'E |

accession with glabrous lemmas, corresponding to *E. amurensis* by diagnoses of Probatova (1985);

* accession with pilose upper surface of LB that corresponds to *E. amurensis* according to diagnosis of Tzvelev & Probatova (2010);
^{MDF} morphologically deviating form, characterized by absolutely glabrous lemmas, the absence of cilia and by elongated paleae

Table 4. Species accessions and their clones, drawn from the GenBank NCBI database.

| Species | Accession (origination) | Accession number in GenBank NCBI | Designation on dendrograms |
|--|-------------------------------|---|--|
| <i>Pseudoroegneria spicata</i> (Pursh) A. Löve | PI 236681 Canada | St AY010998 ¹ | Ps.spi_998 |
| <i>P. spicata</i> | D 2844 USA | St AY011000 ¹ | Ps.spi_000 |
| <i>P. strigosa</i> (M.Bieb.) A. Löve | PI 499637 China | St EU282323 ² | Ps.str_323 |
| <i>P. strigosa</i> | PI 531755 China | St AY360823 ³ | Ps.str_823 |
| <i>Hordeum jubatum</i> L. | RJMG 106 USA | H AY010963.1 ¹ | H.jub_63_H |
| <i>H. bogdanii</i> Wilensky | PI 531760 China | H EU282317 ² | H.bog_17_H |
| <i>H. brevisubulatum</i> (Trin.) Link | PI 401387 Iran | H AY010961 ¹ | H.bre_61_H |
| <i>H. pusillum</i> Natt. | CIho 15654 USA | H EU282321 ² | H.pus_21_H |
| <i>Elymus abolinii</i> (Drob.) Tzvel. | PI 531555 China | St DQ159322 ⁴ Y DQ159323 | abo_9322_S abo_9323_Y |
| <i>E. abolinii</i> | PI 531557 Kyrgyzstan | St GQ847708 ⁵ Y GQ847709 Y GQ847710 | abo_7708_S abo_7709_Y abo_7710_Y |
| <i>E. caucasicus</i> (C. Koch) Tzvel. | PI 531573 Armenia | Y GQ847719 ⁵ Y GQ847720 | cau_7719_Y cau_7720_Y |
| <i>E. ciliaris</i> (Trin.) Tzvel. | PI 531577 Japan | St GQ847721 ⁵ Y GQ847722 | cil_7721_S cil_7722_Y |
| <i>E. ciliaris</i> | PI 531576 Russia, Vladivostok | St GQ847723 ⁵ St GQ847724 Y GQ847725 | cil_7723_S cil_7724_S cil_7725_Y |
| <i>E. ciliaris</i> | PI 531575 China | St DQ159327 ⁴ Y DQ159326 | cil_9327_S cil_9326_Y |
| <i>E. gmelinii</i> (Ledeb.) Tzvel. | PI 499447 China | St GQ847726 ⁵ Y GQ847727 | gme_7726_S gme_7727_Y |
| <i>E. nevskii</i> Tzvel. | PI 314620 Kazakhstan | St GQ847729 ⁵ Y GQ847730 | nev_7729_S nev_7730_Y |
| <i>E. pendulinus</i> (Nevski) Tzvel. | PI 499452 China | St GQ847731 ⁵ Y GQ847732 | pen_7731_S pen_7732_Y |
| <i>Bromus tectorum</i> L. | | AY362757 ³ | Bromus_AY3 |

¹ Mason-Gamer 2001; ² Mason-Gamer 2008; ³ Mason-Gamer 2004; ⁴ Mason-Gamer 2007; ⁵ Mason-Gamer et al. 2010

When considering the general polypeptide polymorphism of *E. ciliaris* and *E. amurensis* within the larger distribution area, the groups of components being specific for both species, were revealed (Fig. 1, marked by arrows and brackets).

Thus, common components distinctly manifested in the zones of 37 and 77 units of REM in accessions of *E. amurensis* from surroundings of MES and of *E. ciliaris* from surroundings of city Vladivostok and town Anisimovka of Partizansky District (shown by arrows). Besides this, the overlapping of two species was revealed within an electrophoretic group, containing 6 components in the range from 50 to 76 units by scale of REM (marked by square brackets). Coincidence of components between *E. amurensis* and North Korean *E. ciliaris* accession COR-8992 was detected in the zone of 77 units of REM. All *E. ciliaris* accessions from MES surroundings were similar in compound of spectra, while *E. amurensis* accessions were slightly polymorphic, what completely coincides with data from expedition collectings of years 2007 and 2011.

Hence, based on the results of study of endosperm proteins polymorphism, no association was detected between the formal

species affiliation of the particular accession and the component structure of protein spectra. The criterion of distance between populations is much stronger expressed

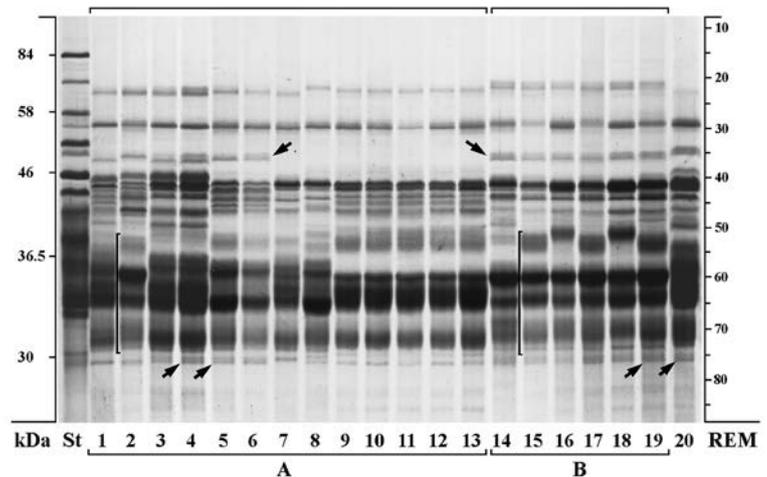


Figure 1 SDS-electrophoregram of endosperm proteins of selected accessions of *Elymus ciliaris* (Trin.) Tzvelev (A) and *Elymus amurensis* (Drob.) Czer. (B) from southern regions of Primorye Territory and *E. ciliaris* accession from northern part of DPRK (20). Polypeptide spectra of separate grains in +Me variant; kDa – approximate scale of molecular masses; REM – the scale of relative electrophoretic mobility; St – the standard spectrum of *E. sibiricus* ALI-8401 line. 1. VBG-8635; 2. VOK-8631; 3. VLA-8630; 4. VLA-8629; 5. ANI-8646; 6. ANI-8616; 7. SLA-8665; 8. SLA-8666; 9. MES-8639; 10. MES-8641; 11. MES-8643; 12. MES-8645; 13. MES-8626; 14. MES-8622; 15. MES-8620; 16. MES-8614; 17. MES-8613; 18. MES-8640; 19. MES-8642; 20. *E. ciliaris* COR-8992

here. At the same time we should note the presence of specific high-molecular components, supposedly glutelin subunits, in the zone of nearly 20 units of REM. These components might be considered as specific ones for *E. amurensis* in a given habitat. The smallest differences appeared in accessions from nearby habitats, which evidences the relatedness of specimens due to the common ancestry. Bit larger differences were in accessions from SLA population, and in ones from closely located, but different populations VBG and VOK. Moreover, populations significantly varied in heterogeneity of this trait.

Variability and specificity on ISSR-markers among the selected accessions of *E. ciliaris* and *E. amurensis*

For the detection of polymorphism and species specificity in *E. ciliaris* and *E. amurensis* we applied the method of analysis of intermicrosatellite DNA sequences (ISSR). Aside from the typical accessions of abovementioned species, the biotypes of MES-1111 population were analyzed, which differed from other *E. ciliaris* and *E. amurensis* accessions by bigger grains, the lack of trichomes and cilia on lemmas and the longer paleas. The *Pseudoroegneria strigosa* (M. Bieb.) A. Löve was taken as an outgroup accession. When compared ISSR-profiles, obtained by using 6 primers (17899B, M1, M2, M11, HB14, HB12), the high polymorphism (80.95–97.2%) of intermicrosatellite DNA sequences was revealed in 46 samples of studied species (Table 5).

Amplified fragments (193 amplicons) varied in range from 200 to 1600 bp. Most ISSR-fragments (42) were obtained when using an M11 primer. The least variable profiles were obtained when applying M1 primer – 21 amplicons. Obtained profiles exhibited visual species specificity (Fig. 2).

Based on the data of polymorphism of six ISSR-PCR profiles the consensus dendrogram was constructed by neighbor-joining (NJ) method (Fig. 3), which clarified inter-

relationships of the studied taxa.

Accessions with hispid and glabrous lemmas formed two large clusters in the dendrogram, that does not contradict to morphological criterion of *E. ciliaris* and *E. amurensis* by Probatova (1985). It evidences that species are separated to some extent on the basis of ISSR-profiles. Nevertheless, the genetical similarity of some MDF with these species was found, that is does not correspond to morphological diagnoses of the separation of these species. For example, MDF with recombinant combination of traits (hispid and ciliated lemmas and pilose LB) correspond on ISSR-profiles to the typical morphotypes of *E. ciliaris* (hispid lemmas with cilia and glabrous LB). In the same time the typical morphotypes of *E. amurensis* (glabrous lemmas with cilia and pilose LB) integrated with MDF, which coincide with them in the basic diagnosis, but differ in the condition of lemmas (absolutely glabrous lemmas without cilia, pilose LB).

Between the two large clades, one of which comprised accessions with hispid lemmas, and other – with glabrous or finely scabrous lemmas, the PNA-0906 morphotype with glabrous lemmas located separately and might be considered as a rare one for its habitat. The fact is that all accessions with glabrous lemmas were found by us only in Khassansky District of Primorye Territory in the SLA and MES populations.

The largest genetic similarity was detected in accessions from the joint populations, where closely related samples with similar morphological traits grow more frequently. Within large clades, the small clusters comprised accessions from joint habitats in locations near Vladivostok, Slavyanka, MES PIBOC and Andreyevka.

Relatively low values of bootstrap support are explained by the fact that accessions of species *E. ciliaris* and *E. amurensis* are closely relative and there are very little genetic differences between them.

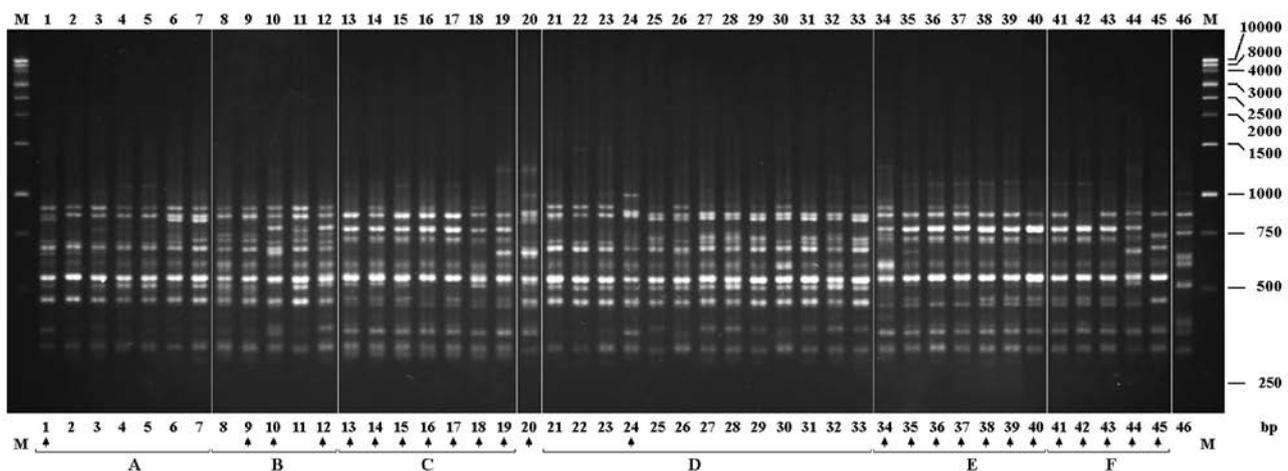


Figure 2 Variability of ISSR fragments within the *Elymus ciliaris* s. l. complex in populations from Primorye Territory when using 17899B primer: A – *E. ciliaris* s. str. from different locations in Nakhodka city and in vicinity of Vladivostok city; B – *E. ciliaris* s. str. in vicinity of Slavyanka township; C – *E. amurensis* s. str. in vicinity of Slavyanka township; D – *E. ciliaris* s. str. in vicinity of Andreyevka settlement and MES; E – *E. amurensis* s. str. in vicinity of MES; F – MDF with glabrous non-ciliated lemmas in vicinity of MES. bp – the scale of DNA fragments sizes. Arrows indicate individuals with pilose LB.

1. PNA-0907; 2. SAD-1030; 3. VOK-1011; 4. VOK-0711; 5. VOK-0725; 6. VLA-0947; 7. RUS-0731; 8. SLA-0705; 9. SLA-0709; 10. SLA-1162; 11. SLA-1211; 12. SLA-1251; 13. SLA-1221; 14. SLA-1227; 15. SLA-1228; 16. SLA-1230; 17. SLA-1231; 18. SLA-1234; 19. SLA-1161; 20. PNA-0906; 21. PRA-1260; 22. ZAR-0708; 23. AND-0707-1; 24. AND-0707-3; 25. AND-1143; 26. AND-1148; 27. MES-0704; 28. MES-0735; 29. MES-1105; 30. MES-1106; 31. MES-1108; 32. MES-1112; 33. MES-1124; 34. MES-0702; 35. MES-1101; 36. MES-1104; 37. MES-1107; 38. MES-1117; 39. MES-1119; 40. MES-1136; 41. MES-1111-10; 42. MES-1111-11; 43. MES-1111-26; 44. MES-1111-29; 45. MES-1111-30; 46. *Ps. strigosa*.

Table 5. The characteristics of primers used for study of the DNA polymorphism. Ta – annealing temperature of primers for PCR; *Y = C or T; **R = G or A.

| Nucleotide sequence 5' - 3' | Primer | Ta, °C | Total number of bands | Number of polymorphic bands | Percentage of polymorphic bands | Sizes of DNA fragments (bp) |
|-----------------------------|--------|--------|-----------------------|-----------------------------|---------------------------------|-----------------------------|
| (CA) ₈ GG | 17899B | 47 | 40 | 37 | 92.50 | 200–1500 |
| (AC) ₈ CG | M1 | 47 | 21 | 17 | 80.95 | 200–1500 |
| (AC) ₈ YG* | M2 | 56 | 36 | 35 | 97.20 | 250–1500 |
| (CA) ₆ R** | M11 | 39 | 42 | 40 | 95.24 | 500–1500 |
| (CTC) ₃ GC | HB14 | 41 | 25 | 23 | 92.00 | 200–1600 |
| (CAC) ₃ GC | HB12 | 42 | 29 | 25 | 86.21 | 200–1200 |

The MDF accession (MES-1111-30) distinctly separated from the whole clade of species *E. ciliaris* and *E. amurensis*, which indicates the genetic difference of this accession from the other ones in subsection *Ciliares*. Such individuals sometimes occur in natural populations, and they probably represent the spontaneous hybrids, which do not produce progeny and are eventually eliminated from the population. But, when getting in favorable conditions, such hybrids might participate in an introgressive process and initiate the development and distribution of a new form.

Accessions, marked by darkening on the dendrogram, were taken for the sequencing of the nuclear gene *GBSS1*

to search the taxonomic specificity at genetic level in individuals with differing morphology.

Sequencing of the nuclear gene *GBSS1* (*waxy*) in selected biotypes of *E. ciliaris* and *E. amurensis* with different morphology

We have obtained results of the comparative study of sequences of *GBSS1* gene in differing morphotypes of two taxonomical species *E. ciliaris* and *E. amurensis* from the Russian Far East for evaluation of levels of divergency between them. The StY-genomic constitution of taxa which was established cytogenetically (Dewey 1984) has been confirmed.

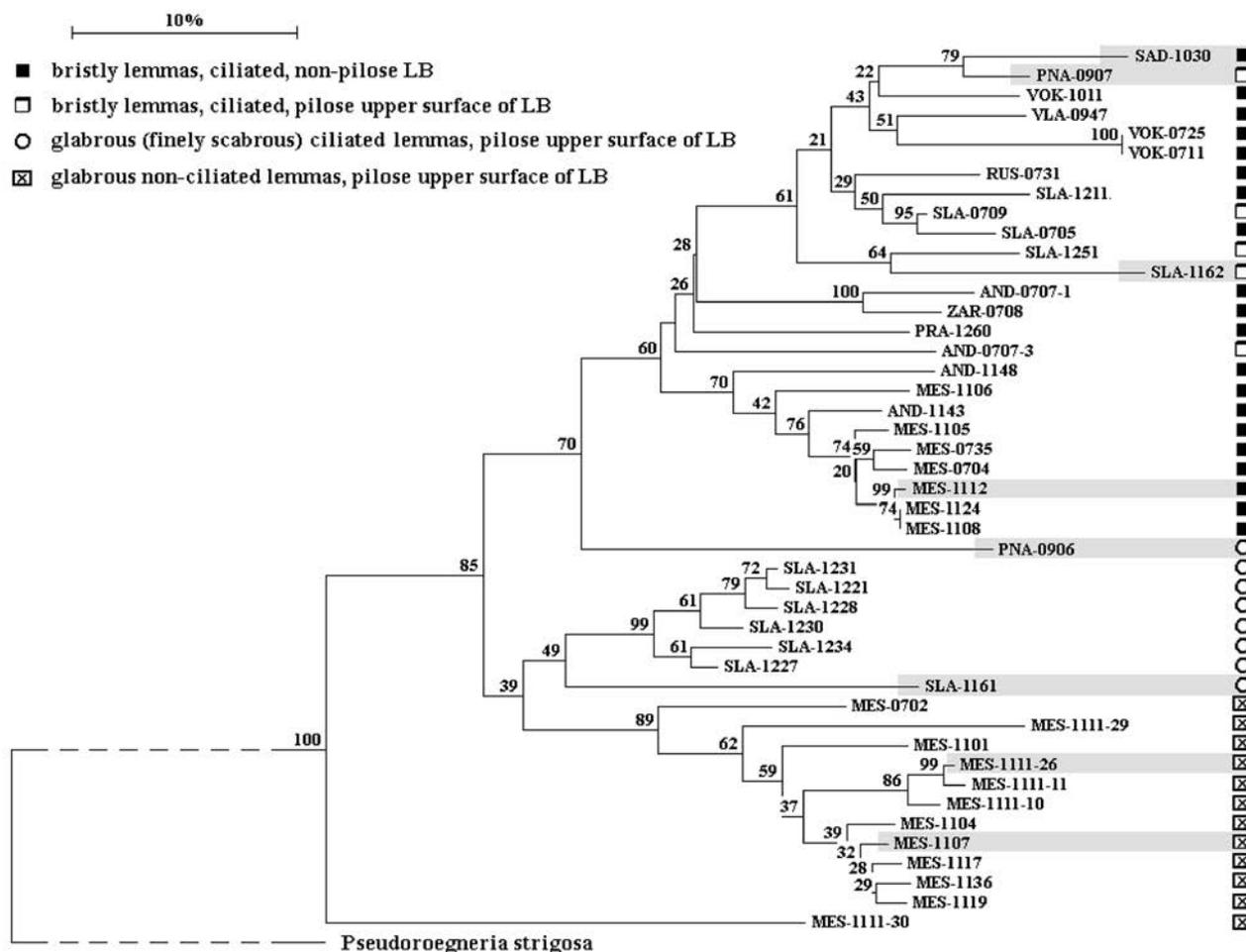


Figure 3 Consensus NJ dendrogram built on the basis of ISSR-PCR profiles of *Elymus ciliaris* (Trin.) Tzvelev and *Elymus amurensis* (Drob.) Czer. when using 6 primers. Bootstrap support values are shown in the nodes. Accessions, taken in the sequencing of the nuclear *GBSS1* gene, marked with darkening. The scale at the top shows genetic distances by Nei & Li (1979)

Dendrogram with bootstrap values higher than 40 were build on the full gene fragments (exons + introns) (Fig. 4).

Cluster NJ-analysis based on Kimura 2-parameter distances (K2P) has confirmed StY-genomic constitution of the studied accessions. The observed close grouping of all *E. ciliaris* and *E. amurensis* sequences, both in St-, and in Y-genomic clade, evidences the belonging of the studied *E. ciliaris* and *E. amurensis* accessions to a single microevolutional complex. St-genomic *GBSS1* sequences are the most informative in terms of evolutionary differentiation. Here becomes apparent the clear separation of *E. ciliaris* – *E. amurensis* complex from other Asian StY-genomic species of the genus *Elymus*. If to follow our conditional classification of St genomes when StH-genomic species of the genus *Elymus* analyzed (Agafonov et al. 2019) and the present literature data (Hu et al. 2013, Dong et al. 2015), one can relegate St genomes of this complex to more advanced St₂ group with North American *P. spicata* species in contrast to older

St₁-species group with Asian species *P. strigosa*. In addition, *E. amurensis* clones located separately and with high bootstrap support within the St₂ group (marked by darkening), what can be the result of partial microevolutional divergency of this species in the St genome. This effect appears also when analyzed more evolutionally significant and conservative exons without introns.

As to Y-subgenomic sequences, the genetic distances between accessions are significantly less, and the *E. ciliaris* – *E. amurensis* Y-clade separated from the St-clade with relatively low bootstrap-support value (Fig. 4).

Distinctions, revealed between the modern St and Y genomes, probably, caused by earlier phylogenetic events, associated with the origin of these genomes from different

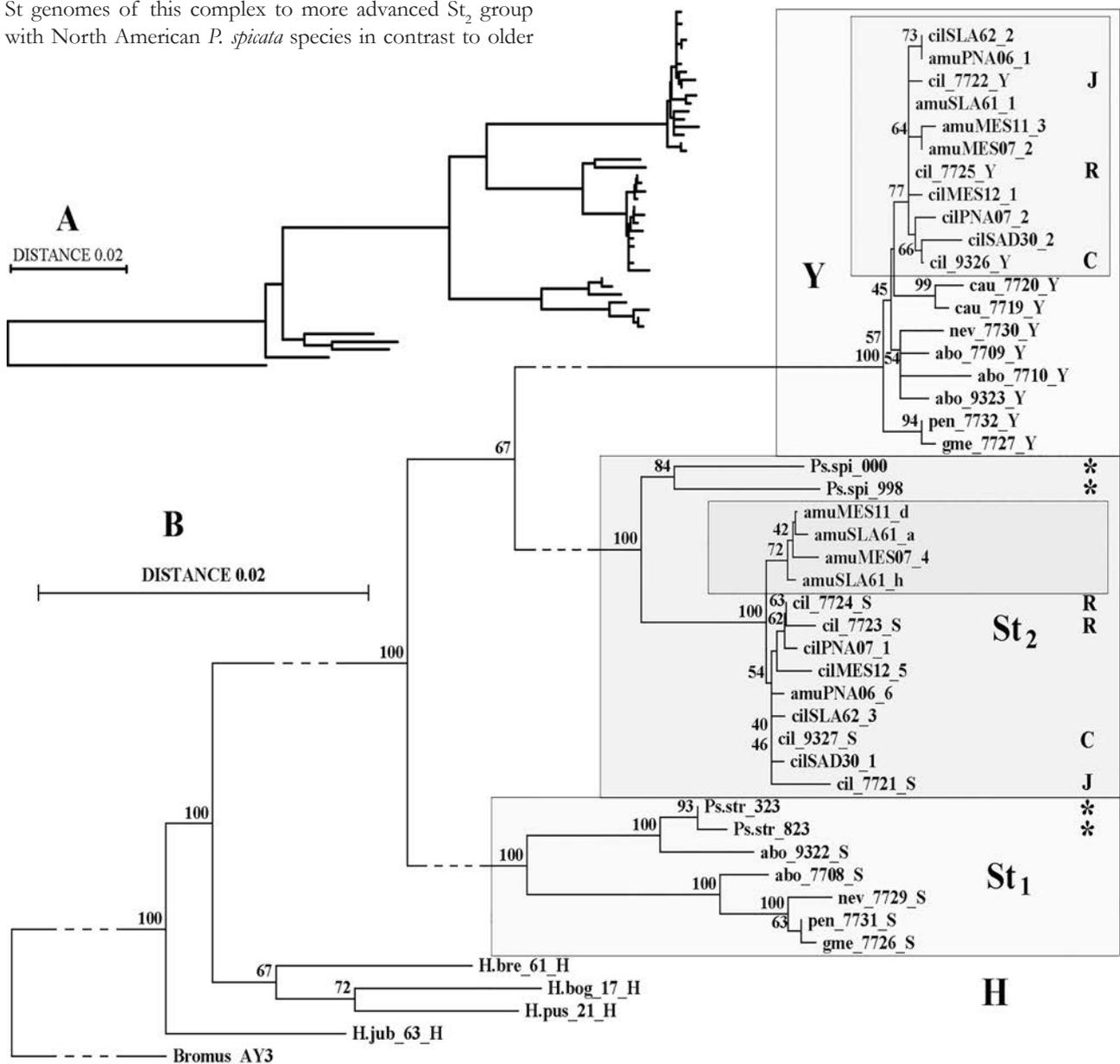


Figure 4 NJ dendrogram, built on the results of analysis of *GBSS1* gene sequences from 9 through 14 exons in *Elymus ciliaris* (Trin.) Tzvelev and *Elymus amurensis* (Drob.) Czer. from Primorye Territory compared to similar sequences of reference species from GenBank NCBI (B). The sequence AY362757 of *Bromus tectorum* L. was taken as an outgroup. Letters to the right of clades indicate countries of origin of reference *E. ciliaris* accessions (Table 4). Reference accessions of *Pseudoroegneria* species – bearing St genome – marked by asterisks. The original dendrogram topology without shortening of branches (dotted line) is shown at the top of figure (A).

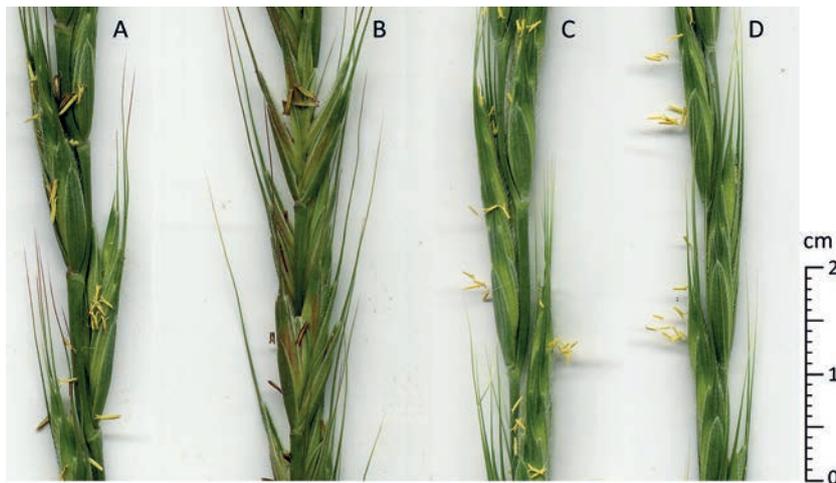


Figure 5 Fragments of flowering spikes from hybrid F_1 plants in different cross combinations between biotypes of some accessions of *Elymus ciliaris* – *Elymus amurensis* complex. Absolutely closed anthers are clearly visible. A – MES-0702 \times RUS-0713; B – VOK-0725 \times MES-0702; C – VLA-0947 \times VBG-0844; D – MES-1111-28 \times AND-0707

monogenomic ancestors, closely related to modern species of the genus *Pseudoroegneria* (Gao et al. 2014). This fact likely determines the differentiation of St genome into St_1 and St_2 variants, related to the modern species *P. strigosa* and *P. spicata*, respectively. Presumably, the differentiation of St_2 genome could have led to the occurrence of a modern Y genome. By other data, the Y genome could have originated directly from the other *Pseudoroegneria* species, close to modern *P. spicata* (Okito 2009), which also is consonant with the model of relationships suggested by us.

Reproductive isolation among *E. ciliaris* and *E. amurensis* biotypes

An important problem for us is the definition of levels of reproductive compatibility between biotypes of complex from different regions of Primorye Territory. For this purpose we created a series of intraspecific hybrids *E. ciliaris* \times *E. ciliaris*, formal interspecific hybrids *E. ciliaris* \times *E. amurensis*, and *E. amurensis* \times *E. ciliaris* (Table 6).

However, all F_1 plants in these combinations, including ones between biotypes with similar morphology but from different populations (*E. ciliaris* VLA-0947 \times *E. ciliaris* VBG-0844), showed totally closed anthers and seed sterility during two growing seasons. Spike fragments of hybrid F_1 plants in different cross combinations are shown in Fig. 5. Absolutely closed anthers are registered in all hybrids, what can indicate significant meiotic disturbances, and as a result failure in formation of fertile pollen. It might mean that *E. ciliaris* as a species is more differentiated than most other *Elymus* species.

Table 6. Hybridization of biotypes, corresponding to *E. ciliaris* and *E. amurensis* species. N_{F_1} – the number of plants were analyzed in F_1 .

| Cross combinations of <i>Elymus ciliaris</i> s. l. | | N_{F_1} | Fertility |
|--|---------------------------------------|-----------|-----------|
| <i>E. ciliaris</i> VLA-0947 | \times <i>E. ciliaris</i> VBG-0844 | 2 | 0 |
| <i>E. amurensis</i> MES-0702 | \times <i>E. ciliaris</i> RUS-0713 | 3 | 0 |
| <i>E. amurensis</i> MES-0702 | \times <i>E. ciliaris</i> RUS-0704 | 1 | 0 |
| <i>E. ciliaris</i> VOK-0725 | \times <i>E. amurensis</i> MES-0702 | 1 | 0 |
| <i>E. amurensis</i> MES-1111-28 | \times <i>E. ciliaris</i> AND-0707 | 1 | 0 |

According to data of Hu et al. (2013), based on the molecular genetic analysis of sequences of nuclear genes *PGKI* (the phosphoglycerate kinase), *RPB2* (the second largest subunit of RNA polymerase II) and *RPS16* (the chloroplast gene encoding ribosomal protein S16), a suggestion was made about the multiple origins of different geographical *E. ciliaris* races with St genome.

Thus, an ambiguous data were obtained when hybridizing *E. ciliaris* and *E. amurensis* biotypes with different morphology. Unlike other *Elymus* species, the criterion of crossability in *E. ciliaris* doesn't work and doesn't allow to research the inheritance of morphological traits. Such type of intraspecific organization is known from the results of study of the hybrids of North American allotetraploid species *Elymus*

glaucus Buckley (Snyder 1950, Stebbins 1957). On the example of this autogamous species the existence of internal groups of specimens, which were called "microspecies", have been shown. It is suggested that *E. glaucus* originated due to the spontaneous hybridization between self-pollinating and cross-pollinating ancestral biotypes of several species. Further inbreeding of the arisen hybrids generates the material for selection of self-fertile biotypes, which combine different traits of parental species (Snyder 1951).

The criterion of reproductive compatibility cannot be applied to these aggregate species, because they represented by the set of self-pollinating lines, between which reproductive barriers were formed on the level of multiple chromosomal rearrangements. The process of gamete formation in hybrids is broken as a result of irregular meiosis, and plants become completely sterile, in spite of common origin. Moreover, some groups of closely relative plants can present a mix of several original species and numerous microspecies, derived from them. In these cases criteria based on traditional taxonomical principles and small morphological distinctions between microspecies do not work, because it is difficult to divide this complex into components (Grant 1981). The subsection *Ciliares* (according to our proposed model = *E. ciliaris* s. l.) likely contains complexes of microspecies, which can appear as a result of spontaneous mutagenesis and recombination speciation, i.e. the formation of daughter microspecies, homozygous for chromosomal rearrangements. Such forms are fertile when self-pollinated, but sterile when crossed with any of the parental species.

It is noteworthy that Yu et al. (2008) when hybridized the accession *E. amurensis* PI 547303 (= MES-8614 from our collection-1986, whose seeds were deposited to living collection in USDA-URS, Logan, Utah, USA), reported the high seed fertility (SF) of interspecific hybrids (cross combinations in author's

nomenclature: *Roegneria amurensis* (PI 547303) × *R. ciliaris* (88-89-227) – SF 53 %; combination *R. japonensis* (88-89-257) × *R. amurensis* (PI 547303) – SF 64 %.

These data leave a number of questions, but it is clear, that more profound researches of reproductive compatibility among differing morphotypes of the set of taxa are needed, which in our opinion should be attributed to the unified microevolutional complex *Elymus ciliaris* s. l.

Taxonomic proposals

We suggest return to earlier classification of the *Elymus* by Tzvelev (1972, 1976), where *E. ciliaris* and *E. amurensis* are considered as subspecies of *E. ciliaris* s. l. In this case it becomes possible to distinguish at least four varieties based on extreme forms according to expression of diagnostic traits.

Key for identification of the subspecies and varieties in *Elymus ciliaris* s. l.

Paleae are 1.2–2 mm shorter than lemmas (without awns), widely rounded at the top ***E. ciliaris* (Trin.) Tzvel. s. l.**

1. Lemmas more or less hispid, margin long ciliated along keels **2 *E. ciliaris* subsp. *ciliaris***

+ Lemmas glabrous or finely scabrous **3 *E. ciliaris* subsp. *amurensis* (Drob.) Tzvel.**

2. Leaf blade glabrous to scabrous **var. *ciliaris***
+ Leaf blade pubescent or pilose ... ***E. ciliaris* (Trin.) Tzvelev var. *pubifolius* A.V. Agaf. & Kobozeva var. nom. provis.**

3. Lemma's margin more or less ciliated **var. *amurensis* (Drob.) S.L. Chen**

+ Lemmas glabrous without cilia ... ***E. ciliaris* (Trin.) Tzvelev var. *glaberrimus* A.V. Agaf. & Kobozeva var. nom. provis.**

Hence, modern *E. amurensis* should be recognized in the previously suggested combination *E. ciliaris* var. *amurensis* (Drob.) C.L. Chen. (Chen 1997), and subsection *Ciliares* must be eliminated due to the inexpediency. Concerning the probable polyphyletic origination of different races of the complex, it is impossible to draw certain conclusions by using material from Russia only.

CONCLUSIONS

Traditionally the main distinctive traits between *E. ciliaris* and *E. amurensis* were the presence and type of trichomes on lemmas and the presence-absence of LB hairiness (both or only upper surfaces). However, the continuity of expression of both traits, i.e. the absence of evident hiatus on any diagnostical trait, does not allow to recognize the independence of both species. Both traits should be regarded as varying on the intraspecific level, although they don't depend from growth conditions. Results of comparative morphological analysis of specimens from different points of distribution area revealed, that all key traits of *E. ciliaris* and *E. amurensis* widely vary and occur in different combinations. Electrophoretic study of polymorphism of endosperm proteins has shown the absence of clear species specificity of *E. ciliaris* and *E. amurensis*. However, given the revealed characteristics of genotypic specificity, it is preferably to indicate some separateness of taxa, but only at the intraspecific level. This conclusion is supported by ISSR analysis and sequencing of the nuclear gene *GBSS1*.

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