Chromosome and molecular analyses reveal significant intraspecific karyotype diversity and provide new evidence on the origin of tetraploid grass *Aegilops columnaris*

Ekaterina D. Badaeva1,2*, Nadezhda N. Chikida3, Andrey N. Fisenko1, Sergei A. Surzhikov2, Maria Kh. Belousova3, Hakan Özkan4, Alexandra Yu. Dragovich1, and Elena Z. Kochieva5

1 – N.I.Vavilov Institute of General Genetics, Russian Academy of Sciences. Gubkina street 3, GSP-1, Moscow 119991, Russia
2 – Engelhardt Institute of Molecular Biology, Russian Academy of Sciences. Vavilova street 34, GSP-1, Moscow 119991, Russia
3 – Federal Research Center, N.I. Vavilov All-Russian Institute of Plant Genetic Resources, Bolshaya Morskaya street 44, St. Petersburg 190121, Russia
4 – Department of Field Crops, Faculty of Agriculture, University of Çukurova, 01330 Adana, Turkey
5 – Federal Research Center "Fundamentals of Biotechnology" of the Russian Academy of Sciences. 60 let Oktyabrya prospect 7, build. 1, Moscow 117312, Russia
* - corresponding author: Badaeva Ekaterina D., e-mail: katerinabadaeva@gmail.com; tel: +7 499 135-0460; Fax: +7 499 132 8962

Abstract:

*Aegilops columnaris* Zhuk. is tetraploid grass species (2n=4x=28, UcUcXcXc) closely related to *Ae. neglecta* and growing in Western Asia and a western part of the Fertile Crescent. Genetic diversity of *Ae. columnaris* was assessed using C-banding, FISH, nuclear and chloroplast (cp)DNA analyses, and gliadin electrophoresis. Cytogenetically *Ae. columnaris* was subdivided into two groups, C-I and C-II, showing different karyotype structure, C-banding and FISH patterns. Group C-I was more similar to *Ae. neglecta*. All types of markers revealed significant heterogeneity of the C-II group, although group C-I was also polymorphic. Two chromosomal groups were consistent with plastogroups identified in a current study based on sequencing of three chloroplast intergenic spacer regions. The similarity of group C-I of *Ae. columnaris* with *Ae. neglecta* and their distinctness from C-II indicate that divergence of the C-I group was associated with minor genome modifications. Group C-II could emerge from C-I relatively recently, probably due to introgression from another *Aegilops* species followed by a reorganization of the parental genomes. Most C-II accessions were collected from the very narrow geographic region, and they might originate from a common ancestor. We suggest that the C-II group is at the initial stage of species divergence and undergoing an extensive speciation process.

Key Words: *Aegilops columnaris*, *Ae. neglecta*, C-banding, FISH, gliadin electrophoresis, sequencing, U31 nuclear DNA fragment, *trnH*(gtg)-*psbA*, *trnT*(ugu)-*trnL*(uaa), and *rpL32-trnL*(tag) spacers regions of the chloroplast DNA; plastogroups, evolution
Introduction

*Aegilops columnaris* Zhuk. is an annual tetraploid (2n=4x=28) grass species naturally growing in Western Asia, mainly in Turkey, Armenia, and in a western part of the Fertile Crescent [1-3]. It is also native to Crete, Iraq, Lebanon, Azerbaijan, and Iran but found as adventive species in France, near Marseille [1]. Despite a relatively broad distribution area, *Ae. columnaris* is uncommon throughout its range. Biodiversity Collecting Mission Database included 816 *Ae. columnaris* site records (https://www.gbif.org/), and according to Genesys, 763 accessions are currently maintained in gene banks worldwide (https://www.genesys-pgr.org/). This number, however, can be overestimated owing to a large number of potentially duplicated and incorrectly classified accessions. On another side, many new sites were recently discovered during collection missions. However, the novel samples (e.g., reported in [4] or materials analyzed in a current study) were not included in these databases.

*Ae. columnaris* was first collected on the Ghalat plateau close to Ankara and on the slopes of Dizgurt-Dagh mountains, Turkey, by the Russian botanist P.M. Zhukovsky during expeditions of 1925-1927 to Asia Minor [5]. Since then, this species was found in other locations, mainly in Turkey, Syria, and Transcaucasia, but also in Lebanon, Iraq, Iran, and Aegean Greece (Rodos, Crete) [1, 6, 7]. *Ae. columnaris* grows in dry fields, roads, and hillsides [1, 5], mainly on limestones, rarer on basalts on more wetter environments than most *Aegilops* L. species [1, 2]. In most locations, *Ae. columnaris* is found together with other *Aegilops* species, often in a mix with *Ae. neglecta* Req. ex Bertol., *Ae. biuncialis* Vis., *Ae. peregrina* (Hack. in J.Fraser) Maire & Weiller, or *Ae. triuncialis* L. [8].

*Aegilops columnaris* is known to be closely related to tetraploid *Ae. neglecta* [9-15], but the origin of these two species was a subject of long debates and is still not clear. Analysis of meiotic chromosome pairing in intraspecific hybrids [15-18], comparison of karyotype structure [19] and C-banding patterns [13, 20], molecular analysis on nuclear [11, 12] and cytoplasmic DNAs [21, 22] showed that one of the *Ae. columnaris* and *Ae. neglecta* genomes was contributed by the diploid species *Ae. umbellulata* Zhuk. (2n=2x=14, UU). Comparative sequence analysis of the nuclear U-genome specific U31 fragment in 48 accessions of each *Ae. columnaris* and *Ae. neglecta* in comparison with 72 accessions of *Ae. umbellulata* allowed to suggest that the U-genomes of *Ae. columnaris* and *Ae. neglecta* may have multiple origins [23]. Cytoplasmic genomes of *Ae. columnaris* (U\(^2\)) and *Ae. neglecta* (U) are also similar to the cytoplasmic genome of *Ae. umbellulata* (U), indicating that *Ae. umbellulata* was the maternal parent of these tetraploid species [22, 24].

The source of the second genome of *Ae. columnaris* and *Ae. neglecta* is still unknown. H. Kihara [17] suggested that it could be related to the M-genome of *Ae. comosa* Sm. in Sibth. & Sm. based on morphological comparisons and analysis of meiotic chromosome pairing in *Ae. columnaris* x *Ae. biuncialis* (2n=4x=28, UUMM) hybrids. He designated this genome as “modified M,” and this symbol is still used in most taxonomical systems [1, 2, 10, 16, 17, 19, 25]. However, the F\(^1\) hybrids of *Ae. columnaris* x *Ae. comosa* exhibited low chromosome pairing [15]. Differences in the patterns of variation of the repetitive nucleotide sequences [11, 26], RAPD-spectra [9], the results of DArTseq-based analysis [12], comparison of karyotype structures [19], C-banding and FISH-patterns [13, 20] contradicted this hypothesis. Taking into consideration the distinctness of *Ae. columnaris* genomes J. Dvořák [26] suggested to change its genome formula from the UM to UX\(^1\). More recent data of DArTseq-based analysis revealed higher similarity of the second genome of *Ae. columnaris* and *Ae. neglecta* with the genome of
**Ae. speltoides** Tausch or **Ae. mutica** Boiss. [12], therefore, a new genomic formula, UTs. was proposed for these tetraploid species.

In the previous publication [13], we uncovered the significant karyotype diversity of **Ae. columnaris**, which was expressed in a variation of the C-banding patterns and, despite a small number of accessions studied, translocation polymorphism. In this paper, however, the translocations were classified tentatively due to the lack of standard genetic nomenclature of **Ae. columnaris** chromosomes. The problem of chromosome classification was solved later when a set of wheat-**Ae. columnaris** (K-1193) introgression lines was developed and cytogenetically characterized [20].

These introgression lines also enabled the identification of gliadin components encoded by particular **Ae. columnaris** chromosomes [27]. Although extensive polymorphism of electrophoretic spectra of gliadins was demonstrated for durum and common wheat [28], these markers are broadly exploited for wheat cultivar identification [29] diversity of gliadin profiles of *Aegilops* species, including **Ae. columnaris**, is much lesser studied. Publications were mainly focused on **Ae. tauschii**, the D-genome donor of common wheat [30, 31], and only a few papers described other *Aegilops* species [32-34].

The aim of the present study was the analysis of intraspecific diversity of **Ae. columnaris** on a broader sample of accessions using cytogenetic (C-banding, FISH with various DNA probes), biochemical (seed storage proteins - gliadins), and molecular (comparative sequence analysis of nuclear and chloroplast DNA fragments) markers.

**Results**

**C-banding analysis of **Ae. columnaris****

We showed that most of **Ae. columnaris** accessions were karyotypically uniform, but two, K-4224 and a sample provided by Drs. E.A. Nazarova and A.G. Gukasyan in 1998 consisted each of three distinct biotypes, while PI 554187 – of two biotypes (Table 1).

**Table 1. The list of material studied and their origins**

| No | Accession # | Duplicates | Country of origin | Collection site | Latitude (N) | Longitude (E) | Alt. (h, m) | Analyzed by FISH |
|----|-------------|------------|-------------------|-----------------|--------------|--------------|------------|-----------------|
| 1  | K-1178      | -          | Armenia           | Aznaburt village, near Dash Agl mountain | 39.4333      | 45.2833      | 1600       | -               |
| 2  | K-1193      | -          | Armenia           | Abovyan, near village of Shor-Bulakh     | 40.1333      | 45.6333      | 1300       | +               |
| 3  | K-1495      | IG 48026   | Armenia           | Naxchivian, Dzulifskii reg., N of Arbakunis vil. | 39.1167      | 45.6333      | 1350       | -               |
| 4  | K-1512      | AE 1188    | Armenia           | near Erevan, valley of Razdan river      | 40.2         | 44.5333      | 136        | -               |
| 5  | K-2344      | -          | Armenia           | Ekhegnadzor reg., along Elpin-Agavnadzor road | 39.7833      | 45.1833      | 776        | -               |
| No | Accession # | Duplicates | Country of origin | Collection site | Latitude (N) | Longitude (E) | Alt. (h, m) | Analyzed by FISH |
|----|-------------|------------|-------------------|-----------------|--------------|---------------|-------------|-----------------|
| 6  | K-4224 (3)  | IG 48738   | Armenia           | Erevan region, 2 km SE of Jrvezh | 40.1667      | 44.6          | 780         | -               |
| 7  | k-4228      | IG 48757   | Armenia           | Shorap, 20 km W of Erevan | 40.25        | 44.3333       | 1350        | -               |
| 8  | K-4229      | IG 126249  | Armenia           | Abovyan reg., Erebuni Natural Reserve | 40.2833      | 44.6333       | 1072        | -               |
| 9  | K-4225      | IG 48740   | Armenia           | Outskirt NE Erevan | 40.1167      | 44.5167       | 1400.0      | -               |
| 10 | K-4366      | IG 48745   | Armenia           | Abovyan reg., NE of Erevan, N. Dzervesh; Gegadir; Muchavan | 40.2833      | 44.6333       | 1045        | -               |
| 11 | K-4551      | -          | Armenia           | Vanadzor, after Gadzor | 39.7833      | 45.3667       | 363         | -               |
| 12 | K-564       | -          | Armenia           | Azizbekovskii reg., around vil. Khandzorut | 39.55        | 45.35         | 1685        | +               |
| 13 | NAZ (3)     | -          | Armenia           | near the village of Urznadzor | -            | -            | -           | -               |
| 14 | PI 499258   | -          | unknown           | obtained from China | -            | -            | -           | -               |
| 15 | PI 574457   | K-512; AE111 | Azerbaijan      | unknown          | -            | -            | -           | -               |
| 16 | IG 48818    | -          | Iran              | Damavand         | 35.7333      | 52.0667       | 2474        | -               |
| 17 | K-4240      | IG 49138   | Iran              | 10 km SW Horand from Ahar | 38.75        | 47.1667       | 1110        | -               |
| 18 | K-4413*     | IG 49087   | Iran              | 20 km W Takestan to Zia Abad road to Zanjan | 36.0333      | 49.5          | 1320        | -               |
| 19 | K-4418      | IG 49107   | Iran              | 31 km Urumiyeh to Oshnaviyeh Kazem Lo Valley | 37.25        | 45.1333       | 1380        | -               |
| 20 | K-3899†     | IG 49010   | Iraq              | Ninawa; Jebel Maqloob near Deir Matti | 36.5         | 43.4167       | 850         | -               |
| 21 | IG 49067    | -          | Lebanon           | Rachaiya, 1 km E of Aita Al Foukhar | 33.6333      | 35.9          | 1350        | -               |
| No | Accession # | Duplicates | Country of origin | Collection site | Latitude (N) | Longitude (E) | Alt. (h, m) | Analyzed by FISH |
|----|-------------|------------|-------------------|----------------|--------------|---------------|------------|----------------|
| 22 | K-4003      | IG 48072   | Lebanon           | Terbol region W slope Anti Lebanon Mts. (zone A) | 33.9         | 36.1          | -          | -              |
| 23 | K-4004      | IG 48091   | Lebanon           | Sanin region E slope Lebanon Mts. (zone B) | 33.9333      | 35.8333       | -          | -              |
| 24 | K-4007      | IG 48107   | Lebanon           | Irsal region; W slope Anti Lebanon Mts. (zone C) | 34.25        | 36.6667       | 0          | -              |
| 25 | K-4406      | IG 49047   | Lebanon           | Baalbek 4 km W Baalbek road to Bcharre laat village | 34.0333      | 36.1667       | 1050       | -              |
| 26 | K-4241a     | i-611188   | Lebanon           | Sanin region E slope Lebanon Mts. (zone B) | 33.9333      | 35.8333       | -          | -              |
| 27 | K-4241b     | i-611189   | Lebanon           | Al Alia; 40 km N of Karak | 31.95        | 35.9333       | 800        | -              |
| 28 | K-4407      | IG 49047   | Lebanon           | Baalbek 4 km W Baalbek road to Bcharre laat village | 34.0333      | 36.1667       | 1050       | -              |
| 29 | K-4409      | IG 49053   | Lebanon           | 3 km from Deir Ahmar road to Ain Ata | 34.1333      | 36.1          | 1370       | -              |
| 30 | K-2680      | PI 487198  | Syria             | 7km from Atareb to Qalaat Samaan, Aleppo | 36.2022      | 36.7758       | 460        | -              |
| 31 | K-4009      | i-571713   | Syria             | Al Hasakah; just N of Jabal Abd El-Aziz | 36.4667      | 40.3333       | 600        | +              |
| 32 | K-4362      | IG 48729   | Syria             | Damascus May Saloun; 4 km before Tukeya | 33.6         | 36.0667       | 1468       | -              |
| 33 | K-4372      | IG 48800   | Syria             | 2 km NE of Sa'an road from Shabki | 32.7         | 36.8417       | 1400       | -              |
| 34 | PI 487196   | -          | Syria             | Aleppo Province | 36.1667      | 36.8333       | 450        | -              |
| 35 | Clae 34     | -          | Turkey            | -              | -            | -            | -          | -              |
| 36 | K-4002      | IG 47875   | Turkey            | 14 km NW Keskin | 39.7167      | 33.4333       | 520        | -              |
| No | Accession # | Duplicates | Country of origin | Collection site | Latitude (N) | Longitude (E) | Alt. (h, m) | Analyzed by FISH |
|----|-------------|------------|-------------------|-----------------|--------------|---------------|------------|----------------|
| 37 | #1          | 2006-6-25-8-2 | Turkey            | 132 km NW from Nevşehir | 39.1622      | 33.9325       | 1060       | -              |
| 38 | #10         | 2006-6-21-5-1 | Turkey            | 50 km NE from Kilis to Gaziantep | 37.315       | 37.7347       | 510        | -              |
| 39 | #2          | 2006-6-17-7-2 | Turkey            | 29 km NE from Kilis to Gaziantep | 36.9242      | 37.0786       | 730        | -              |
| 40 | #3          | 2006-6-21-12-1| Turkey            | 57 km NE from Kilis to Gaziantep | 37.2678      | 37.5208       | 700        | -              |
| 41 | #4          | 2006-7-12-2    | Turkey            | 34 km NE from Kilis to Gaziantep | 37.09667     | 37.0406       | 970        | -              |
| 42 | #6          | 2006-6-21-9-1  | Turkey            | 54 km NE from Kilis to Gaziantep | 37.3739      | 37.8458       | 700        | -              |
| 43 | #7          | 2006-6-17-10-2 | Turkey            | 32 km NE from Kilis to Gaziantep | 36.9944      | 37.9664       | 950        | -              |
| 44 | #8          | 2006-6-25-6-3  | Turkey            | 114 km NW from Nevşehir | 39.0442      | 34.0144       | 880        | -              |
| 45 | #9          | 2006-6-21-1-2  | Turkey            | 46 km NE from Kilis to Gaziantep | 37.2139      | 37.4903       | 831        | -              |
| 46 | i-570045    | PI 554184     | Turkey            | Kars, 5 km S Sivas Malatya border | 39.7333      | 37.05         | 1500       | +              |
| 47 | PI 276968   | -            | Turkey            | Konya            | 37.8333      | 32.5          | -          | -              |
| 48 | PI 486281   | IG 46886     | Turkey            | 42km southeast of Ercis-Karayolları Bakimevi Latitude | 38.9167      | 43.6          | 1700       | +              |
| 49 | PI 542171   | -            | Turkey            | 19 km north of Gaziantep toward Yavuzeli | 37.1833      | 37.4667       | 800        | +              |
| 50 | PI 542191t  | -            | Turkey            | Aegean Agric. Research Inst. Gene Bank, Menemen | -            | -            | 30         | +              |
| 51 | PI 554178   | IG 47040     | Turkey            | 22 km north of Van | 38.7         | 43.3333       | 1734       | +              |
| 52 | PI 554180   | IG 46997     | Turkey            | 35 km west of Tuzluca | 40.15        | 43.3667       | 1010       | -              |
| 53 | PI 554181   | IG 47042     | Turkey            | Aydin, 10 km north of Kusadasi | 37.9167      | 27.2833       | 130        | +              |
| No | Accession # | Duplicates | Country of origin | Collection site | Latitude (N) | Longitude (E) | Alt. (h, m) | Analyzed by FISH |
|----|-------------|------------|-------------------|-----------------|-------------|--------------|------------|-----------------|
| 54 | PI 554182\(\text{II}\) | IG 47048 | Turkey | Icel, 24 km southwest of Erdemli, Mersin across from Boy Scout Recreation Center | 36.4667 | 34.1333 | 30 | - |
| 55 | PI 554185 | IG 47117 | Turkey | 2 km southeast of Van on route to Gürpınar | 38.5 | 43.3667 | 1790 | - |
| 56\(1,2\) | PI 554186 | IG 47166 | Turkey | Van, 6 km southeast of Van | 38.4667 | 43.3833 | 1990 | - |
| 57\(1\) | PI 554187 (2) | IG 47125 | Turkey | Van, 2 km north of Van | 38.5333 | 43.3333 | 1710 | - |
| 58 | PI 554188 | - | Turkey | Van, 29 km north of Van | 38.75 | 43.3667 | 1790 | - |
| 59 | PI 554190 | IG 47170 | Turkey | Van, 29 km north of Van | 38.75 | 43.3667 | - | - |
| 60 | PI 560506 | - | Turkey | Roadside along Lake Van. About 3km W of Ermisler | 38.8667 | 43.4667 | 1630 | - |
| 61 | PI 560507 | - | Turkey | Van, About 2km N of village of Yalnizagac Habitat: Gray soil of S slope on N side of small ravine in valley | 38.7 | 43.5 | 1837 | - |
| 62\(2\) | PI 564179\(\text{III}\) | - | Turkey | 23 km southeast of Manavgat, near Okucahir village | 36.6833 | 31.6333 | 50 | + |
| 63\(1,2\) | PI 564180\(\text{III}\) | - | Turkey | 58 km southwest of Silifke; 4 km west of Ovacik, Mersin | 36.18333 | 33.6333 | 250 | + |
| 64\(1,2\) | PI 564181\(\text{III}\) | - | Turkey | 49 km southwest of Silifke, Mersin | 36.2 | 33.7 | 150 | + |
| 65\(1,2\) | TA 2084\(\text{III}\) | - | Turkey | 1 km N of Iskenderum (Alexandretta) | 36.6001 | 36.1969 | 50 | + |
| 66 | TA 2106 | KU11-2 | Turkey | Konya, collected by Dr. Johnson in 1965. | 37.8667 | 32.4833 | 1030 | - |
| No | Accession # | Duplicates | Country of origin | Collection site | Latitude (N) | Longitude (E) | Alt. (h, m) | Analyzed by FISH |
|----|-------------|------------|------------------|----------------|-------------|-------------|------------|----------------|
| 67 | AE 1521     | -          | unknown          | -              | -           | -           | -          | -              |
| 68 | AE 1607 (2) | -          | unknown          | obtained from UK | -           | -           | -          | +              |
| 69 | TX 01       | -          | unknown          | provided by Dr. M. Feldman | -           | -           | -          | -              |

**Aegilops neglecta**

| 70 | PI 564182*  | -          | Turkey           | 9 km southeast of Ayvacik | 39.583333 | 26.483333 | 420        | +              |
|    | K-4553*     | IG 126975  | Armenia          | Kapan distr. road from Kapan to Charaten | 39.1903   | 46.43      | 970        | -              |
|    | K-1423      | -          | Turkey           | 17 km south of Canakkale | 40.033333 | 26.35      | 100        | +              |

**Aegilops umbellulata**

| 73 | AE 646      | -          | Algeria          | unknown          | -           | -           | -          | +              |

| 74 | AE 155      | K-1234     | Azerbaijan       | unknown          | -           | -           | -          | +              |
|    | AE 820      | -          | Turkey           | 3 km E Kemalpasa | -           | -           | -          | -              |
|    | AE 1339     | -          | Greece           | Kreta            | -           | -           | -          | +              |

Column 1 (No): ¹- accessions used for electrophoretic analysis; ² – accessions used for molecular analysis.

Column 2 (Accession #):* - accessions that were erroneously classified as *Ae. columnaris*; II – accessions belonging to group II.

Three accessions (PI 564182 from Turkey and K-4553 and K-4233 from Armenia) maintained in gene banks under the name *Ae. columnaris* were found to be taxonomically misclassified: and were indeed *Ae. neglecta*. Accession IG 49067 was the mix of *Ae. columnaris* and *Ae. biuncialis*, whereas accessions K-2344 from Armenia and AE 1607 of unknown origin – the mix of *Ae. columnaris* and *Ae. triuncialis*. One K-4224 genotype was the F₁ hybrid between *Ae. columnaris* and *Ae. triuncialis* (Fig. 1, c).
Figure 1. C-banded metaphase cells of accessions representing two karyotypic groups of *Ae. columnaris* (a-c) in comparison with *Ae. neglecta* (d): a – IG 48818 (C-I); b - PI 564180 (C-II), c - the F1 hybrid *Ae. columnaris* (K-4224) x *Ae. triuncialis* (genotype unknown) carrying reciprocal translocations 1Uc:5Uc derived from *Ae. columnaris* and 1Uc:7Ct derived from *Ae. triuncialis*; d - *Ae. neglecta* (K-4553). Chromosomes are designated according to genetic nomenclature; the Uc/Ut chromosomes are shown in dark green, the Xc/Xt chromosomes in dark blue, the Ct of *Ae. triuncialis* - in red, and the Uc in light-green color). Red arrowheads point to satellite chromosomes. Blue arrows show translocated 5Uc:6Xc chromosomes (a).

The C-banding method revealed that *Ae. columnaris* gene pool consists of two distinct karyotypic groups that differed from each other and from *Ae. neglecta* (Fig. 1, a, b, d) in karyotype structure, the number of satellite chromosomes, and C-banding patterns. The first, larger group was designated C-I, whereas the second, smaller one – C-II. Group C-I included 62 accessions collected from an entire distribution range, while C-II comprised only seven accessions - six from the southeast coastal part of Turkey and one from Iraq (Fig. 2). Each *Ae. columnaris* group demonstrated high diversity of the C-banding patterns and, in the case of C-I, broad translocation polymorphism (Figs. 3-7).

Thus, karyotypes of 31 C-I accessions (50%) differed from each other only in the presence/absence or the size of Giemsa C-bands in particular positions; this karyotypic variant was considered basic or “normal (N).” Karyotypes of 31 accessions derived from normal as a result of one or more structural chromosome rearrangements. Accessions collected from geographic closer regions usually had more similar banding patterns than accessions from distant locations, and this similarity remained in genotypes with chromosomal rearrangements. The highest C-banding and translocation polymorphisms were observed in Turkey.
Figure 2. Geographic distribution of the C-I (blue boxes) and C-II (red boxes) accessions of *Ae. columnaris* with known collection sites. Accessions carrying pericentric inversion of 7U\(^c\) are outlined with a green dotted line.

*Ae. neglecta* was similar to *Ae. columnaris* in the amount and distribution of Giemsa-bands on most chromosomes, but differed from it in the morphology of 6X\(^c\), which was more symmetric (arm ratio L/S = 1.173 vs. 1.924). In contrast to the C-II group, *Ae. neglecta* carried three pairs of the satellite chromosomes as the C-I accessions (Fig. 1, a, d). Three accessions of *Ae. neglecta* had similar C-banding patterns (Fig. 3, t-v) and did not possess chromosomal rearrangements.
Figure 3. Polymorphisms of the C-banding patterns of Syrian and Lebanese accessions of *Ae. columnaris* (a-s) in comparison with *Ae. neglecta* (t-v): a – K-4372; b – K-4362; c – PI 486196; d – K-2680; e – PI 487198; f – K-4009 (Syria); g – K-4241a; h – K-4241b; i – K-4004; j – K-4003; k – K-4407; l – K-4406; m – K-4007; n – IG 49067; o – K-4409 (Lebanon); p – TX01; q – Clae34; r – AE 1521; s – AE 1607a (unknown origin); t – PI 564182 (Turkey); u – K-4233; v – K-4553 (Armenia). 1Uc–7Xc – chromosomes; translocated chromosomes are indicated with arrows and designated respectively.

Twenty-six C-I accessions were collected in different regions of Turkey (Table 1; Fig. 4). Nearly half of them (12 accessions) had normal karyotypes (N), and 14 (including segregating accession AE 1607) carried eleven variants of chromosomal rearrangements (Table 2; Figs. 4, 5). Pericentric inversion of the chromosome 7Uc (Fig. 5, Inv3) was the most frequent variant; it was found in three accessions (Fig. 4, u, v, x,
This rearrangement gave rise to the secondary translocation inv7U\(^c\) + T4U\(^c\):4X\(^c\) (T1) identified in the sample H-2 collected in Turkey 132 km NW from Nevşehir (Fig. 4, w). Double translocation T3X\(^c\):7U\(^c\) + T4X\(^c\):6U\(^c\) – T19 (Fig. 4, d) was detected in two non-related accessions, PI 486281 and PI 554181 (Table 2), with identical C-banding patterns. Accession PI 542171 and two AE1607 biotypes carried pericentric inversions of the 2X\(^c\) chromosome (Fig. 4, i; Fig. 6, s), which differed in breakpoint position resulting in a different structure of rearranged chromosomes (Fig. 5, Inv1 and inv2X\(^c\)-2). Five translocation variants: T3U\(^c\):1X\(^c\) (T8) and its derivative T1U\(^c\):5U\(^c\) + T3U\(^c\):1X\(^c\) (T13), T2U\(^c\):2X\(^c\) (T6), T2U\(^c\):4X\(^c\) (T7), T4U\(^c\):5U\(^c\) (T9), T3X\(^c\):7X\(^c\) (T4), and T2X\(^c\):4X\(^c\) – T14 (Fig. 5; Fig. 4, a, b, h, j, m, c, respectively), were found in one accession each (Table 2).

Table 2. Variants of chromosomal rearrangements identified in *Ae. columnaris*, type I accessions

| No | Trans. code | Translocation type | Structure of translocated chromosomes | accessions | origin          |
|----|-------------|--------------------|---------------------------------------|------------|----------------|
| 1  | inv1        | perInv2X\(^c\)     | -                                     | PI 542171; AE 1607b | Turkey         |
| 2  | inv2        | perInv 6X\(^c\)    | -                                     | K-4406     | Lebanon        |
| 3  | inv3        | perInv 7U\(^c\)    | -                                     | i-570045; Clae 34; K-4002; TA2106; AE 1521; H-8; TX01 | Turkey         |
| 4  | T1          | perInv7U\(^c\) + T4U\(^c\):4X\(^c\) | 4U\(^L\):S:4X\(^S\)+4U\(^L\):4X\(^L\) | H-2        | Turkey         |
| 5  | T2          | perInv2X\(^c\)-2 + T6U\(^c\):7X\(^c\) | perInv2X\(^c\)-2 + 6U\(^L\):6U\(^S\):7X\(^S\)+T6U\(^L\):7X\(^S\):7X\(^L\) | AE 1607a   | unknown        |
| 6  | T3          | T3U\(^c\):4X\(^c\) | 3U\(^L\):4X\(^S\)+3U\(^L\):4X\(^L\) | K-4224c; K-564 | Armenia        |
| 7  | T4          | T3X\(^c\):7X\(^c\) | 3X\(^L\):3X\(^S\):7X\(^S\)+3X\(^S\):7X\(^S\):7X\(^L\) | PI 276968; PI 574457 (K-512) | Turkey; Azerbaijan |
| 8  | T5          | T5U\(^c\):6X\(^c\) | 5U\(^L\):6X\(^S\)+5U\(^L\):6X\(^L\) | K-4418     | Iran           |
| 9  | T6          | T2U\(^c\):2X\(^c\) | 2U\(^L\):2U\(^L\)+2U\(^L\)-2X\(^L\):2X\(^S\) | PI 554185   | Turkey         |
| 10 | T7          | T2U\(^c\):4X\(^c\) | 2U\(^L\):4X\(^S\)+2U\(^L\):4X\(^L\) | PI 554187t | Turkey         |
| 11 | T8          | T3U\(^c\):1X\(^c\) | 3U\(^S\):3U\(^L\)-1X\(^S\)+3U\(^L\)-1X\(^S\):1X\(^L\) | K-560506   | Turkey         |
| 12 | T9          | T4U\(^c\):5U\(^c\) | 4U\(^L\):4U\(^S\)-5U\(^L\)+4U\(^S\)-5U\(^L\):5U\(^S\) | K-4002     | Turkey         |
| 13 | T10         | T7U\(^c\):2X\(^c\) | 7U\(^L\)-7U\(^S\)-2X\(^L\):2X\(^S\) | K-4003; K-4407 | Lebanon         |
| 14 | T11         | T1U\(^c\):5U\(^c\) | 1U\(^S\):5U\(^L)+1U\(^L\):5U\(^L\) | PI 499258; K-1178; K-1495; K-4224; K-4366 | Armenia       |
| 15 | T12         | T1U\(^c\):5U\(^c\)+ T4U\(^c\):6U\(^c\) | 1U\(^S\):5U\(^L\)+1U\(^L\):5U\(^L\)+4U\(^L\):4U\(^S\)-6U\(^L\)+4U\(^S\)-6U\(^S\):6U\(^L\) | K-1193 | Armenia       |
| 16 | T13         | T1U\(^c\):5U\(^c\)+ T3U\(^c\):1X\(^c\) | 1U\(^S\):5U\(^L\)+1U\(^L\):5U\(^L\)+3U\(^S\):3U\(^L\)-1X\(^S\)+3U\(^L\)-1X\(^L\):1X\(^L\) | PI 554180 | Turkey         |

\(^1\) i-570045 was the duplicate of PI 554184 and they were further treated as a single accession.
| No | Trans. code | Translocation type | Structure of translocated chromosomes | accessions | origin   |
|----|-------------|--------------------|----------------------------------------|------------|----------|
| 17 | T14         | T2X:4X<sup>c</sup> | 2X'S:4X'S + 2X'L:4X'L                   | PI 560507  | Turkey   |
| 18 | T15         | T2X:4X<sup>c</sup>:6X<sup>c</sup> | 2X'S:4X'S + 2X'L:6X'S + 6X'L:4X'L       | K-4240     | Iran     |
| 19 | T16         | T1U:5U<sup>c</sup> + T3U:5X<sup>c</sup> | 1U'S:5U'S + 1U'L:5U'L + 3U'S:5X'L + 3U'L:5X'S | K-4224B; K-4228 | Armenia |
| 20 | T17         | T1U:5U<sup>c</sup> + T7U:3X<sup>c</sup> + T3U:4U<sup>c</sup> | 1U'S:5U'S + 1U'L:5U'L + 7U'S:3X'L + 7U'L:3X'L + 3U'S:4U'S + 3U'L:4U'L | K-4225; K-4551 | Armenia |
| 21 | T18         | T2U:4X<sup>c</sup> + T4U:6X<sup>c</sup> | 2U'S:4X'S + 2U'L:4X'L + 4U'S:6X'S + 4U'L:6X'L | K-4372     | Syria    |
| 22 | T19         | T6U:4X<sup>c</sup> + T7U:3X<sup>c</sup> | 6U'S:4X'S + 6U'L:4X'L + 7U'S:3X'L + 7U'L:3X'S | PI 486281; PI 554181 | Turkey |
| 23 | T20         | T2U:5X<sup>c</sup> + T4U:2X<sup>c</sup> | 2U'L:2U'S:5X'S + 2U'S:5X'L:5X'S + 4U'S:2X'L + 4U'L:2X'S | K-4362     | Syria    |
Figure 4. Polymorphisms of the C-banding patterns of Turkish C-I accessions of *Ae. columnaris*: a – PI 560506; b – K-PI 554180; c – PI 560507; d – PI 486281; e – PI 554186; f – PI 554188; g – PI 554190; h – PI 554185; i – PI 554187N; j – PI 554187T; k – PI 554178; l – PI 554186; m – K-4002; n – H-6; o – H-7; p – H-10; q – H-3; r – H-9; s – H-4; t – PI 542171; u – PI 554184; v – H-8; w – H-1; x – i-570045; y – TA2106. 1Uc – 7Xc – chromosomes; translocated chromosomes are indicated with arrows and designated respectively.
Translocation variants identified among 62 *Ae. columnaris* accessions belonging to group C-I. T1 – T20 – translocation variants; normal chromosomes are designated with black letters and are shown below the respective chromosomes; arm combinations in translocated chromosomes are designated with red letters. Inversions are identified - with blue. Red arrows point to possible translocation breakpoints, and blue arrows point to possible breakpoint position in inverted chromosomes. Long black arrows define translocation lineages.

Transcaucasia was represented by 19 Armenian and one Azerbaijani accession (Table 1). Nine accessions had normal karyotypes, and five variants of translocations were identified in the remaining ten accessions (Table 2; Fig. 5). Translocation T1U<sup>c</sup>:5U<sup>c</sup> - T10 (Fig. 6, f, g) was present in five Armenian accessions and in PI 488258 of unknown origin. This translocation gave rise to two double translocations: T1U<sup>c</sup>:5U<sup>c</sup> + T3U<sup>c</sup>:5X<sup>c</sup> (T13), T1U<sup>c</sup>:5U<sup>c</sup> + T4U<sup>c</sup>:6U<sup>c</sup> (T12) found in one accession each and one triple translocations T1U<sup>c</sup>:5U<sup>c</sup> + T7U<sup>c</sup>:3X<sup>c</sup> + T3U<sup>c</sup>:4U<sup>c</sup> - T17 (Fig. 6, e, i, j, k) detected in two accessions (Table 2). Interestingly, another complex translocation, the derivative of T1U<sup>c</sup>:5U<sup>c</sup> – T13, was found in Turkey (Fig. 4, b). The only translocation not related to T1U<sup>c</sup>:5U<sup>c</sup> was T3U<sup>c</sup>:4X<sup>c</sup> (T3) identified in two Armenian accessions (Fig. 6, o, p).
Figure 6. Polymorphisms of the C-banding patterns of Transcaucasian (a-r) and Iranian (s-v) accessions of *Ae. columnaris*: a – K-2344; b – K-4224a; c – K-4229; d – K-1512; e – K-4228; f – K-1178; g – K-1495; h – PI 499258; i – K-1193; j – K-4225; k – K-4551; l – N-1; m – N-2; n – N-3; o – K-4224b; p – K-564; q – PI 276457; r – PI 574457; s – K-4240; t – K-4418; u – IG 48818; v – K-4413. 1U: – 7X: chromosomes; translocated chromosomes are indicated with arrows and designated respectively.

Two of the four Iranian accessions analyzed in a current study carried chromosomal rearrangements (Fig. 6, s-v). These were a single translocation T5U:6X (T5) and double cyclic translocation T2X:4X:6X (T15).

Lebanese group of *Ae. columnaris* contained eight accessions, one of which consisted of two karyotypically normal biotypes differing only in the C-banding patterns (Fig. 3, g.
h). Of them, accession K-4241b (Fig. 3, h) was almost identical to K-4004 (Fig. 3, i) in the C-banding pattern. Most Lebanese Ae. _columnaris_ had normal karyotypes, and two types of chromosomal rearrangements were identified in three accessions. Thus, K-4003 and K-4407 carried T7U<sup>c</sup>:2X<sup>c</sup> – T10 translocation (Fig. 3, j, k), while a pericentric inversion of the chromosome 6X<sup>c</sup> (inv2) was detected in K-4406 (Fig. 3, l).

Five accessions were from Syria. Three of them had normal karyotypes, and two different complex translocations were identified in the remaining two accessions (Fig. 3, a, b). K-4372 carried T2U<sup>c</sup>:4X<sup>c</sup> + T4U<sup>c</sup>:6X<sup>c</sup> (T18) and K-4362 – the translocation T2U<sup>c</sup>:5X<sup>c</sup> + T4U<sup>c</sup>:2X<sup>c</sup> (T20). In both cases, the original single translocations were not found.

The origin of four accessions – AE 1512, AE1607, TX01, and Clae 34 was unknown. We found that AE 1607 consisted of two biotypes differing in chromosomal rearrangements (inv2X<sup>c</sup> / inv2X<sup>c</sup>-2 + T6U<sup>c</sup>:7X<sup>c</sup>) and the C-banding patterns. This accession also contained admix of _Ae. triuncialis_ seeds. Three accessions – Clae34, TX01, and AE 1521 carried pericentric inversion of 7U<sup>c</sup> (Fig. 3, p-r). This rearrangement was recorded only in _Ae. columnaris_ collected from Central Anatolian in Turkey (Fig. 2, outlined with green dotted lines); therefore, we suggested that these three accessions may originate from the same region.

Seven _Ae. columnaris_ accessions: six from Turkey and one from Iraq, were karyotypically distinct from all other accessions of the species and exhibited significant variation of the C-banding patterns (Fig. 7). They were assigned to the C-II group. Accession TA2084 carried at least two whole-arm reciprocal translocations; unidentifiable minor translocations may present in other accessions causing variation of the C-banding patterns. Despite heterogeneity, karyotypes of all C-II accessions shared some distinct features discriminating them from the C-I group and _Ae. neglecta_:

1. they had only two pairs of the satellite (SAT) chromosomes;
2. chromosome 1U<sup>c</sup> was more heterochromatic, whereas
3. chromosome 4U<sup>c</sup> of C-II contained less heterochromatin compared to C-I (Fig. 7).
4. chromosome 7U<sup>c</sup> did not possess a prominent C-band complex in a proximal part of the long arm, found in the orthologous chromosomes of all C-I or _Ae. neglecta_ accessions.

Morphology and the C-banding pattern of the chromosome 5U<sup>c</sup> in both C-I and C-II accessions were similar; however, 1U<sup>c</sup> of C-II was more heterochromatic than the 1U<sup>c</sup> in C-I (Figs. 7, 8). Significant differences existed in C-banding patterns of other C-I and C-II chromosomes, although some polymorphisms could result from introgression. Thus, chromosome 3U<sup>c</sup> of PI 554182 (Fig. 7, d) had the C-banding pattern typical for Turkish and Transcaucasian C-I accessions (i.e., PI 554186, PI 554187 on Fig.4, e, i) and may originate via introgression between C-I and C-II groups. A C-banding pattern of the chromosome 4X<sup>c</sup> of PI 564180 was more similar to 4X<sup>i</sup> of _Ae. neglecta_ (Fig. 3, t-v) than other C-II or C-I accessions.
Figure 7. Polymorphisms of the C-banding patterns of *Ae. columnaris* accessions belonging to group C-II (TYPE II) in comparison with TYPE-I accession (i-570045).

a – K-3899 (Iraq); b – PI 542191; c – PI 564181; d – PI 554182; e – PI 564180; f – PI 564179; g – TA2084 (all from Turkey). C-I accession PI 554184 from Turkey (TYPE I) is shown for comparison. The U\(^c\) chromosomes are given on the left half of the figure, the X\(^c\) chromosomes – on the right half. Rearranged chromosomes are indicated with arrows.

**FISH analysis of *Ae. columnaris***

In order to get a deeper insight into genetic differences between groups C-I and C-II of *Ae. columnaris* and to assess their relationship with *Ae. neglecta*, we applied FISH with pTa71 (45S rDNA), pTa794 (5S rDNA), GAA\(_n\), GTT\(_n\), ACT\(_n\), pSc119.2, pAs1, pTa-713 probes for their analysis. The pTa-535 probe was not considered because it produced signals only on a few chromosomes (Fig. S1, o; green signals), uninformative for our analyses.

Hybridization of pTa71 and pTa794 probes revealed three pairs of major, nearly equal pTa71 signals on chromosomes of C-I and *Ae. neglecta*, but only two pairs of major NORs in the C-II accessions (Fig. 8; Fig. S1, a, b; Fig. S2, a, c). Instead, all C-II accessions possessed faint pTa71 signals on a chromosome pair carrying a clear distal 5S rDNA locus. This chromosome was classified as 1X\(^c\) based on results of sequential FISH with 5S + 45S rDNAs followed by GAA\(_n\) + GTT\(_n\)/pTa-713 probes (Fig. S2, b, d). An additional minor NORs were found in the middle of 6U*L (Fig. S3) of all *Ae. columnaris* and *Ae. neglecta*. *Ae. neglecta* differed from *Ae. columnaris* in the presence...
of a minor 45S rDNA site in a distal part of an arm of a pair of large metacentric X*-genome chromosome tentatively classified as 6X1 (Fig. 8; Fig. S1, c, arrowed; Fig. 2, e; Fig. S3a). The application of FAM-labelled oligo-probes allowed us to detect very weak minor pTa71-signals at the terminus of 5X*L, a distal quarter of 1U*L, and in a proximal part of 3Xc*S (Fig. S3). Similar signals were obtained on chromosomes of the C-II accession PI 564181 (data not shown). However, these minor sites never appeared when the plasmid DNA was used as a probe, and they were not considered in the analysis.

Figure 8. Comparison of the C-banding patterns with the distribution of different types of repeats on chromosomes of *Ae. neglecta* (N) and C-I and C-II groups of *Ae. columnaris*. The color of GAA\textsubscript{n}, GTT\textsubscript{n}, pTa71, and pTa794 probes on the respective idiograms corresponds to their color on chromosomal images. Probe combinations: 1 – GAA\textsubscript{n} (green) + pTa-713 (red); 2 – pSc119.2 (green) + pTa-713 (red); 3 – pSc119.2 (green) + pAs1 (red). Positions of pSc119.2 sites on idiograms are shown in green, pAs1 – in pink, and pTa713 – in yellow. Accessions codes: a - PI 564182, e – K-4233; j – PI 170209 (*Ae. neglecta*); b – H-1 (sample provided by Dr. H. Ozkan); d - i-570045; f – PI 554181; g – K-2680; k – AE 1607 (*Ae. columnaris*, C-I); c – PI 564180; h– TA2084; i - PI 564181; l – PI 542181 (*Ae. columnaris*, C-II). Arm combinations on rearranged chromosomes are designated.
Apparent differences between C-I, C-II, and *Ae. neglecta* groups existed in a pattern of 5S rDNA probe. All *Ae. columnaris*, C-I and *Ae. neglecta* accessions contained ten 5S rDNA signals distributed among four chromosome pairs (Fig. S2, a, e; Fig. S3). The chromosome 1X' possessed two pTa794 sites: one located distally to the NOR, while
the second – proximally to it (Fig. 8; Fig. S1, a, c; Fig. S2, a, e). By contrast, four chromosome pairs in all C-II accessions carried a single 5S rDNA signal each.

In *Ae. columnaris* and *Ae. neglecta* labeling patterns of GAA<sub>n</sub> probe were mainly consistent with the C-banding patterns, while the GTT<sub>n</sub> hybridized predominantly on the X<sup>c</sup> chromosomes (Fig. S1, g-i). Only 2U<sup>c</sup>, 4U<sup>c</sup> and 5U<sup>c</sup> contained small GTT<sub>n</sub> sites in pericentromeric/ proximal regions, and a faint signal was present in the middle of the 7U<sup>c</sup>L arm (Fig. 8) in four of the five C-II accessions. By contrast, all X<sup>c</sup> genome chromosomes demonstrated prominent GTT<sub>n</sub> signals located predominantly in the proximal chromosome regions. Position of the GTT<sub>n</sub> clusters on the X<sup>c</sup> chromosomes only partially overlapped with the GAA<sub>n</sub> location; some chromosomes (e.g., 5X<sup>c</sup> or 7X<sup>c</sup>) were poorly labeled with GAA<sub>n</sub>, showed extremely heavy labeling with GTT<sub>n</sub> (Fig. 8). Hybridization patterns of ACT<sub>n</sub> probes were almost identical to that of GTT<sub>n</sub> (Fig. S1, m, n).

The pSc119.2 probe hybridized to subtelomeric regions of one or both arms of most *Ae. columnaris* and *Ae. neglecta* chromosomes except for 7X<sup>c</sup>, which lacks pSc119.2 signals in all C-I and most C-II accessions (Fig. 8; Fig. S1, j, k, o, n). Intercalary sites appeared only on the long arm of 7U<sup>c</sup> and rarely of 6U<sup>c</sup>L, as in a diploid *Ae. umbellulata*. Labeling patterns of the pSc119.2 probe were polymorphic between and among C-I and C-II accessions (Fig. 8). Four of the five C-II accessions studied by FISH possessed intercalary pSc119.2 site also on the chromosome 2U<sup>c</sup>L (Fig. 8, c), but this site was never observed in C-I or *Ae. neglecta*. On the other side, the C-II accession PI 564181 did not possess any pSc119.2 signals on the chromosome 2U<sup>c</sup> (Fig. 8, i).

The D-genome specific probes pAs1 and especially pTa-535 were low informative for chromosome identification of *Ae. columnaris* and *Ae. neglecta*. Distinct pAs1 sites were observed in the pericentromeric region of 6U<sup>c</sup> and 4X<sup>c</sup> chromosomes of all studied species, whereas 2-3 weak signals were present on their 4X<sup>c</sup>S and 7X<sup>c</sup>L arms. The chromosome 5X<sup>c</sup> of C-I also contained a single, small pAs1 site in the distal half of the short arm. Hybridization sites of the pTa-535 probe emerged on the 6U<sup>c</sup>L arm, but only in a few accessions studied (Fig. 8, m; Fig. S3, b).

The pTa-713 probe hybridized to most *Ae. columnaris* (C-I and C-II) and *Ae. neglecta* chromosomes, while the 3U<sup>c</sup>, 4X<sup>c</sup> and 5X<sup>c</sup> (in *Ae. neglecta* – also 7X<sup>c</sup>) lacked the signals completely. In most cases, the distribution of pTa-713 sites on chromosomes of all three groups was similar; however, some differences between them were observed (Fig. 8; Fig. S4). In particular, a large pTa-713 signal was present on the short arm of 1U<sup>c</sup> of all C-I and *Ae. neglecta* accessions, but it was absent in the C-II. Most C-I and one *Ae. neglecta* (K-4233) possessed a distinct site in the proximal half of 2U<sup>c</sup>L, which was not found in C-II and two *Ae. neglecta* accessions (Fig. S4, g, h). We did not observe proximal pTa-713 sites in the short arm of 2X<sup>c</sup>L and 3X<sup>c</sup>L in the C-II group, but they were present in all C-I and *Ae. neglecta* accessions. In turn, a large pTa-713 signal present on the 1X<sup>c</sup>L arm of all C-II accessions was never observed in the C-I group or *Ae. neglecta* (Fig. S4). Position of hybridization sites on 5U<sup>c</sup>, 6U<sup>c</sup>, 7U<sup>c</sup>, was similar in all three groups, but *Ae. columnaris* differed from *Ae. neglecta* in morphology and/or labeling patterns of 6X<sup>c</sup> and 7X<sup>c</sup> chromosomes (Figs. 8).

**Analysis of gliadin spectra of *Ae. columnaris***

Electrophoretic analysis revealed a high diversity of gliadin spectra in 25 *Ae. columnaris* accessions and their distinctness from the spectra of *Ae. neglecta* (Fig. S5). Only two of 25 *Ae. columnaris* accessions, K-4413 and K-4418 from Iran, shared similar gliadin
spectra, whereas four C-II accessions included in our analysis were highly diverse. However, all contained electrophoretic (EP) components, whose position did not match the overall pattern specific for *Ae. columnaris* C-I accessions (Fig. 9).

Thus, electrophoretic profiles of PI 564180 and PI 542191 were characterized by low-intense, virtually invisible (“minor”) components in the α-zone; their intensities and position were distinct from other accessions of *Ae. columnaris* (Fig. 9 c, d; Fig. S5). Based on comparison with the K-1193 spectrum, we proposed that these components can be encoded by both the $X^c$ and $U^c$ genomes (Fig. 9).

![Figure 9. *Ae. columnaris* with the most distinct gliadin spectra](image)

(a) gliadin spectrum of a model *Ae. columnaris* accession K-1193 in comparison with etalon spectrum of wheat cultivar Bezostaya-1 (Bez-1). Blocks of linked electrophoretic gliadin components controlled by a single locus of the particular *Ae. columnaris* chromosome [27] are shown schematically at the right side of the electrophoretic spectrum; (b) EP spectrum of the accession TA2084 in comparison with wheat cultivar Bezostaya-1; (c) EP spectrum of PI 564180; (d) EP spectra of *Ae. columnaris* accessions illustrating protein components presumably encoded by the $X^c$ (red dots) and $U^c$ (yellow dots) chromosomes. The unique components, which were not found in any other *Ae. columnaris* accessions, are shown schematically (parts b, c, and d).

Protein components located in the ω-zone of the spectra of all *Ae. columnaris* C-I accessions were similar in intensity and position (Fig. S5). Among them, components designated as “2” and “3” (Fig. 9, indicated with red dots) corresponded to components detected in the spectrum of K-1193, which were coded by the $1X^c$ chromosome. In
contrast to other materials, accession PI 564181 contained the unique double band instead of “component 3” (Fig. 9d). Besides, it displayed a distinct distribution of components located in the β - γ zone, which, by comparison with the K-1193 spectrum, can be coded by group-6 chromosomes of the Uc and Xc genomes. Such distribution was more typical for common wheat, and the respective zone was controlled by wheat chromosomes 6B and 6D [356].

Protein components encoded by chromosome 1Uc were characterized by low intensities (Fig. 9a, 9c; indicated by yellow dots). By contrast, the spectra of TA 2084 and PI 564180 possessed several intense components in the upper part of ω-zone designated 1', 2', and 3'. By analogy with the spectrum of K-1193, we hypothesized that they could be controlled by the chromosome 1Uc (Fig. 9b, c). TA 2084 and PI 564180 spectra shared components 2' and 3’ with similar mobility and intensity, but they differed in the presence of additional minor component 1’, which showed slower mobility in TA2084.

Variability of the U-genome specific U31 nuclear fragment in Ae. columnaris and Ae. neglecta

Amplification and further sequencing of the U-genome-specific U31 nuclear fragment was performed with primers U31a and U31b in fifteen accessions, including ten Ae. columnaris (K-4225, K-4228, K-4409, K-4413, and PI554186 from different countries and representing chromosomal group C-I, and PI542191, PI564179, PI 564180, PI564181, and TA2084 all from Turkey representing group C-II), two Ae. neglecta from Algeria and Turkey (PI 170209 and AE 646) in comparison with three accessions of their diploid parental species Ae. umbellulata (AE 1339, AE 155, and AE 820) of different geographic origin (Table 1). All accessions analyzed generated 363 bp fragments, except for Ae. columnaris PI 554186. In this accession, the fragment length was reduced to 270 bp due to a 123 bp deletion (Fig. 10; Fig S6).

The sequence of the U31 fragment obtained from Ae. columnaris accessions fall into the types, which corresponded to designations proposed earlier by (Kadosumi et al. 2005) based on fragment length and the presence of MspI restriction site (CCGG). Type-I having the full-size U31 fragment and an intact MspI site was found in seven Ae. columnaris accessions as well as in all analyzed Ae. neglecta and Ae. umbellulata accessions (Fig. S6; Fig. 10).

Figure 10. Nucleotide substitutions in the U31 region in 15 Ae. columnaris (UcUcXcXc), Ae. neglecta (UtUtXtXt), and Ae umbellulata (UU) sequences. Dots correspond to nucleotides identical to consensus sequences. The MspI restriction site is highlighted in red.

The type-II U31 fragment was identified in two Ae. columnaris accessions, both from the C-II chromosomal group (Fig. 10). It emerged as a result of sequence changes at the MspI restriction site: a mononucleotide deletion in position 292 was found in TA2084,
while C/T_290_ substitution in PI 564181. Accession PI 554186 (C-I) possessed the type-III U31 fragment with a 123 bp deletion (Fig. S6). All U31-alleles assigned to type-II corresponded to those reported by Kadosumi et al. (2005) in _Ae. columnaris_ or _Ae. neglecta_. Among U31 type-I accessions of _Ae. columnaris_, four allelic variants were found, three of which were novel alleles (Fig. 10). Two of them were identified in C-II and one in C-I accession.

The U31 sequences of _Ae. umbellulata_ accessions AE 155 and AE 820 of both _Ae. neglecta_ (PI 170209 and AE 646) belonged to type-I and showed just a few (1-2) nucleotide substitutions, while almost 12 SNPs were detected in the U31 sequence of _Ae. umbellulata_, AE 1339 from Greece, which was also assigned to type-I (Fig. S6). Most of the U31 alleles of _Ae. umbellulata_ or _Ae. neglecta_ discovered by us (Fig. 10) were not identified earlier, and only _Ae. neglecta_, PI 170209 carried the same allele as _Ae. columnaris_ (KU-2953A) from Armenia, described earlier by Kadosumi et al. [23].

An ML tree (Fig. 11) shows the possible evolutionary relationship between accessions and species based on comparative sequencing of the U31 alleles. All _Aegilops_ accessions except AE 1339 (_Ae. umbellulata_) formed one common cluster on the tree obtained. No species-specific or ploidy-specific clusters have been observed. Three _Ae. columnaris_ accessions including two of type-II U31 alleles (PI 564179, PI 564180) and one type-III accession (PI 554186) formed a separate sub-cluster with 79% bootstrap support. Other accessions representing different species (_Ae. columnaris_ and _Ae. neglecta_) and different U31 allele types (I and II) fall into one common sub-cluster with _Ae. umbellulata_ (AE 115 and AE 820) showing a closer relationship. _Aegilops neglecta_ accession (AE 646) form an individual branch.

![Figure 11. Maximum-likelihood (Kimura 2-parameter model) tree of the U-genome-specific U31 nuclear sequence.](image)

The numbers above the branches indicate bootstrap values; the C-banding group is shown in red, U31 allele type –in green.
Variability of three plastome intergenic spacers in *Ae. columnaris* and *Ae. neglecta*

Variability of three plastome fragments – *trnH*(gtg)-*psbA*, *trnT*(ugu)-*trnL*(uaa), and *rpL32-trnL*(tag) DNA were assessed on the same set of 10 *Ae. columnaris* accessions as for nuclear U31 fragment. The total length of plastome sequences obtained corresponded to 1825 bp (*trnH-psbA* – 558 bp, *trnT-trnL* – 577 bp, and *rpL32-trnL* -690 bp). Polymorphism levels differed between the analyzed fragments: only three SNPs were found in the *trnT-trnL* spacer, while *rpL32-trnL* and *trnH-psbA* sequences were much more polymorphic. In contrast to *Ae. columnaris*, spacer sequences of two *Ae umbellulata* accessions (AE 155 and AE 1339) were invariable (Fig. 12).

**Fig. 12. Nucleotide substitutions in *trnH-psbA*, *trnT-trnL*, *rpL32-trnL* plastome regions in 13 *Ae. columnaris* (U°U°X°X°), *Ae. neglecta* (U°U°X°X°), and *Ae umbellulata* (UU) sequences.** Dots correspond to nucleotides identical to consensus sequences.

| C-band group | *trnH-psbA* | *trnT-trnL* |
|--------------|-------------|-------------|
| *Ae. columnaris* K-4225 | I | G |
| *Ae. columnaris* K-4228 | I | C |
| *Ae. columnaris* K-4409 | I | T |
| *Ae. columnaris* K-4413 | I | T |
| *Ae. columnaris* PI564186 | I | T |
| *Ae. columnaris* PI564180 | II | C |
| *Ae. columnaris* PI564191 | II | T |
| *Ae. columnaris* PI564179 | II | A |
| *Ae. columnaris* TA2084 | II | C |
| *Ae. columnaris* PI564181 | II | C |
| *Ae neglecta* AE 146 | | |
| *Ae. umbellulata* AC 100 | | |
| *Ae. umbellulata* AE 1339 | | |

According to the analysis of all three plastome regions, ten *Ae. columnaris* accessions split into two groups (plastogroups). Four C-I accessions (K-4225, K-4228, K-4409, and PI 554186) had identical sequences of the plastome spacers, while K-4413 (C-I, Iran) differed at a single site: substitution of the hexamer sequence CCTCAT by ATGAGG at position 470-475 of the *rpL32-trnL* spacer (Fig. 12). Accessions of group C-II: PI542191, PI564179, PI564180, PI564181, and TA2084, showed significantly higher sequence polymorphisms at all three plastome regions. Nevertheless, they all shared the same deletion of one of the two AAGAA 5-bp repeats, as well as the deletion of the mononucleotide T at position 446. (Fig. 12). Besides, they all carried G/T substitution at position 468 of *trnT-trnL*, as *Ae neglecta* and *Ae. umbellulata* accessions.

C-II accession PI 564179 possessed the highest number of mutations, especially in the *rpL32-trnL* sequence. Together with C-II accession PI 564181 and Iranian C-I K-4413, it carried ATGAGG/CCTCAT sequence substitution. The same substitution was also identified in *Ae. umbellulata* and *Ae. neglecta* (Fig. 12). Comparison of the observed plastogroups with groups discriminated based on C-banding and FISH analyses showed that all *Ae. columnaris* accessions characterized by an increased variability (PI542191, PI564179, PI 564180, PI564181, and TA2084) belonged to karyotypic group
C-II, while low polymorphic accessions (K-4225, K-4228, K-4409, K-4413, PI 554186) fall in C-I.

On ML tree (Fig. S7), all Ae. columnaris accessions with invariable plastome sequences clustered together, whereas K-4413 formed a separate branch in a common sub-cluster with two Ae. umbellulata accessions (bootstrap = 67). Five genetically variable Ae. columnaris accessions fall either in a common sub-group with Ae. neglecta (TA2084, PI 542191, PI 564180), or formed separate branches (PI 564179, PI 564181) (Fig. S7).

**Discussion**

Cytogenetic (C-banding, FISH), biochemical (seed storage proteins – gliadins), and molecular (sequence analysis of polymorphic U31 nuclear fragment and three intergenic regions of cpDNA) analyses showed close genetic relationships of Ae. columnaris and Ae. neglecta, in agreements with previous studies [2, 4, 11, 12, 15, 19, 23]. From another side, chromosome analysis revealed higher genetic diversity of Ae. columnaris compared to that reported for Ae. neglecta [4, 13, 23], which was expressed in higher C-banding/ FISH-polymorphisms and broader spectra of chromosomal rearrangements as well as by a higher number of U31 alleles and higher variability of cpDNA identified in these species.

Two karyotypic groups, the C-I and C-II, have been discriminated within Ae. columnaris based on chromosome analysis and each group displayed characteristic C-banding and FISH patterns, enabling their discrimination. Group C-I was mainly similar to Ae. neglecta, whereas C-II differed from the C-I group of Ae. columnaris and from Ae. neglecta in karyotype structure, heterochromatin distribution, and in the patterns of rDNA loci. Such heterogeneity of ribosomal loci was not reported for other Aegilops species [13, 36-39]. Although these karyotypic groups were not supported by comparing gliadin profiles or sequences of the U31 nuclear fragment, they fully coincided with plastogroups discriminated based on cpDNA analysis.

Groups C-I and C-II karyotypically differed from each other, but the divergence level varied between individual chromosomes. Thus, no significant changes were observed in 2Uc, 5Uc, 2Xc, and 6Xc, while 3Uc, 4Uc, 7Uc, 1Xc, 5Xc, 7Xc of the C-II were modified. Despite it, we found chromosomes among C-II accessions, which matched chromosomes of C-I (e.g., 3Uc of PI 554182) or Ae. neglecta (e.g., 4Xc of PI 564180), which can be caused by introgressions. Another evidence of gene flow between species and chromosomal groups came from the analysis of the U31 nuclear fragment: Type-II U31 allele identified in C-II accession PI 564181 (Fig. 12) was earlier detected by Kadosumi et al. [23] in four accessions of Ae. neglecta and three Ae. umbellulata, but not in Ae. columnaris. A similar trend was observed in the presence of ATGAGG/CCTCAT substitution in the rpl32-trnL spacer region, which was present in one C-I and two C-II accessions of Ae. columnaris, but also in Ae. neglecta and Ae. columnaris (Fig. 12).

All methods used in our study highlighted significant genetic diversity of both C-I and C-II chromosomal groups, but each of them exhibited a different type of polymorphisms. Karyotype divergence in the C-I group was associated with variation in the presence and size of C-bands in particular positions and chromosomal rearrangements identified here in 55% of the accessions studied. However, no polymorphisms that could be associated with introgressions or unbalanced rearrangements have been found. The results of electrophoretic analysis of seed storage proteins led to the same conclusion. Although 25 accessions of Ae. columnaris had unique gliadin profiles; the spectra of most C-I genotypes shared several characteristic bands, especially in the α-zone. The number of U31 alleles identified here in the C-I accessions (Fig. 10) was relatively
small, and this group displayed very low polymorphism of the intergenic spacers of cpDNA: only one 6-bp-substitution in position 470 of rpl32-trnL was found (Fig. 12).

By contrast, accessions constituting the C-II group were highly heterogeneous. Although karyotypes of all accessions carried several diagnostic features discriminating them from the C-I group and Ae. neglecta, the observed variation cannot be explained by polymorphism of heterochromatic regions only. The emergence of some variants can be due to introgressions and heterochromatin re-patterning. In contrast to group C-I, chromosomal rearrangements did not play such an essential role in the divergence of the C-II group: translocations were detected only in TA2084, which is geographically distant from others (Table 1; Fig. 2). However, minor translocations may present in other C-II accessions, but they skip identification due to the lack of appropriate markers. Significant heterogeneity of the C-II group was also shown by gliadin analysis. All four C-II accessions had different gliadin profiles, which did not possess any common components. The spectra of each of the C-II accessions (PI 564180, PI 564181, TA2084, and PI 542191), however, carried a number of features (band loss or gain; bands that differed in intensity or position) which were not observed in the C-I group.

The comparative sequence analysis of the U31 nuclear fragment and three plastome intergenic spacer regions also revealed the highly heterogeneous composition of the C-II groups. Thus, the U31 fragment of type-I was found in three C-II accessions (Fig. 10), but two of them carried mutant alleles. All accessions with type-II U31 fragment belonged to the C-II group. It was an interesting observation because, according to Kadosumi et al. [23], type-II U31 fragment occurred extremely rare in Ae. columnaris, although frequently in Ae. umbellulata. Both type-II alleles identified here in the C-II accessions corresponded to those described earlier by these authors, but they found one allele in Ae. columnaris from Syria, while the second - in Ae. neglecta. Kadosumi et al. [23] also identified an additional type-II U31 allele, not found in this work, in Ae. columnaris from Iran; however, the karyotypic group of this accession was not determined.

In contrast to the relatively conservative C-I group, from three to 27 SNP’s covering all three intergenic spacer regions of cpDNA were identified among accessions of the C-II group.

An interesting fact uncovered by molecular analysis of the U31 nuclear fragment was an unexpectedly high number of SNPs (12) identified in Ae. umbellulata accession AE 1339 from Greece (Fig. S6), which showed no changes in the cpDNA (Fig. 12). According to FISH [40], this accession was karyotypically normal and similar to other Ae. umbellulata genotypes in the distribution of repetitive DNA probes [4, 41-46]. All these indicate that the observed mutations of AE 1339 were not caused by chromosomal rearrangement. From another side, Kawahara [47] has already uncovered the distinctness of Ae. umbellulata population from Greek Islands based on morphological and isozyme markers.

Summarizing our results, we can conclude that Ae. columnaris is phylogenetically very close to Ae. neglecta, and probably derived from it (or their common ancestor). It is supported by the following observations.

1. Owing to species-specific inversion of the chromosome 6Xc, the karyotype of Ae. columnaris becomes more “asymmetric” compared to Ae. neglecta. According to Stebbins [48], an increase of karyotype asymmetry is a trend of evolution in plant species and, therefore, Ae. neglecta karyotype should be considered “more primitive,” while Ae. columnaris – “more advanced;”
2. The chromosome 6X of *Ae. neglecta* possesses a minor 45S rDNA locus, which probably pre-existed in the progenitor *Aegilops* species; however, this locus is absent in *Ae. columnaris*;

3. *Ae. columnaris* is characterized by chromosome instability expressed in a higher proportion and broader diversity of chromosomal rearrangements (20 variants in more than 55% of accessions). Chromosome instability is an essential factor of speciation [49, 50] and is usually more expressed in phylogenetically new species. In addition, we found significant intraspecific polymorphism of *Ae. columnaris* plastome, although the only low variation of the chloroplast DNA sequences was recorded in *Triticum* and *Aegilops* species [51, 52].

The similarity of rDNA and repetitive DNA patterns of chromosomes of *Ae. neglecta* and group C-I of *Ae. columnaris* and their distinctness from chromosomes of the C-II accessions indicate that the C-I group diverged from *Ae. neglecta* or their common ancestor as a result of minor genome modifications. Group C-II could derive from a progenitor presumably belonging to group C-I of *Ae. columnaris* relatively recently, probably due to introgression from another *Aegilops* species, accompanied by significant reorganization of the parental genomes. As most C-II accessions with known collection sites originated from the very narrow geographic region of the southeastern coastal part of Turkey (Fig. 2, red boxes), they might originate from one common ancestor. Significant heterogeneity of the C-II accessions in karyotype structure, C-banding and FISH patterns, gliadin composition, and nuclear and chloroplast DNA sequences may indicate that they are currently at the initial stage of species divergence; most likely, this group is undergoing an extensive speciation process.

Materials and Methods

Intraspecific diversity of *Aegilops columnaris* Zhuk. (2n=4x=28, UcUtXcXc) was assessed on a set of 69 accessions of various geographic origin in comparison with the related tetraploid species *Ae. neglecta* Req. ex Bertol. (2n=4x=28, UUtUUtUt) – 4 accessions and *Ae. umbellulata* Zhuk. (2n=2x=14, UU), the diploid U-genome progenitor of *Ae. columnaris* and *Ae. neglecta* – 3 accessions. All 69 accessions were analyzed using C-banding, while 16 *Ae. columnaris*, three *Ae. neglecta*, and two *Ae. umbellulata* accessions were studied by FISH (Fluorescence in situ hybridization). Gliadin profiles were examined in 25 *Ae. columnaris* accessions of various geographic origins and one *Ae. neglecta* (Table 1), whereas 10 *Ae. columnaris* (five from C-I and five from C-II groups), two *Ae. neglecta* and three *Ae. umbellulata* accessions were selected for subsequent molecular analysis.

The materials were obtained from the gene banks of the N.I. Vavilov Institute of Plant Genetic Resources, S.-Petersburg, Russia; USDA-ARS (Aberdeen, Idaho, USA); Wheat Genetics and Genomics Resource Centre, Kansas State University (WGGRC), Kansas, USA; and Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. One sample of *Ae. columnaris* was collected by Drs. E.A. Nazarova and A.G. Gukasyan (, Erevan, Armenia) during an expedition of Takhtadjan Institute of Botany (1997) near the village Urznadzor, Armenia. Nine samples were collected in 2005-2006 by Dr. H. Özkan during expeditions to different regions of Turkey.

**DNA probes**

The following DNA probes were used:
(1) pTa71 was used as plasmid DNA (a 9 kb long sequence of common wheat encoding 18S, 5.8S and 26S rRNA genes including spacers [53] or the 5’ FAM-end-labeled (Syntol, Moscow, Russia) oligo-probe 5’- GGG CAA AAC CAC GTA CGT GGC ACA CGC CGC CTA-3’ [54];

(2) pTa794 was used as plasmid DNA (a 420 bp long sequence of wheat containing the 5S rRNA gene and intergenic spacer [55] or as the 5’ Cy-3-end-labeled (Evrogen, Moscow, Russia) oligo-probe 5’-TCA GAA CTC CGA AGT TAA GCG TGC TTG GCC GAG AGT AGT AC-3’ [56];

(3) pSc119.2– a 120 bp long sequence isolated from rye [57];

(4) pAs1 - a 1 kb fragment derived from *Ae. tauschii* and belonging to *Afa* family [58];

(5) pTa535-1 was used as 5’ 6-carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (TAMRA) end-labeled (MWG, Germany) oligo-probe (5’-AAA AAC TTG ACG CAC GTC ACG TAC AAA TTG GAC AAA CTC TTT CGG AGT ATC AGG GTT TC-3’) [54, 59];

(6) pTa-713 was used as 5’ 6-carboxytetra-methylrhodamine (TAMRA) or Cy3 end-labeled oligo-probe (5’- GTC GCG GTA GCG ACG ACG GAC GCC GAG ACG ACG TGA CAC CAT TCC CAC CCT GTC TA-3’) [54, 59];

(7) The oligo-(GTT)9 probe labeled at the 3’-end with fluorescein-12-dUTP was synthesized in the laboratory of biological microchips at the Engelhardt Institute of Molecular Biology, Moscow, Russia.

(8) The oligo-(GAA)10 probe labeled at the 3’-end with fluorescein-12-dUTP or Cy3 was synthesized in the laboratory of biological microchips at the Engelhardt Institute of Molecular Biology, Moscow, Russia.

(9) The oligo-(ACT)10 probe labeled at the 3’-end with Cy3 was synthesized in the laboratory of biological microchips at the Engelhardt Institute of Molecular Biology, Moscow, Russia.

**Giemsa C-banding method**

The C-banding procedure was carried out as described in Badaeva et al. [60]. Chromosomes of *Ae. columnaris* were classified according to genetic nomenclature developed earlier by [20] based on analysis of introgressive lines. Chromosomes of *Ae. neglecta* were classified according to similarity with *Ae. columnaris* chromosomes. Designation of *Ae. umbellulata* chromosomes followed the nomenclature suggested by [41].

**Fluorescence in situ hybridization**

FISH was carried out according to the protocol described in [61]. The probes labeled with fluorescein were detected using anti-fluorescein/Oregon green®, rabbit IgG fraction, Alexa Fluor® 488 conjugate (Molecular Probes, USA). The slides were counter-stained with DAPI (4’,6-diamidino-2-phenylindole) in Vectashield mounting media (Vector Laboratories, Peterborough, UK) and examined on a Zeiss Imager D-1 microscope. Selected metaphase cells were captured with AxioCam MRm digital camera using software AxioVision, version 4.6. Images were processed in Adobe Photoshop®, version CS5 (Adobe Systems, Edinburgh, UK).
Seed storage protein (Gliadin) analysis

Electrophoresis (EP) in polyacrylamide gel (PAAG) according to the previously published protocol [62] was employed to obtain gliadin spectra of the 25 Ae. columnaris and one Ae. neglecta accessions. The spectra of wheat cultivar Bezostaya-1 (a standard of gliadin spectra of common wheat) and Ae. columnaris, K-1193 with the known genetic control of gliadin components [27] were used to compare gliadin profiles of other Aegilops accessions (Fig. 2, a).

DNA extraction, PCR amplification, and DNA sequencing

Ten accessions of Ae. columnaris (five C-I representing five countries and five C-II from Turkey), Ae. umbellulata (3 accessions) and Ae. neglecta (2 accessions) were selected for analyses by molecular methods. Genomic DNA was extracted from 10-day-old seedlings using the DNeasy Plant Mini kit (QIAGEN, Hilden, Germany). DNA quantitative and qualitative evaluation was performed using NanoDrop 2000c spectrophotometer (Thermo-Scientific, Madison, USA).

Amplification of the U-genome-specific U31 nuclear fragment was performed using primers U31a and U31b [23] with PCR reaction conditions: an initial denaturation step of 95 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C -1 min, and 72 °C for 1 min with a final extension step at 72 °C for 3 min. The amplified fragments were sequenced directly from both ends with the same U31a and U31b primers.

Amplification of the three intergenic spacers regions (trnH(ugu)-psbA, rpl32-trnL(tag), trnT(ugu)-trnL(uaa)) of the plastome DNA of Aegilops accessions was performed using primer sets listed in Table S1. PCR amplification was performed in a 15-μl reaction mixture containing approximately 50 ng genomic DNA, 1.5 μl of 10× PCR buffer, 1.5 mM MgCl₂, 0.2 mM of dNTPs, 0.3 μM of each primer, and 0.5 unit of Taq DNA polymerase. The PCR conditions were as follows: an initial denaturation step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, annealing at the appropriate Tm for 1 min, and 72 °C for 1 min with a final extension step at 72 °C for 5 min. Amplification temperatures for trnH-psbA was 58 °C, trnL-rpl32 – 56 °C, trnT-trnL- 55 °C. The same primers were used to sequencing the obtained chloroplast DNA fragments; PCR products were cleaned before sequencing using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). PCR products were sequenced using standard protocols with the ABI Prism Big Dye Terminator cycle sequencing kit v. 3.1. Sequences were resolved on an ABI Prism 3100 automated sequencer.

A phylogenetic tree was constructed based on U31 data and combined chloroplast sequence data using MEGA 7 [63] based on ML (maximum likelihood) method. Kimura 2-parameter model was used for U31 and Tamura-3 parameter model for cpDNA, which was selected using Modeltest; 1000 bootstrap replicates were applied for the branch support evaluation. The SNP data from 10 Ae. columnaris genotypes were taken for subsequent analyses. The SNP position was determined from the first nucleotide of U31 or of each of the analyzed chloroplast spacers.
Funding

The work was performed within the framework of the State Task according to the theme plan, Projects No. 0662-2019-0006 (VIR), No 0112-2019-0002 (VIGG), No. 19-119021490115-2 (Research Center of Biotechnology), and with partial support from the Russian Foundation for Basic Research (project No. 20-04-00284a).

Acknowledgments

Materials for analysis were obtained from collections of N.I. Vavilov Institute of Plant Genetic Resources (VIR), St-Petersburg, Russia; small grain collection of USDA-ARS, Aberdeen, Idaho, USA; Wheat Genetics and Genomics Resource Centre, Kansas State University, Kansas, USA; IPK, Gatersleben, Germany. Nine samples were collected by Dr. H. Özkan in Turkey, and one sample was kindly provided by Drs. E.A. Nazarova and A.G. Gukasyan, Institute of Botany after A. Takhtajyan, Academy of Sciences of the Republic of Armenia, Erevan.

Authors’ contribution

EDB – planned experiments; performed C-banding and FISH analyses, wrote the ms; NNC, MKhB, HO – provided material for the analysis, SAS – synthesized oligo-probes for FISH analysis; ANF and AYuD – performed gliadin analysis; EZK – performed the analysis of nuclear and chloroplast DNA, wrote the ms. All authors read and approved the final version of the manuscript.

Data Availability Statement

The data presented in this study are available in Supplementary Material, Figure S6.

Conflicts of Interest

The authors declare no conflict of interest
References

1. Van Slageren, M.W. *Wild wheats: A monograph of Aegilops L. and Ambylopomum (Jaub. et Schach) Eig (Poaceae)*. Wageningen Agricultural University, Wageningen and ICARDA, Aleppo, Syria: Wageningen, 1994; p 514.

2. Kilian, B.; Mammen, K.; Millet, E.; Sharma, R.; Graner, A.; Salamini, F.; Hammer, K.; Ozkan, H. *Aegilops*. In *Wild crop relatives: Genomics and breeding resources*. Cereals, Kole, C., Ed. Springer: Berlin Heidelberg, 2011; pp 1-76. DOI: 10.1007/978-3-642-14228-4

3. Witcombe, J.R. *A guide to the species of Aegilops l. Their taxonomy, morphology and distribution*. IBPGR Secretariat: Rome, 1983; p 74.

4. Abdolmalaki, Z.; Mirzaghdari, G.; Mason, A.S.; Badaeva, E.D. Molecular cytogenetic analysis reveals evolutionary relationships between polyploid *Aegilops* species. *Plant Syst Evol 2019*, 305, 459-475. DOI: 10.1007/s00606-019-01585-3

5. Zhukovsky, P.M. A critical-systematical survey of the species of the genus *Aegilops* L. *Bul. Appl. Bot. Genet. Pl. Breed 1928*, 18, 417-609 (in Russian)

6. Eldarov, M.; Aminov, N.; van Slageren, M. Distribution and ecological diversity of *Aegilops* L. In the greater and lesser Caucasus regions of Azerbaijan. *Genet Res Crop Evol 2015*, 62, 265-273. DOI: 10.1007/s10722-014-0151-0

7. Haruntyunyan, M.; Dulloo, M.E.; Yeritsyan, N.; Danielyan, A. Red list assessment of nine *Aegilops* species in Armenia. *Genet Res Crop Evol 2010*, 57, 1177-1189. DOI: 10.1007/s10722-010-9558-4

8. Ohta, S.; Iwasaki, R.; Mori, N.; Ozkan, H. *Geographical distribution of two varieties of Aegilops neglecta and Ae. columnaris in southern Turkey revealed by the field researches from 2003 to 2005*. Fukui Prefectural University: Japan, 2006; pp 38-43.

9. Goryunova, S.V.; Chikida, N.N.; Kochieva, E.Z. RAPD analysis of the intraspecific and interspecific variation and phylogenetic relationships of *Aegilops* L. Species with the U genome. *Rus J Genet 2010*, 46, 841-854. DOI: 10.1134/s1022795410070094

10. Kimber, G.; Feldman, M. *Wild wheat, an introduction*. College of Agriculture University of Missouri: Columbia, Mo, U.S.A., 1987; Vol. Special Report 353, p 142.

11. Resta, P.; Zhang, H.B.; Dubkovsky, J.; Dvořák, J. The origin of the genomes of *Triticum biunciale*, *T. ovatum*, *T. neglectum*, *T. columnare*, and *T. rectum* based on variation in repeated nucleotide sequences. *Am J Bot 1996*, 83, 1556-1565. [https://doi.org/10.2307/2445829](https://doi.org/10.2307/2445829)

12. Edet, O.U.; Gorafi, Y.S.A.; Nasuda, S.; Tsujimoto, H. DArTseq-based analysis of genomic relationships among species of tribe *Triticeae*. *Sci Rep 2018*, 8, 16397. DOI: 10.1038/s41598-018-34811-y

13. Badaeva, E.D.; Amosova, A.V.; Samatadze, T.E.; Zoshchuk, S.A.; Shostak, N.G.; Chikida, N.N.; Zelenin, A.V.; Raupp, W.J.; Friebe, B.; Gill, B.S. Genome differentiation in *Aegilops*. 4. Evolution of the U-genome cluster. *Plant Syst Evol 2004*, 246, 45-76. DOI: 10.1007/s00606-003-0072-4

14. Cui, L.; Ren, Y.; Murray, T.D.; Yan, W.; Guo, Q.; Niu, Y.; Sun, Y.; Li, H. Development of perennial wheat through hybridization between wheat and
wheatgrasses: A review. *Engineering* 2018, 4, 507-513. https://doi.org/10.1016/j.eng.2018.07.003

15. Kihara, H. Genomanalyse bei *Triticum* und *Aegilops*. IX. Systematischer aufbau der gattung *Aegilops* auf genomanalytischer grundlage. *Cytologia* 1949, 14, 135-144. DOI: 10.1508/cytologia.14.135

16. Kihara, H. Considerations on the evolution and distribution of *Aegilops* species based on the analyser-method. *Cytologia* 1954, 19, 336-357. DOI: 10.1508/cytologia.19.336

17. Kihara, H. Interspecific relationship in *Triticum* and *Aegilops*. *Seiken Ziho* 1963, 15, 1-12.

18. Kimber, G.; Yen, Y. Hybrids between wheat relatives and autotetraploid *Triticum umbellulatum*. *Genome* 1989, 32, 1-5. DOI: 10.1139/g89-401

19. Chennaveeraiah, M.S. Karyomorphologic and cytotaxonomic studies in *Aegilops*. *Acta Horti Gotoburgensis* 1960, 23, 85-186.

20. Badaeva, E.D.; Ruban, A.S.; Shishkina, A.A.; Sibikeev, S.N.; Druzhin, A.E.; Surzhikov, S.A.; Dragovich, A.Y. Genetic classification of *Aegilops columnaris* Zhuk. (2n=4x=28, U³³X³³X³³X³³) chromosomes based on fish analysis and substitution patterns in common wheat × *Ae. columnaris* introgressive lines. *Genome* 2018, 61, 131-143. DOI: 10.1139/gen-2017-0186

21. Ogihara, Y.; Tsunewaki, K. Molecular basis of the genetic diversity of the cytoplasm in *Triticum* and *Aegilops*. I. Diversity of the chloroplast genome and its lineage revealed by the restriction pattern of ct-DNAs. *Jpn J Genet* 1982, 57, 371-396. https://doi.org/10.1266/jjg.57.371

22. Tsunewaki, K. Plasmon analysis as the counterpart of genome analysis. In *Methods of genome analysis in plant: Their merits and pitfalls*, Jauhar, P.P., Ed. CRC Press: Boca Ration, 1996; pp 271-299.

23. Kadosumi, S.; Kawahara, T.; Sasanuma, T. Multiple origins of U genome in two UM genome tetraploid *Aegilops* species, *Ae. columnaris* and *Ae. triaristata*, revealed based on the polymorphism of a genome-specific PCR fragment. *Genes Genet Syst* 2005, 80, 105-111. https://doi.org/10.1266/ggs.80.105

24. Tsunewaki, K.; Mukai, Y.; Endo, T.R.; Tsuji, S.; Murata, M. Genetic diversity of the cytoplasm in *Triticum* and *Aegilops*. V. Classification of 23 cytoplasms into eight plasma types *Jpn J Genet* 1976, 51, 175-191.

25. Kimber, G.; Tsunewaki, K. In *Genome symbols and plasma types in the wheat group*, Proceedings of the 7th International Wheat Genetics Symposium, 13-19 July 1988, Cambridge, England, 1988; Miller, T.E.; Koebner, R.M.D., Eds. Bath Press, Avon: Cambridge, England, pp 1209–1210.

26. Dvořák, J. In *Genome analysis in the Triticum-Aegilops alliance*, Proceedings of the 9th International Wheat Genetics Symposium, 2-7 August 1998, Saskatoon, Saskatchewan, 1998; Slinkard, A.E., Ed. Printcrafters Inc.: Saskatoon, Saskatchewan, pp 8-11.

27. Novoselskaya-Dragovich, A.Y.; Yankovskaya, A.A.; Badaeva, E.D. Alien introgressions and chromosomal rearrangements do not affect the activity of gliadin-coding genes in hybrid lines of *Triticum aestivum* L. × *Aegilops columnaris* Zhuk. *Vavilov J Genet Breed* 2018, 22, 507-514. DOI 10.18699/VJ18.388
28. Metakovsky, E.V.; Branlard, G.P.; Graybosch, R.A. Gliadins of common wheat: Polymorphism and genetics. In Gliadin and glutenin. The unique balance of wheat quality, Wrigley, C.; Békés, F.; Bushuk, W., Eds. AACC Internat: 2006; pp 35-84. https://doi.org/10.1094/9781891127519.004

29. Metakovsky, E.V.; Graybosch, R.A. Gliadin alleles in wheat: Identification and application. In Gliadin and glutenin. The unique balance of wheat quality, Wrigley, C.; Békés, F.; Bushuk, W., Eds. AACC Internat: 2006; pp 85-114. https://doi.org/10.1094/9781891127519.005

30. Dudnikov, A.J. Polymorphism of gliadins in Aegilops tauschii Coss. Local populations in two primary habitats in Dagestan. Genet Res Crop Evolut 2018, 65, 845-854. DOI: 10.1007/s10722-017-0575-4

31. Yan, Y.M.; Hsam, S.L.K.; Yu, J.Z.; Jiang, Y.; Zeller, F.J. Genetic polymorphisms at Gli-D' gliadin loci in Aegilops tauschii as revealed by acid polyacrylamide gel and capillary electrophoresis. Plant Breed 2003, 122, 120-124. DOI: 10.1046/j.1439-0523.2003.00824.x

32. Konarev, V.G. Wheat proteins. Kolos: Moscow, 1980; p 351 (in Russian).

33. Cole, E.W.; Fullington, J.G.; Kasarda, D.D. Grain protein variability among species of Triticum and Aegilops: Quantitative SDS-PAGE studies. Theor Appl Genet 1981, 60, 17-30. DOI: 10.1007/BF00275173

34. Medouri, A.; Bellil, I.; Khelifi, D. The genetic diversity of gliadins in Aegilops geniculata from Algeria. Czech J Genet Plant Breed 2015, 51, 9-15.

35. Metakovsky, E.V. Gliadin allele identification in common wheat. II. Catalogue of gliadin alleles in common wheat. J Genet Breed 1991, 45, 325-344.

36. Badaeva, E.D.; Friebe, B.; Gill, B.S. Genome differentiation in Aegilops. 2. Physical mapping of 5S and 18S-26S ribosomal RNA gene families in diploid species. Genome 1996, 39, 1150-1158. DOI: 10.1139/g96-145

37. Badaeva, E.D.; Amosova, A.V.; Muravenko, O.V.; Samatadze, T.E.; Chikida, N.N.; Zelenin, A.V.; Friebe, B.; Gill, B.S. Genome differentiation in Aegilops. 3. Evolution of the D-genome cluster. Plant Syst EvoL 2002, 231, 163-190. DOI: 10.1007/s00606-020-01731-2

38. Yamamoto, M. Distribution of ribosomal RNA genes in Aegilops and Triticum chromosomes. Bulletin of Kansai Women’s College 1992, 2, 25-37 (in Japanese)

39. Yamamoto, M. Detection of ribosomal RNA genes in Aegilops by in situ hybridization. Bulletin of Osaka Private College Association 1992, 29, 77-82 (in Japanese).

40. Badaeva, E.D.; Chikida, N.N.; Belousova, M.K.; Ruban, A.S.; Surzhikov, S.A.; Zoshchuk, S.A. A new insight on the evolution of polyploid Aegilops species from the complex Crassa: Molecular-cytogenetic analysis. Plant Syst Evo 2021, 307, 3. DOI: 10.1007/s00606-020-01731-2

41. Friebe, B.; Jiang, J.; Tuleen, N.; Gill, B.S. Standard karyotype of Triticum umbellulatum and the characterization of derived chromosome addition and translocation lines in common wheat. Theor Appl Genet. 1995, 90, 150-156. DOI: 10.1007/BF00221010

42. Molnár, I.; Molnár-Láng, M. Visualization of U and M genome chromosomes by multicolour genomic in situ hybridization in Aegilops biuncialis and Triticum
aestivum-Ae. biuncialis amphiploids. Acta Agronom Hung 2010, 58, 195-202. http://dx.doi.org/10.1556/AGr.58.2010.3.1

43. Molnár, I.; Kubaláková, M.; Šimková, H.; Cseh, A.; Molnár-Láng, M.; Doležel, J. Chromosome isolation by flow sorting in Aegilops umbellulata and Ae. comosa and their allotetraploid hybrids Ae. biuncialis and Ae. geniculata. PLOS ONE 2011, 6, e27708. DOI: 10.1371/journal.pone.0027708

44. Molnár, I.; Vrána, J.; Burešová, V.; Cápal, P.; Farkas, A.; Darkó, É.; Cseh, A.; Kubaláková, M.; Molnár-Láng, M.; Doležel, J. Dissecting the U, M, S and C genomes of wild relatives of bread wheat (Aegilops spp.) into chromosomes and exploring their synteny with wheat. Plant J 2016, 88, 452-467. DOI: 10.1111/tpj.13266

45. Song, Z.; Dai, S.; Bao, T.; Zuo, Y.; Xiang, Q.; Li, J.; Liu, G.; Yan, Z. Analysis of structural genomic diversity in Aegilops umbellulata, Ae. markgrafii, Ae. comosa, and Ae. uniaristata by fluorescence in situ hybridization karyotyping. Front Plant Sci 2020, 11, 710-710. DOI: 10.3389/fpls.2020.00710

46. Badaeva, E.D.; Friebe, B.; Gill, B.S. Genome differentiation in Aegilops. 1. Distribution of highly repetitive DNA sequences on chromosomes of diploid species. Genome 1996, 39, 293-306. DOI: 10.1139/g96-040

47. Kawahara, T. Morphological and isozyme variation in genebank accessions of Aegilops umbellulata Zhuk., a wild relative of wheat. Genet Res Crop Evol 2002, 49, 89-94. DOI: 10.1023/A:1013895912971

48. Stebbins, L. Chromosomal evolution in higher plants. Addison-Wesley: London, UK, 1971; p 216.

49. Dion-Côté, A.-M.; Barbash, D.A. Beyond speciation genes: An overview of genome stability in evolution and speciation. Curr Op Genet Dev 2017, 47, 17-23. DOI: 10.1016/j.gde.2017.07.014

50. Fishman, L.; Statthos, A.; Beardsley, P.M.; Williams, C.F.; Hill, J.P. Chromosomal rearrangements and the genetics of reproductive barriers in Mimulus (monkey flowers). Evolution 2013, 67, 2547-2560. https://doi.org/10.1111/evo.12154

51. Gornicki, P.; Zhu, H.; Wang, J.; Challa, G.S.; Zhang, Z.; Gill, B.S.; Li, W. The chloroplast view of the evolution of polyploid wheat. New Phytol 2014, 204, 704-714. DOI: 10.1111/nph.12931

52. Bernhardt, N.; Brassac, J.; Kilian, B.; Blattner, F.R. Dated tribe-wide whole chloroplast genome phylogeny indicates recurrent hybridizations within Triticeae. BMC Evol Biol 2017, 17, 141. DOI: 10.1186/s12862-017-0989-9

53. Gerlach, W.L.; Bedbrook, J.R. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. 1979, 7, 1869-1885. DOI: 10.1093/nar/7.7.1869

54. Tang, Z.; Yang, Z.; Fu, S. Oligonucleotides replacing the roles of repetitive sequences pAs1, pSc119.2, pTa-535, pTa71, CCS1, and pAWRC.1 for FISH analysis. J Appl Genet 2014, 55, 313-318. DOI: 10.1007/s13353-014-0215-z

55. Gerlach, W.L.; Dyer, T.A. Sequence organization of the repeated units in the nucleus of wheat which contains 5S-RNA genes. Nucleic Acids Res. 1980, 8, 4851-4865. DOI: 10.1093/nar/8.21.4851

56. Yu, Z.; Wang, H.; Xu, Y.; Li, Y.; Lang, T.; Yang, Z.; Li, G. Characterization of chromosomal rearrangement in new wheat—Thinopyrum intermedium addition
lines carrying Thinopyrum—specific grain hardness genes. *Agronomy* 2019, 9, 18. doi:10.3390/agronomy9010018

57. Bedbrook, R.J.; Jones, J.; O’Dell, M.; Thompson, R.J.; Flavell, R.B. A molecular description of telomeric heterochromatin in *Secale* species. *Cell* 1980, 19, 545-560. DOI: 10.1016/0092-8674(80)90529-2

58. Rayburn, A.L.; Gill, B.S. Isolation of a D-genome specific repeated DNA sequence from *Aegilops squarrosa*. *Plant Mol Biol Rep* 1986, 4, 102-109. DOI: /10.1007/BF02732107

59. Komuro, S.; Endo, R.; Shikata, K.; Kato, A. Genomic and chromosomal distribution patterns of various repeated DNA sequences in wheat revealed by a fluorescence in situ hybridization procedure. *Genome* 2013, 56, 131-137. DOI: 10.1139/gen-2013-0003

60. Badaeva, E.D.; Badaev, N.S.; Gill, B.S.; Filatenko, A.A. Intraspecific karyotype divergence in *Triticum araraticum* (Poaceae). *Plant Syst Evol* 1994, 192,117-145. DOI: 10.1007/BF00985912

61. Badaeva, E.D.; Ruban, A.S.; Aliyeva-Schnorr, L.; Municio, C.; Hesse, S.; Houben, A. In Situ Hybridization to Plant Chromosomes. In: *Fluorescence In Situ Hybridization (FISH) Application Guide*; Liehr, T. Ed.; Springer: Berlin, Germany, 2017; pp. 477-494.

62. Metakovsky, E.V.; Novoselskaya, A.Y. Gliadin allele identification in common wheat 1. Methodological aspects of the analysis of gliadin patterns by one-dimensional polyacrylamide gel electrophoresis. *J Genet Breed* 1991, 45, 317-324.

63. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis Version 7.0 for bigger datasets. *Mol Biol Evol* 2016, 33,1870-1874. DOI: 10.1093/molbev/msw054