Hybrid Necrosis of Wheat: Systematic Genetic Analysis, Fine Mapping, Distribution and Evolution in China

Min Zhang

Northwest A&F University: Northwest Agriculture and Forestry University

Shikai Lv

Northwest A&F University: Northwest Agriculture and Forestry University

Yanzhen Wang

Northwest A&F University: Northwest Agriculture and Forestry University

Siwen Wang

Northwest A&F University: Northwest Agriculture and Forestry University

Chunhuan Chen

Northwest A&F University: Northwest Agriculture and Forestry University

Changyou Wang

Northwest A&F University: Northwest Agriculture and Forestry University

Yajuan Wang

Northwest A&F University: Northwest Agriculture and Forestry University

Hong Zhang (✉ zhangh1129@nwafu.edu.cn)

Northwest Agriculture and Forestry University <https://orcid.org/0000-0002-3249-0935>

Wanquan Ji

Northwest A&F University: Northwest Agriculture and Forestry University <https://orcid.org/0000-0003-0663-4568>

Research Article

Keywords: Hybrid necrosis of wheat, Ne1 and Ne2, genetic maps, necrotic symptom, wild emmer

Posted Date: July 9th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-665444/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Hybrid necrosis of wheat is caused by two incompatible natural alleles *Ne1* and *Ne2* residing in normal phenotype parents. It was regarded as a barrier of gene flow between crop species. However, the necrosis alleles were still frequent in modern wheat varieties. Here, we constructed two high-density genetic maps of *Ne1* and *Ne2* in winter wheat. Of which, *Ne1* locus was located in a span interval of 0.50 centimorgan (cM) on chromosome 5BL delimited by markers *Nwu_5B_4137* and *Nwu_5B_5114*, while *Ne2* co-segregated with markers *Lseq102* and *TC67744* on 2BS. Genetic allelic tests confirmed that the *Ne1* allele in N9134 is different from that in Spcia and TA4152-60, while *Ne2* allele in Zhoumai 22 (ZH22), Manitou, WL711 and Pan555 are also varied. The statistics substantiated that the dosage effect of *Ne* alleles also existed in moderate and severe hybrid-necrosis system, and the necrotic symptom may also be affected by genetic background. Furthermore, we clarified the discrete distribution and proportion of the *Ne1* and *Ne2* in China's major wheat regions, and concluded that introduced modern cultivars directly affect the frequencies of necrosis genes in modern Chinese cultivars (lines), especially that of *Ne2*. Taking investigation in spring wheat together, we proposed that hybrid necrotic alleles could positively affect breeding owing to their linked excellent genes. Additionally, based on the pedigree, we speculated the *Ne1* and *Ne2* in winter wheat may directly originate from wild emmer and rye, respectively. Overall, this study makes an important step toward better understanding hybrid necrosis in wheat.

Introduction

In genetics, F_1 -generation plants from two normal parents unexpectedly phenotyping gradually premature senescence or death is not uncommon, and called as hybrid necrosis. At the first glance, this kind of necrosis is considered to be postzygotic hybrid incompatibility, and coincide with Bateson–Dobzhansky–Muller (BDM) model (Orr 1996). So far, several hybrid-necrosis systems have been found in plants (Bomblies and Weigel 2007) according to the extent of necrosis. The severest type of hybrid necrosis resulted in death at seedling stage resembling a kind of reproductive isolation, so that it was considered to play a possible vital role in speciation in nature (Bomblies and Weigel 2007; Chen et al. 2014; Hermesen 1963a). For breeding practice, hybrid necrosis does limit the use of certain combinations of parents from diverse germplasm pools, therefore, it was thought as a barrier in crop improvement through combining desirable traits from different varieties or transferring genes from related species to commercial cultivars (Bizimungu et al. 1998; Hermesen 1963a). In bread wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD), the inheritance of hybrid necrosis is caused by the interaction of two dominant complementary genes *Ne1* and *Ne2* located on chromosome arms 5BL and 2BS, respectively (NISHIKAWA et al. 1974). The necrosis could start at any development stages from 1–2 leaves stage onwards depending on the nature of crosses (Hermesen 1963a). When leaf tissues reach a certain physiologic maturity, the necrosis program was activated, then the leaves are gradually yellowing, wilting and necrosis from tip progressively to base orderly developed from the lowest leaf to flag leaf (Caldwell and Compton 1943). Finally, hybrid necrosis would result in wheat plant dwarf, reducing growth rate, poor fertility, infertility or even death before heading period (Caldwell and Compton 1943). Intriguingly, it was proposed that hybrid necrosis could result from autoimmunity of hypersensitive response (HR) associated with superoxide, signal transduction regulation disorder and so on (Chu et al. 2006; Kandel 2016; Khanna-Chopra et al. 1998; Zhang et al. 2014; Zhou et al. 2015). This hinted that the necrosis might be a pleiotropic effect of evolution of genes involved in pathogen response (Bomblies and Weigel 2007). Hence, understanding the molecular basis and mechanisms of hybrid necrosis will be helpful to fill the gap of acknowledge in hybrid breeding and also in plant pathology.

The first genetic map of either *Ne1* or *Ne2* was mapped by Chu, et al. (2006) in spring wheat using microsatellite (simple sequence repeats, SSR) markers screening two backcross populations consisting of 100 and 94 individuals, respectively. On which, the genetic distances separately were 2.0 centimorgan (cM) and 3.2cM from *Ne1* and *Ne2* to their respective nearest SSR marker *Xbarc74* and *Xbarc55* (Chu et al. 2006). Apart from, subsequently studies linked *Ne2* with two leaf rust resistance genes (*Lr13*, *LrLC10*) in spring wheat (Qiu et al. 2020; Zhang et al. 2016). However, even if all wheat lines with *Lr13* appear to carry *Ne2m*, not all *Ne2m*-carrier wheat lines carry *Lr13* (Wamishe and Milus 2004; Zeven 1981). For example, wheat cultivars, Kalyansona (Zhang et al. 2016), 'Penjamo 62' and 'Siete Cerros 66' (Singh and Rajaran 1991) are *Ne2* carriers but none of them carries *Lr13*. This means that the *Ne2* locus maybe have different alleles or haplotypes. It is reported that there is high level of genetic differentiation between spring and winter wheat lines (Chao et al. 2010; Singh et al. 2000). Moreover, the *Ne1* and *Ne2* in winter wheat and those in spring wheat should be derived from two different groups which had been introduced into different regions (Zeven 1981). Therefore, it is necessary to carry out targeted study of these two genes in winter wheat.

The surveys on the distribution (Hermesen 1963b; Oetmann and Zeller 1989; Pukhalskiy et al. 2008a; Pukhalskiy et al. 2000; Zeven 1965) of *Ne1* and *Ne2* showed both of them are widely distributed among different wheat species, subspecies and cultivars throughout the world (Bomblies and Weigel 2007). And the clear distribution of them were established according to the global lists of *Ne* carriers published from 1963 to 1981 (Hermesen 1963b; Zeven 1965, 1967, 1968, 1969, 1971, 1973, 1976, 1981). In which, up to 5541 worldwide wheat cultivars (lines) were tested, but only 154 Chinese wheat cultivars were involved (Hermesen 1963b; Zeven 1965, 1967, 1968, 1969, 1971, 1973, 1976, 1981). As frequent international cooperation, the frequencies of *Ne1* and *Ne2* have changed in wheat populations of different regions, although the distribution of them continue to be discrepant in different countries (Bomblies and Weigel 2007; Pukhal'sky and Bilinskaya 1998; Pukhalskiy et al. 2000; Pukhalskiy et al. 2008b; Vikas et al. 2013). Notably, both of *Ne1* and *Ne2* in bread wheat are still widely distributed (Bomblies and

Weigel 2007; Pukhalskiy et al. 2018; Vikas et al. 2013). China is the largest wheat-production country, however, the distribution and proportion of necrotic genes in China were not systematically analyzed.

According to the intensity of necrosis, Hermesen distinguished hybrid necrosis into nine grades (0–8) and three levels (grades 6–8 are indicated as severe necrosis, 3–6 as moderate necrosis and 0–3 as weak necrosis) in 1962 based on the distinct variation of necrosis in different F₁-generation (*Ne1ne1Ne2ne2*). These variations were determined by the occurrence of three alleles of *Ne1* and five alleles of *Ne2* in wheat (Hermesen 1963a). However, to date, there is no directly genetic proof to definite their relationship of allelism (Zhang et al. 2016). Moreover, the dosage effect of *Ne* genes in weak-necrosis system was demonstrated by Hermesen (1963a). But it is unclear whether the dosage effect is still true in the moderate and severe hybrid necrosis.

During wheat breeding for improving the resistance of high-yield cultivar, we observed several necrotic F₁ combinations crossing normal green wheat lines. Considering the importance of wheat breeding and the gap of knowledge in the hybrid necrosis of wheat, here we report a series of genetic analysis on hybrid necrosis in winter wheat and mainly focus on the moderate and severe types. Firstly, through systemic genetic analysis, we confirmed the multiple alleles of *Ne1* and that of *Ne2* should be residing in different loci, respectively. Meanwhile, we revealed the dosage effect on the intensity of necrosis in the moderate and severe necrotic system. Then, two fine genetic maps of *Ne1* and *Ne2* were constructed in winter wheat mainly using the KASP markers and AS-PCR markers. Furthermore, we clarified the discrete distribution, proportion and genotype frequencies of the two *Ne* genes in China's major wheat areas. Finally, we traced the origin of *Ne1* and *Ne2* in the two backcross mapping populations of this study.

Materials And Methods

Plant materials

Common wheat line N9134 was selected from hybrid progeny of 5B Nullisomic (5BN) of Abbondanza / wild emmer (WE) accession AS846 (*T. dicoccoides*). N9134 and N0439 are both excellent winter wheat lines developed at Northwest A&F University. Zhoumai 22 (ZH22) and Xinong 509 (XN509) are both main commercial cultivars in Yellow and Huai River valleys winter wheat region of China. The F₁ generations of reciprocal crosses (e.g., N9134 / ZH22 and ZH22 / N9134, etc.), the corresponding F₂ populations and BC₁F₁ populations were engaged to find, verify, and determine the hybrid necrosis in our wheat cultivars (Table S1). And the two BC₁F₁ populations were also employed for finely mapping the two necrotic genes *Ne1* and *Ne2*. Specifically, the BC₁F₁ population, derived from the backcrosses of ZH22 (*Ne2* carrier) // [(N9134 / ZH22) or (ZH22 / N9134)], was used to map the *Ne1* allele, and it was consisted of necrotic plants (*Ne1ne1Ne2_*) and normal plants (*ne1ne1Ne2_*). Meanwhile, the other BC₁F₁ population, from N9134 (*Ne1* carrier), was used for mapping the *Ne2* alleles on the basis of necrotic and normal plants in this population genotyped *Ne1_Ne2ne2* and *Ne1_ne2ne2*, respectively.

The five cultivars (lines), an Australian spring wheat cultivar Spica carrying *Ne1s* (Zhang et al. 2016), the CIMMYT synthetic hexaploid wheat (SHW) lines TA4152-60 carrying *Ne1* (Chu et al. 2006), the Chinese winter wheat cultivar Pan555 carrying *Ne2* (Jiang et al. 2008), the WL711 and the Canadian cultivar Manitou both carrying *Ne2m* and *Lr13* (Zhang et al. 2016), were used for allelism tests with *Ne1* in N9134 and *Ne2* in ZH22 carrying correspondingly.

The seeds of other cultivars (lines) used for genotyping the two necrotic genes (Table S2) were kept in Chromosome Engineering Laboratory, Northwest A&F University or obtained from the program of "Precise identification and innovative utilization of wheat germplasm resources" (2016YFD0100102-6).

Allelism Tests

To test the relationship between *Ne1* in N9134, Spica and TA4152-60, N9134 was firstly crossed with these two *Ne1*-allele carriers to obtain the F₁ gametes, which were further crossed with ZH22 to form two populations of test cross F₁ (TF₁), i.e., ZH22 // (N9134 / Spica or Spica / N9134) and ZH22 // (N9134 / TA4152-60 or TA4152-60 / N9134). Similarly, for the relationship between *Ne2* in ZH22 and the other *Ne2* alleles, ZH22 was crossed with WL711 (*Ne2m*), Manitou (*Ne2m*) and Pan555 (*Ne2*) respectively, and then the F₁ plants were employed as male parents to cross with N9134 forming the corresponding TF₁ populations, i.e., N9134 // (ZH22 / WL711 or WL711 / ZH22), N9134 // (ZH22 / Manitou or Manitou / ZH22) and N9134 // (ZH22 / Pan555 or Pan555 / ZH22). Each TF₁ population was consisted of more than 250 progeny plants.

Investigation of the phenotype and statistical analysis

All crosses, backcrosses and self-crosses were conducted with bagged ears. With one spike per row, the seeds were planted in the experimental field of Northwest A&F University, Yangling, Shaanxi, China. In the past years, they were generally planted at beginning of October, then, each of them was numbered in early December when plants grew to about 15cm tall. Subsequently, the first investigation of the phenotype at seedling

stage was conducted in the early stage of winter tillering. The second investigation was conducted around the Vernal Equinox in following year when wheat plants were at the early jointing stage after returning to green. Finally, the third was carried out when plants were at heading stage in mid-April. The investigation was conducted with directly observing the obviously contrasted phenotypes between necrotic and normal plants, and also comparing to the normal parents. After summarizing the survey data, Chi-squared (χ^2) tests were conducted to determine the goodness of fit of segregation ratios to theoretical Mendelian ratios.

Sampling and DNA extraction

Following the second investigation, the second leaf of plants were sampled and numbered at jointing stage. After being freeze-dried, the samples (about 0.5 g of each fresh leaf in a 2.0 ml centrifuge tube) were powered using medium throughput ball mill (TL2010, DHS Life Science & Technology Co., Ltd, Beijing, China). The CTAB method was applied for genomic DNA (gDNA) extraction (Saghai-Maroo et al. 1984) but just with some improvements to the extraction buffer due to the leaf tissue was relatively older in this study. The specific working composition of the modified gDNA extraction buffer is: 0.8 M NaCl, 0.14M D-Sorbitol, 0.22 M Tris (pH 8.0), 22 mM EDTA (pH 8.0), 0.8% CTAB, 0.8% Sarcosine, 3% PVP-K30, 2% 2-Mercaptoethanol, to reduce the content of secondary metabolites (e.g., polysaccharide, polyphenols, etc.).

Screening and developing of molecular markers

The flanking molecular markers of *Ne1* (Chu et al. 2006) and *Ne2* (*Lr13*, *LrLC10*) (Chu et al. 2006; Qiu et al. 2020; Zhang et al. 2016) in previous reports were applied on the assays in the present study. Moreover, the other SSR markers were obtained from the GrainGenes Database (<https://wheat.pw.usda.gov/GG3/>). Specific-Locus Amplified Fragment Sequencing (SLAF-Seq) (Sun et al. 2013) was applied for typing and screening SNP sites between the three pools (Table S3). For the *Ne1* allele, SNP sites were searched according to the SLAF-Seq result of the positive pool (a) and negative pool (b) (Table S3). The upstream and downstream 500 bp flanking sequence of those SNP sites were obtained from the Chinese Spring reference genome sequence (IWGSC RefSeq v1.0). It was same for the *Ne2* locus using the positive pool (a) and negative pool (c) (Table S3). Furthermore, the SNP sites were converted into Allele-Specific PCR (AS-PCR) or Kompetitive Allele-Specific PCR (KASP) markers. The primers' sequences of these markers were designed using PolyMarker (<http://www.polymarker.tgac.ac.uk>). The SLAF-Seq was conducted by Biomarker Technology Co., Ltd (Beijing, China). The primers were synthesized by Beijing AuGCT DNA-SYN Biotechnology Co. (Beijing, China). The markers used in this study were listed in Table S4. The molecular marker assays of SSR, AS-PCR and InDel were performed following the protocol described in previous reports (Xue et al. 2012). KASP assays were performed following the manufacturer's protocol (LGC Genomics; <http://www.lgcgroup.com>).

The polymorphic markers were screened using bulked segregant analysis (BSA), and they were separately screened for *Ne1* and *Ne2* among the DNA pools consisting of the two parents, the heterozygous F_1 , and the corresponding one of the two BC_1F_1 populations. Here, in either of the two BC_1F_1 populations, the necrotic plants and normal plants constitute the corresponding positive and negative pool, respectively. And each sample pool was made by pooling equal amounts of gDNA from each of the 20 plants with specific phenotype and genotype. Then, the selected markers were further independently tested in a small number of individuals (23 plants of necrosis and 22 plants of normal phenotype) from the corresponding BC_1F_1 population. Afterwards, those markers which were still polymorphic would be picked out for finely mapping the two necrotic genes with all the individuals of the two BC_1F_1 populations, respectively.

Drawing the genetic linkage maps

Linkage analysis was established using QTL IciMapping V4.2 (<http://www.isbreeding.net/software/>) with grouping based on a threshold LOD score of 3.0, ordering by the algorithm of nnTwoOpt (nearest neighbor was used for tour construction, and two-opt was used for tour improvement) and rippling by the criterion of SARF (Sum of Adjacent Recombination Frequencies) (Meng et al. 2015). And then genetic distances were calculated using the Kosambi mapping function (Kosambi 1943).

Pedigree analysis and genotype detection of wheat cultivars for hybrid necrosis

N9134 and ZH22 were separately regarded as the two fundamental carriers (cultivars) of *Ne1* and *Ne2* in winter wheat. The pedigrees of them were traced using hybrid test to determine whether their parents and ancestors contain the hybrid necrosis genes, and if so, which one it is. Hybrid tests were also applied for classifying the genotype of the wheat cultivars (lines) for *Ne1* and *Ne2* through carrying out cross with the carriers of *Ne1* or *Ne2* (including but not limited to N9134 and ZH22). Additionally, the independent genotype detection was also conducted using the developed close linkage KASP markers (*Nwu_5B_4137* and *Nwu_5B_5114* for *Ne1* locus; *Nwu_2B_4204* and *Nwu_2B_4249* for *Ne2* locus).

Fluorescence in situ hybridization (FISH)

The FISH was engaged to identify the differences in the karyotype of the cultivars carrying the *Ne* alleles. This was performed following the protocol described by Tang et al. (2014) and using the oligonucleotide probes Oligo-pTa535 (Tamra-5', red) and Oligo-pSc119.2 (6-FAM-5'

green), which were synthesized by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China). Fluorescent signals were scanned and photographed with an Olympus BX53 microscope equipped with a Photometrics SenSys CCD DP80 camera (Wang et al. 2016).

Results

It was verified to be hybrid necrosis that presented in winter wheat of this study.

In practice of wheat breeding, we found the F_1 -generation plants of certain crosses derived from two normal-phenotyping cultivars (lines) showed gradual premature necrosis from seeding stage. Luckily, the F_1 plants could produce premature but fertile seeds. Then, through systematic genetic analysis (Table S1) of winter wheat cultivars (lines) (N9134, N0439, ZH22, Z8425B, XN509, and so on) using the populations of F_1 , F_2 , multiple cross F_1 and BC_1F_1 , we confirmed the hybrid necrosis presented in the winter wheat of this study was also controlled by two complementary dominant alleles, and its genetic pattern was same to that reported in spring wheat. The specific results are summarized as follows: the F_1 -generation plants derived from crosses of N9134 (or N0439) / ZH22 (or Z8425B) were necrosis consistently; the F_2 populations, from the self-pollinated F_1 individuals with necrotic phenotype, segregated with the ratio of 9 necrosis : 7 normal at seedling stage; all of the BC_1F_1 populations from the separate backcrosses (N9134, N0439 and ZH22 were as female parents and their corresponding F_1 individuals with necrosis were as male parents) showed a segregation ratio of 1:1 for necrosis Vs. normal phenotype; all plants of the F_1 generations of XN509 / ZH22 and XN509 / N0439 were with normal phenotype; and the populations from XN509 // N0439 / ZH22 and XN509 / N0439 // ZH22 showed the segregation ratios of 1:3 and 1:1 with necrotic to normal phenotype, respectively. Furthermore, we concluded that N9134 (or N0439) should contain the allele of *Ne1*, while ZH22 (or Z8425B) should contain the allele of *Ne2*, by performing test crosses with WL711, Manitou and Pan555 (*Ne2*-carriers) and Spica and TA4152-60 (*Ne1*-carriers) (Table S1), respectively. The traits of hybrid necrosis at different growth stages and conditions in this study were shown in Fig. 1.

The dosage effect still exists in the moderate and severe hybrid-necrosis system.

The degree of the weak necrosis would be affected by the number of dominant *Ne* alleles (gene dosage) (Hermesen 1963a). Here we did some detailed investigation and confirmed that not only in the weak necrosis but also in the moderate and severe hybrid necrosis, the dosage effect of dominant *Ne* alleles still exists. The F_1 plants derived from N9134 and ZH22 are belonged to the moderate or severe necrosis according to the criteria of necrosis grades stated by Hermesen (1963a). Through genetic analysis (Table 1, Table S1), we hypothesized that these necrotic plants having genotype *Ne1Ne1Ne2Ne2* of F_2 population would die before heading stage and could not produce seeds. Under this premise and according to Mendel's law of inheritance, in the F_2 population, the phenotypic separation ratio of necrotic plants to normal plants should be between 8:7 and 9:7 at different growth stage. Meanwhile, in the F_3 population generated from the randomly selected seeds of necrotic F_2 plants, the phenotypic segregation ratio should be 16:11 rather than 25:11 after heading. In addition, in the $F_{2:3}$ lines generated from the necrotic F_2 plants, all the lines should contain both necrotic and normal plants instead of only having the necrotic plants. Specifically, the 140 spikes used to cultivate the $F_{2:3}$ lines were randomly selected (one spike per plant) just from the necrotic F_2 plants with varying the necrotic degree from light to heavy. Among the 140 $F_{2:3}$ lines, one line had no living plants (There were only four seeds in this spiker.); three lines contained necrotic plants only, and they had four, four and five seeds of the spiker, respectively, with the living plants rate of 50%, 20% and 20%; the other 136 lines segregated necrotic and normal plants (Table 1, Table S5). This means that there were no more than four double dominant homozygous lines. Even the ratio of double dominant homozygous lines Vs. heterozygous lines was 4:136, it was not fit to, 1:8 ($p = 7.83E^{-04}$), the theoretical Mendelian ratios without dosage effect.

Table 1
Genetic analysis of dosage effect associated with the moderate and severe necrosis.

Years	Female Parent	Male Parent	Genetic population	Necrotic plants	Normal plants	Seeds	Investigation condition	Theoretical Mendelian ratios (Necrotic: Normal)	<i>p</i> value	Theoretical Mendelian ratios with dosage effect (Necrotic: Normal)	<i>p</i> value
2017-18	N0439	ZH22	F ₂	110	71	264	Seedling, Field	9:7	0.220	8:7	0.045 *
2012-13	ZH22	N0439	F ₂	137	132	380	Jointing, Field	9:7	0.079	8:7	0.429
2015-16	ZH22	N0439	F ₂	128	132	420	Heading, Field	9:7	0.023 *	8:7	0.185
2013-14	ZH22	N0439	F ₃	241	172	800 ^a	Heading, Field	25:11	9.93E ⁻⁰⁷ **	16:11	0.708
2018-19 ^b	N9134 (ZH22)	ZH22 (N9134)	F ₃ (F _{2:3})	986	717	2637	Heading, Field	25:11	4.44E ⁻²⁵ **	16:11	0.253
2018-19 ^b	N9134 (ZH22)	ZH22 (N9134)	F _{2:3}	3 ^c	136 ^c	140 ^c	Heading, Field	1:8	7.83E ⁻⁰⁴ **	0:8	—
Notes: a. The seeds were randomly selected from the mixed seeds of the F ₂ necrotic plants. b. The details of F _{2:3} population were shown in Table S5. c. 3 was the number of the F _{2:3} lines only having necrotic plants; 136 was the number of F _{2:3} lines containing both necrotic and normal plants; 140 was the number of all the selected spikes from necrotic F ₂ plants. <i>p</i> -values were from chi-square test calculated between the ratios of genetic population and theoretical Mendelian ratios. * <i>p</i> <0.05; ** <i>p</i> <0.001.											

Taken the results together, including the F₂ populations, the F₃ populations and the F_{2:3} lines (Table 1, Table S5), all of them proved the inference and revealed the dosage effect of the *Ne* alleles associating with moderate and severe hybrid necrosis. That is, regardless of the necrosis grades, the *Ne1ne1Ne2ne2*-plants (double heterozygous, dosage 2) should be the weakest generally and also coincide with the corresponding F₁-plants in the necrotic intensity, while the plants with genotype *Ne1Ne1Ne2Ne2* (double dominant homozygous, dosage 4) would always have the strongest degree of necrosis, but just would die before heading stage in the moderate and severe hybrid necrosis.

The multiple alleles of *Ne1* and that of *Ne2* are indeed different, respectively.

There were three alleles of *Ne1* (*Ne1w*, *Ne1m* and *Ne1s*) and five alleles of *Ne2* (*Ne2w*, *Ne2wm*, *Ne2m*, *Ne2ms* and *Ne2s*) (*w*= weak, *m*= moderate, *s*= strong, *wm* and *ms* are inter-mediate strengths) classified by Hermesen (1963a). It was regarded as the common recognized classification all the time. In this study, the results of allelism tests (Table 2) indicated that the particular allele *Ne1* in N9134 should be different from both the alleles of *Ne1* in TA4152-60 and *Ne1s* in Spica. Likewise, the particular allele of *Ne2* in ZH22 should also be different from the reported three alleles of *Ne2* in Manitou (*Ne2m*), WL711 (*Ne2m*) and Pan555 (*Ne2*). Furthermore, we compared their karyotypes by FISH using oligonucleotide probes oligo-pTa535 and pSc119.2. For the alleles of *Ne1* (Fig. 2a), the cytogenetics results of oligo-pTa535 probe signal showed distinct difference on chromosome 5BL between N9134, TA4152-60 and Spica, as well as several visible differences on chromosomes 1A, 2A, 2B, 4A, 5A and 7B. Meanwhile, the distinct differences could be observed on chromosomes 2B among the four cultivars, Zhoumai 22, Manitou, WL711 and Pan55, carrying the alleles of *Ne2* (Fig. 2b), as well as the chromosomes 1B, 1D and 5A. This gave an additional evidence for the differences of these *Ne* alleles, and hinted that their carriers should be derived from different pedigree.

Table 2
Allelism tests for distinguishing the multiple alleles of *Ne1* and *Ne2*.

Years	Cultivars containing <i>Ne</i> genes in this study	Cultivars containing <i>Ne</i> genes in previous reports	Genetic population	Female parent	Male parent_F ₁	Seeds	Necrotic plants	Normal plants	Ratio (%)	Investigation Condition
2019-20	N9134 (<i>Ne1</i>)	Spica (<i>Ne1s</i>)	multiple cross F ₁	ZH22	Spica / N9134	328	178	1	0.56	Heading; Field
2019-20	N9134 (<i>Ne1</i>)	TA4152-60 (<i>Ne1</i>)	multiple cross F ₁	ZH22	TA4152-60 / N9134	251	148	1	0.67	Heading; Field
2019-20			multiple cross F ₁	N9134	ZH22 / Manitou	378	282	3	1.05	Heading; Field
	ZH22 (<i>Ne2</i>)	Manitou (<i>Ne2m</i>)								
2019-20	ZH22 (<i>Ne2</i>)	Pan555 (<i>Ne2</i>)	multiple cross F ₁	N9134	ZH22 / Pan555	777	557	6	1.07	Heading; Field
2019-20	ZH22 (<i>Ne2</i>)	WL711 (<i>Ne2m</i>)	multiple cross F ₁	N9134	ZH22 / WL711	450	319	4	1.24	Heading; Field

Apart from, we also compared the necrotic degree of different F₁ plants to distinguish the alleles of *Ne1* and *Ne2*. Their necrotic intensity could be ordered as: Spica (*Ne1s*) / ZH22 > N9134 / ZH22 ≈ TA4152-60 / ZH22; ZH22 / N9134 > Manitou (*Ne2m*) / N9134 ≈ WL711 (*Ne2m*) / N9134 > Pan555 / N9134. And the reciprocal crosses absolutely showed the same as the above comparison of positive crosses.

High-density genetic maps of the *Ne1* and *Ne2* in winter wheat.

Based on the *Ne1* (Chu et al. 2006) and *Ne2* (*Lr13* and *LrLC10*) (Chu et al. 2006; Qiu et al. 2020; Zhang et al. 2016) reported in spring wheat, we constructed two genetic maps of the *Ne1* and *Ne2* in winter wheat using 269 and 264 BC₁F₁ plants, respectively (Fig. S1). Then, some new linkage markers were developed and more BC₁F₁ plants were employed here. Ultimately, the two high-density genetic linkage maps of them (Fig. 3) were constructed mainly using the developed KASP markers and AS-PCR markers with taking advantage of the two BC₁F₁ populations consisted of 1006 and 1143 plants, respectively. The fine genetic map of *Ne2* locus contained 25 molecular markers including one SSR marker, three InDel markers, seven AS-PCR markers and 14 KASP markers (Figs. 3A and B). Both the InDel marker *Lseq102* (co-segregated with *LrLC10*) and SSR marker *TC67744* are co-segregated with *Ne2*. They are located on the sites of about 156.59 and 157.76 million bases (Mb) on chromosome arm 2BS in Chinese Spring RefSeq v1.0, respectively. Furthermore, a genetic interval of 0.18 cM (0.09 cM on either side) is just for the *Ne2* locus between the two-flanking tightly linked InDel markers, *Lseq54* and *Lseq22*, and the physical distance is only about 4.45Mb. Moreover, the genetic distance is 0.44cM (0.17 and 0.27 cM on either side) between the two flanking tightly linked KASP markers (*Nwu_2B_4204* and *Nwu_2B_4249*), and the corresponding physical distance is about 5.45Mb. Similarly, there were 16 markers including seven AS-PCR markers and nine KASP markers in the fine genetic map of *Ne1* locus (Figs. 3C and D). The two closest linked markers on either side of *Ne1* locus are SNP markers *Nwu_5B_4137* and *Nwu_5B_5114* with the genetic interval of 0.50 cM (0.20 and 0.30 cM), and both are separately located on the sites of about 383.40 Mb and 388.01 Mb on chromosome arm 5BL in Chinese Spring RefSeq v1.0 with the physical interval 4.61 Mb.

Distribution, proportion and genotype frequencies of *Ne1* and *Ne2* are discrete in China's wheat regions.

To investigate the distribution and proportion of *Ne1* and *Ne2* genes in China's wheat areas, we further tested the *Ne* genotypes among 1364 wheat cultivars (lines) consisting of 1178 modern Chinese cultivars (lines; MCC), 65 Chinese landraces (CL), and 121 introduced modern cultivars (IMC) (Table S2) using molecular marker genotyping and/or hybrid test. Totally, a panel of 1261 wheat cultivars (lines) consisting of 1075 MCC, 65 CL and 121 IMC (Table S2) was genotyped with the four closely linked KASP markers. Meanwhile, 202 wheat cultivars (lines) including 195 MCC, two CL and five IMC (Table S2) through hybrid test. Among them, 99 were detected by two means, and 98 out of the 99 had the consistent genotyping for *Ne1* or *Ne2* (Table S2). This means that the reliability of genotyping results should be at least 98.99% (98 / 99) between the two tested means. Correspondingly, the two groups of genotype frequencies separately obtained from the two means were also highly positive correlated ($r = 0.919$, Fig. 4a). After de-redundancy, there were 357 cultivars (lines) with genotype of *Ne1Ne1ne2ne2*, accounting for 26.2%, while 453 out of 1364 were genotyped with *ne1ne1Ne2Ne2*, accounting for 33.2% (Fig. 4b).

Further, a total of 1243 Chinese cultivars (MCC and CL) were analyzed according to the 10 China's agro-ecological production zones (Fig. 4c, Table S2). The number of cultivars in the region I–IV reach to 1197, accounting for 87.76% (1197/1364) of the cultivars in this study, while the

number of cultivars in the region V–X was only 46. Accordingly, the planting area and the ultimate production in the I–IV accounted for about 85% and 90% of wheat in China, respectively (CHINA STATISTICAL YEARBOOK 2019, <http://www.stats.gov.cn/tjsj/ndsj/2019/indexch.htm>). Consequently, the number of cultivars here we selected should be representative enough for each wheat region of China. The results demonstrated that the distribution and proportion of *Ne* genes from different wheat region were discrete (Fig. 4c), and the correlation coefficients of them were highly variable (Fig. 4d). Typically, the frequency of *Ne1Ne1ne2ne2* was significantly dominant in the region IV with a proportion of 46.9%, and that of *ne1ne1Ne2Ne2* was significantly dominant in the IMC population with a proportion of 54.5%. While, cultivars (lines) in the region III have the highest frequency of *ne1ne1ne2ne2*, up to 65.3%, and the relatively lower frequencies of both the genotypes *Ne1Ne1ne2ne2* and *ne1ne1Ne2Ne2* than the other regions (Fig. 4e).

Similarly, according to the classification of CL, IMC and MCC (Fig. 4f), we found that the CL population had the highest genotype frequency of *ne1ne1ne2ne2* as well as *Ne1Ne1ne2ne2*, while the IMC population showed the relatively lowest frequency of *ne1ne1ne2ne2*, but with the highest frequency of *ne1ne1Ne2Ne2*, up to 54.5%. Intriguingly, the frequency of *Ne2* gene in the MCC population was 2.3 times higher than that in the CL since the IMC were introduced into wheat breeding program of China. Moreover, the proportions of the three genotypes distributed in the MCC population were highly correlated with the average of that in the CL and the IMC ($r = 0.971$; Fig. 4g). Therefore, the genotype frequencies of *Ne* genes in the MCC should be formed by the interaction of the CL (contributing *Ne1* allele) and the IMC (contributing *Ne2* allele).

The *Ne1* in N9134 was inherited from WE As846 while the *Ne2* in ZH22 should be from the IMC or rye.

In order to clarify the origins of *Ne1* in N9134 and *Ne2* in ZH22, we traced the pedigree of them separately. It's easy and clear to determine that the *Ne1* in N9134 was inherited from WE As846 according to the pedigree of N9134 (Fig. 5a). Because the chromosome 5B of N9134 could only be inherited from WE As846, which could confirm by the previous study of *PmAS846* (Xue et al. 2012). While, it could only infer that the *Ne2* in ZH22 should be inherited from one of the five ancestral parents, four introduced modern cultivars and one hexaploid triticales. Because the F_1 -plants from the cross of N9134 and Z8425B showed hybrid necrosis in F_1 generation instead of that from the other three crosses between N9134 and Zhoumai 12 Yumai 49, and Zhoumai 9, we proposed that the *Ne2* in ZH22 was inherited from Z8425B. However, since the ancestral parents of ZH22 included two Italian cultivars (lines) St2422/464 (Zhengyin 4) and St1472/506 (Zhengyin 1), a cultivar named Прелгопная 2 (*Erythrospermum*-315H160 / Wumang 1) from Soviet Union, a Mexican cultivar Nainari60 and a hexaploid triticales line Guangmai 74 (Fig. 5b), it still needs to be verified furtherly which one of these five cultivars (lines) did the *Ne2* exactly donor.

Discussion

The criteria of hybrid-necrosis grades should be improved with reference to the percentage of 1000-grain weight (KGW).

The intensity or degree of hybrid necrosis in the F_1 -generation (*Ne1ne1Ne2ne2*) varied greatly from cross to cross, and was distinguished into nine necrosis grades (0–8). In which, the grades 6–8 are indicated as severe necrosis (no seed is produced), 3–6 as moderate necrosis (premature seed), 0–3 as weak necrosis (normal seed) (Hermesen 1963a). In this study, both the F_1 -plants from N9134 / ZH22 and ZH22 / N9134 would appear the first necrotic symptoms at 2–3 leaves stage (grade 6; Fig. 6a). However, they could finish the cycle of life but only premature about 10 days (grade 4; Figs. 1-II-c, 1-III-c and d), and the two 1000-grain weight of the F_1 -generations reach to 25.40g and 20.05g (grade 4; Fig. 6b), respectively. Meanwhile, the KGW of N9134 and ZH22 were 49.9g and 46.5g, respectively. From above, it could not determine the necrosis grade occurred in the F_1 -generation of N9134 / ZH22 and ZH22 / N9134 according to the only criteria of necrosis grades established by Hermesen (1963a). Meanwhile, it also could not determine that for the other crosses (*Spica* (*Ne1s*) / ZH22, WL711 (*Ne2m*) / N9134 and so on). Moreover, extra favorable conditions, such as a high fertility level, could improve and alleviate the moderate and severe necrosis of F_1 plants with a striking increase of the seed production (Hermesen 1963a). Therefore, it was considered that the necrosis grades of the F_1 -generation should be determined based on results of multi-point experiments for at least three years, and the two parents should be as control with the same treat to obtain the credible 1000-grain weight. Furthermore, we considered the percentage of 1000-grain weight between the F_1 -generations and the average of their two parents should be more reasonable for grading of hybrid necrosis, so mainly with referring to that, we formulated improved grading criteria (Table 3) in order to better fit the necrosis here.

Table 3

The criteria for the necrosis grades mainly based on the traits of plants and the percentage of 1000-grain weight.

Grades of necrosis	Whether it can produce fertile seeds	Stage when first symptoms appear	The number of leaves keeping normal from necrosis appearance to maturity death	Percentage of 1000-grain weight between F ₁ generation and the average of their parents
W	Yes	From jointing	>3	81–100%
M	Yes	From tillering to jointing	2–3 (included)	51–80%
mS	Yes	Seedling	1–2 (included)	21–50%
S	No (almost none)	Seedling	≤ 1	≤ 20%
Notes: W = weak, M = moderate, mS = mediate severe, and S = severe.				

Genetic background should be another effect for the differences of hybrid necrosis.

Theoretically, hybrid necrosis is only caused by the two *Ne* genes, but in practice, the intensity of hybrid necrosis phenotype and the developmental stage time-point when symptoms start were affected by a number of factors, including genetic and environmental factors. It is noted that the effect of environmental factors, such as water and fertilizer conditions, temperature and light, is limited, because the symptoms of hybrid necrosis could be generally alleviated, but would not disappear even in a superior environment (Kandel 2016; Zhang et al. 2016). Whereas, different symptoms of hybrid necrosis could be easily observed in F₁-generations (*Ne1ne1Ne2ne2*) from cross to cross. It is no doubt that the difference of phenotype was mainly caused by various alleles of *Ne1* (three) and *Ne2* (five) (Hermesen 1963a). While, for F₂-plants from the same cross with the same *Ne1* and *Ne2* alleles, the differences should be caused by the dosage effect of *Ne* genes (Hermesen 1963a).

In our study, we observed the different necrotic phenotypes between the two BC₁F₁ populations [N9134 // (N9134 / ZH22 or ZH22 / N9134) and ZH22 // (N9134 / ZH22 or ZH22 / N9134)] only with different female parents (Fig. 7). Specifically, the necrosis processes in the BC₁F₁ plants with female parent of N9134 were: the leaves became yellow from the tip to base but the whole leaf kept fresh; and then they would gradually become dry from the tip again; i.e., this kind of necrosis might go through two independently different processes, yellowing and dryness (Fig. 7a). While, the processes in the BC₁F₁ plants from backcross of ZH22 were: the leaves firstly became yellow at the tip and turned to dry immediately; then the synchronized yellowing and dryness developed from tip to base orderly; i.e., the necrosis should go through only one process (Fig. 7b). Since the dosage and environment are same here, therefore, we inferred that there might be two possible reasons for this: one is that the efficiency of *Ne1* is a little weaker than *Ne2* functioned in hybrid necrosis; while the second is differential sensitivity to the activated senescence progress conferred by different genetic background of N9134 and ZH22. Whatever, it set an example for the effect of genetic background on the manifestation of hybrid necrosis.

The *Ne1* and *Ne2* in common wheat might directly originate from wild emmer and rye, respectively.

The two necrosis genes of common wheat were hypothesized to be differently originated (Mori and Tsunewaki 1992; Tsunewaki 1970). It is inferred that the *Ne1* was originated from the B genome donor (a diploid ancestor) but there is still no direct clue. An accepted opinion was that the multiple alleles of *Ne1* should have been differentiated genetically before their domestication because it was showed different frequencies between tetraploid (emmer, durum and timopheevi) and the hexaploid wheat in a wide geographical distribution (Hermesen 1963a; Mori and Tsunewaki 1992; Tsunewaki 1970; Zeven 1969). In this study, it was clarified that the *Ne1* of N9134 was directly derived from wild emmer (tetraploid wheat). This implies the common wheat could also be possible to inherit the *Ne1* alleles from the tetraploid species with the introgression after being domesticated (He et al. 2019). As for *Ne2*, no allele was found in tetraploid wheat and the donor or ancestor remained an enigma (Mori and Tsunewaki 1992). Here, we found the rye genome might be another possible origin of the *Ne2* alleles in common wheat because the common wheat cultivars (lines), ZH22 and Z8425B, carry the chromosome 1RS.1BL (Fig. 2) and derived from a hexaploid triticale, Guangmai 74 (Fig. 5b). Additionally, the necrosis genes in rye genome (*Ner*) could interact with the dominant complementary necrotic genes of common wheat to cause hybrid necrosis (Ren and Lelley 1988, 1989). These two facts both support the above inference, although a further detailed investigation should be given. Whatever, it is worthy to note that *Ne* genes could be pedigree markers in wheat. Moreover, both the two inferences might also hint the common wheat is still in the dynamic process of the adaptive evolution in genome. This evolution should attribute to the artificial selection of breeding with prompting introgressions from wild related species and alien species (Cheng et al. 2019; He et al. 2019).

The *Ne1* in N9134 and *Ne2* in ZH22 might be novel hybrid necrosis alleles.

For the differences of the multiple alleles of *Ne1* and *Ne2*, not only the evidence at the phenotypic level of allelism tests but also the linkage molecular markers (Chu et al. 2006; Huang et al. 2020; Qiu et al. 2020; Zhang et al. 2016) should also provide some other clues. This should

naturally arouse the concern about whether the linkage molecular markers of an allele are widely effective. Of course, the reliability of the linkage molecular markers might be another explanation for the multiple alleles of the two necrosis genes.

The *Ne1* in N9134 was directly introgressed into common wheat from wild emmer accession As846 and the *Ne2* in ZH22 was from Z8425B. Hereafter, we named them as *Ne1-nw* and *Ne2-zh* for convenience to description. Until now, up to three *Ne1* alleles (*Ne1w*, *Ne1m* and *Ne1s*) and five *Ne2* alleles (*Ne2w*, *Ne2wm*, *Ne2m*, *Ne2ms* and *Ne2s*) have been reported in wheat (Hermesen 1963a). In this work, we confirmed that the loci of *Ne1s* in Spica and *Ne1* in TA4152-60 were definitely different to *Ne1-nw* by allelism tests. The Spica and TA4152-60 are spring wheat cultivars (lines), so *Ne1-nw* should have different origination from the other two *Ne1* alleles. Another allele of *Ne1* in Shaannong 33 was reported to be different from that in the Mexican carries (Huang et al. 2020). Therefore, the multiple alleles of *Ne1* mentioned here are indeed different, and which means *Ne1-nw* derived from wild emmer should be a new hybrid necrosis gene. Similarly, here the allelism tests evidenced that *Ne2-zh* was different to *Ne2m* in WL711 and Manitou, and *Ne2* in Pan555. For the *Ne2* locus, there were two slightly different linear orders of the linkage molecular markers in the three reported genetic maps (Chu et al. 2006; Qiu et al. 2020; Zhang et al. 2016). Furthermore, we found that the two markers, *kwh37* closely linked to *Lr13* (*Ne2m*) (Zhang et al. 2016) and *Lseq302* co-separated with *LrLC10* (*Lr13*) (Qiu et al. 2020), both have no polymorphism in our BC₁F₁ populations (Fig. S2). These might imply that the allele locus of *Ne2-zh*, might be from rye, should be possible different from those previously reported. But further studies will be still needed to provide more powerful evidences in different aspects for proving that *Ne1-nw* and *Ne2-zh* here are novel hybrid necrosis genes.

N9134 and ZH22 are currently important germplasm in China. Apart from *Ne1-nw*, the resistance gene *PmAs846* carrying by N9134 remains effective against all Chinese *Bgt* races, while ZH22 is the major wheat cultivar in Henan provinces which having many excellent agronomic traits, such as lodging resistance and high yield in addition to stripe rust resistance, and it is an attractive parent for breeders. Moreover, hybrid necrosis was thought to be linking with autoimmunity of hypersensitive response (HR). Therefore, an effort should be made to understand the two *Ne*-genes. For both MAS and eventual cloning of *Ne*-genes, closer markers than reported will be essential. Hopefully, the current multi-omics will provide the tools to find those markers or to sequence the genes without the need for map-based cloning.

The IMC should directly affect frequencies of the necrotic genes in MCC, especially for *Ne2*.

In the nine reports from 1963 to 1981, 1467 out of the 5541 cultivars (26.5%) carried *Ne1* and 1189 (21.5%) were *Ne2* carriers all over the world, while there were 60 *Ne1* carriers (39.0%) and 15 *Ne2* carriers (9.7%) among the 154 involved Chinese wheat cultivars (lines) (Hermesen 1963b; Zeven 1965, 1967, 1968, 1969, 1971, 1973, 1976, 1981). In contrast, among the 1178 MCC in this study, there are 311 cultivars (26.4%) with the *Ne1Ne1ne2ne2* genotype and 378 cultivars (32.1%) with *ne1ne1Ne2Ne2* genotype (Table S2, Fig. 4f). At last century, wheat breeding was carried out by breeder using empirical science. During wheat breeding, it is common that the crosses phenotyping hybrid necrosis were eliminated through selection or competition. This means that *Ne* genes have not chance to propagate though phenotype, conversely would be purged from wheat gene pool. Strikingly, comparing with previous reports, the *Ne1* carriers in China showed a decrease of 12.6% (from 39.0–26.4%), while the frequency of *Ne2* rises considerably from 9.7–32.1%. And the *Ne*-carriers increased by 9.8% in total. The trends tie well with that in previous reports (Bomblies and Weigel 2007). These imply that these two *Ne*-genes were unconscious selected by breeder, and the selective advantages of *Ne2* are obviously greater than *Ne1*. These advantages should owe to the tightly linked loci conferring excellent traits (such as rust fungus resistance), as well they caused breeding efforts to purge the necrosis alleles from the wheat gene pool had failed apparently indeed (Bomblies and Weigel 2007). This inference could also be supported by modern cultivars derived from the backbone parent, Z8425B, in wheat breeding of China (Fig. 5b). Moreover, considering the frequency variation between the CL, IMC and MCC population (Fig. 4f), we could infer that the increase of the necrosis allele *Ne2* in China is caused by the decisive effect of IMC. This hinted that the IMC gave a lot contribution to wheat breeding in China, as well as the constitution of genome in MCC (Chen et al. 2019; Hao et al. 2020).

The alleles brought hybrid necrosis could also positively affect wheat breeding.

Hybrid necrosis is generally considered to be the barrier of gene flow at interspecific or intraspecific level (Presgraves 2010; Rieseberg and Willis 2007; Zhou et al. 2020). Specifically, it is usually regarded as negative effects for breeding (Hermesen 1963a; Vikas et al. 2013) which needs to be avoided. Nevertheless, the necrosis only occurs when both *Ne1* and *Ne2* are present in the same single plant (Hermesen 1963a), which alleviates the necessity to eliminate the two alleles at the same time. In fact, both the 'negative effectors' *Ne1* and *Ne2* are linked with excellent genes (Bomblies and Weigel 2007; Xue et al. 2012; Zeven 1981; Zhang et al. 2016). That is to say that both of them could positively affect breeding when they are independent being in an individual. Therefore, if breeder could reserve F₁ plants showing hybrid necrosis, the excellent plants might be separated in F₂ generation, and there would be more chance to obtain progenies with excellent comprehensive traits. Taken together, we hope that all of information presented here could inspire breeders to adjust the traditional concept in hybrid necrosis, or at least help wheat breeder to select parents reasonably for designing combination in breeding to avoid hybrid necrosis in F₁ generation. In addition, the *Ne1* and *Ne2* are still widely distributed all over the world instead of being eliminated (Bomblies and Weigel 2007; Vikas et al. 2013). This fact could also support this view, meanwhile it also hints that the diversity of the excellent germplasm resources should be not sufficient enough for wheat breeding (Hao et al. 2020; Zhou et al. 2020).

Declarations

Conflict of interest:

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Acknowledgments

This work was financially supported by the National Key Research and Development Program of China (grant no. 2016YFD0100302, 2016YFD0102004 and 2016YFD0100102), and by the Key Research and Development Program of Shaanxi Province (grant no. 2019ZDLNY04-06).

We thank Dr. Peng Zhang (Plant Breeding Institute, University of Sydney, Cobbitty, NSW, Australia) for providing the seeds of Spica, TA4152-60 and WL711; thank Dr. Yonggui Xiao (Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China) for providing the seeds of Manitou; thank Prof. Xinglai Pan (Food Crop Science Department, Cotton Research Institute, Shanxi Agriculture Science Academy, Yuncheng, Shanxi, China) for providing the seeds of Pan555; and thank Dr. Xiaojie Chen (Institute of isotope research, Henan Academy of Sciences, Zhengzhou, Henan, China) for providing the seeds of Yutong 68-2, Yutong 194 and Yutong 198. We also thank Prof. Steven S. Xu, Dr. Andrew Green, Dr. Ahmed Amri, Dr. Mergoum Mohamed, Dr. Horsley Richard, Dr. Efren Rodriguez Carranza, Dr. Nicole Boyer and others not mentioned here for the kindly assistance in requesting of the wheat seeds which carrying the necrotic genes, even if it finally fails to obtain them.

In addition, we are grateful to Cong Li, Bo Liu, Jingxuan Chen and others for the help in investigation and the preliminary experiment preparation. And also, we thank Dr. Miaomiao Nie and Dr. Xudan Kou for the advices on and critical reading of this manuscript.

References

1. Bizimungu B, Collin J, Comeau A, St-Pierre CA (1998) Hybrid necrosis as a barrier to gene transfer in hexaploid winter wheat x triticale crosses. *Can J Plant Sci* 78:239-244
2. Bomblies K, Weigel D (2007) Hybrid necrosis: autoimmunity as a potential gene-flow barrier in plant species. *Nature reviews Genetics* 8:382-393
3. Caldwell RM, Compton LE (1943) Complementary lethal genes in wheat: causing a progressive lethal necrosis of seedlings. *Journal of Heredity* 34:67-70
4. Chao S, Dubcovsky J, Dvorak J, Luo MC, Baenziger SP, Matnyazov R, Clark DR, Talbert LE, Anderson JA, Dreisigacker S, Glover K, Chen J, Campbell K, Bruckner PL, Rudd JC, Haley S, Carver BF, Perry S, Sorrells ME, Akhunov ED (2010) Population- and genome-specific patterns of linkage disequilibrium and SNP variation in spring and winter wheat (*Triticum aestivum* L.). *BMC Genomics* 11:727
5. Chen C, Chen H, Lin YS, Shen JB, Shan JX, Qi P, Shi M, Zhu MZ, Huang XH, Feng Q, Han B, Jiang L, Gao JP, Lin HX (2014) A two-locus interaction causes interspecific hybrid weakness in rice. *Nature communications* 5:3357
6. Chen H, Jiao C, Wang Y, Wang Y, Tian C, Yu H, Wang J, Wang X, Lu F, Fu X, Xue Y, Jiang W, Ling H, Lu H, Jiao Y (2019) Comparative Population Genomics of Bread Wheat (*Triticum aestivum*) Reveals Its Cultivation and Breeding History in China. *bioRxiv*:519587
7. Cheng H, Liu J, Wen J, Nie X, Xu L, Chen N, Li Z, Wang Q, Zheng Z, Li M, Cui L, Liu Z, Bian J, Wang Z, Xu S, Yang Q, Appels R, Han D, Song W, Sun Q, Jiang Y (2019) Frequent intra- and inter-species introgression shapes the landscape of genetic variation in bread wheat. *Genome Biol* 20:136
8. Chu CG, Faris JD, Friesen TL, Xu SS (2006) Molecular mapping of hybrid necrosis genes *Ne1* and *Ne2* in hexaploid wheat using microsatellite markers. *Theor Appl Genet* 112:1374-1381
9. Hao C, Jiao C, Hou J, Li T, Liu H, Wang Y, Zheng J, Liu H, Bi Z, Xu F, Zhao J, Ma L, Wang Y, Majeed U, Liu X, Appels R, Maccaferri M, Tuberosa R, Lu H, Zhang X (2020) Resequencing of 145 Landmark Cultivars Reveals Asymmetric Sub-genome Selection and Strong Founder Genotype Effects on Wheat Breeding in China. *Molecular plant*
10. He F, Pasam R, Shi F, Kant S, Keeble-Gagnere G, Kay P, Forrest K, Fritz A, Hucl P, Wiebe K, Knox R, Cuthbert R, Pozniak C, Akhunova A, Morrell PL, Davies JP, Webb SR, Spangenberg G, Hayes B, Daetwyler H, Tibbits J, Hayden M, Akhunov E (2019) Exome sequencing highlights the role of wild-relative introgression in shaping the adaptive landscape of the wheat genome. *Nat Genet* 51:896-904
11. Hermesen JT (1963a) Hybrid necrosis as a problem for the wheat breeder. *Euphytica* 12:1-16
12. Hermesen JT (1963b) Sources and distribution of the complementary genes for hybrid necrosis in wheat. *Euphytica* 12:147-160
13. Huang S, Liu S, Zhang Y, Xie Y, Wang X, Jiao H, Wu S, Zeng Q, Wang Q, Singh RP, Bhavani S, Kang Z, Wang C, Han J, Wu J (2020) Genome-wide Wheat 55K SNP-based Mapping of Stripe Rust Resistance Loci in Wheat Cultivar Shaannong 33 and Their Alleles Frequencies in Current Chinese Wheat Cultivars and Breeding Lines. *Plant Dis* doi: 10.1094/PDIS-07-20-1516-RE

14. Jiang QY, Chen H, Pan XL, Pan QY, Shi YH, Li XR, Zhang GY, Wang YJ, Xie SG, Shen SH (2008) Proteomic analysis of wheat (*Triticum aestivum* L.) hybrid necrosis. *Plant Sci* 175:394-401
15. Kandel DR (2016) A Molecular Genetic and Physiological Comparison of the Hybrid Necrosis Response in Wheat (*Triticum Aestivum* L.) to Biotic and Abiotic Stress Responses. *Agronomy, Horticulture, and Plant Science*. South Dakota State University, South Dakota State University, p 227
16. Khanna-Chopra R, Dalal M, Kumar GP, Laloraya M (1998) A genetic system involving superoxide causes F₁ necrosis in wheat (*T. aestivum* L.). *Biochem Biophys Res Commun* 248:712-715
17. Kosambi DD (1943) The Estimation of Map Distances from Recombination Values. *Annals of Eugenics* 12:172-175
18. Meng L, Li HH, Zhang LY, Wang JK (2015) QTL IciMapping: Integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. *Crop J* 3:269-283
19. Mori N, Tsunewaki K (1992) Distribution of the necrosis and chlorosis genes in two wild tetraploid wheats, *Triticum dicoccoides* and *T. araraticum*. *The Japanese Journal of Genetics* 67:371-380
20. NISHIKAWA K, MORI T, TAKAMI N, FURUTA Y (1974) Mapping of progressive necrosis genes, *Ne1* and *Ne2* of common wheat by the telocentric method. *Japanese Journal of Breeding* 24:277-281
21. Oetmann A, Zeller FJ (1989) Distribution and Origin of Hybrid Necrosis Genes in German Winter Wheat (*Triticum aestivum* L.) Cultivars. *Plant Breeding* 103:207-211
22. Orr HA (1996) Dobzhansky, Bateson, and the genetics of speciation. *Genetics* 144:1331-1335
23. Presgraves DC (2010) The molecular evolutionary basis of species formation. *Nature reviews Genetics* 11:175-180
24. Pukhal'sky VA, Bilinskaya EN (1998) Identification of hybrid necrosis genes in winter varieties of common wheat *Triticum aestivum* L. *Genetika* 34:1639-1647
25. Pukhalskiy VA, Bilinskaya EN, Kudryavtsev AM (2018) Analysis of Hybrid Necrosis Genes in Winter Common Wheat (*Triticum aestivum* L.) Cultivars. *Russ J Genet+* 54:1487-1492
26. Pukhalskiy VA, Bilinskaya EN, Martynov SP, Dobrotvorskaya TV, Obolenkova GA (2008a) New data on the distribution of hybrid necrosis genes in winter bread wheat (*Triticum aestivum* L.) cultivars. *Russ J Genet+* 44:171-179
27. Pukhalskiy VA, Martynov SP, Dobrotvorskaya TV (2000) Analysis of geographical and breeding-related distribution of hybrid necrosis genes in bread wheat (*Triticum aestivum* L.). *Euphytica* 114:233-240
28. Pukhalskiy VA, Udachin RA, Bilinskaya EN (2008b) Hybrid necrosis genes in aboriginal wheats of Middle Asia in the light of the problem of the primary centers of biodiversity of the *Triticum* L. genus. *Euphytica* 165:533-543
29. Qiu L, Wang H, Li Y, Wang W, Liu Y, Mu J, Geng M, Guo W, Hu Z, Ma J, Sun Q, Xie C (2020) Fine Mapping of the Wheat Leaf Rust Resistance Gene *LrLC10* (*Lr13*) and Validation of Its Co-segregation Markers. *Frontiers in plant science* 11:470
30. Ren ZL, Lelley T (1988) Genetics of Hybrid Necrosis in Rye. *Plant Breeding* 100:173-180
31. Ren ZL, Lelley T (1989) Hybrid necrosis in triticale and the expression of necrosis genes in allopolyploids. *Theor Appl Genet* 77:742-748
32. Rieseberg LH, Willis JH (2007) Plant speciation. *Science* 317:910-914
33. Saghai-Maroo MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci U S A* 81:8014-8018
34. Singh RP, Rajaran S (1991) Resistance to *Puccinia recondita* f. sp. *tritici* in 50 Mexican Bread Wheat Cultivars. *Crop Sci* 31:1472-1479
35. Singh S, Chaudhary HK, Sethi GS (2000) Distribution and allelic expressivity of genes for hybrid necrosis in some elite winter and spring wheat ecotypes. *Euphytica* 112:95-100
36. Sun X, Liu D, Zhang X, Li W, Liu H, Hong W, Jiang C, Guan N, Ma C, Zeng H, Xu C, Song J, Huang L, Wang C, Shi J, Wang R, Zheng X, Lu C, Wang X, Zheng H (2013) SLAF-seq: an efficient method of large-scale *de novo* SNP discovery and genotyping using high-throughput sequencing. *PloS one* 8:e58700
37. Tang Z, Yang Z, Fu S (2014) Oligonucleotides replacing the roles of repetitive sequences pAs1, pSc119.2, pTa-535, pTa71, CCS1, and pAWRC.1 for FISH analysis. *J Appl Genet* 55:313-318
38. Tsunewaki K (1970) Necrosis and chlorosis genes in common wheat and its ancestral species. *Seiken Zihou*:67-75
39. Vikas VK, Tomar SMS, Sivasamy M, Kumar J, Jayaprakash P, Kumar A, Peter J, Nisha R, Punniakotti E (2013) Hybrid necrosis in wheat: evolutionary significance or potential barrier for gene flow? *Euphytica* 194:261-275
40. Wamishe YA, Milus EA (2004) Genes for Adult-Plant Resistance to Leaf Rust in Soft Red Winter Wheat. *Plant Dis* 88:1107-1114
41. Wang Y, Quan W, Peng N, Wang C, Yang X, Liu X, Zhang H, Chen C, Ji W (2016) Molecular cytogenetic identification of a wheat–*Aegilops geniculata* Roth 7M⁹ disomic addition line with powdery mildew resistance. *Mol Breeding* 36:1-10

42. Xue F, Ji W, Wang C, Zhang H, Yang B (2012) High-density mapping and marker development for the powdery mildew resistance gene *PmAS846* derived from wild emmer wheat (*Triticum turgidum* var. *dicoccoides*). Theor Appl Genet 124:1549-1560
43. Zeven A (1965) First supplementary list of genotypes of hybrid necrosis of wheat varieties. Euphytica 14:239-243
44. Zeven A (1967) Second supplementary list of genotypes of hybrid necrosis of wheat varieties. Euphytica 16:18-22
45. Zeven A (1968) Third supplementary list of wheat varieties classified according to their genotype for hybrid necrosis. Euphytica 17:46-53
46. Zeven A (1969) Fourth supplementary list of wheat varieties classified according to their genotype for hybrid necrosis. Euphytica 18:43-57
47. Zeven A (1971) Fifth supplementary list of wheat varieties classified according to their genotype for hybrid necrosis and geographical distribution of *Ne*-genes. Euphytica 20:239-254
48. Zeven A (1973) Sixth supplementary list of wheat varieties classified according to their genotype for hybrid necrosis and geographical distribution of *Ne*-genes. Euphytica 22:618-632
49. Zeven A (1976) Seventh supplementary list of wheat varieties classified according to their genotype for hybrid necrosis and geographical distribution of *Ne*-genes. Euphytica 25:255-276
50. Zeven A (1981) Eighth supplementary list of wheat varieties classified according to their genotype for hybrid necrosis. Euphytica 30:521-539
51. Zhang P, Hiebert CW, McIntosh RA, McCallum BD, Thomas JB, Hoxha S, Singh D, Bansal U (2016) The relationship of leaf rust resistance gene *Lr13* and hybrid necrosis gene *Ne2m* on wheat chromosome 2BS. Theor Appl Genet 129:485-493
52. Zhang Y, Cheng Y, Guo J, Yang E, Liu C, Zheng X, Deng K, Zhou J (2014) Comparative transcriptome analysis to reveal genes involved in wheat hybrid necrosis. International journal of molecular sciences 15:23332-23344
53. Zhou J, Cheng Y, Yin M, Yang E, Gong W, Liu C, Zheng X, Deng K, Ren Z, Zhang Y (2015) Identification of novel miRNAs and miRNA expression profiling in wheat hybrid necrosis. PloS one 10:e0117507
54. Zhou Y, Zhao X, Li Y, Xu J, Bi A, Kang L, Xu D, Chen H, Wang Y, Wang YG, Liu S, Jiao C, Lu H, Wang J, Yin C, Jiao Y, Lu F (2020) *Triticum* population sequencing provides insights into wheat adaptation. Nat Genet 52:1412-1422

Figures

Figure 1

Hybrid-necrosis plants derived from N9134 and Zhoumai 22 (ZH22) in different growth stages. The seedling plants of F2 with normal and necrotic phenotypes were growth in greenhouse (I-a) and in field (I-b), respectively. The jointing plants of N9134 (II-a), ZH22 (II-b), F1 (II-c), F2 with normal (II-d) and necrotic (II-e, f) phenotypes were growth in field. The filling plants of N9134 (III-a), ZH22 (III-b), F1 (III-c, d), F2 with normal (III-e) and necrotic (III-f, g, h) phenotypes, BC1F1 with normal (III-i) and necrotic (III-j) phenotypes derived from N9134 // N9134 / ZH22, and BC1F1 with normal (III-k) and necrotic (III-l) phenotypes derived from ZH22 // N9134 / ZH22 were also growth in field. The plants marked II and III were photographed following after being transplanted into the pots.

Figure 2

Fluorescence in situ hybridization (FISH) analysis using oligonucleotide probes Oligo-pTa535 (red) and Oligo-pSc119.2 (green).

Figure 3

Genetic linkage maps of Ne1 and Ne2 with high-density molecular markers in winter wheat. (A) shows the genetic linkage map of Ne2 in ZH22, and markers are indicated in the right as well the genetic distances are indicated with centimorgan (cM) along the left. (B) shows the corresponding physical location of the polymorphic linkage markers on 2BS of Chinese Spring RefSeq v1.0, and the physical distances are indicated with million bases (Mb) along the right. (C) and (D) are shown for Ne1 on 5BL (N9134) in the same way as Ne2.

Figure 4

Distribution, proportion and genotype frequencies of hybrid necrosis genes in China. The genotype frequencies obtained from linkage KASP markers detection and hybrid test were highly positive correlated (a). (b) shows the frequencies of hybrid necrosis genes for all the 1364 wheat cultivars (lines). The distribution and genotype frequencies of hybrid necrosis genes in each of the 10 China's agro-ecological production zone were different for the 1246 Chinese wheat cultivars (lines) (c), [I, north China winter wheat region (230); II, Yellow and Huai River valley winter wheat region (681); III, middle and lower Yangtze River valley winter wheat region (124); IV, south-western winter wheat region (162); V, south China winter wheat region (3); VI, north-eastern spring wheat region (11); VII, northern spring wheat region (2); VIII, north-western spring wheat region (9); IX, Qinghai-Tibet spring-winter wheat region (12); X, Xinjiang winter-spring wheat region (9)]. The different wheat regions were with discrepant correlation coefficients (d) and comparison (e) for the frequencies of hybrid necrosis genes. The frequencies of hybrid necrosis genes (f) and their correlation coefficients (g) were analyzed according to the classification of Chinese landraces (CL), introduced modern cultivars (IMC) and modern Chinese cultivars (lines; MCC).

Figure 5

The pedigrees of N9134 (A) and Zhoumai 22 (B).

Figure 6

Figure 7

Necrotic flag leaves of BC1F1 plants only with the different female parent at filling stage. (a) Flag leaves of the cross of N9134 // (ZH22 / N9134 or N9134 / ZH22). (b) Flag leaves of the cross of ZH22 // (ZH22 / N9134 or N9134 / ZH22).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Fig.S1.tif](#)
- [Fig.S2.tif](#)
- [TableS1.xlsx](#)
- [TableS2.xlsx](#)
- [TableS3.xlsx](#)
- [TableS4.xlsx](#)
- [TableS5.xlsx](#)