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IDENTIFICATION OF COLD TOLERANCE AND ANALYSIS OF GENETIC DIVERSITY FOR MAJOR WHEAT VARIETIES IN JIANGHUAI REGION OF CHINA

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Abstract

Late spring coldness is one of the mainly disastrous weather affecting wheat development in the central Jianghuai region of China. In this study, 18 major wheat varieties were selected to investigate their adaptability under cold stress in year of both 2018 and 2019, and the genetic diversity by SSR makers. The results showed that cold tolerance and yield-related traits significantly differed among the 18 wheat varieties. The freeze injury of young spikes was greatly and negatively correlated with plant height, spikelet number, grain numbers and grain weight of main stem spike. The stronger cold-tolerant wheat varieties showed the higher SOD activity and proline content than control. A total of 594 polymorphic bands were amplified by 28 pairs of SSR primers in 18 wheat varieties. The polymorphism information content (*PIC*) for genetic loci varied from 0.179 to 0.983, as well as the genetic diversity index (*Nei*) ranged from 0.105 to 0.697. The 18 wheat cultivars were divided into four groups, Group I-1 comprised 8 wheat cultivars with abundant polymorphic loci, a high gene diversity index, strong cold tolerance, and high yield per plant. These cultivars can be emphatically utilized in the breeding of cold-resistant wheat. Seven varieties, including Yannong 5158, Huaimai 28, Huaimai 33, Jinan 17, Fanmai 5, Yannong 19, and Xumai 35, had good comprehensive yield-related traits and cold tolerance. They can be used as the major wheat varieties in central regions of Jianghuai in Anhui province.

Key words: Cluster analysis, Cold tolerance, Genetic diversity, SSR molecular marker, Wheat

Introduction

The cold injury of wheat in late spring (late-spring-coldness injury) is one of the main meteorological disasters in the central regions of Jianghuai, China. The phenomenon refers to the injury caused by a suddenly low temperature or a severe change in temperature after jointing stages. The jointing stage of wheat is the key phase of young spikes differentiation, which is sensitive to low temperature (Martino & Abbate, 2018). If the late spring coldness happens, the differentiation of young spikes probably stops, forming a "bare rod" and a "bald tip", leading to a reduced seed setting rate and significant reduction of wheat yield (Zhu *et al*., 2018). Therefore, the selection and identification of major wheat varieties with strong cold-resistance are basic steps to the effective prevention or mitigation of cold damage within the major wheat-cultivated region of Jianghuai.

Low-temperature stress causes a series of cellular physiological changes, such as the production of a large number of O^{-2}, H_2O_2, OH, and other reactive oxygen species in plants (Ashraf *et al*., 2013). These reactive oxygen species could lead to membrane lipid peroxidation, thereby causing oxidative damage to the membrane system. Reactive oxygen species are scavenged by antioxidant enzymes, especially for superoxide dismutase (SOD) and peroxidase (POD), which help plants tolerate, slow down, or resist low-temperature stress to a certain extent (Cheng *et al*., 1994; Li *et al*., 2013). Proline (Pro) is highly hydrophilic and can stabilize protoplast colloids and metabolic processes in tissues, proline content reflects the cold resistance of plants to a certain extent (Chen *et al.*, 2014). Simple sequence repeats (SSR) markers are usually used to detect the genetic diversity of germplasm resources and analyze the origin and genetic relationship among wheat varieties (Yousaf *et al*., 2015; Singh *et al*., 2017). In this study, 18 major wheat varieties in the Jianghuai region were selected to systematically evaluate the cold resistance of different wheat varieties by investigating the freezing injury degree at the jointing and flowering stage, detecting the oxidase activity and proline content of wheat under natural low-temperature stress, and determining the yield-related traits after wheat harvest in 2018 and 2019. The genetic diversity of the wheat population was identified in terms of SSR markers related to cold tolerance. This study provides a theoretical basis for selecting optimal wheat varieties and breeding new varieties with strong cold tolerance in the Jianghuai region.

Materials and Methods

Materials and plant condition: 18 wheat germplasm resources (semi-winter wheat), with greatly variable cold tolerance, are the major wheat varieties in the Jianghuai region of Anhui province. The varieties were collected from the various provinces in China. In early November of both 2017 and 2018, the varieties were continuously planted at Jinzhai Crop Variety Resistance Identification Test Station in Anhui province for two years. Each variety was planted in four rows with a row length of 1.5 m, a row spacing of 25 cm, and a plant spacing of 6.7 cm. In 2018, when the main stem of wheat plants grew to five leaves stage (Z23) (Zadoks *et al.*, 1974), the leaves were collected and frozen in liquid nitrogen. The leaves of four plants of each variety were randomly prepared for replication (n=4), and DNA was extracted via the CTAB method (Sambrook *et al.*, 2001).

Low-temperature treatment and investigation of physiology-related traits: In 2018 and 2019, the temperature in the field suddenly dropped when the wheat varieties started to return green period at spring. On February 2018, the average temperature dropped up to 1.7°C, with the lowest temperature of -16.2°C, this condition lasted for 10 days, while the average temperature on February 2019 dropped to 3.5°C, with the lowest temperature of -2°C and lasting for 12 days, Then wheat gradually turn green with the increase of field temperature. However, from the 5^{th} to 11^{th} of March 2018, the field temperature dropped sharply again, with an average temperature of 4.4℃ and a minimum temperature of −4.8°C, while from the 1^{th} to 15^{th} of March 2019, the daily minimum temperature was average 5.1°C and lasted for 15 days. During the late spring, the freezing injury of wheat plants in the two years was investigated when the young plants grew to the eight tillers stage (Z29) (Zadoks *et al.*, 1974), as well as the freezing injury to young spikes at the flowering stage (Z60). The injury degree of wheat was recorded on the basis of the cold-tolerance criteria of wheat (Supplement Table 1). In 2019, the wheat leaves (mixture of the top three leaves) were collected to determine the activities of SOD, POD, and content of Pro among the 18 wheat varieties. The wheat was harvested and yield-related agronomic traits were investigated in 2018 and 2019. Ten replicates were used for each variety in annual growth.

Determination of oxidase activity and proline content: SOD and POD activities were determined via methods of nitroblue tetrazolium (NBT) and guaiacol colorimetry, respectively, and proline content was determined via technical steps of ninhydrin colorimetry (Jahantigh *et al.*, 2016).

Diversity detection of simple sequence repeats (SSR) markers: SSR primers (Supplement Table 2.) related to the cold tolerance of wheat were selected, and synthesized by TaKaRa company (Dalian, China). 28 pairs of SSR primers are evenly distributed on wheat chromosomes A, B, and D. PCR reaction system: 1 µl PCR Buffer (10×), 1 µl dNTP (2.5 mM), 1.5 µl primer (2 µmol, mixture with forward and reverse primers), 2 µl DNA template (100 ng), 0.5 µl Taq polymerase, and 4 µl ddH_2O, for a total volume of 10 µL. PCR procedure: 94℃ for 5 min, 35 cycles (94℃ for 30s, 58℃ for 30s, 72℃ for 1min), 72℃ for 10min, incubation at 4℃. PCR products was detected by polyacrylamide gel electrophoresis (Bassam *et al.*, 1991), and the results were observed under an incandescent lamp.

Data analysis: According to PCR results, the band in the same migration position within the survey interval was labeled as 1, and no band was recorded as 0. Double-type bands were marked by letters A, B, C, and D. Popgen 32 software was used to calculate the number and ratio of polymorphic loci, the Shannon information diversity index (*I*), Nei genetic diversity index (*H*), gene flow (*Nm*), genetic distance, and genetic consistency among populations. SPSS12.0 software was utilized for the statistical test and cluster analyses of wheat varieties based on the cold tolerance and yield-related traits. Genetic diversity and varieties cluster by SSR evaluation was identified in accordance with UPGMA method. The cold resistance of different wheat varieties was comprehensively evaluated using the following formula (Xie, 1983):

$$D = \sum_{j=1}^{n} [u(X_j) \times \omega_j], \quad j=1,2,3, \ldots, n$$

where D is the comprehensively evaluated value of the cold resistance of wheat varieties, u is the subordinate function of each comprehensive index, X_j is the j^{th} comprehensive index, and ω_j is the weight of the j^{th} index in all comprehensive indices.

Supplement Table 1. Classification grade of freezing injury of wheat plants.

Grade of freezing injury	Characteristics and classification grade of freezing injury of wheat plants after late spring coldness	Ratio of freeze to death of wheat young spikes
1	Ratio of yellow tips of freezing leaves was below 1/4, and the rarely dead tillers (seedlings)	Ratio of freeze to death of young spikes was below 20%
2	Ratio of yellow tips of freezing leaves was from 1/4 to 1/2, and the ratio of the dead tillers (seedlings) was below 10%	Ratio of freeze to death of young spikes was from 20% to 50%
3	Ratio of yellow tips of freezing leaves was from 1/2 to 1/4, and the ratio of the dead tillers (seedlings) was from 10% to 30%	Ratio of freeze to death of young spikes was from 50% to 80%
4	Ratio of leaves freezing to death was above 3/4, and the ratio of the dead tillers (seedlings) was from 30% to 50%	Ratio of freeze to death of young spikes was above 80%
5	Leaves almost all died from the coldness, and the ratio of the dead tillers (seedlings) was above 50%	Young spikes almost all died from the coldness

Supplement Table 2. Name and sequence of 28 pairs of SSR primers.

Code	Name	Forward primers	Reverse primers	Location
1	Barc4	GCGTGTTTGTGTCTGCGTTCTA	CACCACACATGCCACCTTCTTT	5BS
2	Barc32	GCGTGAATCCGGAAACCCAATCTGTG	TGGAGAACCTTCGCATTGTGTCATTA	3AS
3	Barc40	GCCGCCTACCACAGAGTTGCAGCT	GCGGCATTGACAAGACCATAGC	5BS
4	Barc59	GCGTTGGCTAATCATCGTTCCTTC	AGCACCCTACCCAGCGTCAGTCAAT	5AS
5	Barc61	TGCATACATTGATTCATAACTCTCT	TCTTCGAGCGTTATGATTGAT	5BL
6	Barc76	ATTCGTTGCTGCCACTTGCTG	GCGCGACACGGAGTAAGGACACC	4AL
7	Barc79	GCGTTGGAAAGGAGGTAATGTTAGATAG	TCGTGGGTTACAAGTTTGGGAGGTCA	7DL
8	Barc105	CAGGAAGAAAAGGAAAGCATGTTAGATAG	GCGGTGTGGCAATAATTACTTTTT	2BS
9	Barc127	TGCATGCACTGTCCTTTGTATT	AAGATGCGGGCTGTTTTCTA	7DS
10	Barc170	CGCTTGACTTTGAATGGCTGAACA	CGCCCACTTTTTACCTAATCCTTTTGAA	2BL
11	Barc174	TGGCATTTTTCTAGCACCAATACAT	GCGAACTGGACCAGCCTTCTATCTGTTC	4AL
12	Barc177	GCGATCCTGTTGTTGAGCGTTTGCATAA	TCCCGTTTTCCCGTGTGTTAGTCTA	1BL
13	Barc180	GCGATGCTTGTTTGTTACTTCTC	GCGATGGAACTTCTTTTTGCTCTA	5DL
14	Barc181	CGCTGGAGGGGGTAAGTCATCAC	CGCAAATCAAGAACACGGGAGAAAGAA	5AS
15	Barc206	GCTTTGCCAGGTGAGCACTCT	TGGCCGGGTATTTGAGTTGGAGTTT	6BS
16	Barc302	GCGAACAGACTTTGCCTTCTTCCAG	CGCCTAAAGGTGTGCTATATTGAG	2DS
17	Barc319	GCAGAGCTACGGCAATGT	GCGTAAGTCCCGGAAGTAACAGAA	4DS
18	Barc320	CGTCTTCATCAAATCCGAACTG	AAAATCTATGCGCAGGAGAAAC	5AL
19	Barc321	TGCACTTCCCACAACACATC	TTGCCACGTAGGTGATTTATGA	5DL
20	Gwm136	GACAGCACCTTGCCCTTTG	CATCGGCAACATGCTCATC	7AS
21	Gwm148	GTGAGGCAGCAAGAGAGAAA	CAAAGCTTGACTCAGACCAAA	1AS
22	Gwm149	CATTGTTTTCTGCCTCTAGCC	GTAGCATCGAACCTGAACAAG	2BS
23	Gwm190	GTGCTTGCTGAGCTATGAGTC	GTGCCACGTGGTACCTTTG	5AL
24	Gwm273	ATTGGACGGACAGATGCTTT	AGCAGTGAGGAAGGGGATC	7AS
25	Gwm369	CTGCAGGCCATGATGATG	ACCGTGGGTGTTGTGAGC	1DS
26	Gwm512	AGCCACCATCAGCAAAAATT	GAACATGAGCAGTTTGGCAC	5BL
27	Gwm526	CAATAGTTCTGTGAGAGCTGCG	CCAACCCAAATACACATTCTCA	2AS
28	Wmc388	AGCCACCATCAGCAAAAATT	GAACATGAGCAGTTTGGCAC	7AS

Results

Temperature fluctuation and cold tolerance of different wheat varieties: It showed that the average temperature during wintering period was 3.5°C and 6.8°C in 2017 and 2018, respectively (Fig. 1), which can ensure the safe overwintering of semi-winter wheat varieties in Jianghuai region. In early February, the wheat varieties started to return green, and suddenly suffered from the lower field temperature: in 2018, the temperature below 0°C lasted for 8 days, with average temperature of -3.9°C and the lowest temperature of -16.2℃; in 2019, the lower temperature lasted for 14 days, with average temperature of 1.4°C and the lowest temperature of -1℃ , which resulted in the delayed development process of wheat. After mid-February, as the field temperature increased, the wheat turned green and the seedlings grew rapidly. The wheat plants began to joint and booting, and developed normally. However, from March 5 to 11 in 2018, the field temperature in the test station dropped sharply with an average temperature of 4.4°C and a minimum temperature of −4.8°C, while dropping to the average temperature of 5.1°C in the daily minimum temperature from March 1 to 15 in 2019.

Low-temperature weather reoccurred for 4 days with average of 5.3°C in 2018 and daily minimum 5.8°C in 2019. Late spring coldness influenced the early differentiation, leading the hindered development of wheat organs at the later stage, some wheat varieties had yellow leaf tips, lethal young spikes, or no grain of spikes, which seriously affected the yield of wheat at the harvest stage.

After exposure to low-temperature stress, wheat plants showed that the number of death tillers increased, the seedlings had a yellowish tip, the leaves drooped and wilted, and the young spikes froze to various degrees (Fig. 2).

Among the wheat varieties (Table 1), in two years, Anke157, Yannong19, Annong0711, and Xumai35 expressed the lowest degree of freezing injury, only with the curled and withered leaves in the bottom parts of plants, and the lower freezing death rate of young spikes at later stage, injury grade of late spring coldness and young spikes coldness were all Grade 1. Liangxing 66 and SXM208 varieties were seriously damaged by coldness, most of leaves lost green appearance, withered, drooped seriously, and almost all the young spikes were injured and killed at the later stage. Injury grade of late spring coldness and young spikes coldness were Grade 3 and Grade 4 above, respectively. Zhoumai27 was the most severely damaged, it had weak seedlings and local necrosis, some plants died, and some spikes of surviving plants had no grains. The results showed that the wheat growth was seriously damaged under cold stress, but the cold resistance of different wheat varieties was significantly different.

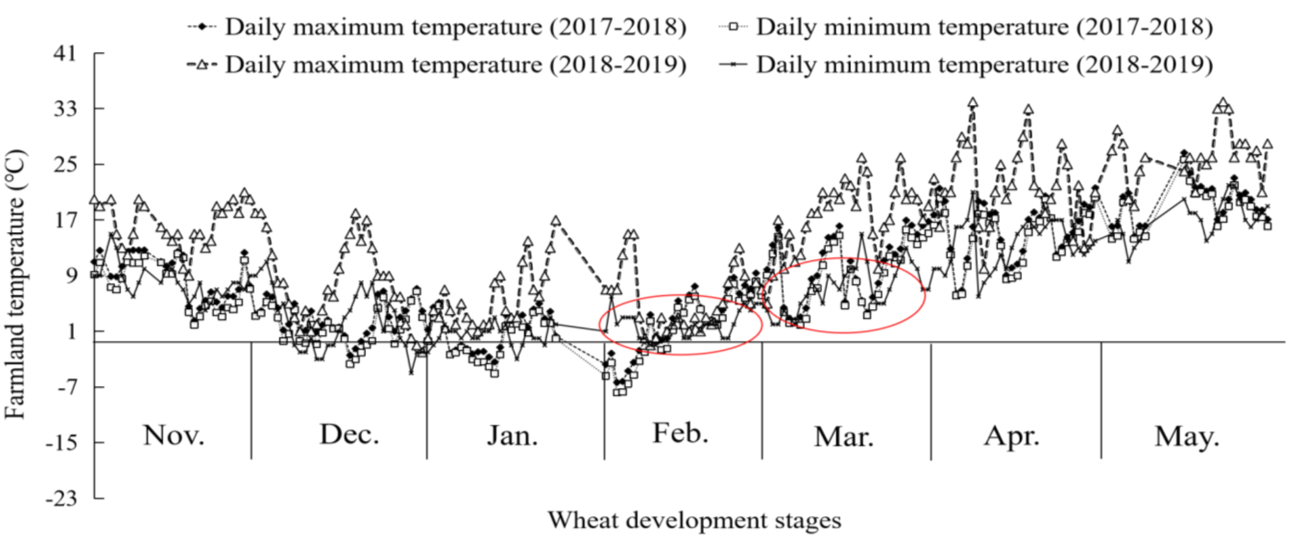

Fig. 1. Temperature distribution during the wheat-growing period in the year of 2017-2018 and 2018-2019.

Fig. 2. Phenotype of wheat plants under low-temperature stress.

Table 1. Investigation on freezing injury of wheat plants after late spring coldness in the year of 2017-2018 and 2018-2019.

Wheat varieties	2017-2018		2018-2019		Wheat varieties	2017-2018		2018-2019	
	IGLSC	IGYSC	IGLSC	IGYSC		IGLSC	IGYSC	IGLSC	IGYSC
1. Yannong 5158	1	2	1	1	10. Anke 157	1	1	1	1
2. Yannong 5286	2	3	2	2	11. Yannong 19	1	1	1	1
3. Jimai 22	2	4	2	3	12. Guomai 9	2	4	2	3
4. Huaimai 22	2	4	2	4	13. Annong 0711	1	1	1	1
5. Huaimai 28	2	2	2	2	14. Liangxing 66	3	5	3	4
6. Huaimai 33	2	3	2	2	15. Xinmai 26	1	2	1	2
7. Jinan 17	2	3	2	2	16. Xumai 35	1	1	1	1
8. Fanmai 5	2	2	2	1	17. Zhoumai 27	4	5	4	5
9. Wanmai 52	1	2	1	2	18. SXM 208	3	5	3	5

Note: IGLSC was the injury grade of late spring coldness, IGYSC was the injury grade of young spikes coldness

Effects of low-temperature stress on the yield-related traits of wheat: In both 2018 and 2019, after the wheat was harvested, yield-related traits of 18 varieties were investigated in two years and obtained the mean value. The results showed that the grain yield per plan (GYPP) of wheat Yannong5158, Huaimai28, Huaimai33, Jinan17, Fanmai5, Yannong19 and Xumai35 was high, with an average yield of 5.36 g (Table 2). By contrast, the GYPP of wheat Yannong 5286, Guomai9, Annong0711, Liangxing 66, and Zhoumai27 was low, with an average yield of 1.78 g.

Although the damage to cultivar Huaimai33 and Jinan17 was greater than the damage to other cultivars, they had more spike numbers per plant (SNPP) and a higher GYPP. The cultivar Anke157 and Annong0711 were slightly damaged, while SNPP was poor, resulting in a low GYPP. The variety SXM208 suffered from the most serious damage, with a high freezing death rate of young spikes, few grain number of main stem spike (GNMSS), and lowest GYPP of 0.2 g. There was significant differences of the yield-related traits among the 18 wheat varieties under low-temperature stress ($p<0.01$), and it was necessary to further clarify the genetic differences among wheat varieties at a molecular level. Evaluating the cold resistance of wheat was difficult when a single index was used, because the yield-related traits of the 18 wheat varieties differed in various ranges. According to the cold tolerance grade and yield-related traits of wheat in 2018 and 2019, the corresponding subordinate functions were obtained, and weighted analysis was conducted. The cold tolerance of the 18 wheat varieties was comprehensively evaluated: Jinan17 > Huaimai28 > Huaimai33 > Fanmai5 > Yannong5158 > Yannong19 > Xumai35 > Wanmai52 > Xinmai26 > Womai9 > Liangxing66 > Huaimai22 > Anke157 > Yannong5286 > Jimai22 > Annong0711 > Zhoumai27 > SXM208.

Correlation analysis showed that the correlation of injury grade between late spring coldness and young spikes coldness was significantly positive (r = 0.80, $p<0.01$), and the correlation of injury grade of young spikes coldness with PH, SNMSS, GNMSS and GWMSS was greatly and significantly negative ($p<0.01$), whereas the significantly negative with GYPP ($P < 0.05$) (Table 3). These indicated that the injury of late spring coldness was not a direct factor affecting a decrease of wheat yield, and mainly affected the formation of young spikes and grains at the later stage of wheat growth, the poor development of spikes probably led to the yield reduction of wheat harvest. At the early stage, if the wheat population had a large plant type, great strength and strong cold resistance, the young spikes show the good resistance to freezing injury, the seedlings should be properly strengthened under adversity.

Effects of cold stress on SOD and POD activities and proline content of wheat: SOD and POD play an important role in scavenging reactive oxygen species (ROS) and can make plants resist stress to a certain extent. Proline is an important physiological index to improve plant cold resistance. In order to confirm whether the wheat varieties with cold tolerance performed a superior physiology process, wheat varieties with better or worse cold tolerance were selected to determine their oxidase activity and proline content in 2019.

The results showed that POD and SOD activities and the proline content of seven wheat varieties with strong cold tolerance were significantly higher than those of wheat varieties with poor cold tolerance (Table 4). The average SOD activity of wheat varieties with strong cold tolerance was 461 U g^{-1} FW min^{-1}, and the difference of wheat between poor and strong cold tolerance was significant ($p<0.05$). However, the proline content of 6 varieties with strong cold tolerance was significantly higher than that of wheat varieties with poor cold tolerance ($p<0.05$), except of the variety Yannong 5158 (No.1). These results indicated that high oxidase activity and proline content in wheat tissues were helpful in improving the cold tolerance of wheat.

Correlation analysis showed that the SOD activity of wheat varieties was positively correlated with the POD activity and the proline content ($p<0.01$), negatively correlated with injury grade of late spring coldness (IGLSC) and young spikes coldness (IGYSC) ($p<0.01$). The proline content was positively correlated with the POD activity ($p<0.01$), and negatively correlated with injury grade of young spikes coldness ($p<0.01$) (Table 5). These indicated that the SOD activity and proline content were the important physiological indexes reflecting the cold tolerance of wheat, and proline was a major identification index to injury degree of wheat coldness at the later growth stage.

Cluster analysis and yield-related characteristics of wheat varieties: Cluster analysis was conducted on the basis of the freeze injury grade and yield-related traits of 18 wheat varieties in both 2018 and 2019. The results showed that 18 wheat cultivars could be divided into four groups when the Euclidean distance was equal to 5 (Fig. 3). The wheat varieties number in groups I and III was large and divided into two subgroups.

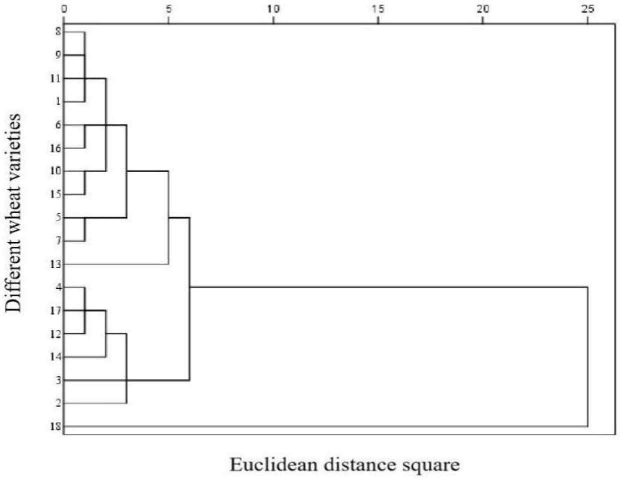

Fig. 3. Cluster analysis of different wheat varieties.
Note: Wheat varieties were represented as code number, the details were as Table 2 above.

Group I, including total 10 wheat varieties. The wheat plant height (PH) are taller, grain number of main stem spike (GNMSS) was more, grain weight of main stem spike (GWMSS) was heavier, thousand kernel weight (TKW) is larger, grain yield per plant (GYPP) is higher, the injury grade of late spring coldness is lighter, the lethal rate of young spikes coldness is lower, and the comprehensive agronomic traits is better. The Group I was divided into two subgroups: Subgroup I-1 had eight varieties, and wheat Anke 157 (10), Yannong 19 (11) and Xumai 35 (16) showed the strongest cold tolerance and the similar yield traits, subgroup I-2 had two varieties, Huaimai 28 (5) and Jinan 17 (7), the GNMSS was relatively small within the group I.

Group II, including only variety Annong 0711 (13), has more spikelet number of main stem spike (SNMSS) and GNMSS, the smallest TKW, the lowest GYPP, and a strong cold tolerance.

Group III, including total 6 wheat varieties, these varieties showed fewer GNMSS, lighter GWMSS, smaller TKW, lower GYPP, greater injury grade of late spring coldness, a higher lethal rate of young spikes coldness and worse comprehensive agronomic traits. Group III was divided into two subgroups. Group III-1 had four varieties with fewer spikes, lighter TKW, and more serious disease at the later growth stage. Group III-2 had two cultivars with more spikes and fewer diseases at the later growth stage.

Table 2. Variance analysis of yield-related traits of the 18 wheat varieties.

Wheat varieties	PH (cm)	SNPP (No.)	MSSL (cm)	SNMSS (No.)	GNMSS (No.)	GWMSS (g)	TKW (g)	GYPP (g)	Rank of cold resistance
1. Yannong 5158	55.0 ± 1.7	7 ± 0	7.7 ± 0.2	19 ± 0	40 ± 6	1.22 ± 0.15	30.49 ± 1.2	8.54 ± 1.08	5
2. Yannong 5286	52.3 ± 0.9	4 ± 1	6.4 ± 0.1	17 ± 0	19 ± 1	0.60 ± 0.02	32.18 ± 1.0	1.61 ± 0.77	14
3. Jimai 22	39.8 ± 1.4	3 ± 0	6.6 ± 0.2	15 ± 1	24 ± 4	0.71 ± 0.13	29.89 ± 0.9	2.12 ± 0.39	15
4. Huaimai 22	47.3 ± 2.0	3 ± 1	6.9 ± 0.4	21 ± 1	24 ± 1	0.69 ± 0.16	28.25 ± 1.3	2.16 ± 0.68	12
5. Huaimai 28	61.2 ± 3.1	4 ± 1	8.1 ± 0.1	19 ± 1	33 ± 5	1.02 ± 0.15	30.94 ± 0.6	4.20 ± 1.16	2
6. Huaimai 33	51.5 ± 2.5	4 ± 1	7.9 ± 0.7	18 ± 2	44 ± 8	1.24 ± 0.32	28.08 ± 0.5	5.28 ± 1.84	3
7. Jinan 17	54.8 ± 3.6	5 ± 0	8.4 ± 0.5	17 ± 1	33 ± 4	1.17 ± 0.12	35.56 ± 2.1	5.50 ± 0.78	1
8. Fanmai 5	59.0 ± 1.5	4 ± 0	7.2 ± 0.4	18 ± 0	39 ± 4	1.11 ± 0.17	28.64 ± 0.3	4.83 ± 0.88	4
9. Wanmai 52	59.8 ± 2.2	3 ± 0	9.2 ± 0.5	21 ± 0	40 ± 9	1.23 ± 0.22	30.73 ± 1.2	3.69 ± 0.66	8
10. Anke 157	49.0 ± 1.7	2 ± 0	7.2 ± 0.6	19 ± 1	40 ± 4	1.28 ± 0.07	31.79 ± 0.9	3.01 ± 0.51	13
11. Yannong 19	60.7 ± 2.2	3 ± 0	8.4 ± 0.2	19 ± 1	44 ± 4	1.28 ± 0.13	29.02 ± 0.8	4.28 ± 0.65	6
12. Womai 9	50.7 ± 2.4	3 ± 0	8.3 ± 0.5	18 ± 1	28 ± 4	0.63 ± 0.21	22.32 ± 0.4	1.90 ± 0.63	10
13. Annong 0711	53.3 ± 2.6	3 ± 0	6.4 ± 0.3	20 ± 0	37 ± 2	0.63 ± 0.04	17.15 ± 1.4	1.66 ± 0.16	16
14. Liangxing 66	46.3 ± 2.6	2 ± 0	6.3 ± 0.1	16 ± 1	34 ± 1	0.80 ± 0.10	23.60 ± 1.2	1.81 ± 0.02	11
15. Xinmai 26	52.0 ± 1.5	2 ± 0	7.7 ± 0.3	20 ± 1	36 ± 4	1.22 ± 0.22	34.23 ± 0.6	2.76 ± 0.36	9
16. Xumai 35	54.7 ± 2.3	3 ± 0	7.7 ± 0.1	21 ± 1	48 ± 1	1.47 ± 0.10	30.47 ± 1.1	4.93 ± 0.66	7
17. Zhoumai 27	48.7 ± 1.5	2 ± 0	8.3 ± 0.3	18 ± 1	29 ± 7	0.85 ± 0.28	29.45 ± 0.9	1.94 ± 0.55	17
18. SXM 208	33.7 ± 0.7	2 ± 0	4.9 ± 0.2	11 ± 0	4 ± 1	0.10 ± 0.02	25.50 ± 0.5	0.20 ± 0.05	18
MS among cultivars	150.63	4.22	3.27	17.29	340.39	0.37	57.39	11.87	—
Error MS of different cultivars	13.81	0.41	0.40	2.20	64.72	0.08	10.01	1.82	—
F	10.90**	10.35**	8.10**	7.84**	5.26**	4.45**	4.70**	6.53**	—

Note: Values were represented as means ± E.E. in 2018 and 2019 (n=20). MS was mean square of statistics. PH was plant height, SNPP was spike number per plant; MSSL was main stem spike length, SNMSS was spikelet number of main stem spike, GNMSS was grain number of main stem spike, GWMSS was grain weight of main stem spike, TKW was thousand kernel weight, GYPP was grain yield per plan. Asterisk indicated significant differences at a 0.01 level (** $p<0.01$)

Table 3. Correlation analysis of freezing injury with yield-related traits of wheat.

	LGLSC	PH (cm)	SNPP (No.)	MSSL (cm)	SNMSS (No.)	GNMSS (No.)	GWMSS (g)	TKW (g)	GYPP (g)
IGLSC		-0.35	-0.22	-0.12	-0.43	-0.42	-0.415	-0.10	-0.36
IGYSC	0.80^{**}	-0.62^{**}	-0.24	-0.29	-0.61^{**}	-0.67^{**}	-0.64^{**}	-0.17	-0.47^{*}

Note: Value was presented with correlation coefficient (n=20). IGLSC was injury grade of late spring coldness, IGYSC was injury grade of young spikes coldness. PH was plant height, SNPP was spike number per plant; MSSL was main stem spike length, SNMSS was spikelet number of main stem spike, GNMSS was grain number of main stem spike, GWMSS was grain weight of main stem spike, TKW was thousand kernel weight, GYPP was grain yield per plant. Asterisk indicated significant differences (* $p<0.05$ and ** $p<0.01$)

Table 4. SOD and POD activities and proline content of the selected wheat varieties at seedling stage under low-temperature stress.

Cold resistance	Varieties	SOD ($U \cdot g^{-1}$ FW$\cdot min^{-1}$)	POD ($U \cdot g^{-1}$ FW$\cdot min^{-1}$)	Proline (%)
Strong	1 Yannong 5158	504.4 ± 14.8d	108.8 ± 31.5b	14.5 ± 0.4bcd
	5 Huaimai 28	406.9 ± 43.7cd	110.3 ± 30.9b	22.9 ± 0.4g
	6 Huai mai 33	435.5 ± 22.4cd	98.2 ± 26.4ab	15.4 ± 0.2cde
	7 Jinan 17	429.6 ± 23.9cd	91.7 ± 27.0ab	15.0 ± 0.5cde
	8 Fanmai 5	488.3 ± 72.8d	113.4 ± 32.5b	17.3 ± 0.7f
	11 Yannong 19	490.5 ± 25.2d	67.3 ± 5.0ab	16.6 ± 0.8ef
	16 Xumai 35	469.2 ± 21.0d	96.1 ± 24.8ab	16.4 ± 1.3def
Poor	4 Huaimai 22	197.9 ±49.9a	30.0 ± 2.5a	10.9 ± 0.2a
	12 Womai 9	337.7 ±14.8b	49.2 ± 13.1ab	13.7 ± 0.4bc
	18 SXM 208	263.6 ±14.6ab	56.3 ± 7.5ab	12.7 ± 0.0b

Note: Values were represented as means ± S.E. (n=6), and lowercase letters indicated significant differences at 0.05 level ($p<0.05$)

Table 5. Correlation analysis of SOD, POD, proline content and wheat freezing injury.

	SOD ($U \cdot g^{-1}$ FW$\cdot min^{-1}$)	POD ($U \cdot g^{-1}$ FW$\cdot min^{-1}$)	Proline (%)
SOD			
POD	0.50^{**}		
Proline	0.47^{**}	0.49^{**}	
IGLSC	-0.51^{**}	-0.13	-0.07
IGYSC	-0.70^{**}	-0.36	-0.56^{**}

Note: Value was presented with correlation coefficient (n=6). IGLSC was injury grade of late spring coldness, IGYSC was injury grade of young spikes coldness. Asterisk indicated significant differences at 0.01 level (** $p<0.01$)

Group IV, including SXM 208 (18), had the lowest PH, the shortest main stem spike length (MSSL), the least SNMSS and GNMSS, the lowest GYPP, the lowest cold tolerance, and the worst comprehensive agronomic traits. Wheat varieties and yield-related characteristics in each group are as follows (Table 6).

Genetic diversity of 18 wheat varieties by SSR markers: Eighty-one pairs of SSR primers related to cold tolerance were selected on the basis of the wheat genomic DNA. A total of 28 pairs of SSR primers with abundant polymorphism were obtained, they were evenly distributed on 21 chromosomes of wheat, and these primers were used to analyze the molecular genetic diversity of different wheat varieties.

28 pairs of primers with the effective polymorphic loci detected a total of 594 polymorphic PCR bands, the proportion of polymorphic bands was 54.6%. The polymorphic bands with a size of 100- 300 bp were counted, and 1 to 4 alleles were detected in each pair of primers due to the small investigation interval. A total of 87 alleles were detected in 28 pairs of primers, with an average of 3 alleles for each pair of primers. The polymorphism information content (*PIC*) ranged from 0.179 to 0.983, the Shannon's information index (*I*) was between 0.215 and 1.255. The genetic diversity index (*Nei*), with an average of 0.469, ranged from 0.105 to 0.697 (Table 7). These suggest that the genetic diversity among the wheat varieties was relatively low at the molecular level.

Table 6. Cluster analysis and agronomic characteristics of 18 wheat varieties.

Group	Wheat varieties	Total No. of varieties	Main agronomic characteristics
I	(1) 1, 6, 8, 9, 10, 11, 15, 16	8	Higher PH, larger GNMSS, higher TKW, heavier GYPP, stronger cold resistance, and better comprehensive agronomic traits
	(2) 5, 7	2	
II	13	1	Lighter TKW, lower GYPP and strong cold tolerance
III	(1) 4, 12, 14, 17	4	Fewer GNMSS, lighter TKW, lower GYPP, weak cold resistance, and poor comprehensive agronomic traits.
	(2) 3, 2	2	
IV	18	1	Lowest PH and GYPP, and worst cold tolerance

Note: Wheat varieties were represented as code number, the details were as Table 2 above. PH was plant height, GNMSS was grain number of main stem spike, TKW was thousand kernel weight, GYPP was grain yield per plant

Table 7. Molecular diversity and genetic differentiation of 18 wheat varieties.

Code	SSR primer	*PIC* value	*I*	*Nei*	*Fis*	*Fit*	*Fst*	*Nm*
1	Barc4	0.748	1.255	0.697	-0.401	-0.248	0.109	2.048
2	Barc32	0.573	0.865	0.539	-0.585	-0.305	0.177	1.164
3	Barc40	0.679	0.934	0.549	-0.623	-0.350	0.169	1.234
4	Barc59	0.878	1.036	0.628	0.607	0.865	0.656	0.131
5	Barc61	0.895	1.030	0.620	1.000	1.000	0.670	0.123
6	Barc76	0.197	0.215	0.105	1.000	1.000	0.077	3.000
7	Barc79	0.440	0.965	0.593	-0.651	-0.296	0.215	0.911
8	Barc105	0.953	0.693	0.500	-1.000	-0.044	0.478	0.273
9	Barc127	0.643	1.015	0.607	-0.765	-0.636	0.073	3.180
10	Barc170	0.928	1.010	0.615	-0.252	0.183	0.347	0.470
11	Barc174	0.765	1.173	0.635	-0.152	0.496	0.563	0.194
12	Barc177	0.820	0.693	0.500	-1.000	0.429	0.714	0.100
13	Barc180	0.554	0.678	0.484	-0.688	-0.659	0.017	14.482
14	Barc181	0.983	1.040	0.625	1.000	1.000	0.840	0.048
15	Barc206	0.972	0.377	0.219	-1.000	0.704	0.852	0.044
16	Barc302	0.651	0.806	0.518	-0.886	-0.581	0.162	1.296
17	Barc319	0.740	0.562	0.375	0.288	0.798	0.716	0.099
18	Barc320	0.762	1.024	0.622	-0.781	-0.675	0.059	3.974
19	Barc321	0.947	0.892	0.520	0.515	0.819	0.626	0.149
20	Gwm136	0.903	0.668	0.475	0.576	0.946	0.872	0.037
21	Gwm148	0.817	0.722	0.403	0.218	0.729	0.653	0.133
22	Gwm149	0.590	0.410	0.245	1.000	1.000	0.929	0.019
23	Gwm190	0.288	0.133	0.057	-0.059	-0.014	0.042	5.667
24	Gwm273	0.906	0.483	0.305	-0.579	-0.263	0.200	1.000
25	Gwm369	0.886	0.591	0.401	-0.905	-0.667	0.125	1.750
26	Gwm512	0.590	0.683	0.490	-0.875	-0.818	0.030	8.000
27	Gwm526	0.928	0.287	0.153	-1.000	0.704	0.852	0.044
28	Wmc388	0.845	1.152	0.640	0.304	0.589	0.410	0.360
Average		**0.746**	**0.764**	**0.469**	**-0.203**	**0.204**	**0.415**	**1.783**

Note: *PIC* was the polymorphism information content, *I* was the Shannon's information index, *Nei* was the genetic diversity index, *Fis* was the inbreeding coefficient, *Fit* was the total inbreeding coefficient, *Fst* was the genetic differentiation coefficient, *Nm* was the gene flow

Table 8. Nei's genetic identity and genetic distance among 4 wheat groups.

Groups	I	II	III	IV
I		0.713	0.889	0.552
II	0.339		0.748	0.586
III	0.118	0.291		0.648
IV	0.594	0.534	0.434	

Note: *Nei's* genetic identity (above diagonal) and genetic distance (below diagonal)

The inbreeding coefficient (*Fis)* in the population ranged from −1.000 to 1.000, with an average of -0.203, the variation in total inbreeding coefficient (*Fit*) was between -0.818 and 1.000, with an average of 0.204. The average value of *Fit* was positive, indicating that the inbreeding frequency of these varieties was seriously high at the SSR loci, these varieties are basically homozygous, only 10 loci with the negative *Fit* value are hybridized (Nagylaki, 1998). These suggested that self-crossing of wheat within each variety was common, and hybridization is less occured among varieties, possible due to the self-pollination characteristic of wheat. The genetic differentiation coefficient (*Fst*) ranged from 0.017 to 0.929, with an average of 0.415, implying that 41.5% and 58.5% of the genetic variation occurred among wheat varieties and within wheat varieties, respectively. Gene flow (*Nm*) is an important factor affecting population differentiation, the variation of *Nm* at different SSR loci ranged from 0.019 to 14.482, with an average of 1.783. This finding indicated that the *Nm* level at these loci was low, and the whole population was easily differentiated by genetic drift. The *Nm* levels of 12 loci in the population were greater than 1, accounting for 43%, the *Nm* levels of primers Barc180 and Gwm512 loci were 14.48 and 8.00, respectively, and sufficient to resist genetic differentiation caused by genetic drift in the population.

In accordance with the genetic distance of *Nei*, the wheat varieties were clustered into four groups by the UPGMA method, the genetic distance between groups varied greatly, ranging from 0.118 to 0.594. The genetic distance between groups I and IV was large, indicating that groups I and IV had a far genetic relationship. The genetic identity of the four groups ranged from 0.552 to 0.889, suggesting that the genetic consistency among different wheat groups was relatively homogeneity, and wheat varieties between Group I and Group III showed the high genetic consistency at a molecular level (Table 8). This finding was basically consistent with the clustering results of the agronomic traits.

The ratio of polymorphic loci within the four class groups was low, accounting for 23.09% on average (Table 9). Among them, Group I-1 and Group III-1 had the most abundant polymorphic loci, reaching 49.09% and 41.82%, respectively. Group IV had fewer polymorphic loci (only the 10 loci). The Shannon’s information index (*I*) of different groups ranged from 0.208 to 0.384, and the genetic diversity indexe (*Nei*) were between 0.150 and 0.246, indicating that the genetic diversity among different class groups was relatively small. The *Nei* of the four groups were ranked as follows: I-1 > III-1 > III-2. > IV > I-2 > II. The varieties in group I-1 had a high yield, strong cold tolerance, and abundant genetic resources, suggesting that these varieties could be properly selected and utilized in wheat breeding.

Table 9. Genetic diversity of four wheat class groups.

Group	Samples	Polymorphic loci numbers	Ratio of polymorphic loci (%)	***I***	***Nei***
I	10	27	49.09	0.384	0.246
	3	14	25.45	0.233	0.162
II	2	12	21.82	0.208	0.150
III	5	23	41.82	0.371	0.240
	3	10	18.18	0.243	0.174
IV	2	10	18.18	0.239	0.172
Average	4.17	16	23.09	0.280	0.190

Note*: I*: Shannon’s information index; *Nei*: genetic diversity index

Discussion and Conclusion

The study on the physiological properties variation of wheat under cold stress and evaluation on the cold resistance of different wheat varieties have been widely explored, but the results varied greatly due to diverse wheat varieties and different ecological environments within wheat cultivation regions. The cold tolerance of wheat have been conducted by indoor potting and locally artificial cold treatments, to detect the physiological and biochemical characteristics, oxidase activity, hormone content of wheat plants (Xiao *et al.*, 2018; Kosova *et al.*, 2012). However, these showed the great limitation to select the major wheat varieties with strong cold tolerance, because wheat growth condition is more complex around, and natural resources, such as light, temperature, and water, probably affect the stress resistance and final yield of wheat. This study was conducted at Jinzhai Crop Variety Resistance Identification Test Station in Anhui province, which belongs to the mid-regions of Jianghuai, the altitude is high, and the coldness injury of late spring frequently occured. Under the natural cold stress, the cold-resistant of selected wheat varieties is much more reliable, due to the similar planting condition with wheat cultivation regions of Jianghuai.

Different wheat varieties have different genetic mechanisms, stress resistance is a complex quantitative trait, wheat varieties under adverse conditions have various responses to some specific physiological process, and it is unilateral to evaluate the cold resistance of wheat by using any single index (Li *et al.*, 2018). Our results found that three wheat varieties of Liangxing66 (14), Zhoumai27 (17) and SXM208 (19) showed severely freezing injury of late spring coldness, whereas six wheat varieties had severely freezing injury of young spikes at later stage, suggesting the phenotype of freezing injury in late spring could not accurately reflect the later harmful influence on wheat development. After harvest, yield-related traits showed that the freezing injury in late spring coldness caused serious damage to the development of wheat young spikes, and remarkably affecting the formation of yield-related traits. The injury degree under cold stress varied greatly among different varieties, as well of the different performance of yield-related traits, it was difficult to determine the cold tolerance of wheat only by one single index. In this study, to avoid the redundant information provided by multiple indicators, ten single indicators were used for principal component analysis to obtain three comprehensive indicators. On the basis of the contribution rate of the three comprehensive indicators, the corresponding subordinative function values were calculated, and the cold resistance of 18 wheat varieties was comprehensively ranked through weighted analysis based on their relative importance of the three comprehensive indicators. Among them, seven varieties (Yannong 5158, Huaimai 28, Huaimai 33, Jinan 17, Fanmai 5, Yannong 19, and Xumai 35) had a good comprehensive performance of high grain yield, oxidase activity and proline content, and strong cold resistance, these varieties were suggested as the candidates for the popularization and application in Jianghuai region of Anhui Province.

SSR markers provide an ideal method for finding new germplasm resources and broadening the germplasm base, ensuring considerable genetic distance among different germplasms is the basis for improving the stress resistance of crops (Sun *et al.*, 2015; Talukder *et al.*, 2010). In this study, the wheat population showed few hybrids among different varieties, low gene flow levels at different SSR loci, and the differentiation among populations possibly occurred by genetic drift. The total inbreeding coefficient (*Fit*) of the population was large, indicating the obvious inbreeding trend within varieties, leading to a low *Nei* index. Wheat is a self-pollinating crop with an excessive directional selection, producing a narrow genetic range in the specific population. 18 wheat varieties can be classified into four groups, the Group I-1 had abundant polymorphic loci, a high genetic diversity, strong cold tolerance, a good performance of yield-related traits and rich genetic resources, in which the varieties could be used as the key germplasm resources to improve the cold resistance of bread wheat.

The genetic variation detected by SSR data is not absolutely consistent with that reflected by agronomic traits (Naushad *et al.*, 2012; Carputo *et al.*, 2013). The yield-related traits were significantly different among the 18 wheat varieties, whereas the genetic diversity was general, and the genetic basis was relatively simple as to SSR identification. It was speculated that yield-related traits were susceptible not only to environmental conditions but also to gene dominance and recessiveness, their genetic expression was unstable, but SSR markers detected the non-functional region in the genome, and the results were stable at different developmental stages or in various tissues of plants. Although our study investigated the main yield-related traits and cold tolerance of wheat, the sequences of functional genes targeted by SSR markers were unclear, possibly leading to a deviation between phenotypic characteristics and molecular results. As such, the related characteristics of wheat after exposure to cold stress should be combined with the molecular markers identification to evaluate the relationship between the cold tolerance of wheat and the genetic diversity of different varieties.

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