

Hybridization Domestication and Molecular Cytogenetic Characterization of New Germplasm of *Thinopyrum intermedium* with smGISH at Northeastern China

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Abstract

Thinopyrum intermedium ($2n = 6x = 42$, StStJ^rJ^rJ^{vs}J^{vs}), a perennial relative of wheat, is resistant or tolerant to biotic and abiotic stresses, making it an important genetic material for breeding perennial crops. It is also known as intermediate wheatgrass (IWG). The morphological performance, chromosome composition, and yield components of 24 newly developed perennial germplasm, designated 19HSC-Q and 20HSC-Z lines, were investigated from 2019 to 2022. Six lines of 19HSC-Q had higher values of yield component traits, such as thousand-kernel weight, grains per spike, and tiller number than that of *Th. intermedium*. 19HSC-Q14, 19HSC-Q18 and 19HSC-Q20 had the best agronomic traits with grain number per spike and thousand-kernel weight of 115, 97, and 102 and 7.674, 7.516, and 7.371 g in 2022, respectively. Three 20HSC-Z lines had been growing for two years that the grain number per spike was 116, 88, and 81, and the thousand-kernel weight was 7.146, 6.523, and 5.916 g, respectively. The results of sequential multicolor GISH analysis revealed that the J^{vs}-subgenome of *Th. intermedium* were divided into two karyotypes, type-I J^{vs} with 3 pairs of chromosomes and type-II J^{vs} with 4 pairs of chromosomes. Both IWG and 24 perennial germplasm had the similar chromosome composition but the translocation between subgenome chromosomes were detected in some lines with prominent comprehensive agronomic traits, such as 19HSC-Q6, 19HSC-Q11, 19HSC-Q18, and 20HSC-Z. The chromosome aberration could be distinguished into two types: the large fragment translocation with J^r-St, J^{vs}-St, J^r-IIJ^{vs}, and J^{vs}-J^r and the small fragment introgression of St-IJ^{vs}. Results of this study provide important materials for the next breeding cycle of cold resistant perennial wheatgrass.

Background

The continuous growth of the global population increases human demand for food and livestock, and plenty of lands that are most suitable for annual crops have been already used. The competition for arable lands between non-food products (e.g., biofuels) and food crops is becoming more and more fierce (Glover et al., 2010; Godfray et al., 2010; Springmann et al., 2018). Although today's farming methods have enabled us to achieve unprecedented yields, they have also exacerbated ecosystem problems, such as soil erosion, greenhouse gas emissions, and water pollution (Monfreda et al., 2008). The strategy of "Ecological intensification of agriculture" was proposed to enhance the production of food while expanding ecosystem services and reducing inputs (DeHaan et al., 2018). Many studies have demonstrated that perennial crops are important to realize intensification of agricultural ecology, because they have advantages over annual crops in soil carbon balance, nutrient retention, soil water uptake efficiency, functional utilization of soil microbiome and weed suppression (Cox et al., 2006; Batello et al., 2013; DeHaan, 2015; Kantar et al., 2016; Crews and Cattani, 2018; Ryan et al., 2018). Therefore, it is a common objective of breeding for perennial crops with acceptable grain yields without environmental penalty.

Perennial grass *Thinopyrum intermedium* (Host) Barkworth and Dewey ($2n = 6x = 42$, StStJ^rJ^rJ^SJ^S), also known as intermediate wheatgrass (IWG), is one of the most promising gene reservoirs in the tribe Triticeae. IWG has lush plant growth, high stature and leaf protein content, cold-hardness, drought resistance, and salt tolerance (Wagoner, 1995; Harmoney, 2015). It has been used extensively as a cool-season forage in the USA and Canada and across its native range in Eurasia (Wagoner, 1995). Because of its developed perennial rhizome system, IWG can improve quality of soil, surface and ground water, provides habitat for wild animals (such as Waterfowl nesting sites), isolate carbon sources in the atmosphere, and strong impacts on ecosystem improvement (DeHaan and Ismail, 2017; DeHaan et al., 2018;). Moreover, IWG was initially selected as a target for domestication as a perennial grain because it has synchronous seed maturity, edible grain, moderate shattering and reasonable thresh ability similar as common wheat, but with higher contents of grain protein and fiber contents (Wagoner, 1990, 1995). Although protein characteristics of the flour were different from that of ordinary wheat flour, IWG grain has the same nutritional composition and unique flavor. Mixed with common wheat flour in a certain proportion, it can be used to make baking products (Becker et al., 1991; Zhang et al., 2014; Zhang et al., 2015; Marti et al., 2015; Marti et al., 2016). IWG has many outstanding advantages as a wild perennial species, but the yield was still a big gap between IWG and ordinary annual wheat.

Since 1983, in order to domesticate and improve the yield components of *Th. intermedium* and select its plants as a perennial crop, the Rodale Research Center, Kutztown, PA, USA, has initiated a project to domesticate *Th. intermedium* in order to improve its yield components and perenniality (Wagoner, 1990). From 1991 to 1995, Rodale Research Center and Big Flats Plant Materials Center (USDA) completed two cycles of selection for IWG, and the average seed yield per plant was 25% higher than that of the whole population (Wagoner and Schaeffer, 1990; Wagoner, 1995). Subsequently, scientists at The Land Institute (TLI), Saline, KS, USA, have worked on some of the selected plants aiming at improving yield per head, increased seed size, free threshing, reduced height, and early maturity since 2003 (DeHaan et al., 2018; Bajgain et al., 2020). The first IWG cultivar, Kernza®, was commercialized after nine cycles of selection (DeHaan et al., 2018; Bajgain et al., 2020). In 2011, the University of Manitoba, Winnipeg, Canada, and the University of Minnesota, St. Paul, USA, also initiated a project to directly domesticate wheatgrass germplasm from TLI (Zhang et al., 2016; Cattani, 2016). The first commercial food-grade intermediate wheatgrass cultivar MN-Clearwater (Reg. no. CV-287, PI 692651) was released as a synthetic population by University of Minnesota in 2019

(Bajgain et al., 2020). In variety trials across Minnesota, MN-Clearwater produced 696 kg ha⁻¹ of grain with minimal lodging and negligible disease levels (Bajgain et al., 2020). An IWG breeding program was also initiated in Utah, USA, and Uppsala, Sweden (Bajgain et al., 2020).

As an allohexaploid species, *Th. intermedium* carries three subgenomes with chromosomes sorted in seven homoeologous groups. The genome composition of *Th. intermedium* has been extensively studied with molecular cytogenetic methods resulting in different hypotheses (Chen et al., 1998; Chen, 2005; Mahelka et al., 2011, 2013; Li et al., 2016). Using EST-SSR primers developed from the putative progenitor diploid species *Pseudoroegneria strigosa* (Pursh) (2n = 2x = 14, StSt), *Th. bessarabicum* (Savul. & Rayss) (2n = 2x = 14, JJ = J^bJ^b) and *Th. elongatum* (Host) (2n = 2x = 14, EE = J^eJ^e), *Th. intermedium* is proposed to be formed by an ancient hybridization event between the diploid *Ps. strigosa* (2n = 14, StSt) and a segmental tetraploid carrying J^r and J^{vs} genomes (Wang et al., 2015). The J^r and J^{vs} genomes represent ancestral genomes of J^b of *Th. bessarabicum* and J^e of *Th. elongatum*, respectively. Genome J^{vs} is distinct from genome J^b as it retains repetitive sequences from the V genome of *Dasyprum villosum* (L.) P. Candargy, while J^r carries a long terminal repeat (LTR) originating from the R genome of rye (*Secale cereale* L., 2n = 2x = 14, RR) (Kishii et al., 2005; Mahelka et al., 2011; Wang et al., 2015). Cseh et al. (2019) suggested that the *Th. intermedium* chromosomes originated from three genomes, which were *Ps. strigosa*, *Th. bessarabicum*, and *D. villosum* and the genome composition of *Th. intermedium* was proposed to be StStJ^rJ^rJ^{vs}J^{vs}.

Currently, the direct domestication strategy based on recurrent phenotypic selection is widely used in most breeding programs for domestication and improvement of *Th. intermedium*. This method relies on the variations of plants within a population. Dehaan et al. (2014) suggested that wheat with grass-like traits or perennial grass with wheat-like traits were possible to realize the future perennial grains. It is less difficult to introduce domesticated genes from common wheat into perennial plants to improve wild traits, such as seed and head shattering, infinite flowering, and larger kernels (Abbo et al., 2014). This offers a new strategy to accelerate the domestication of *Th. intermedium*.

We developed 34 perennial wheatgrass lines with vigorous growth and cold hardiness by recurrent phenotypic selection of the cross between octoploid tritelytrigia (2n = 8x = 56, AABBDD EE) and *Th. intermedium* (Yan et al., 2020). Two progeny lines 19HSC-Q and 20HSC-Z with promising agronomic performances and seed setting were developed after five selection cycles from 2007 to 2022. The objectives of this study were to (1) analyze the morphology of these perennial lines and the potential for grain production; (2) characterize their chromosome compositions using sequential multicolour genomic *in situ* hybridization (smGISH) for understanding the subgenome chromosome variation type of new germplasm.

Methods

Plant Materials

Annual wheat-*Th. intermedium* partial amphiploids Maicao 8 (919-C /Yuan16-3) and Maicao 9 (2A2/Xiaoyan68) (2n = 8x = 56, AABBDDXX, X was the mix genome with St-J^r-J^{vs} chromosomes) as the female parents were crossed with *Th. intermedium* (2n = 6x = 42, StStJ^rJ^rJ^{vs}J^{vs}) originating from Heilongjiang Academy of Agricultural Sciences, Harbin, China in 2006. Twenty lines of perennial wheatgrass were obtained after two rounds of three-year artificial and natural selection based on recurrent phenotypic evaluation (HNU-C1 and HNU-C2) in the field of Harbin Normal University, Harbin, Heilongjiang province (126°57'E, 45°87'N) from 2007 to 2013 (Yan et al. 2020). Four hundreds of cold-hardy perennial plants were obtained during the 3rd cycle of selection (HNU-C3) from 2013 to 2016. Twenty plants were selected because they performed high seed setting, cold tolerance, and strong post-harvest regrowth ability. In the 4th cycle of selection (HNU-C4) from 2016 to 2019, 200 perennial plants were selected. Finally, 24 perennial cold-hardy wheatgrass lines, designated 19HSC-Q and HSC-Z, were developed. From 2019 to 2022 as HNU-C5, 21 19HSC-Q and 3 20HSC-Z lines were subjected to molecular cytogenetic analysis (Fig. 1). Accessions of *Ps. strigosa* (2n = 2x = 14, StSt), *Th. bessarabicum* (2n = 2x = 14, J^bJ^b) and *D. villosum* (2n = 2x = 14, VV) were included as the sources of DNA to be used as probes to detect *Th. intermedium* subgenomes.

Evaluation of Perennial Cold-hardy Ability and Agronomic Traits

From 2007, the newly developed IWG lines were investigated in plots in an experimental farm of Harbin Normal University, Harbin, China. The agricultural climate of Harbin is warm semiarid, with spring drought and a semi-humid summer. During 2013–2022, the annual mean temperature ranged from 5.37°C to 17.2°C in January and 21.27°C to 28.3°C in July. The extreme winter temperature was – 30.11°C.

In autumn of 2019 and 2020, 19HSC-Q and 20 HSC-Z perennial wheatgrass lines were sown in the experimental field for HNU-5C. The field management, seeding, and agronomic characterization were carried out as described previously (Yan et al. 2020). Defoliation was performed in August for harvesting both grains and forages each year. Post-harvest regrow ability and the regrowth of underground

rhizomes were observed from harvest to the onset of winter in October and November. From November to early April in the following year, plants in the field were exposed to the natural cold environment to examine their cold hardiness based on their survivability during winter and regrowth in spring. Plants that were able to regrow for at least three consecutive years in the field were regarded as cold-hardy perennials. Maicao 8, Maicao 9, and the *Th. intermedium* accession were grown in 1-m rows and 0.3-m spacing as the annual and perennial controls, respectively.

During the growing seasons, the agronomic traits, including plant height (cm), tiller number, spike length (cm), seed set percentage, grain number per spike and 1000-kernal weigh (g), were investigated for the 19HSC-Q and 20HSC-Z perennial wheatgrass lines in the sampled individuals from each plot. Plant height was determined from the ground level to the top of a spike. Spike length was measured from the base of rachis to the top of spike. Numbers of kernels and spikelets per spike were enumerated and spikes were threshed in a bench micro-thresher to determine 1000-kernel weight. Common practices of irrigation (one before over-winter stage immediately after defoliation, and the other after grain and forage harvesting at July), fertilizers (nitrogen, phosphorus, and potassium), and pesticides were conducted following the local farm system (Yan et al. 2020).

Chromosome preparation

Seeds were germinated at 23.5°C for 24 h on moist filter paper in Petri dishes, incubated at 4°C for 48 h, and then incubated at 23.5°C for 27.5 h. Root tips were treated with ice water at 0–4°C for 24 h, fixed in Carnoy's fixative (anhydrous alcohol: acetic acid = 3:1, v/v) for 24 h. Root tips were squashed in 45% acetic acid and observed under a phase contrast microscope (BH-2, Olympus, Tokyo, Japan).

Sequential multicolor GISH

Genomic DNA was isolated from young leaves of *Ps. strigosa* (St-genome), *Th. bessarabicum* (J^b-genome) and *D. villosum* (V-genome) to prepare the St-, J^b-, and V-genome probes using a CTAB (Cetyltrimethylammonium Bromide) method (Doyle and Doyle, 1987). Five seeds randomly selected from each line were subjected for smGISH analysis. In the first round of GISH, the genomic DNA of *Ps. strigosa* and *Th. bessarabicum* were separately labelled with fluorescein-12-dUTP (green) (BioNick Labeling System; Invitrogen, Waltham, MA, USA) and Tetranmethyl-Rhodamine-5-dUTP (red) (DIG-Nick Translation Mix, Roche Diagnostics, Mannheim, Germany). In the second round of GISH, the genomic DNA of *D. villosum* and *Ps. strigosa* were separately labelled with fluorescein-12-dUTP (green) and Tetranmethyl-Rhodamine-5-dUTP (red). The protocol of smGISH was described previously by Yan et al. (2020) and Cseh et al. (2019). All slides were visualized using a Leica DM5500B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with different filters for detecting DAPI [2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride] (blue), Alexa Fluor 488 (green), and Alexa Fluor 594 (red). A Leica DM6000B fluorescence microscope (Leica, Mannheim, Germany) was used for observing the hybridization signals, and photographs were captured with a Leica digital camera (Model DFC480) and processed by Photoshop 8.0 (Adobe, city, state, USA).

Statistical analysis

Values of the agronomic traits investigated were subjected to analysis of variance (ANOVA) using genotype, year, and genotype by year interaction as main factors. The Fisher's Least Significance Difference (LSD) was used to perform multiple comparisons for differentiating the significant difference among genotypes and plant ages (i.e., the first, second, and third-year plants) based on means of each parameter examined. Statistical analysis was performed in the IBM SPSS Statistics for Windows Version 26.0 (IBM Corp., Armonk, NY, USA). Significant differences in the growth years and various traits of different materials were identified by the *t*-test of multiple samples at $P < 0.05$ using IBM SPSS Statistics 19 software (SPSS Inc., Chicago, IL, USA).

Results

Cold-hardiness and maturity of the perennial wheatgrass lines

From 2019 to 2022, the twenty-one 19HSC-Q perennial lines were evaluated for their cold hardiness, perennial habit, and maturity. All of them survived for more than 3 years and were perennial, which resembled their perennial parent *Th. intermedium*. The coleoptile was purple and the plants were grown before November. The seedlings stopped growing and went into dormancy as snow covering in natural cold winter, but the leaves and stems remained green during the whole winter. All the 19HSC-Q plants resumed growth normally in April 2020 and entered the flowering stage on June 22nd lasting for a week. They matured by the end of July and showed the post-harvest regeneration after cutting in early August 2020. From September to October, the plants grew vigorously, and new stems and leaves emerged from crowns or roots. The growth of plants was slowed down in November and entered a dormant period after snow-cover in December (Fig. 2a). From November 2020 to the end of March 2021, some stems and leaves near the ground remained green in all the regenerated plants, and the new rhizomes appeared in the soil. In April, the leaves and stems were withered. After cutting, new leaves regrew from the original crowns and the plants returned to the vegetative state for a new life cycle in May 2021. The plants resumed growth again, and the underground

rhizomes developed into new plants. The rates of returning green was 100% for all the 19HSC-Q plants. The subsequent performance of the plants was consistent with the first life cycle. All plants were harvested in the end of July 2021. The 19HSC-Q plants still maintained the post-harvest regeneration. In the third growth cycle from 2021 to 2022, the plants kept a stable rate of returning green and regeneration after cutting (Fig. 2b), and the dates to flowering and maturity were consistent with the traits of the previous two living years. Among the twenty-one 19HSC-Q lines, 19HSC-Q5 and 19HSC-Q17 also passed the cold tolerance and perennial test, but the vitality of plants weakened. The ability of regeneration and returning green were decreased also, and the plant's cluster gradually shrunk. Currently, all the 19HSC-Q plants have entered the fourth life cycle.

From 2020 to 2022, three 20HSC-Z lines in HNU-5C were also tested for the cold tolerance and maturity in the natural cold winter. These lines had better cold tolerance and post-harvest regrowth and their growth habits were consistent with other 19HSC-Q lines. At present, they have entered the third growth cycle.

Twenty-one 19HSC-Q and three 20HSC-Z perennial wheatgrass lines with better cold tolerance and post-harvest regeneration (HPR) were seeded in Harbin. Compared with the previous four breeding cycles, dates to flowering and maturity of the 19HSC-Q plants were about 40 days earlier. It was more adapted to the cold winter of Harbin.

Performance of morphological and yield components

The agronomic traits of 21 19HSC-Q perennial lines were investigated for three consecutive growing seasons from 2019 to 2022. Except 19HSC-Q5 and 19HSC-Q17, these lines were maintained the wheatgrass characteristics in plant height, heading date and tillering ability. However, the agronomic performances of these plants were different and the land cover area per plant was inconsistent after three growth cycles.

The average plant height of 19HSC-Q lines over 3 years ranged from 109.8 cm to 163.5 cm (Fig. 3a). The average spike length ranged from 18.04 cm to 26.89 cm (Fig. 2d, 3b). The average number of spikelets ranged from 15.77 to 23.87 with 4–5 florets per spikelet, and the highest number of 24 spikelets was observed in 19HSC-Q14 in 2022 (Table 1). The average spike length and spikelet number of 19HSC-Q5, 19HSC-Q7, 19HSC-Q9, 19HSC-Q12, 19HSC-Q15, and 19HSC-Q17 were lower than those of the control Th. intermedium. Although 19HSC-Q5 and 19HSC-Q7 have grown for three years, 19HSC-Q5 was sterile and 19HSC-Q7 produced a few seeds in 2022.

Table 1
Agronomic traits, yield composition traits data and grain morphological characteristics and protein content for nine cold-hardiness perennial wheatgrass lines and IWG in 2022.

Lines	Years of growth	Plant height (cm)	Spike length (cm)	Spikelet number	Tiller number	Grain number per spike	Thousand-kernel weigh (g)	Seed setting rate (%)	Yield quadrat size (m ²)	Seed length (mm)	Seed width (mm)	Protein Content (%)
IWG	3	131.8	19.41	17	102	54.6	5.128	42.68	0.093	3.415	0.918	15.47
19HSC-Q2	3	155.4	26.75	22	147	94.67	6.314	52.9	0.267	4.572	1.355	17.21
19HSC-Q11	3	144.9	21.27	23.3	98	82.33	6.521	43.0	0.204	4.128	1.218	17.11
19HSC-Q14	3	154.6	25.58	24	335	115	7.674	50.3	0.335	5.535	1.472	17.36
19HSC-Q18	3	134.4	24.76	21	225	97.33	7.516	55.9	0.272	4.584	1.246	19.14
19HSC-Q20	3	145.1	23.73	23	467	102	7.371	54.5	0.615	4.657	1.347	19.89
19HSC-Q21	3	156.9	27.61	23.3	249	92.33	6.895	43.5	0.247	4.697	1.245	17.73
20HSC-Z1	2	155	27.97	21	131	116	7.146	52.46	0.058	6.314	1.218	20.01
20HSC-Z2	2	133.3	27.17	24	149	88.67	6.523	52.94	0.119	4.346	1.365	17.85
20HSC-Z3	2	132.7	26.41	19	173	81	5.916	55.69	0.151	3.728	1.146	18.52

The tiller number of 19HSC-Q plants increased over years. For example, line 19HSC-Q2 produced 57 tillers in the first growth cycle, while all the other plants had 14 to 38 tillers. In the third growth year, the tiller number of 19HSC-Q2 was 147, which was much lower than that of 19HSC-Q14 (335) and 19HSC-Q20 (467). However, the tiller numbers of 19HSC-Q14 and 19HSC-Q20 in the first life year were only 33 and 31. In the third growth cycle, there were nine plants with more than 200 tillers (Fig. 3d). In 2022, the plants with the largest number of tillers was 19HSC-Q20, which also had the largest of land cover area (0.615 m²), followed by 19HSC-Q14 (0.335 m²) and 19HSC-Q8 with (0.272 m²). While the other plants ranged from 0.080 m² to 0.615 m² for the land cover area per plant.

From 2020 to 2022, the agronomic characteristics of three 20HSC-Z lines were measured. The average plant height ranged from 55 cm to 138 cm in two growth cycles. The spike length and the number of spikelets per spike ranged from 18.37 cm to 27.97 cm and 19 to 24, respectively. The number of florets per spike were 4 to 6. In 2022, the plant height and spike length of 20HSC-Z1 was 155 cm and 27.97 cm, respectively (Fig. 2d, Table 1). The highest number of spikelets was 24 for 20HSC-Z2 (Table 1). In 2022, the largest tiller number of the three 20HSC-Z lines was 173 from 20HSC-Z3 (Table 1). The largest land cover area per plant was also observed in 20HSC-Z3 (0.151m²), followed by 20HSC-Z2 (0.119 m²) and 20HSC-Z1 (0.058 m²) (Table 1).

The yield components from 2019 to 2022 showed that the number of grains per spike and thousand-grain weight varied significantly among the 21 19HSC-Q lines, and the grain protein content was greater than 15%. In the first life year, the grains per spike of 15 lines exceeded 60, but 14 of them decreased each year. Only 7 lines increased in the number of grains per spike during the three living cycles. In the third life cycle, lines 19HSC-Q2, 19HSC-Q11, 19HSC-Q14, 19HSC-Q18, 19HSC-Q20, and 19HSC-Q21 had more than 80 grains per spike in comparison with 54.6 of *Th. intermedium* (Fig. 3e, Table 1). Moreover, grain numbers per spike of the top three lines 19HSC-Q14, 19HSC-Q20 and 19HSC-Q18 in 2022 were 115, 102 and 97, respectively (Fig. 2c, Fig. 3e, Table 1). The average seed set for these lines was around 50% and the thousand-grain weight in 2022 was more than 6 g, higher than 5.128 g of IWG in the same year. Lines 19HSC-Q14, 19HSC-Q18 and 19HSC-Q20 with the highest grains per spike also had the largest thousand-grain weight of 7.67, 7.52 and 7.37 g, respectively (Fig. 3f, Table 1). In 2022, the average grain length and width of 6 lines were 4.13–5.54 and 1.22–1.47 mm, which were higher than those of the IWG (3.42 mm and 0.92 mm, respectively). All materials, The average length and width of 19HSC-Q14 were the largest with 5.54 mm in length and 1.47 mm in width (Fig. 2e, 2f, Table 1). In addition, the protein content of 10 materials was more than 17%, and 19HSC-Q14, 19HSC-Q18 and 19HSC-Q20 with the best grains per spike and thousand-grain weight had protein content of 17.36%, 19.14% and 19.89%, respectively (Table 1).

During 2022, the yield components results of three 20HSC-Z plants revealed that the number of grains per spike was above 80, and the seed setting rate was above 50%. The highest number of grains per spike was 116 of 20HSC-Z1. 20HSC-Z3 had the highest set rate of 55.69% (Table 1). The thousand grain weight of 20HSC-Z1, 20HSC-Z2 and 20HSC-Z3 were 7.146, 6.523 g and 5.916 g, respectively (Table 1). The grain length of the three materials ranged from 3.728mm to 6.314mm, and the average width ranged from 1.146mm to 1.365mm among which 20HSC-Z1 had the longest seed length of 6.314 mm (Fig. 2e, Table 1) and 20HSC-Z2 had the widest width of 1.365 mm (Table 1). The protein content assay showed that all three plants contained more than 17% protein, and 20HSC-Z1 had the highest protein content with 20.01% (Table 1).

In conclusion, we obtained 6 key lines in 19HSC-Q series, 19HSC-Q2, 19HSC-Q11, 19HSC-Q14, 19HSC-Q18, 19HSC-Q20 and 19HSC-Q21, with the most outstanding agronomic traits and yield component character in HNU-5C. 19HSC-Q14, 19HSC-Q18, and 19HSC-Q20 had the best comprehensive traits (Fig. 4).

Sequential multicolor GISH analysis

SmGISH of *Th. intermedium*

During the deduction of *Th. intermedium* using the whole genome DNA from the diploid *Th. bessarabicum* (J^b-genome, red) and *Ps. strigosa* (St-genome, green) as the probes, 14 St-subgenome chromosomes with complete light-green fluorescent signals were observed in IWG at the first round of GISH analysis. Another 28 of chromosomes had the light-green fluorescent signals at the ends or centromeric regions of chromosomes. The light-red fluorescence signals were divided into two types, totaling 28 chromosomes. One was a dark-pink signal with a total of 14 chromosomes that was spread throughout the long and short arms of chromosome and had a green St-genome probe signal at the end of chromosomes. The other was a light pink hybridization signal in 14 chromosomes. Similarly, there were the green St-genome probe signals at the telomeric region of a chromosome, but 6 of them were covered by light pink signals. Besides the telomeric regions, 8 chromosomes also had St-genome probe signals at the centromeric regions, and the other parts were covered by a light pink signal (Fig. 5a).

When using the whole genome DNA from the diploid *D. villosum* (V-genome, green) and *Ps. spicata* (St-genome, red) as the probes in the second round of GISH analysis, 14 chromosomes with the J^b-genome dark-red signals in the first round of GISH analysis had no hybridization signals if the St-genome probe signal channel was turned off. However, 14 chromosomes with the light pink J^b-genome probe

hybridization signals in the first round of GISH showed the green hybridization signal by the V-genome probe. The positions of the signals on chromosomes were identical to those generated by the J^b-genome probe. Six chromosomes were completely covered by the V-genome hybridization signals, and 8 chromosomes were covered by V-genome hybridization signals except for the telomeric regions and the centromeric portions (Fig. 5b). When opening the signal channel of the St-genome probe, the locations of the hybridization signals were consistent with those of the St-genome probe during the first round of GISH analysis (Fig. 5c).

The results of smGISH analyses for IWG showed that 14 chromosomes with complete St-genome probe signals were the St-subgenome of IWG. Fourteen chromosomes were only stained dark-red by the J-genome probe, belonging to the J^r-subgenome. The remaining 14 chromosomes that showed both light-pink fluorescent signals of the J^b-genome probe and the V-genome probe signals were another subgenome of IWG, namely the J^{vs}-subgenome (Fig. 5c). According to the patterns of the hybridization signals, 14 chromosomes of the J^{vs}-subgenome could be divided into two types. One type of 6 chromosomes fully covered by the V-genome probed signals except the telomeric regions were known as Type-I J^{vs} (I J^{vs}). The other 8 chromosomes, named Type-II J^{vs} (II J^{vs}), had the St-genome probe hybridization signals at the centromeric portions and telomeric regions, and the rest regions of chromosomes were covered with the V-genome probe signals (Fig. 5d). The results of smGISH analysis also showed that the J^b-genome probe was able to distinguish the J^r- and J^{vs}-subgenomes based on the patterns of the hybridization signals. However, the V-genome probe only stained the J^{vs}-subgenome chromosomes but did not produce any hybridization signals on the J^r-subgenome chromosomes. The St-genome probe stained not only the St-subgenome chromosomes at the whole arm regions, but also the J^r- and J^{vs}-subgenome chromosomes at the telomeric and/or centromeric regions. Therefore, the genome of IWG could be expressed as $2n = 6x = 42 = 14St + 14J^r + 14J^{vs}$ (6 IJ^{vs}+8 IIJ^{vs}), when probed by the combination of probes prepared by the St- and V- genomic DNA.

Analysis of variant chromosomes on HSC-lines

Using smGISH analysis for 19HSC-Q and 20HSC-Z lines with the same combination of probes, we found that the chromosome compositions of most plants were the same as IWG, i.e., $2n = 6x = 42$, StStJ^rJ^rJ^{vs}J^{vs}. However, the translocation and introgression between subgenome chromosomes were observed in some lines with prominent comprehensive agronomic traits, such as 19HSC-Q6, 19HSC-Q11, 19HSC-Q18 and 20HSC-Z.

The smGISH analysis detected an intercalary translocation of J^r-St subgenome chromosome in 19HSC-Q6, in which a J^r-chromosome segment was inserted into the proximal end of the long arm of a St-subgenome chromosome (Fig. 6a1, b1, and c1). This translocation was also observed in the root tip cells of 20HSC-Z1 (Fig. 6a2, b2, and c1), 20HSC-Z2 (Fig. 6a3, b3, and c1), and 20HSC-Z3 (Fig. 6a4, b4, and c1). The pattern of smGISH analysis for 19HSC-Q11 showed that an IJ^{vs} chromosome had the introgression of St-subgenome chromosomes in the centromeric region (Fig. 6a5, b5, and c4). The first round of mGISH analysis of 19HSC-Q18 showed that a St-subgenome chromosome had a large light red hybridization signal produced by the J^b-genome probe at the proximal end of the long arm. Based on the results of IWG smGISH analysis, it was inferred that the light red hybridization region should contain the components from the J^{vs}-subgenome. The second round of mGISH analysis for 19HSC-Q18 with the V-genome probe proved the presence of the conjecture. This chromosome belongs to the terminal translocation of a large segment with the J^{vs}-St subgenome chromosome (Fig. 6a6, b6, and c2).

The results of smGISH analysis for lines 19HSC-Q and 20HSC-Z detected more than five types of chromosome variations based on the size of the translocated fragments. The translocation of large fragments included three types, J^r-St, J^{vs}-St, and J^r-IIJ^{vs}, and the introgression of small fragments contained two types, St-IJ^{vs} and J^{vs}-J^r. The introgression of J^{vs}-J^r was mainly concentrated on the terminal parts of both long and short arms, as well as centromeric regions. The introgression of St-IJ^{vs} was observed at the centromeric regions of the chromosomes (Fig. 6c1-6).

Discussion

The breeding methods of perennial crops include the domestication breeding and cross breeding (DeHaan et al., 2018). As an important candidate plant species for perennial crops, *Th. intermedium* has some advantages, such as regeneration, cold-hardiness, and grain quality, but the performance of yield components is still far lower than those of annual cultivated crops (DeHaan et al., 2020). Two factors must be considered in the breeding process for the selection of IWG new germplasm. One is to keep the wheatgrass characteristics, such as cold-hardiness, regenerative capability after harvest and perennial. The other is to improve the yield component traits, including the grain number per spike, seed setting rate, thousand kernel weight, etc.

For the cross breeding, how to sustainably screen plants with potential breeding goals among many genetically unstable hybrid offspring is the key point. New IWG germplasm has been developed through the cross breeding of octoploid Trititrigia and *Th. intermedium* since 2006

(Yan et al., 2020). Due to the existence of six subgenomes in the cross between *Tritrigia* and IWG, the genomes of the progenies may not be quickly balanced within a limited generations of selfing, resulting in the genetic instability. It is difficult to achieve the breeding objectives by artificial selection alone. In the cold regions, however, perennial crops must be tolerant to cold stress for living continuously, so that they can be viable in cold winter before resuming growth in spring to complete their life cycle. Therefore, we have carried out five breeding cycles under the cold winter climate condition in Harbin. From HNU-1C to HNU-5C, using the natural cold winter conditions for screening plants, those progenies were selected against because they were able to regrow after the cold winter.

Long-term of low-temperature screening and domestication gradually advance the growing period of plants under the cold winter condition in Harbin. The time of florescence and maturity of the plants for HNU-5C were about 40 days earlier than that for HNU-1C and - 2C. But the wheatgrass traits are still excellent, such as thousand-kernel weight, grains per spike, and tiller number. At similar latitudes, the new *Th. intermedium* variety "MN Clearwater" bred by the University of Minnesota was also tolerant to cold (Cattani et al., 2016). This suggests that to select and domesticate perennial crops according to the growth environment in winter is effective.

The yield constitutes, including the grain number per spike, seed setting, thousand-kernel weight, etc., are the core indicator to measure the value of crop production (Alterndorf et al., 2021; Bajgain and Anderson, 2021). The small grain size and low seed setting are the disadvantage of IWG, which are also important traits that should be improved in domestication and breeding. In HNU-5C, both tiller number and grain number per spike increased in the 6 lines investigated. In the third living year, the grain number per spike exceeded 80, and two of these lines had more than 100 grains, much higher than *Th. intermedium*. Similarly, the seed setting of these lines was also significantly higher than that of IWG. In addition, thousand-kernel weights of these lines were above 6 g and protein contents were above 17%. These properties resembled "MN-Clearwater" and derived lines reported by Bajgain et al. (2020). Moreover, the average seed area of these perennial lines was comparable to the MN-series lines.

Tiller number and area occupied by individual plant are also the main indicators to evaluate the vitality and yield constitutes of wheatgrass (Altendorf et al., 2021). The tiller number and grain number per spike for the 19HSC-Q plants were increased over years. However, the area occupied by the individual plant showed obvious differences, spanning from 0.080 m² to 0.615 m². This demonstrates that tillering and the covered area of perennial grain crops embodies the vitality of the underground rhizome in the cold regions. These traits, combined with the performance of yield components, can comprehensively reflect the growth characteristics and genetic differences of plants, as well as the adaptability of plants to the environment. The plants with strong tillering ability and large covered area can be used as forage grass for the ecological modification and improvement of cold grassland, as well as for the low yield fields or the poor land to improve the land use.

The characteristics of plant phenotype is the result of the combination of genotype and environment. Cytogenetic analysis of *Th. intermedium* genome has been controversial, and its classification is relatively diverse. With the development of genomics research technology, the chromosome composition of *Th. intermedium* has been identified as E^eE^eE^bStSt or StStJJJ^sJ^s (Ji. et al, 2001; Chen, 2005). We used smGISH to analyze IWG with *Ps. strigose* (2n = 14, StSt), *Th. bessarabicum* (2n = 2x = 14, J^bJ^b) and *D. villosum* (2n = 2x = 14, VV) genomic DNA as probes and obtained the same results as StStJ^rJ^rJ^{vs}J^{vs} 2n = 6x = 42 by Cseh et al. (2019). According to the hybridization signals of the J^{vs}-subgenome, we divided it into type-I and type-II, which were 3 pairs of chromosomes and 4 pairs of chromosomes, respectively. At the same time, we found that the J^b-genome probe could stain and distinguish chromosomes of the J^r- and J^{vs}-subgenomes based on the intensity of hybridization signals. The V-genome probe could only stain the J^{vs}-subgenome chromosomes. The signal patterns were consistent with the light color area from the J^b-genome probe hybridization signal, and no hybridization signals were observed on the J^r-subgenome chromosomes. Therefore, whether the combination of the St- and J^b-genome probe or the St- and V-genome probe is used alone, the subgenomes of IWG can be effectively distinguished.

The smGISH analysis in HNU-5C showed that most of the lines had the same chromosomal compositions as IWG, but there were some translocations between the subgenomes of 19HSC-Q and 20HSC-Z, including the insertion of small segment on the short arm with the J^r-St chromosome, the half of arm translocation with the J^r-J^{vs} chromosomes. It suggests that the subgenomes of IWG was impacted by the alien chromosome during hybridization breeding and domestication under the extremely cold environments. To better adapt to the environment, chromosome recombination occurred during the process of genome rebalancing. Because of the adaptation to cold growing conditions that the dominant IWG chromosomes were preserved, and the wheat chromosomes may be eliminated in most plants (Yan et al., 2020). Based on the current results, the observed translocations of subgenome chromosomes were existed in some lines with prominent comprehensive agronomic traits, such as 19HSC-Q6, 19HSC-Q11, 19HSC-Q18, and 20HSC-Z, which warrants further study to reveal the relationship between chromosomal variation and phenotype. Meanwhile, the smGISH results provide a new reference for the genome analysis of progenies of wheat and *Th. intermedium*, such as the chromosome variation in the mixed genome of wheat-*Th. intermedium* partial amphidiploids, as well as the genome selection breeding of IWG.

Conclusions

Using the domestication method of distant hybridization, we selected 6 key lines from 24 new germplasm of *Th. intermedium* at the end of the fifth breeding cycle. The perenniality, cold tolerance, and yield components of these lines maintained to be stable for 3 consecutive years and were superior to *Th. intermedium*. It suggested that the hybrid domestication breeding had improved yield characteristics of *Th. intermedium*. The subgenome chromosome variations of new germplasm enriches the chromosome karyotype of *Th. intermedium*, which the lines with prominent comprehensive agronomic traits carried the translocation chromosomes warrants further study to reveal the relationship between chromosomal variation and phenotype. The analysis strategy with smGISH for the genome provides a new idea for further analyzing the relationship between yield traits and genomes of *Th. intermedium*.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

YL and WS conceived the project. AS, CW, JD, XY, JS, and ML, conducted the cold-hardy perennial feature and morphological observation. XY, CJ, HZ and XL performed smGISH experiments and the yield components analysis. LC conducted the resequencing and alignment data analysis. HL and YZ reviewed the manuscript. All authors reviewed and approved the manuscript.

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Figures

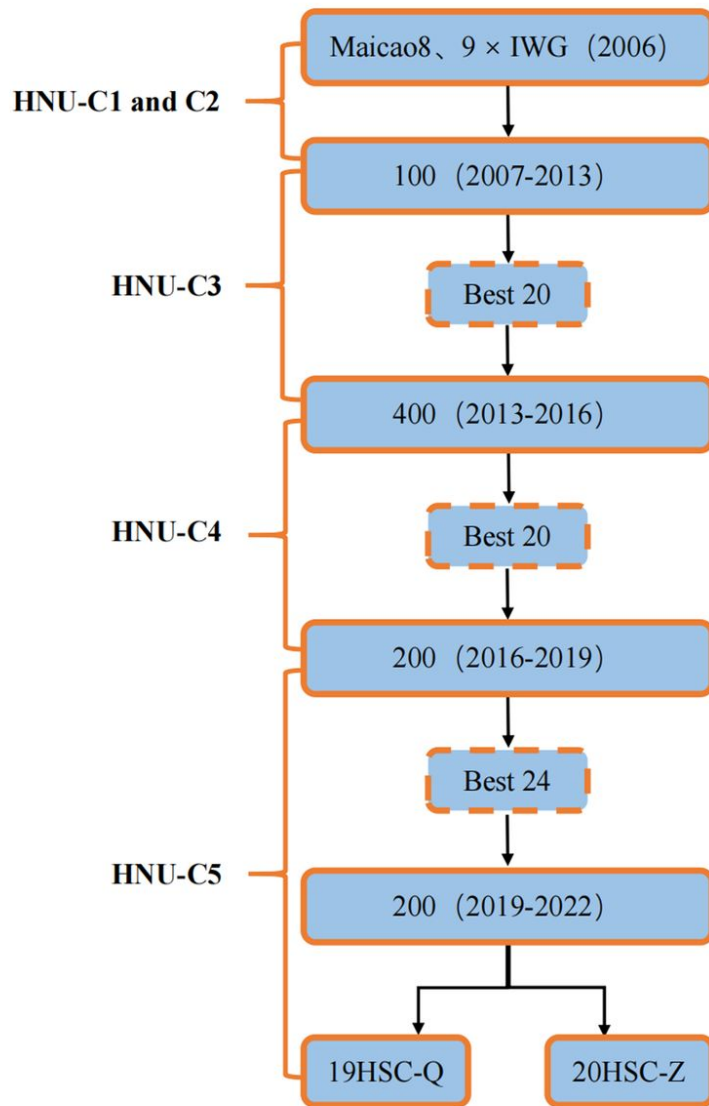


Figure 1

Breeding process of new germplasm of *Thinopyrum intermedium*. The new IWG germplasm has been developed through the cross breeding of octoploid *Trititrigia* and *Th. intermedium* since 2006. From 2007 to 2022, it took three years as a breeding cycle and carried out five breeding cycles in Harbin Normal University under the cold winter climate. At 2022, two populations named 19HSC-Q and 20HSC-Z were selected for molecular cytogenetic analysis.

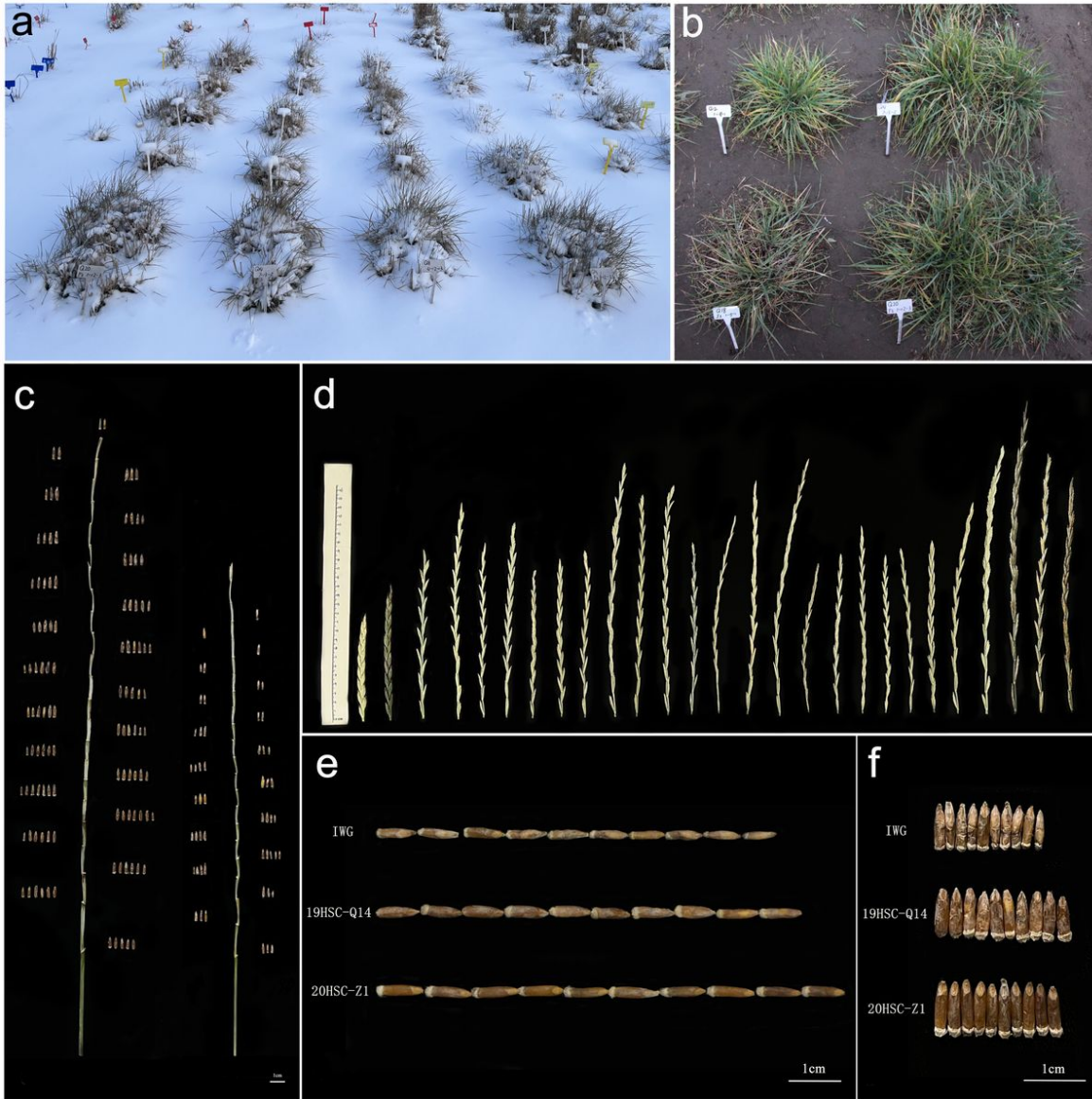


Figure 2

The observation of plant growth and morphological characteristics of spike and grain. **(a)** Lines of 19HSC-Q was covered with snow (photo taken in November 2022). **(b)** The cluster area of 19HSC-Q12 (top left), 19HSC-Q14 (top right), 19HSC-Q18 (bottom left) and 19HSC-Q20 (bottom right) (photo taken in October 2022). **(c)** Grain number per spike of 19HSC-Q14 (left) and IWG (right). **(d)** Spikes of the wheatgrass plants from 19HSC-Q to 20HSC-Z generations and parents. The first from left to right was Maicao 8, Maicao 9 and *Th. intermedium*, followed by lines 19HSC-Q from 19HSC-Q2 to 19HSC-Q23. The last three was 20HSC-Z1, 20HSC-Z2, 20HSC-Z3 (photo taken in July 2022). **(e)** and **(f)** Seed characteristics of perennial wheatgrasses. **(e)** From top to bottom were the grain lengths of IWG, 19HSC-Q14 and 20HSC-Z1, respectively. **(f)** From top to bottom were the grain widths of IWG, 19HSC-Q14 and 20HSC-Z1, respectively. Scale bars=1cm.

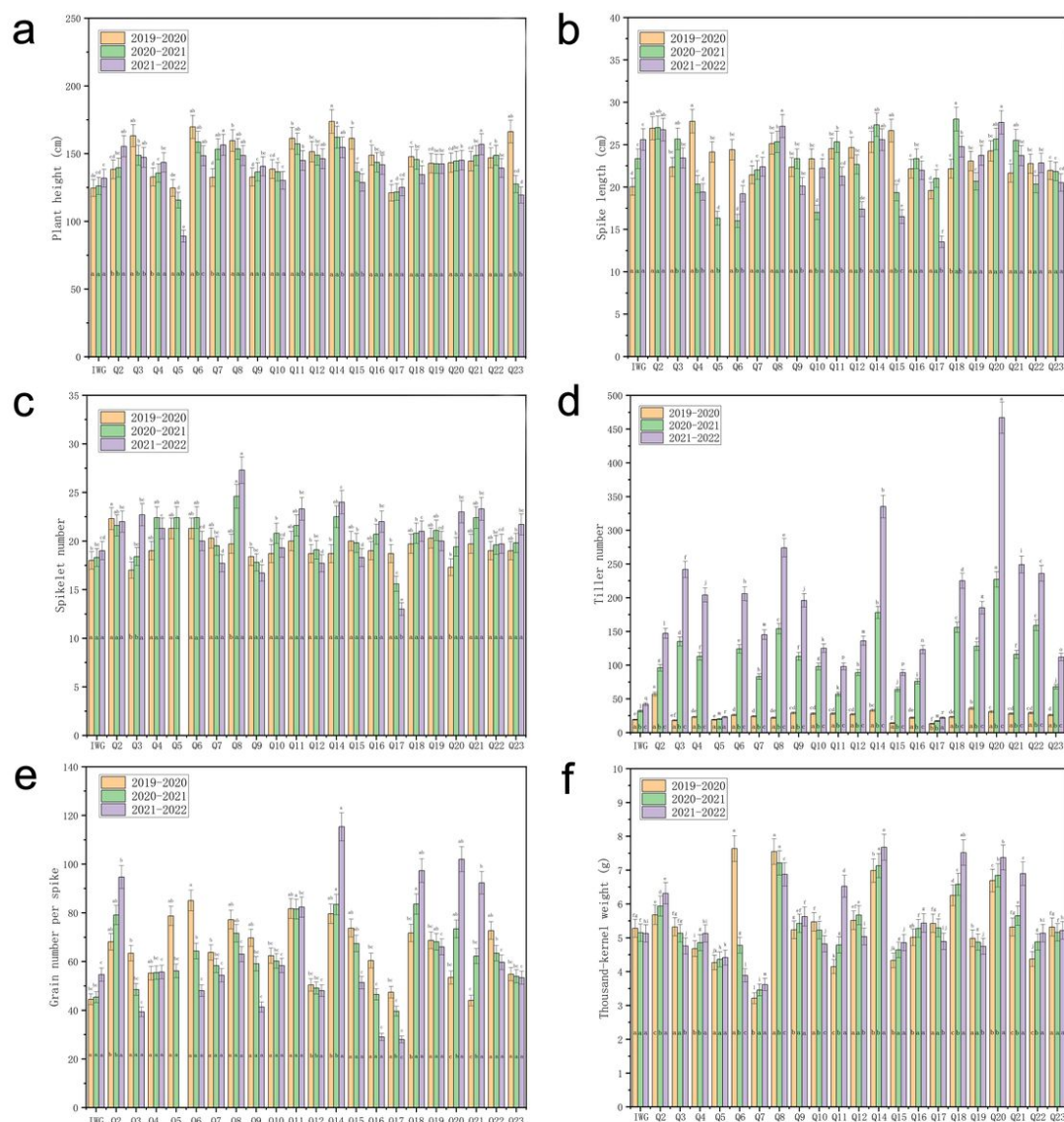


Figure 3

Assessment of agronomist traits including (a) plant height and (b) spike length and (c) spikelet number and (d) tiller number and yield components including (e) grain number per spike and (f) thousand-kernel weight of perennial wheatgrass lines developed from present study and control IWG over the three growing seasons 2019-2020, 2020-2021, and 2021-2022 in Harbin, China. For each factor per growing season, different letters inside bars indicate a significant different among lines using the Fisher's LSD ($P < 0.05$). For the tested variables of each line, different letters above bars represent difference among growing seasons ($P < 0.05$).

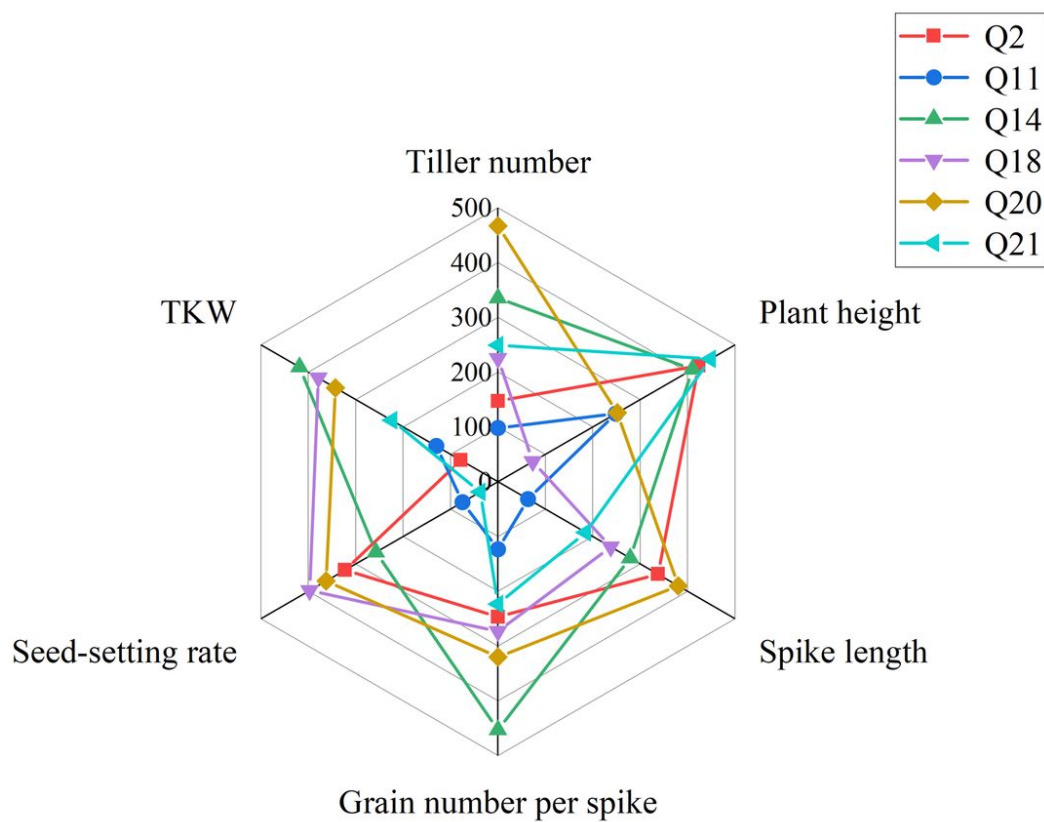


Figure 4

Radar map of six agronomic traits. There were tiller number, plant height, spike length, grain number per spike, seed-setting rate, and thousand grain weight. The red line represented 19HSC-Q2, the blue line represented 19HSC-Q11, the green line represented 19HSC-Q14, the purple line represented 19HSC-Q18, the brown line represented 19HSC-Q20 and the yellow line represented 19HSC-Q21.

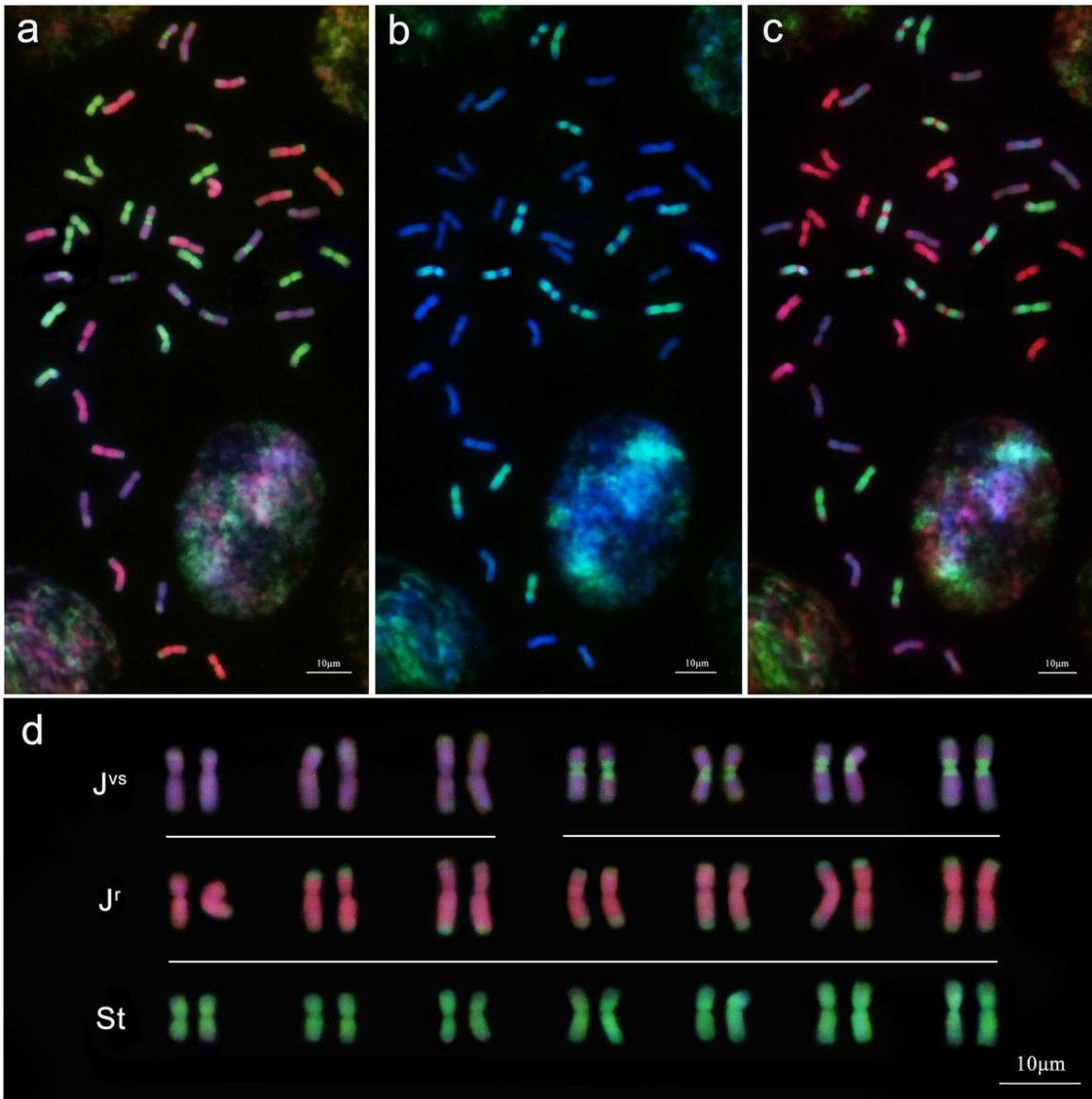


Figure 5

Sequential multicolor GISH analysis of *Th. intermedium* (StStJ^rJ^rJ^{vs}J^{vs}, 2n=6x=42) **(a)** *Pseudoroegneria spicata* (StSt) probe showed 14 St-subgenome chromosomes with bright-green signals along the entire chromosomes. *Th. bessarabicum* (J^bJ^b) probe detected 14 J^r-subgenome chromosomes with dark-pink signals over the whole chromosomes except for the terminal regions that was covered by the bright-green signals of St-probe. Both St- and J^b- probe displayed 14 J^{vs}-subgenome chromosomes with light-pink signals and bright-green signals. **(b)** *Dasypyrum villosum* (VV) probe detected 14 J^{vs}-subgenome chromosomes with bright green signals along the entire chromosomes or the whole chromosome arms except the centromeric region. **(c)** *Pseudoroegneria spicata* (StSt) probe (red) labelled the same 14 chromosomes as seen on, while *Dasypyrum villosum* probe (VV) probe (green) detected 14 J^{vs}-subgenome chromosomes together with St-probe. **(d)** Karyotype of *Th. intermedium* chromosomes based on GISH signals from (a), J^{vs} = Type-I (top left), J^{vs} = Type-II (top right). The chromosomes were counterstained with DAPI (blue). Scale bars = 10 μm.

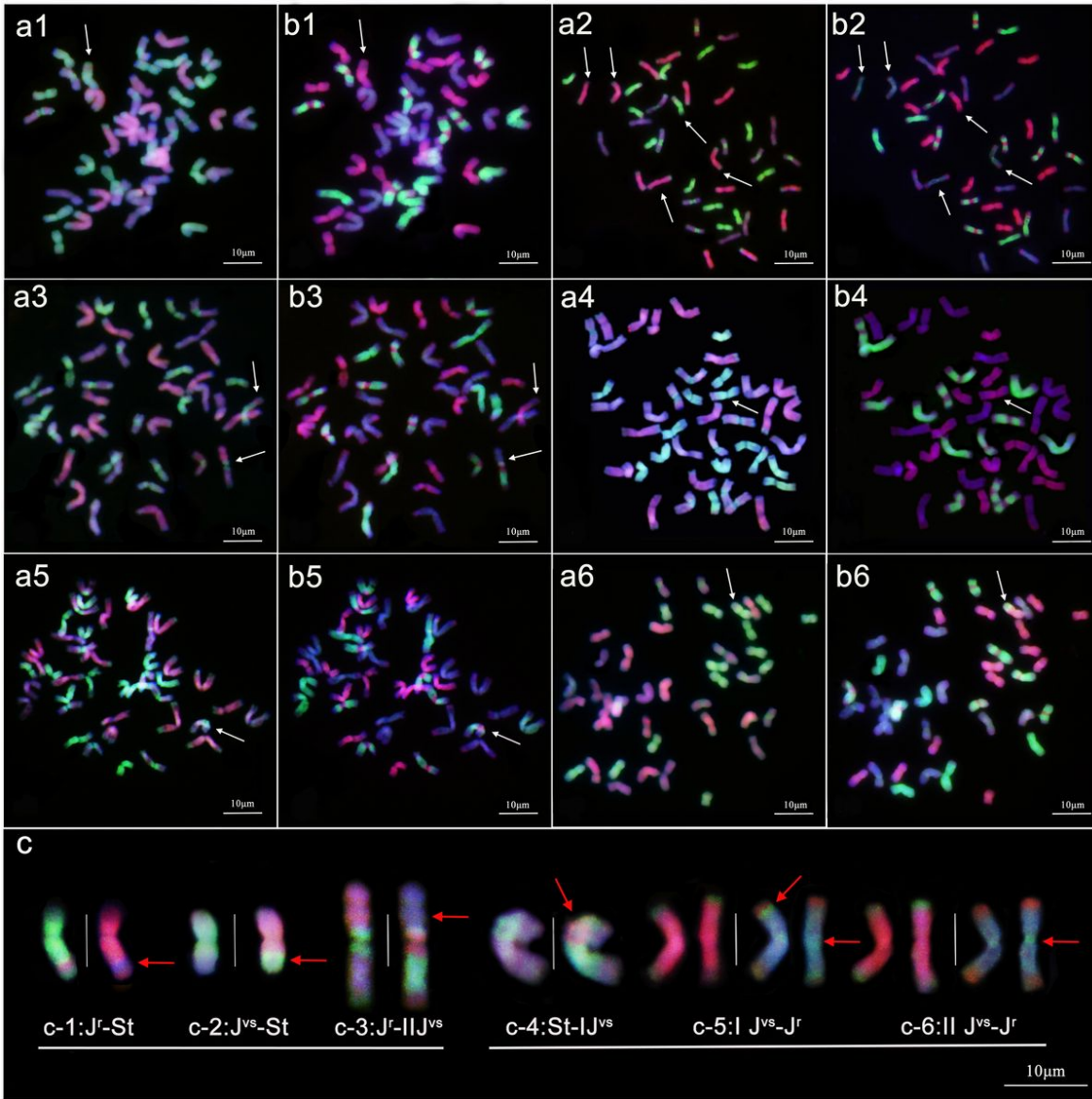


Figure 6

Sequential multicolor GISH analysis of 19HSC-Q lines and 20HSC-Z lines. **(a1-a6)** The probe combination was diploid *Th. bessarabicum* (J^b-genome, red) and *P. strigosa* (St-genome, green). **(b1-b6)** The probe combination was diploid *D. villosum* (V-genome, green) and *P. strigosa* (St-genome, red). (a1, b1) 19HSC-Q6. (a2, b2) 20HSC-Z1. (a3, b3) 20HSC-Z2. (a4, b4) 20HSC-Z3. (a5, b5) 19HSC-Q11. (a6, b6) 19HSC-Q18. Both (a) and (b) patterns were from the same root tip cell. **(c)** Sequential multicolor GISH analysis of recombinant chromosomes in new germplasm of 19HSC-Q lines and 20HSC-Z lines. The white arrows in (a) and (b) patterns indicated the recombinant chromosomes. The red arrows indicated the location of chromosome recombination and the white vertical line used to separate chromosomes in (c) pattern. The chromosomes were counterstained with DAPI (blue). Scale bars = 10 μm.