

Monitoring of *Aegilops* L. local species genetic diversity of Kazakhstan's flora

R. Urazaliev¹, M. Yessimbekova¹✉, K. Mukin¹, A. Chirkin², G. Ismagulova²

¹ Kazakh Scientific Research Institute of Agriculture and Plant Growing, Almaty, Kazakhstan

² M.A. Aitkhozhin Institute of Molecular Biology and Biochemistry, Almaty, Kazakhstan

Cereal Crop Wild Relatives (CWR) are a very important gene pool for cereal/wheat improvement. New genes for resistance to diseases and pests are urgently needed to avoid using pesticides and to raise adaptivity to the environmental stresses caused by global climate change. In this regard, the study is aimed at *ex situ* conservation of *Aegilops* L. genus local ecotypes' genetic diversity, which is very relevant and promising for breeding. In order to establish breeding utility and form an *ex situ* collection reflecting the intra- and inter-specific diversity, the phenotypic screening of Kazakhstan's local populations of *Aegilops* L. genus (*Ae. cylindrica*, *Ae. tauschii*, *Ae. triuncialis* and *Ae. crassa*) was conducted on the basis of multiple indicators. For the first time molecular-genetic analysis of 50 representatives of *Aegilops* L. genus from Kazakhstan's flora was performed. The microsatellite analysis with the use of 11 EST-SSR markers revealed eight of them to be most effective. For each marker, allele frequency and average heterozygosity was calculated. For the most informative markers the presence of 5 and 6 respective allelic variations was found. A bank of genomic DNA was created and kept in *ex situ* storage (–70 °C, long-term) in the IMBB of the MES of RK.

Key words: *Aegilops* L.; local population; phenotyping; molecular genetic analysis.

Мониторинг генетического разнообразия местных популяций рода *Aegilops* L. флоры Казахстана

Р. Уразалиев¹, М. Есимбекова¹✉, К. Мукин¹, А. Чиркин², Г. Исмагулова²

¹ Казахский научно-исследовательский институт земледелия и растениеводства, Алматы, Казахстан

² Институт молекулярной биологии и биохимии им. М.А. Айтхожина, Алматы, Казахстан

Дикие сородичи – это таксоны диких растений, относящиеся к культурам, которые потенциально могут использоваться в качестве доноров генов многих полезных признаков, улучшая урожайность и/или качество, помогая избежать применения пестицидов и повышая адаптивность растений к экологическим стрессам в условиях глобального изменения климата. В этой связи актуальны исследования, направленные на сохранение и изучение генетического разнообразия местных экотипов рода *Aegilops* L. Проведен скрининг казахстанских местных популяций рода *Aegilops* L. (*Ae. cylindrica*, *Ae. tauschii*, *Ae. triuncialis*, *Ae. crassa*) для формирования коллекции *ex situ*, отражающей меж- и внутривидовое разнообразие. Мониторинг проведен на основе нескольких индикаторов для установления селекционной полезности (фенология, продуктивность, устойчивость к стрессам абиотического и биотического характера). Идентифицированы источники устойчивости к болезням, раннеспелости. Впервые выполнен молекулярно-генетический анализ 50 представителей рода *Aegilops* L. казахстанской флоры. Микросателлитный анализ выявил наиболее информативные EST-SSR маркеры (пять и шесть аллельных вариантов), была рассчитана частота аллелей и средняя гетерозиготность. Создан банк геномной ДНК для хранения *ex situ* (–70 °C).

Ключевые слова: *Aegilops* L.; местные популяции; фенотипирование; молекулярно-генетический анализ.

HOW TO CITE THIS ARTICLE:

Urazaliev R., Yessimbekova M., Mukin K., Chirkin A., Ismagulova G. Monitoring of *Aegilops* L. local species genetic diversity of Kazakhstan's flora. Vavilovskii Zhurnal Genetiki i Selektzii = Vavilov Journal of Genetics and Breeding. 2018;22(4):484-490. DOI 10.18699/VJ18.386

УДК 576:631.527(574)

Поступила в редакцию 12.12.2017

Принята к публикации 28.04.2018

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Crop Wild Relatives (CWR) are wild plant taxa related to crops that have a potential use as gene donors as they possess many beneficial traits conferring pest and/or disease resistance, improve yield or quality. These traits can be bred into cereal crops to meet changing environmental or market demands. CWR have greater intrinsic genetic diversity due to their wide adaptation to a variety of habitats (Maxted, Kell, 2009; Maxted et al., 2012, 2013). Wild species are unique genetic resources especially for corresponding cultivated species, but in general sense, even distantly related species can serve as good gene donors of target characteristics. Many populations and localized sub-specific taxa of CWR are severely endangered by human activities such as overgrazing, especially in arid regions and by industrial and construction activities. It must be a responsibility of each country to take measures for their sustainable conservation (Holubec, 2005, 2010a, b). CWR represent a very important gene pool for cereal improvement. New genes for resistance to diseases and pests are urgently needed to avoid using pesticides and to raise adaptivity to environmental stresses in connection with global climate change. *Aegilops* L. genus is closely related to the cultivated wheat (donor of B and D genomes) and has an important potential for improving wheat resistance to various biotic and abiotic stresses. Many regions in Kazakhstan, rich in CWR, are severely endangered by human activities. Local ecotypes of *Aegilops* L. genus are often used in Kazakhstan as a grazing culture that may lead to their complete destruction (Sitpaeva et al., 2004; Yessimbekova et al., 2004, 2017; Urazaliev et al., 2007; Alimgazinova, Yessimbekova, 2013). Long-term expeditions helped to collect more than

200 local ecotypes of *Aegilops* L. genus species. The lack of knowledge about the phenotypic and genetic diversity of the collected germplasm was a serious obstacle for its rational use in bread wheat programs and preservation *ex situ*. More comprehensive knowledge is required to identify the potential sources of their useful traits.

The present study investigates the diversity of four local species – *Ae. cylindrica*, *Ae. tauschii*, *Ae. triuncialis* and *Ae. crassa*. The results reveal the variation of agronomic traits, resistance to abiotic and biotic stresses, and genetic diversity of the collected germplasm. This data will be useful in future cultivation and breeding of domesticated wheat, especially where the goal is to increase adaptability of wheat in diverse environmental conditions. The goals of the study included: (1) phenotypic screening of agronomic traits diversity; (2) assessment for resistance to rust (yellow, brown, stem) on the artificial background; and (3) creation of genomic DNA bank for long term *ex situ* storage.

Materials and methods

Four provinces in North Kazakhstan, two provinces in West Kazakhstan, two provinces in South Kazakhstan, and South-Eastern Kazakhstan were surveyed for collecting the accessions. During the expeditions, a collection of more than 200 accessions of *Aegilops* L. was formed. The present study involves 50 accessions (local ecotypes) of four species *Ae. cylindrica*, *Ae. tauschii*, *Ae. triuncialis* and *Ae. crassa* from South and South-Eastern Kazakhstan. The geographical distribution of these four *Aegilops* L. species is shown in Fig. 1 (GPS data, MAPSOURCE program, USA).

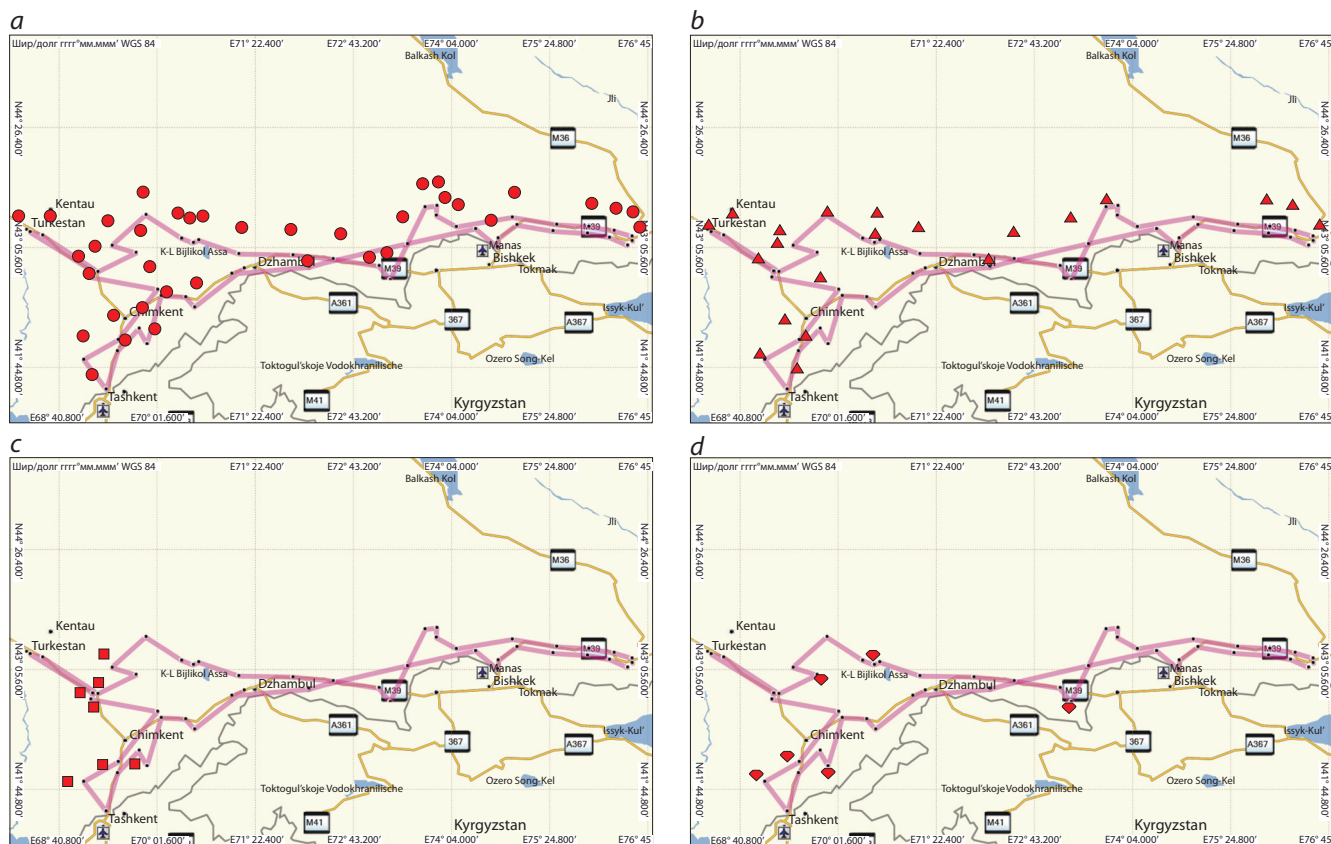


Fig. 1. Maps of four local *Aegilops* L. species distribution: a – *Ae. cylindrica*; b – *Ae. tauschii*; c – *Ae. crassa*; d – *Ae. triuncialis*.

Table 1. Sequences of EST-STS markers

Marker	Sequence (5'→3')	Annealing temperature, °C
PK1	F – GCCTTCGCCACCACTTC R – CACGACGTACAAGTACACATGC	53
PK3	F – TTGCTGAGGTGGTGTTCCT R – TAAGGGCTCCTCAAGCTCCT	51
PK5	F – GAGGCGGCCTACAAGAAGAT R – GAGGCGGCCTACAAGAAGAT	51
PK8	F – GCTAGCAACCAGAGACTCAC R – GTCGTCCTCGCTGTTGTTG	50
PK9	F – GTCCAGCTCTCGGTACTTGG R – TGCATCCAACAAGCCATGC	49
PK29	F – TACTAGAGGAGATGCAGACG R – TGGCAATAGCCTGAACCACT	52
PK18	F – AGACGGCGCATGGCGA R – AAGTCTACGCGGTGAGGT	49
PK31	F – GATGGCTCCCGTAAAGATCA R – TTTCCATTTTCGAAAGCAC	50
PK32	F – GCGAGGCTCAACCAAGAG R – GCAAGGCTGGCTGAATCTAC	52
PK34	F – CGCAAATCGCAACCTTATTT R – CCTTGGAGATCCACAGATCC	50
PK57	F – AATTGGAAAAGGTGGTGACG R – GTAACCTACCCTGGGGAAAA	50

Two species: *Ae. cylindrica*, *Ae. tauschii* were collected from 3 provinces of Kazakhstan – Almaty, Zhambyl, South-Kazakhstan, *Ae. triuncialis*, *Ae. crassa* were mainly collected from South-Kazakhstan province at various altitudes: *Ae. cylindrica* – 198–2200 m above sea level, *Ae. tauschii* and *Ae. triuncialis* – 223–934 m, *Ae. crassa* – 214–710 m. All plant accessions were maintained by the Department of Field Crop Genefund, Kazakh Scientific Research Institute of Agriculture and Plant Growing. The specimens representing each accession were grown in the experimental field (the foothill zone of the Zailiysky Alatau – 48° N, 77° E, 740 m above sea level) with the number of days with temperatures below 0 °C ranged from 125 to 130 and the average perennial precipitation of 360–400 mm. The soil cover of the experimental plot was light chestnut soils.

For phenotyping the protocols and technical guidelines of management in the field were used (Prescott et al., 2002; Engels, Visser, 2003; Reed et al., 2004). Such parameters as height of plants, spike length, number of spikelets, number of kernels and spike density were determined on randomly selected spikes from healthy mature plant specimens of each accession. The height of plants was measured from the ground to the top of the terminal spikelets on the spike (excluding awns), the spike length – from the base of the lowermost spikelet to the top of a spike excluding awns. The spike density was calculated from the number of spikelets divided by the spike length. Data on heading time were recorded according to the international methodology (from 01.01.), when approximately

50 % of the plants in a plot showed spikes. Winter hardiness was calculated as the percentage of plants having survived in the spring after the last date of a possible winter kill report. Evaluation of the collections for leaf rust resistance was carried out using the modified Cobb scale (Peterson et al., 1948), where R marked resistance, MR – moderate resistance, MS – moderate susceptibility, S – susceptibility.

The genomic DNA from five- or seven-day seedlings was isolated according to the protocol of Thermo Scientific (EU) DNA isolation kit. The purity of the preparations was checked by electrophoretic separation in 1 % agarose gel and by spectrophotometry. A genomic DNA bank was put for *ex situ* storage (–70 °C, long-term). For molecular genetic analysis microsatellite markers (SSR) that had previously provided good results in the phylogeny of the genera *Triticum* and *Aegilops* were used (Bandopadhyay et al., 2004). PCR with STS primers was performed in a mixture containing 1 unit of HotTaq polymerase (Sileks), 4 pmol of forward and reverse primers, 200 μM each of dNTP and 100 ng of DNA. The PCR conditions were as follows: 95 °C – 5 min, 94 °C – 1 min, 49–53 °C (depending on the primer sequence, see Table 1) – 1 min, 72 °C – 1 min, repeated for 35 cycles, the last elongation – 5 min at 72 °C. Primers of the given sequence were synthesized in the ASM 800 machine (Biosset, Russia) using the phosphoimide method according to the Manufacturer's Instruction (Manual of oligonucleotides synthesizer ASN800. Novosibirsk, Russia, "Biosset", 2002). Sequences of the primers used in the study are presented in Table 1.

The amplification reaction products were fractionated in 10 % polyacrylamide gel (PAGE). Electrophoresis was performed at the voltage of 10 V/cm in 1×TBE buffer (50 mM Tris-H₃BO₃, 2 mM EDTA, pH 8.0).

Results

Winter hardiness is the main adaptive feature of winter crops highly correlating with productivity (Grabovets, Fomenko, 2015; Gorash et al., 2017). Depending on the species and the site of collection, germination in autumn sowing ranged from 32 % (*Ae. tauschii*) to 73 % (*Ae. triuncialis*), the winter hardiness varied from 10 to 100 %, see Fig. 2. High winter hardiness (up to 100 %) marked 45.5 % of *Ae. triuncialis* accessions and 38.5 % of *Ae. tauschii* accessions. 14 accessions with high level (80–100 %) of winter hardiness were selected: 2 accessions of *Ae. cylindrica* (T. Ryskulov, Sairam districts); 5 accessions of *Ae. tauschii* (T. Ryskulov, Saryagash, Arys, Otrar districts, Taldykorgan); 5 accessions of *Ae. triuncialis* (T. Ryskulov, Sairam, Bayzak, Talas, Jualinsky districts); 2 accessions of *Ae. crassa* (Ordabasinsky, Shardara districts).

Boxplots for six agronomical traits of *Ae. cylindrica*, *Ae. tauschii*, *Ae. triuncialis* and *Ae. crassa* are shown in Fig. 3 and 4. In each boxplot, the lower and upper boundaries of the box indicate 25 and 75 percentiles, the line within the box indicates the median, the lower and upper whiskers – the minimum and maximum within 1.5 times range of the box, the circles – the data points outside 1.5 times range of the box.

Vegetation period is one of the plants' basic biological properties that determine their suitability for environmental conditions. The vegetation period, in particular, the speed of development up to heading is associated with many properties that determine the reaction of plants to frost and drought, rust,

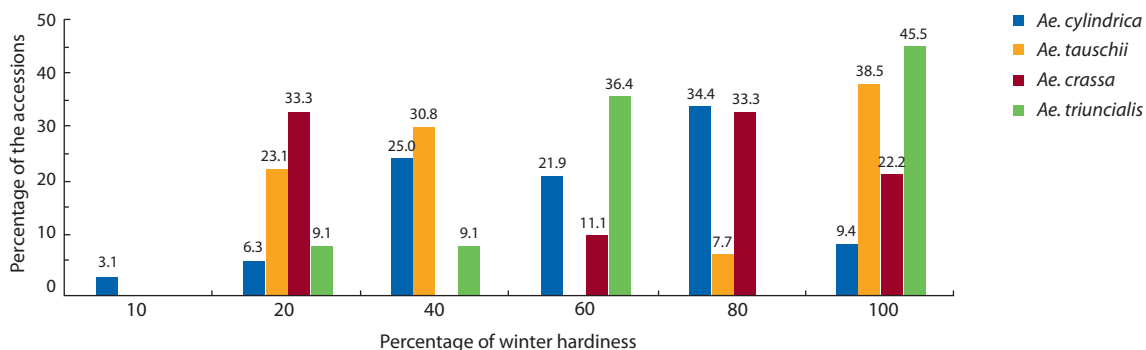


Fig. 2. The range of winter hardiness variability of Kazakhstan *Aegilops* L. species.

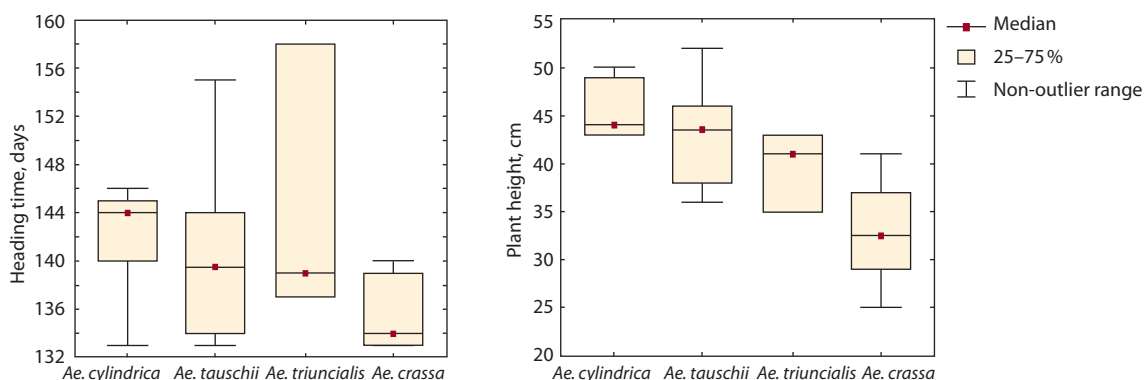


Fig. 3. Boxplots for 2 agronomical traits (heading time and plant height) in *Ae. cylindrica*, *Ae. tauschii*, *Ae. triuncialis* and *Ae. crassa*.

insect damage, productivity and grain quality (Kamran et al., 2014; Likhenko et al., 2014; Chen et al., 2016).

Depending on the species and the site of collection, significant intra- and inter-specific differences were observed in heading time. The range of variability between the variants is marked as 7 days (133–140) for *Ae. crassa*; 13 days (133–146) for *Ae. cylindrica*; 21 days (137–158) for *Ae. triuncialis*; 22 days (133–155) for *Ae. tauschii* (see Fig. 3). There were selected early headed (133–135 days) 8 accessions from *Ae. cylindrica*, *Ae. crassa* from Zhambyl, South Kazakhstan and *Ae. tauschii* from the Almaty province. Early headed accessions of *Ae. tauschii* from the Sarkand and Taldykorgan districts of the Almaty province were clearly separated from *Ae. tauschii* (155 days) accessions from the Baizak, Merken and T. Ryskulov districts of the Zhambyl province.

The plant height of local populations in most cases was presented by dwarf accessions: *Ae. tauschii* (35–46 cm), *Ae. cylindrica* (43–50 cm), *Ae. crassa* (25–41 cm) and *Ae. triuncialis* (41–52 cm) (see Fig. 3).

The length of the spike varied from short to very long (5–14 cm) (see Fig. 4). According to the length of the spike, all accessions were divided into 2 groups. A short spike (5–7 cm) was registered in *Ae. triuncialis* and *Ae. tauschii*; long (10–12 cm) and very long (≥ 14 cm) length of the spike in three species: *Ae. cylindrica* (Almaty, Zhambyl, South Kazakhstan provinces), *Ae. tauschii* (Almaty province) and *Ae. crassa* (Zhambyl, South Kazakhstan provinces). *Ae. cylindrica* was characterized by high intra-specific variation of spikelet number (5–13 pcs.) and spike density (0.5–1.1) (see Fig. 4).

The average number of spikelets per spike were: *Ae. cylindrica* – 8.8 pcs., *Ae. tauschii* and *Ae. crassa* – 8.2 pcs., *Ae. triuncialis* – 3.3 pcs. A relatively high number of spikelets per spike (13.0 pcs.) with a spike length of 12.0 cm was a characteristic for *Ae. cylindrica* species, collected in the Almaty province (spike density was 1.1). The average number of kernel per spike was generally low with intra-specific variations, depending on the site of collection: from 4 pcs. (*Ae. triuncialis*, Zhambyl province) to 14 pcs. (*Ae. tauschii*, Almaty province) (see Fig. 4). Phenotypic monitoring using an optical sensor “Greenseeker” showed that the accessions had a low NDVI biomass index – from 0.23 to 0.34, which is explained by their low productivity.

Rust fungi of wild cereals, as well as their host plants, are distinguished by a great variety of species. Wild relatives, in particular *Aegilops* L. genus can serve as sources and donors of wheat resistance. The introgression of rust resistance genes from wild species-relatives into commercial varieties is one of the genetic methods for increase of the bread wheat resistance (*Triticum aestivum* L.) (Koishibayev, 2002). Though a large number of resistance genes are available for breeding, the search for novel sources of resistance is of particular importance because of the changing virulence in the population of the pathogen as stressed by R.A. McIntosh and Z.A. Pretorius (2011). Susceptibility (MS – 40 %) to leaf rust was noted for the accessions of *Ae. cylindrica* from 2 districts (T. Ryskulov, Merken) of the Zhambyl province and *Ae. crassa* from the Ordabasy district of South Kazakhstan. Resistance to leaf rust on an artificial background was recorded for the accessions

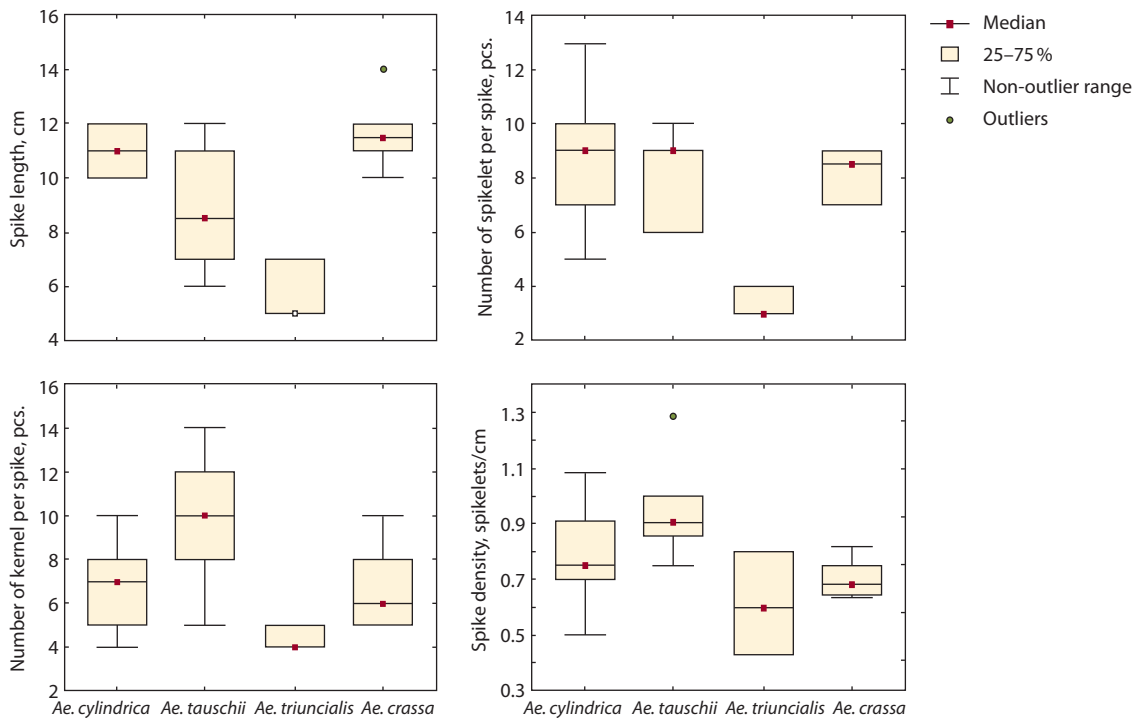


Fig. 4. Boxplots for 4 agronomical traits (spike length, number of spikelets per spike, number of kernel per spike and spike density) in *Ae. cylindrica*, *Ae. tauschii*, *Ae. triuncialis* and *Ae. crassa*.

of *Ae. triuncialis*, *Ae. tauschii*, *Ae. cylindrica* (Saryagash and Sayram districts) and *Ae. crassa* (Shardara and Sayram districts) from South Kazakhstan (R – 0–5 %).

Development and improvement of the methods of molecular genetic analysis opens up new opportunities for study of the population structure and the principles of spatial distribution of wild plant species. Polymorphism analysis of different sections of genomic DNA enables one to identify intraspecific genetic diversity, to reconstruct phylogenetic relationships between species and spatial relationships between populations, and also assess the well-being of the population and its ability to withstand adverse external influences, including anthropogenic ones. Molecular genetic studies make it possible to assess the level of DNA polymorphism in populations, intra-population genetic diversity, to establish the degree of genetic similarity between species, populations and individuals directly at the genetic level, in some cases to assess the heterozygosity of the population, and to study genetic variability in individual loci and gene alleles. Analysis of 50 accessions of local and foreign ecotypes of *Aegilops* spp. was carried out to assess the use of EST-SSRs microsatellite repeats, characterized by localization only in the coding part of the genome of the studied organisms. As shown by R. Bandopadhyay et al. (2004), these microsatellite markers are highly conserved for *Triticeae* and can be used in study of the genetic diversity of both intraspecific and intergeneric relationships between certain species of the same tribe. From the 64 EST-SSR markers, eleven were selected for our study: PK1, PK3, PK5, PK8, PK9, PK18, PK29, PK31, PK32, PK34, and PK57. The analysis of the informativeness and, thus, the justification of use of a specific marker in the future, was determined by statistical processing of the results for all the studied accessions as a whole. Of the eleven mark-

ers, eight were effective and provided the amplicons of the right size. The results of DNA amplification with markers PK3, PK8 and PK57 were difficult to interpret. For the rest, allelic polymorphic variants, frequency of occurrence for each of them separately, heterozygosity for each marker separately, and the average heterozygosity for the studied group were established. The most informative of all the studied markers were PK1 and PK5. So, for the PK1 marker the presence of five allelic variants was shown, and six were identified for PK5. Four alleles were detected for the PK18, PK31 and PK32 markers. For the remaining PK9 and PK29, PK34 markers, the presence of three and two alleles was demonstrated. High heterozygosity was detected for PK1 and PK18. It was found that 56 % of the accessions were heterozygous for the PK1 marker and 68 % – for PK18. This index for the other markers did not exceed 34 %. The PK29 and PK34 markers in the examined group showed only the presence of homogeneous accessions. In the studied group of plants, the average number of alleles was 2.73 per locus and the average heterozygosity reached 20 %. The frequency of occurrence, calculated for each allele, is presented in Table 2. The presence of three allelic variations in the PK9 marker was established in most accessions of *Ae. triuncialis* and *Ae. crassa* species. These species are characterized by hexaploid DMS genome for *Ae. triuncialis* species and DCDM for *Ae. crassa* (<http://herbarium.usu.edu/triticeae/genomesaegilops.htm>, circulation date 10.09.2016). Perhaps, the gene from which PK9 was developed is present in all three genomes of these species. Thus, it was established that the studied group of wild representatives of *Aegilops* genus was characterized by the presence of more variants of alleles in many of the studied markers, in comparison with the results obtained for *Ae. cylindrica* and

Table 2. Allele frequencies of polymorphic loci for all studied accessions

Marker	Allele	Frequency of occurrence
PK1 $H_o = 0.56$	1	0.3
	2	0.36
	3	0.04
	4	0.25
	5	0.05
PK5 $H_o = 0.18$	1	0.1
	2	0.46
	3	0.07
	4	0.27
	5	0.06
	6	0.04
PK9 $H_o = 0.32$	1	0.1233
	2	0.5533
	3	0.3234
PK18 $H_o = 0.68$	1	0.16
	2	0.47
	3	0.19
	4	0.18
PK29 $H_o = 0$	1	0.489
	2	0.511
PK31 $H_o = 0.16$	1	0.14
	2	0.17
	3	0.61
	4	0.08
PK32 $H_o = 0.34$	1	0.28
	2	0.5
	3	0.15
	4	0.07
PK34 $H_o = 0$	1	0.58
	2	0.32
$H_{ave} = 0.2; H_{ave exp} = 0.428$		

Note: H_o – heterozygosity of the locus, H_{ave} – average heterozygosity, $H_{ave exp}$ – average expected heterozygosity.

Ae. tauschii species. The revealed difference is most likely caused by high heterogeneity in the species, genomic and geographic features of the accessions.

Discussion

Agronomic traits. Wild plants have a capacity to adapt to environmental conditions. Phenotypic analyses revealed a significant variation within and between the populations. Since the heading time is an important adaptive trait in wild species, it remains possible that there is some relationship between geographical distribution and suitability for environmental conditions (Kato et al., 1998; Kooyers, 2015). In the present study, differences in the heading time were observed and it was assumed that these variations had been shaped in response to differences in the habitat environments of each species. Heading time coincide with the general strategy of adaptation to dry

conditions observed frequently in annual plants. Therefore, candidates for the drought tolerance traits can be selected from early heading (133–135 days) of the *Ae. cylindrica*, *Ae. tauschii* and *Ae. crassa* accessions that grow in areas with lower rainfall (South Kazakhstan province). These species could be useful as genetic resource for creating varieties that tolerate to the drought. In contrast, the *Ae. tauschii* accessions from Baizak, Merken, T. Ryskulov districts of the Zhambyl province had a longer heading time (155–158 days). The *Ae. cylindrica* and *Ae. tauschii* accessions from the same geographical region (Zhambyl province) were differed on heading time. The difference in a sympatric area could promote divergence of the species (i. e. act as a lead speciation event (Ohta et al., 2017)). This suggests that difference on heading time for *Ae. cylindrica* and *Ae. tauschii* reflects genetic differences between the two species rather than a phenotypic response to the environment. The heading time in these species will also improve the understanding of similar processes in wheat. The understanding of genetic control of such adaptation will be useful for future attempts to breed high stress-tolerant varieties of agriculturally-important crops, such as wheat (Bandou et al., 2009).

In the present study, clear differences were detected in agronomic traits (see Fig. 2–4). Accessions were distinguished successively by their spike length, number of spikelets per spike, number of kernel per spike, and spike density. The positive correlations indicating the possibility of using these species in wheat breeding was identified. The heading time was positively correlated in *Ae. triuncialis* with: (1) the number of spikelets per spike and the number of kernel per spike ($r = 0.89$); in *Ae. crassa* with: (1) number of kernel per spike ($r = 0.84$); (2) the length of the spike ($r = 0.56$). Plant height of *Ae. tauschii* highly correlated with: (1) the number of spikelets per spike ($r = 0.84$); (2) the number of kernel per spike ($r = 0.73$), and (3) the length of the spike ($r = 0.51$). The length of the spike had a high positive correlation with: (1) the number of spikelets per spike ($r = 0.82$) – *Ae. cylindrica*; ($r = 0.65$) – *Ae. crassa*; ($r = 0.76$) – *Ae. tauschii*; (2) the number of kernel per spike ($r = 0.84$) – *Ae. cylindrica*; ($r = 0.93$) – *Ae. tauschii*; ($r = 0.69$) – *Ae. crassa*.

Thus, by the conducted investigations, it was formation of *ex situ* collection of the *Aegilops* L. genus local populations reflecting the intra- and inter-population diversity. The sources of early ripeness, winter hardiness and resistance to diseases were identified.

Molecular-genetic analysis. The microsatellite analysis was carried out using 11 EST-SSR markers (PK1, PK3, PK5, PK8, PK9, PK18, PK29, PK31, PK32, PK34, and PK57). Of all these markers, eight markers were effective. For each marker, allele frequency and heterozygosity were calculated. The mean number of alleles was determined to be 2.73, and the average heterozygosity was 20 % for the studied group. The most informative of all the studied markers were PK1 and PK5. So, for PK1 marker, the presence of 5 allelic variants was demonstrated, and six were identified for PK5. The remaining markers showed the presence of 4 to 2 alleles. For hexaploid accessions of *Ae. crassa* species with PK9 marker three simultaneous allelic variants were established. The differences may enhance species divergence and could represent a lead speciation event. The results of this study will facilitate identification of populations or accessions of

wild wheat with favorable traits and/or novel adaptive genes. A bank of genomic DNA was created and put for *ex situ* storage (-70°C , long-term) at the Institute of Molecular Biology and Biochemistry of the MES of the RK. Further studies on these traits in the complex will provide new insight into species differentiation and diversification.

Conflict of interest

The authors declare to have no conflict of interest.

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