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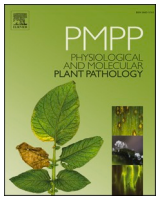

Molecular screening of stripe rust and powdery mildew resistance genes in European bread wheat using the validated gene-specific SSR markers

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ABSTRACT

Wheat is susceptible to fungal diseases that significantly impact yield. Characterizing wheat germplasm for innate resistance is a sustainable strategy against fungal pathogens. This study evaluates the resistance potential of 60 bread wheat cultivars against *Puccinia striiformis* f. sp. *tritici* (*Pst*, causing stripe rust, or yellow rust, Yr) and *Blumeria graminis* f. sp. *tritici* (causing Powdery mildew, Pm) using six validated gene-specific SSR markers. The results of molecular screening revealed high amplification rates for the *Pst* resistance genes *Yr15* (98.55 %) and *Yr5* (95 %), suggesting the potential presence of both resistance genes markers among the tested Cultivars. In contrast, *Pm* resistance genes showed varying allele frequencies: *Pm41* (77.33 %), *Pm24* (31.66 %), and *Pm38* (8.77 %). Notably, *Pm60* was absent across all tested samples. Serbian cultivar Simonida showed the highest genetic potential, harboring five resistance genes. Genotype-wise screening revealed clear genetic and geographical patterns; French cultivars had more *Pst* resistance genes, Serbian ones carried a broader range across *Yr* and *Pm* genes, and Croatian cultivars, though limited in number, showed significant amplification for *Pm41*, highlighting breeding potential. Despite significant progress, the absence of durable resistance in some genes (e.g., *Pm60*) suggests a need for incorporating additional resistance sources from wheat relatives. This study highlights the potential of gene-specific markers as a reliable strategy for enhancing disease resistance in wheat breeding programs, while also highlighting the valuable contribution of region-specific germplasm to the global development of resilient cultivars for ensuring food security under increasing environmental and biotic stresses.

1. Introduction

Predictions for 2050 estimate a global population increase to 9 billion, driving a projected 60 % rise in food demand, including staple crops such as wheat. To meet this challenge, not only must wheat yield increase, but its grain quality, particularly nutritional value must also improve. With a total harvesting area of 215.9 million hectors, wheat contributes approximately 20 % of the global dietary calorie and protein intake for nearly 4.5 billion people. However, yield growth has stagnated approximately 37 % of wheat-producing areas, posing a significant challenge to keeping pace with rising global demand [1]. Due to its adaptability to various agroecological conditions, wheat is widely cultivated and remains a critical pillar of global food security. Enhancing wheat production requires the selection of genotypes with high genetic potential and critical genes for disease resistance. Identifying high-quality germplasm with valuable traits is vital for breeding

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programs aimed at improving both quality and disease resistance in wheat [2,3].

Plant diseases significantly effect crop yield, with global losses ranging from 11 to 30 %, posing a significant threat to food security [4]. Among wheat diseases, Powdery mildew (Pm), caused by *Blumeria graminis* f. sp. *tritici*, ranks sixth among the top 10 fungal pathogens and causes the eighth highest yield loss from pests and pathogens worldwide [5]. In Serbia, annual virulence research of *B. graminis* f. sp. *tritici* populations has been carried out since 1961. In the beginning, physiological races were determined based on reactions of a few standard wheat varieties, since the 1980s, differential wheat genotypes with known genetic backgrounds have been used to identify virulence pathotypes [6]. To date, over 240 Pm resistance genes/loci have been discovered, and more than 60 have been identified and mapped [7]. The major *Pm* resistance genes are located on the A subgenome (1A, 2A, and 7A) and B subgenome (2B, 5B, and 6B) [8]. Similarly, stripe rust (yellow rust,Yr), caused by *Puccinia striformis* f. sp. *tritici*, (*Pst*) can reduce yield by 10–70 % depending on climatic conditions, disease pressure, and the susceptibility of cultivars. Currently, about 88 % of the global wheat production is susceptible to *Pst*, leading to over 5 million tons of yield losses annually. More than 80 *Pst* resistance genes have been reported [9,10]. In Serbia, the first detection of *Pst* occurred in 1997 at Rimski Šančevi [11]. Although, it was not a major wheat rust pathogen in Serbia until 2014, warmer winter temperatures during the 2013/2014 production season led to yellow rust becoming dominant, and jeopardizing wheat production [12–14]. To date several effective resistance genes have been identified in wheat germplasm, including *Yr5, Yr10, Yr15, Yr24/Yr26, Yr32,* and *YrSp* for *Pst* resistance, and *Pm1a, Pm2, Pm3/Pm8/Pm17, Pm5e, Pm21/Pm12, Pm24, Pm33, Pm41, Pm51, Pm60, Pm64, Pm69, MlZec1* and *MlAB10* for resistance against *B. graminis* [15].

Two main types of *Pm* resistance in wheat have been identified: resistance conferred by disease resistance genes and resistance resulting from mutations in negative regulators of *Pm* resistance [16]. However, the progress in breeding and deploying resistant wheat cultivars remains slow due to several challenges. These include the complexity of screening for Pm resistance, a limited understanding of its genetic basis, and the polygenic nature of resistance that is heavily influenced by environmental factors. Resistance gene expression can vary significantly across location and season, making it difficult to maintain consistent disease pressure, which can complicate the identification and selection of highly resistant genotypes. Generally, few genes provide resistance to all pathogen races, resulting in the development of short-lived, non-durable resistance genes. Although gene pyramiding—combining multiple resistance genes—has been suggested as the most effective strategy to enhance durable resistance, implementing this approach through conventional breeding remains highly complex [8].

Therefore, the screening resistance genes to develop the disease resistance wheat cultivars is a major concern of all pathologists and the plant breeders. The present study aimed to screen for *Pm* and *Pst* resistance genes using validated gene-specific primers in 60 bread wheat cultivars collected from the Faculty of Agriculture (UNSFA) and the Institute of Field and Vegetable Crops, Novi Sad, Serbia. This work highlights the importance of integrating molecular tools in breeding programs to enhance disease resistance and ensure sustainable wheat production under increasing biotic stress.

2. Materials and methods

2.1. Plant materials

This study utilized 60 bread wheat cultivars, released by leading European institutes, and sourced from the Faculty of Agriculture (UNSFA) and the Institute of Field and Vegetable Crops in Novi Sad, Serbia. These cultivars were selected for screening Pm and *Pst* resistance genes based on their genetic background and agronomic importance. Among them, 20 genotypes originated from Serbia, while the remaining 40 were from France (25), Croatia (7), Italy (4), Mexico (2), Hungary (1), and Romania (1). Table 1 provides detailed information on the wheat cultivars, including their collection regions and origin.

2.2. DNA isolation from fresh wheat leaf samples

Bread wheat cultivars were sown in a greenhouse at the Department of Plant Protection, Sivas University of Science and Technology, for 35 days before DNA extraction. Genomic DNA was isolated from the fresh leaf samples using the Cetyltrimethylammonium Bromide (CTAB) method, following the protocol of Doyle and Doyle [17], with modification as recommended by Diversity Arrays Technology [18]. The quality and quantity of extracted DNA were assessed using a Nanodrop spectrophotometer (model DS11 FX, DeNovix, Wilmington, DE, USA).

2.3. Primer optimization and PCR amplification

Two gene-specific primers (*Yr15* and *Yr5*) were used for Yr resistance screening, while four primers (*Pm24*, *Pm38*, *Pm41*, and *Pm60*) were used for Pm resistance. Primer optimization was performed using a gradient PCR with annealing temperature ranging from 54 °C to 60 °C to determine optimal conditions for amplification (Table 2). Each PCR reaction was carried out in a 10 μL reaction volüme, consisting of 5 μL of 2 × Phanta Max Master Mix (Vazyme, Nanjing, China), 0.5 μL of each forward and reverse primer (10 Pmol), 3 μL of nuclease-free water (Bio-Labs), and 1 μL of template DNA (100 ng/μL).

The PCR amplification conditions for *Yr15* and *Yr5* involved an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 30 s (for both primers), and extension at 72 °C for 30 s. A final extension was performed at 72 °C for 4 min. The successful amplification of *Yr15* and *Yr5* was confirmed by electrophoresis on a 2 % agarose gel, where a distinct band of 991 bp and 320 bp was observed.

The PCR amplification of the *Pm* primers was carried out using the following conditions: an initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturation at 95 °C for 15 s, annealing at 57–59 °C for 15 s, and extension at 72 °C for 45 s with a final extension at 72 °C for 4 min. PCR products were validated through electrophoresis on a 2 % agarose gel, where bands of the expected sizes were observed for all four *Pm* primers.

2.4. Data analysis

For data analysis, the obtained band with a different size of each resistance gene was scored as presence (1) or absence (0) and the data was recorded in Excel software. Analysis of Molecular Variance (AMOVA) was performed to assess the genetic variation among and within the wheat genotypes originating from different countries based on the presence or absence of six resistance genes (*Yr15, Yr5, Pm24, Pm38, Pm41* and *Pm60*). A binary data matrix was prepared by scoring the amplified bands for each gene as '1' (present) and '0' (absent). The genotypes were grouped based on their country of origin (France, Croatia, Italy, Mexico, Serbia, Hungary, and Romania). The AMOVA was conducted using the GenAlEx 6.5 add-in for Microsoft Excel.

3. Results

3.1. A summary of the amplification of resistance genes

PCR-based screening of 60 bread wheat cultivars for *Pst* and *Pm* resistance genes revealed valuable genetic resources for resistance breeding aimed at developing resistance wheat cultivars. Screening for the *Yr15* using the validated gene specific markers (Y15K1_F2/uhw301R) resulted in successful amplification in 58 out of 60 cultivars, indicating a frequency of 98.55 %. Similarly, the new design primers (Yr5F/Yr5R) for the *Yr5* gene amplified in 57 out of 60 cultivars, with a

Table 1
Plant Material used during the present study.

No. Sr.	Genotypes	Year	Country	Institution
1	Falado	–	France1	Syngenta
2	Cellule	2011	France2	Florimond Desprez
3	KWS Marvel	2019	France3	KWS
4	Osmose	2015	France4	Caussade
5	Sonahine	2020	France5	Caussade
6	KWS Criterium	1995	France6	Hybritech
7	KWS Feria	2009	France7	KWS
8	Sofolk	2014	France8	Caussade
9	Solveig	2011	France9	Caussade
10	Solindo	2016	France10	Caussade
11	Centurion	2014	France11	Saaten Unıon
12	Sofru	2009	France12	Caussade
13	Sothys	2014	France13	Caussade
14	LG Aigle	2012	France14	LG
15	Sosthene	2012	France15	Caussade
16	Solenzara CS	2014	France16	Caussade
17	Providence	2018	France17	Florimond Desprez
18	LG Airbus	2014	France18	LG
19	Presnatce	2020	France19	Florimond Desprez
20	LG Anapurna	2013	France20	LG
21	Nogal	2010	France21	Florımond Desprez
22	Alhambra	2010	France22	LG
23	LG Alcantara	2013	France23	LG
24	Winner	2018	France24	Florimond Desprez
25	KWS Modern	2012	France25	KWS
26	BC Lorena	2011	Croatia1	Bc Institut d.d. Zagreb
27	Renan	2012	Croatia2	GRI OBTENTIONS
28	BC Bernarda	2012	Croatia3	Bc Institut d.d. Zagreb
29	BC Anica	2009	Croatia4	Bc Institut d.d. Zagreb
30	BC Darija	2011	Croatia5	Bc Institut d.d. Zagreb
31	BC Opsesija	2015	Croatia6	Bc Institut d.d. Zagreb
32	BC Ljepotica	2015	Croatia7	Bc Institut d.d. Zagreb
33	Katou	2014	Italy1	Apsovsementi
34	Apsov Katon	2014	Italy2	Apsovsementi
35	Marinello	2008	Italy3	KWS Momont
36	Algeri	2020	Italy4	Apsovsementi
37	Eswyt 50	1992	Mexico1	CIMMYT Line
38	Sawyt 47	1992	Mexico2	CIMMYT Line
39	BG Converta	2020	Serbia1	Biogranum
40	Quattrona	2021	Serbia2	AgroSava
41	BG Flexa	2020	Serbia3	Biogranum
42	NS Igra		Serbia4	Institute of Field and Vegetable Crops, Novi Sad
43	NS Modena		Serbia5	Institute of Field and Vegetable Crops, Novi Sad
44	Nataša	2003	Serbia6	Institute of Field and Vegetable Crops, Novi Sad
45	Mohikana (line)		Serbia7	***Line, still not recognized
46	NS Lenija		Serbia8	Institute of Field and Vegetable Crops, Novi Sad
47	Simonida	2003	Serbia9	Institute of Field and Vegetable Crops, Novi Sad
48	NS Epoha		Serbia10	Institute of Field and Vegetable Crops, Novi Sad
49	NS Grivna		Serbia11	Institute of Field and Vegetable Crops, Novi Sad
50	PKB Pahuljica		Serbia12	Institut PKB Agroekonomik
51	Zvezdana	2006	Serbia13	Institute of Field and Vegetable Crops, Novi Sad
52	PKB Ratarica		Serbia14	Institut PKB Agroekonomik
53	PKB Talas		Serbia15	Institut PKB Agroekonomik
54	BG Klimatika	2020	Serbia16	Biogranum
55	BG Ikona	2019	Serbia17	Biogranum
56	BG Logika	2020	Serbia18	Biogranum
57	Bisenija	2021	Serbia19	Agrosava
58	BG Elastika	2020	Serbia20	Bigranum
59	GK Koros	2010	Hungary1	GK
60	Amicus	2016	Romania1	Saatzucht Donau

Table 2
A list of the validated gene-specific primers used during the study to screen the targeted resistance genes.

Primer ID	Sequence F and R	Band size	References
Yr15 (Y15K1_F2/ uhw301R)	F: GGAGATAGAGCACAATTACAGAC R: TTTCGCATCCCA CCCTACTG	991	Klymiuk et al. [19]
Yr5 (*Yr5F/Yr5R*)	F: CTTTGAAGGTAGATGGGTGTAGG R: TTGAGTGCCTGCAGAGATG 3′	320 bp	New design
Pm24 (Xgwm337F/ Xgwm337R)	F: TGAGTCCAAACCGGTGC R: GACTGCGTACGAATTCAA	218	Cheng et al. [20]
Pm38 (CsLv34F/ CsLv34R)	F: GTTGGTTAAGACTGGTGATGG R: TGCTTGCTATTGCTGAATAGT	150	Cheng et al. [20]
Pm41 (Pm41-645F/Pm41-645R)	F: TCGGGTACATCTGACTGTTCA R: TGGCCAGAGTAATTATCGCCA	1690	Li et al. [4]
Pm60 (Pm60SIF/ Pm60SIR)	F: CTCACAGTTCCACACTGATAT R: CTCCATCAATCTCAAGTTCTTCG	831	Cheng et al. [20]

frequency of 95 %. For *Pm* resistance, gene-specific markers revealed that *Pm24* was present in 19 cultivars (31.66 %), *Pm38* in 5 cultivars (8.77 %), *Pm41* in 44 cultivars (77.33 %). However, no amplification was observed for the *Pm60* in any of the screened wheat samples (Fig. 1).

A total of 183 resistance genes amplification was recorded. Among these, *Yr15* was the most frequently present with 58 out of 183 detected genes (31.69 %) followed by *Yr5* present in 57 out of 183 genes (31.14 %), *Pm41* found in 44 out of 183 genes (24.04 %), *Pm24* detected with 24 of 183 genes (10.38 %), *Pm38* found with 19 of 183 genes (9.63 %), and the *Pm60* was absent in any of tested cultivar (Fig. 2).

3.2. Genotype-wise detection of resistance (targeted) genes

The genotype-wise analysis using the six validated primers revealed a total of 183 resistance gene amplifications across the tested genotypes (Fig. 3). Among them, the Serbian genotypes ''Simonida'' exhibited the highest number of resistance genes, with five detected-excluding *Pm60*. Four resistance genes were identified in in 14 genotypes, three genes in 22 genotypes, two genes in another 22 genotypes. Notably, only single gene, *Yr15*, was detected in the French genotype Solindo.

3.3. PCR amplification results for Yr15 and Yr5 genes

The molecular analysis for the presence of *Yr15* genes revealed that the majority of the tested bread wheat cultivars amplified a specific 991 bp band, indicating the presence of resistance gene (Fig. 4, Table 3). Out

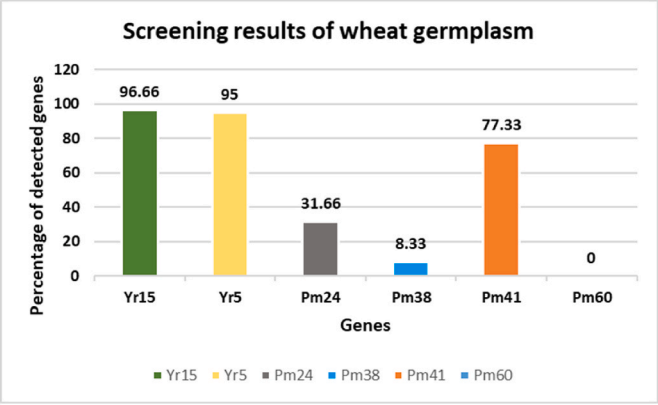

Fig. 1. PCR screening results of all targeted genes from the tested bread wheat germplasm.

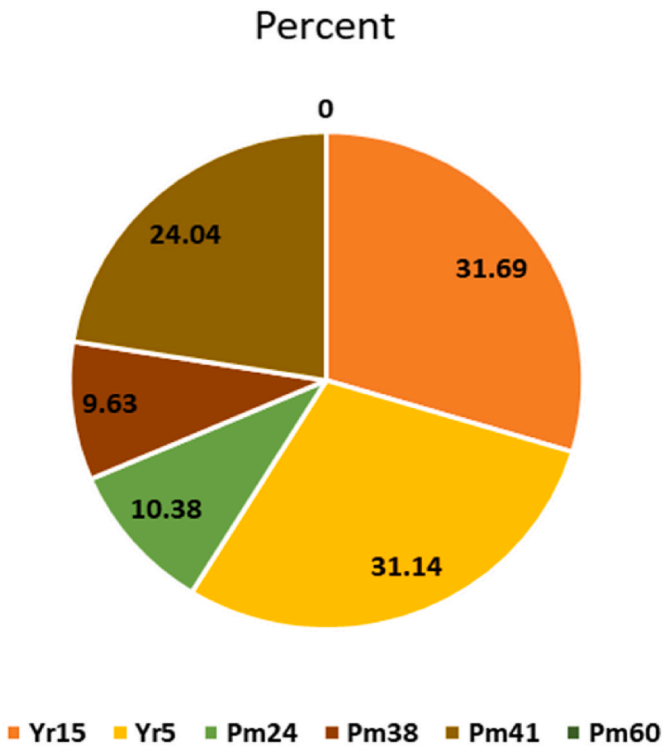

Fig. 2. The gene frequencies for all targeted genes out of the detected genes (183).

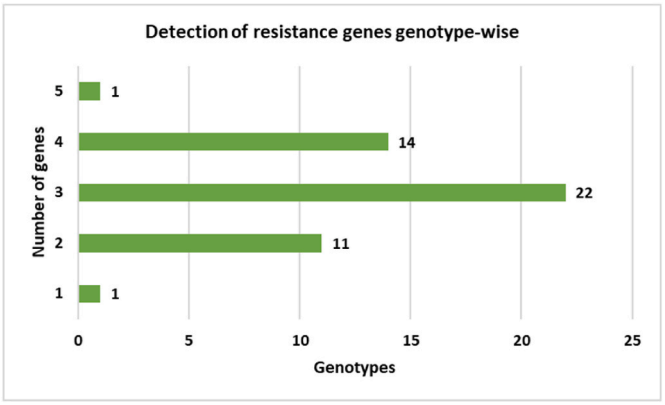

Fig. 3. Genotype-wise detection and graphical presentation of all six resistance genes.

of the 60 tested bread wheat cultivars examined, 58 showed successful amplification of the 991 bp fragment, Corresponding to a 98.66 % detection rate. Notably, the Serbian genotypes NS Igra and NS Lenija did not amplify the *Yr15* gene, however, they possessed other screened resistance genes. Similarly, molecular screening for the *Yr5* gene demonstrated that 57 out of 60 cultivars produced the expected 320 bp band, representing a 95 % amplification rate. The French genotypes KWS Marvel, Solindo, and Alhambra, failed to show amplification for the *Yr5* gene.

3.4. PCR amplification results for Pm24 and Pm38 genes

The amplification results of the *Pm24* gene using the Xgwm337F/Xgwm337R primers revealed that 19 out of 60 bread wheat genotypes produced a positive band of 204 bp, indicating the presence of the *Pm24* gene (Fig. 5). Among the 25 genotypes released from France, only 6

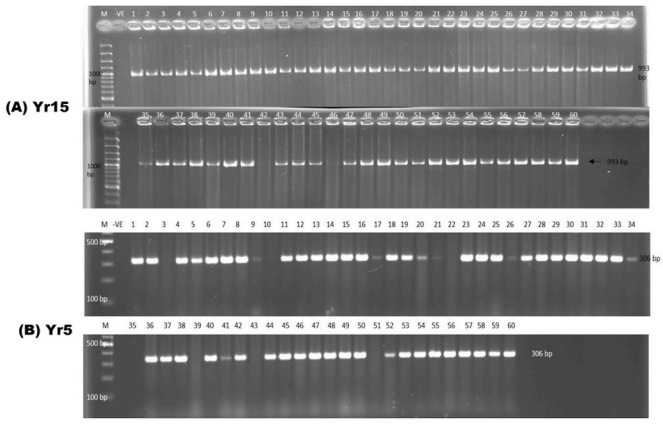

Fig. 4. The agarose gel (2 %) was used to check the PCR amplification results of both genes. The *Yr15* PCR product had a 991 bp band (A), and the *Yr5* PCR product had a 320 bp band(B). Lane M is a 100 bp marker, lane -ve is a negative control (water), and lanes 1–60 are the amplified samples corresponding to the applied primers.

(Osmose, KWS Criterium, KWS Feria, Sofolk, Nogal, and Alhambra) amplified the expected 3204 bp band. A total of seven bread wheat genotypes were collected and screened for *Pm24*. Three (BC Darija, BC Opsesija, and BC Ljepotica) out of 7 genotypes produced a positive band size. Among the 2 genotypes obtained from Mexico, one showed the presence of the *Pm24* gene. Additionally, 8 out of 20 Serbian genotypes tested positive for the gene. In contrast, none of the genotypes collected from Italy, Hungary, or Romania amplified the *Pm24*-specific band under the conditions used in this study.

The amplification results of the *Pm38* resistance gene using the primer pair CsLv34F/CsLv34R revealed that five out of sixty tested bread wheat genotypes showed positive amplification, producing the an expected 150 bp band (Fig. 5, Table 3). Among the 25 genotypes originating from France, only one genotype showed positive amplification for the targeted gene. Out of the 20 genotypes collected from Serbia; four genotypes produces the 150 bp band corresponding to the *Pm38* resistance gene. In contrast, none of the genotypes from Italy, Mexico, Hungary, or Romania amplified the expected 150 bp fragment, indicating the absence of the *Pm38* gene in those samples.

3.5. PCR amplification results for Pm41 and Pm60 genes

PCR-based screening for the *Pm41* gene revealed it to be the most prevalent among the tested genotypes, followed by *Pm24*, *Pm38*, and *Pm60*, respectively (Table 3). The positive amplification for Pm41 was observed in 44 of 60 bread wheat samples, corresponding to a 77.33 % detection rate, with a specific band size of 1690 bp (Fig. 6). Among the regional collections, 20 out of 25 wheat samples from France showed positive amplification for Pm41. All seven samples from Croatia also tested positive for Pm41. Of the four samples collected from Italy, only one genotype ('Katou') exhibited positive amplification. From the two samples originating from Mexico, one tested positive. In the case of Serbia, Pm41 was detected in 7 out of 20 samples. Additionally, one sample from Hungary ('GK Koros') tested positive, while the single sample from Romania showed no amplification for Pm41.

Regarding the *Pm60* gene, no positive amplification was detected in an of the wheat genotypes during this study (Fig. 6).

The Analysis of Molecular Variance (AMOVA) revealed that the majority of genetic variation (97 %) was found within populations, while only 3 % of the variation was attributed to differences among populations (Table 4, Fig. 7). The estimated variance among populations was relatively low (0.021), compared to the within-population variance (0.567), indicating limited genetic differentiation among the populations analyzed. The PhiPT value (0.035) indicated low genetic

Table 3
PCR amplification and screening results of all targeted resistance genes during the study.

No.Sr.	Genotypes	*Yr15*	*Yr5*	*Pm24*	*Pm34*	*Pm41*	*Pm60*	Total number of detected genes
1	Falado	■	■					2
2	Cellule	■	■			■		3
3	KWS Marvel	■				■		2
4	Osmose	■	■	■		■		4
5	Sonahine	■	■			■		3
6	KWS Criterium	■	■	■		■		4
7	KWS Feria	■	■	■		■		4
8	Sofolk	■	■	■		■		4
9	Solveig	■	■		■	■		4
10	Solindo	■						1
11	Centurion	■	■					2
12	Sofru	■	■			■		3
13	Sothys	■	■			■		3
14	LG Aigle	■	■			■		3
15	Sosthene	■	■					2
16	Solenzara CS	■	■			■		3
17	Providence	■	■			■		3
18	LG Airbus	■	■			■		3
19	Presnatce	■	■			■		3
20	LG Anapurna	■	■			■		3
21	Nogal	■	■	■		■		4
22	Alhambra	■		■		■		3
23	LG Alcantara	■	■			■		3

differentiation among populations. The PhiPT max (0.812) and Phi'PT (0.043) values also point to minimal population structure. Overall, the results reveal high genetic homogeneity, with most variation occurring within populations, likely due to high gene flow or recent common ancestry.

4. Discussion

Fungal diseases represent a persistent challenge to the wheat cultivation globally [2]. Rust and powdery mildew resistance is typically assessed through greenhouse trials with artificial inoculation, as well as field evaluations under both natural and artificial infection conditions. However, these phenotyping approaches are inherently time-intensive, constrained by the growing season, and require the continuous maintenance of diverse pathogen races to enable detection of specific resistance genes [21]. Compounding this issue, pathogen races capable of differentiating between the resistance genes *Yr* (especially *Yr5* and *Yr15*) and *Pm* have not yet been identified, rendering conventional greenhouse or field-based phenotyping ineffective for distinguishing genotypes carrying both genes—except through laborious methods such as progeny testing and genetic crosses. In contrast, marker-assisted selection (MAS) offers a robust and efficient alternative, enabling precise and accelerated identification of genotypes harboring desired resistance loci, thereby facilitating improved breeding outcomes [22].

Resistance genes play a crucial role in providing broad-spectrum and durable protection against plant pathogens [23]. The use of molecular

24	Winner	■	■					2
25	KWS Modern	■	■					2
26	BC Lorena	■	■			■		3
27	Renan	■	■			■		3
28	BC Bernarda	■	■			■		3
29	BC Anica	■	■			■		3
30	BC Darija	■	■	■		■		4
31	BC Opsesija	■	■	■		■		4
32	BC Ljepotica	■	■	■		■		4
33	Katou	■	■	■		■		4
34	Apsov Katon	■	■					2
35	Marinello	■	■			■		3
36	Algeri	■	■			■		3
37	Eswyt 50	■	■			■		3
38	Sawyt 47	■	■	■				3
39	BG Converta	■	■			■		3
40	Quattrona	■	■	■		■		4
41	BG Flexa	■	■	■				3
42	NS Igra		■			■		2
43	NS Modena	■	■			■		3
44	Nataša	■	■			■		3
45	Mohikana (line)	■	■			■		3
46	NS Lenija		■			■		2
47	Simonida	■	■	■	■	■		5
48	NS Epoha	■	■	■		■		4

markers to identify these genes represents a powerful approach for developing advanced disease-resistant wheat cultivars. In this study, resistance genes against *Pst* and *Pm* were comprehensively assessed in 60 bread wheat cultivars using the six validated gene-specific markers. The results revealed a high frequency of amplification for *Pst* resistance genes, with *Yr15* and *Yr5* detected in 58 (98.55 %) and 57 (95 %) cultivars, respectively. Among the *Pm* resistance genes, *Pm41* was the most prevalent, identified in 44 cultivars (77.33 %), followed by *Pm24* in 19 cultivars (31.66 %) and *Pm38* in 5 cultivars (8.77 %). The absence of *Pm60* in the tested wheat cultivars suggests that this resistance gene is not present in the studied germplasm, indicating a potential gap in genetic resistance to PM. These findings highlight that the studied germplasm possess significant genetic resource for resistance breeding and can be effectively utilized in the development of disease-resistance wheat cultivars. Understanding the regional disease pressure further emphasizes the importance of integrating resistance genes into breeding programs.

Yr continues to pose a significant threat to global wheat production. In France, recurrent epidemics since the 1980s, particularly in the northern regions, are fueled by favorable climates and varietal diversity [24]. Italy faces severe Yr outbreaks, with Sicily also experiencing stem and leaf rust epidemics (RustWatch). In Serbia, Yr surpassed leaf rust in 2014, reaching up to 90 % severity in trials and causing 60 % yield losses [25]. Mexico also suffered devastating yield reductions of up to 70 % in key wheat regions [26]. In Hungary, Yr escalated from rarity in 1999 to widespread epidemics by 2001 [27]. Regarding Pm, consistent threats are reported in Romania and Serbia, causing yield losses ranging from 3 to 40 % depending on infection severity [28,29]. However, specific data

49	NS Grivna	■	■	■				3
50	PKB Pahuljica	■	■			■		3
51	Zvezdana	■	■		■			3
52	PKB Ratarica	■	■		■			3
53	PKB Talas	■	■	■				3
54	BG Klimatika	■	■		■	■		4
55	BG Ikona	■	■	■				3
56	BG Logika	■	■	■		■		4
57	Bisenija	■	■					2
58	BG Elastika	■	■			■		3
59	GK Koros	■	■			■		3
60	Amicus	■	■					2
Total number of detected genes		58	57	19	5	44	0	183
Frequency (%)		96.66	95	31.66	8.33	77.33	0	

* The green-filled boxes indicate the presence of resistance genes, while the white boxes represent their absence.

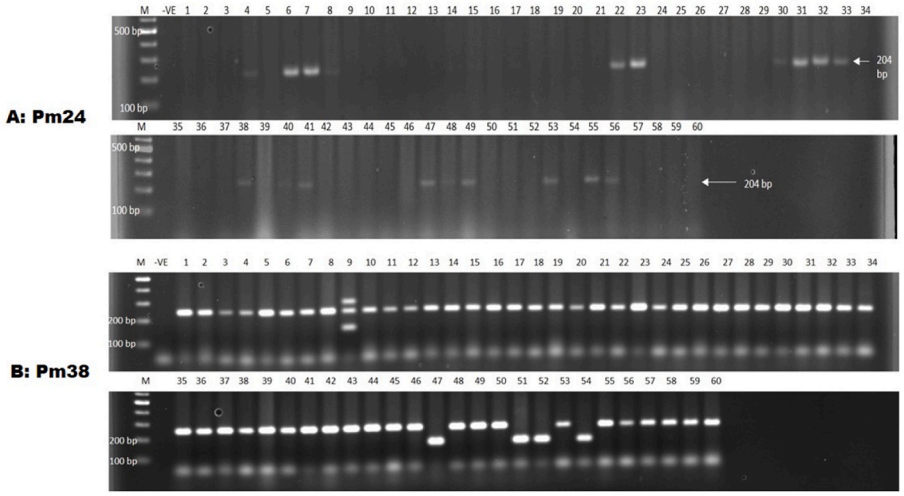

Fig. 5. The agarose gel (2 %) was used to check the PCR amplification results for *Pm24* resistance gene that amplified with a band size of 204 bp (A), and the PCR results for Pm38 was obtained with a band size of 150 bp (B). Lane M is a 100 bp marker, lane -VE is a negative control (water), and lanes 1–60 are the amplified samples corresponding to the applied primers.

on Pm impact in France, Italy, Croatia, and Hungary remain scarce, underlining the need for further monitoring and research in these regions.

Yr resistance genes are broadly classified into two major categories: Class I, which encompasses genes encoding nucleotide-binding site–leucine-rich repeat (NBS-LRR) proteins, and Class II, which comprises genes that lack the NBS-LRR domain. The NBS-LRR-containing genes serve as key components of the plant innate immune system, functioning as molecular sentinels that detect pathogen-derived effectors and initiate robust defense signaling cascades. Both classes contribute significantly to the activation and regulation of host defense mechanisms against yellow rust infection [30]. The results of this study revealed a high detection rate for the *Yr15* gene, with a 98.55 % amplification rate (58 out of 60 bread wheat cultivars) using the Y15K1_F2/uhw30_1R markers, underscoring the reliability of the gene-specific SSR markers employed. This finding significantly exceeds the detection rates reported in several previous studies. For instance, Pal et al. [31] achieved a 100 % detection rate for *Yr15* using SSR markers in a smaller germplasm set of 12 bread wheat genotypes, suggesting that *Yr15* may exhibit consistent amplification when tested in smaller and

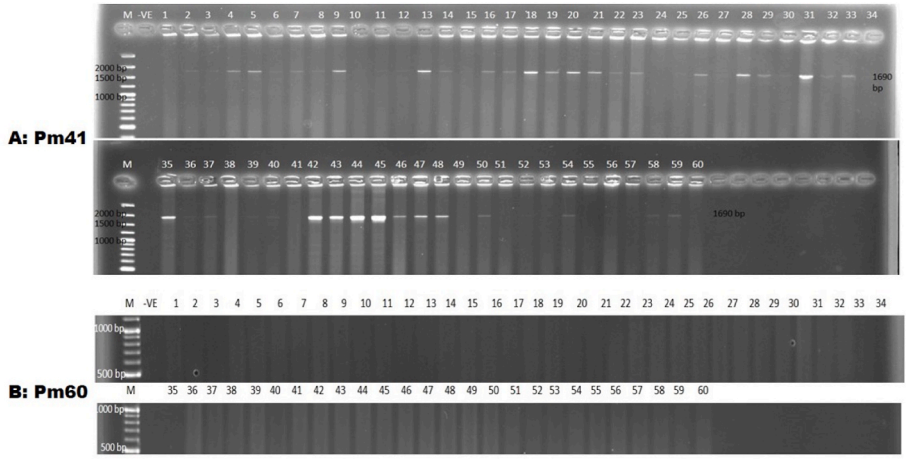

Fig. 6. PCR results on a 2 % agarose gel: (A) The *Pm41* gene product at 1690 bp, (B) the *Pm60* gene was absent (None). Lane M: 100 bp marker, Lane -VE: a negative control (water), Lanes 1–60: PCR products with respective primers.

Table 4
AMOVA results summary for wheat resistance genes data.

Source	SS (Sum of Squares)	df (Degrees of Freedom)	MS (Mean Square)	Estimated Variation	Percentage (%)
Among Pops	4.310	6	0.718	0.021	3
Within Pops	31.174	54	0.567	0.567	97
Total	35.484	60	–	0.587	100

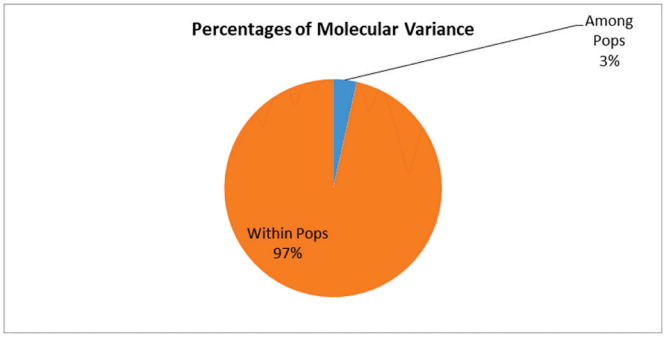

Fig. 7. Graphical presentation of AMOVA results among and within populations.

controlled germplasm groups. In contrast, Rani et al. [32] detected the *Yr15* gene in only 44.1 % of 68 wheat genotypes, indicating variability in gene prevalence across different wheat populations. Similarly, Zhang et al. [33] reported a much lower detection rate of 10 % in 70 wheat genotypes, highlighting possible differences in the genetic background of the tested germplasm or environmental influences affecting gene expression. Interestingly, studies utilizing larger germplasm collections reported drastically reduced detection rates. For instance, Pirko et al. [34] screened 558 bread wheat samples and reported a detection percentage of only 0.53 %, while Hu et al. [35] found no amplification of *Yr15* in a set of 305 bread wheat genotypes using STS markers. These findings suggest that while *Yr15* may be rare in broader populations, specific marker systems like SSRs can be highly effective in amplifying its presence when properly optimized. Additionally, Haider et al. [36] found *Yr15* in 31.3 % of 45 wheat genotypes, which falls between the high detection rates observed in controlled studies and the lower rates reported for large germplasm collections. Moreover, studies on wild relatives of wheat, such as the wild emmer wheat tested by Turgay [37], detected the *Yr15* gene in 57.1 % of 140 genotypes, highlighting its higher prevalence in wild germplasm compared to cultivated varieties.

Yr5 exhibits a broad-spectrum resistance profile against *Pst*, effectively conferring resistance to nearly all known stripe rust isolates worldwide, with the notable exceptions of some virulent strains reported in Australia [38]and India [39,40]. Its effectiveness extends across diverse geographic regions, including North America, Iran, China, Türkiye, India, and Kazakhstan [34]. Despite its strong resistance, *Yr5* is categorized as a race-specific seedling resistance gene, which makes it vulnerable to potential breakdown under high pathogen variability. To ensure long-term durability, it is recommended that *Yr5* be strategically deployed in gene pyramiding schemes, particularly in combination with other effective race-specific genes and/or race-non-specific adult plant resistance (APR) genes. This integrative approach enhances the genetic resilience of wheat cultivars and mitigates the risk of resistance erosion. In the current study, *Yr5* was detected in 57 out of 60 tested bread wheat cultivars, with a frequency of 95 %. This result demonstrates the robust presence of the *Yr5* gene in the studied germplasm compared to previous reports. For instance, Zhang et al. [33] utilized STS markers across 70 wheat samples and reported detection percentages ranging from 20 % to 27.1 %, depending on the specific marker used (e.g., S19M93: 25.7 %, S23M41: 27.1 %, and STS-9/10: 20 %). In contrast, Hu et al. [35] found no detection of *Yr5* using SSR markers in 305 bread wheat samples, highlighting the variability in results based on marker choice. Haider et al. [36] screened 45 wheat samples with both SSR and STS markers and reported detection rates of 35.5 % and 28.8 % for Xwmc175 and Xgwm120, respectively, which were significantly lower than the 95 % amplification achieved in this study. Similarly, Al-Maaroof and Ali [41] analyzed 46 samples, including wheat and triticale, and reported a low detection rate of 13 %, emphasizing the limited occurrence of *Yr5* in certain populations. Kokhmetova et al. [42] investigated 16 bread wheat samples using STS markers, detecting *Yr5* in only 6.25 % of cases, while Mukhtar et al. [43] achieved a relatively higher detection rate of 35.9 % in a collection of 39 wheat samples.

The *Pm24* gene, situated on chromosome 1DS in the wheat cultivar Chiyacao, encodes the resistance protein WTK3, which contains two tandemly arranged kinase domains. A distinctive feature of WTK3—a deletion of two amino acids—confers its resistance capability [44]. In the current study, *Pm24* was detected in 19 of the 60 tested bread wheat cultivars, resulting in a detection frequency of 31.66 %. This value is substantially higher than the previously reported detection frequencies for *Pm24*. Chang et al. [20] reported a low detection percentage of only 4.8 % in 332 wheat accessions using SSR markers, while Jin et al. [45] and Wang et al. [46] reported no detection of *Pm24* in 659 wheat and

137 wheat relatives, respectively, using STS markers. Similarly, Emara et al. [47] and Elsayed and Elkot [48] failed to detect *Pm24* in their bread and durum wheat studies. These contrasting results highlight the efficacy of the SSR marker used in this study and suggest potential differences in the genetic background of the germplasm or marker specificity.

The *Pm38* gene, associated with Pm resistance in wheat, is located on chromosome 7DS and functions as a pleiotropic locus. It encodes an ATP-binding cassette (ABC) transporter and confers resistance to multiple pathogens. The amplification of *Pm38* in only five of the 60 bread wheat cultivars (8.77 %) in this study was notably lower than the 35.8 % detection frequency reported by Chang et al. [20] for 332 wheat samples using SCAR markers. The relatively lower detection of *Pm38* in the current study could be attributed to differences in the genetic makeup of the tested germplasm or environmental factors influencing the expression of this gene.

Pm41 is the first Pm resistance gene to be cloned from the wild emmer wheat accession IW2 and provides all-stage resistance against Pm. It encodes a classical coiled-coil, nucleotide-binding site, and leucine-rich repeat (CNL) protein. Susceptibility to Pm in EMS-induced mutants, which exhibited either missense or nonsense mutations within the CNL domain, confirmed that the functional integrity of this domain is essential for *Pm41*-mediated resistance [49]. The *Pm41* gene demonstrated a high detection frequency of 77.33 % in this study, with 44 out of 60 cultivars showing positive amplification. This result far exceeds the 4.8 % detection frequency reported by Chang et al. [20] using dCAPs markers in 332 wheat samples. The significantly higher detection percentage of *Pm41* in this study suggests that the bread wheat germplasm analyzed may harbor a greater prevalence of this resistance gene compared to the germplasm studied by Chang et al. [20]. This finding underscores the value of region-specific studies in uncovering genetic diversity for resistance breeding.

The Pm resistance gene *Pm60* was first identified in diploid wild wheat *Triticum urartu* (*T. urartu*). *Pm60* represents the first Pm resistance gene that has been cloned and characterized in *T. urartu*. Previously, a Pm resistance locus in *T. urartu* was mapped to a similar position at chromosome arm 7AL as *Pm60* [50]. Interestingly, *Pm60* was not detected in any of the 60 bread wheat cultivars tested in the current study. This absence contrasts with the detection percentages reported in previous research. Chang et al. [20] recorded a 6.6 % detection gene frequency of *Pm60* in 332 wheat accessions using SCAR markers. Zhao et al. [51], however, reported a significantly higher frequency of 40.9 % in 227 einkorn wheat samples, indicating that *Pm60* may be more prevalent in certain wild wheat relatives than in modern bread wheat. Furthermore, it suggests that *Pm60* may have been lost during the domestication and breeding of elite bread wheat lines or has not yet been introgressed into European cultivars. Additionally, environmental factors and selection pressure during domestication may have contributed to its loss in elite bread wheat lines. The absence of *Pm60* could also be attributed to the limited use of wild wheat relatives, such as *T. urartu*, as donors in European breeding programs.

Identifying multiple resistance genes within a single genotype presents a promising strategy for enhancing wheat disease resistance [52]. In the present study, the Serbian cultivar Simonida, which harbors five resistance genes, emerged as an excellent candidate for gene pyramiding approaches. Stacking these resistance genes into a single genotype can provide durable and broad-spectrum protection, thereby reducing dependence on chemical fungicides and promoting sustainable agriculture practices. However, the absence of *Pm60* gene, highlights the need to incorporate additional resistance sources to effectively manage the diverse range of Pm pathotypes. Genotype-wise detection of resistance genes revealed notable genetic and geographical patterns. French cultivars exhibited a higher frequency of *Pst* resistance genes, while Serbian cultivars showed a broader range of resistance across both *Yr* and *Pm* genes. Although Croatian cultivars were fewer in number, they demonstrated significant amplification of *Pm41*, suggesting their potential value in resistance breeding. These finding emphasize the importance of conserving and utilizing diverse germplasm from different regions to strengthen the resilience of global wheat production.

The use of validated gene-specific SSR markers in this study demonstrated a reliable and efficient approach for identifying resistance genes. PCR-based screening enabled high-resolution detection of target genes, providing valuable insights into the genetic diversity of the tested germplasm [53–57]. Optimization of primer conditions ensured robust amplification, as reflected by the clear and consistent results obtained for most genes. This methodology can serve as a model for future studies aimed at assessing disease resistance across large germplasm collections. Based on these findings, future research should focus on broadening the scope of resistance gene screening by incorporating additional markers and targeting other important pathogens affecting wheat.

5. Conclusion

In conclusion, the screened bread wheat germplasm exhibits strong resistance potential against *Pst* and Pm pathogens. The successful amplification of the *Yr15* and *Yr5* resistance genes indicates robust resistance to *Pst*, while the detection of *Pm41*, *Pm24*, and *Pm38* suggest varying levels of resistance to *Pm*. The absence of the *Pm60* gene in screened germplasms highlights the need for its integration from related species. Among the cultivars, the Serbian cultivar Simonida, which carries five resistance genes, stand out as a promising candidate for resistance breeding and gene pyramiding. Additionally, cultivars such as NS Epoha, KWS Feria, and BC Opsesija also demonstrated multiple resistance genes, making them valuable assets for breeding programs aimed at achieving durable resistance. The genetic diversity present in this germplasm offers a strong foundation for developing wheat varieties resistant to major pathogens, contributing to global food security.

CRediT authorship contribution statement

Rada Šućur: Writing – original draft, Methodology. **Amjad Ali:** Writing – original draft, Investigation, Data curation. **Parnaz Mortazavi:** Writing – original draft, Formal analysis, Data curation. **Velimir Mladenov:** Writing – review & editing, Supervision. **Bojan Jocković:** Resources, Data curation. **Jin Ying Gou:** Writing – review & editing, Funding acquisition, Conceptualization. **Muhammad Azhar Nadeem:** Writing – review & editing, Formal analysis. **Faheem Shehzad Baloch:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Yong Suk Chung:** Writing – review & editing, Funding acquisition.

Data availability statement

All data needed to conduct this study is provided within the manuscript.

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Declaration of competing interest

I am pleased to submit my research article for consideration in *Physiological and Molecular Plant Pathology*.

I have thoroughly reviewed and adhered to the journal's guidelines for submission and acknowledge the importance of transparency regarding any potential conflicts of interest. I hereby confirm that we have no conflicts of interest to disclose in relation to this manuscript.

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Data availability

All data is within the manuscript.

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