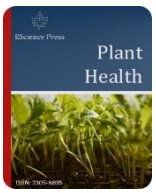
Plant Health 03 (01) 2024. 103-110 DOI: 10.33687/planthealth.03.01.5152



Available Online at EScience Press
Plant Health
ISSN: 2305-6835
https://esciencepress.net/journals/planthealth

Microsatellite Co-dominant Marker Xstm773-2 for the Detection of Sr36 Gene in Pakistani Wheat Landraces

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ARTICLE INFO

Article History
Received: Februry 13, 2024
Revised: June 01, 2024
Accepted: June 11, 2024

Keywords
Stem rust
Sr genes
Wheat landraces
Co-dominant marker

ABSTRACT

Stem rust, induced by *Puccinia graminis f.sp. Tritici* Eriks. and E. Henn., stands as a predominant affliction in global wheat cultivation. The emergence of novel races within *P. graminis f. sp. Tritici*, notably Ug99, poses a substantial threat due to its virulent impact on resistant especially on Sr31 gene. This perilous variant, originating from Uganda, menaces wheat cultivation worldwide due to the vulnerability of germplasm to its influence. Approximately 90% of global wheat cultivars exhibit susceptibility to Ug99 and its derivatives, alongside indigenous stem rust races, intensifying the risk to wheat (*Triticum aestivum*) production in Pakistan. This study aimed to evaluate 86 Pakistani wheat landraces by co-dominant micro satellite PCR marker Xstm 773-2 for their potential possession of the stem rust resistant Sr36 gene. The results showed that co-dominant marker xstm773-2 tagged the presence of gene Sr36 in 36% landraces holds promise for future breeding activities, potentially adding it with modern cultivars through marker assisted selection.

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INTRODUCTION

Pakistan, being an agrarian country relies significantly on crops for its economic sustenance, with key contributions from cereals, cotton, pulses, and vegetables. Wheat (*Triticum aestivium*.) among cereals, holds the pinnacle position, covering over 46,000 hectares. Globally, Pakistan ranks sixth in wheat production, securing the third position in Asia after China and India (FAO, 2010). Renowned for its soft endosperm, wheat, also known as bread wheat, is widely used for bread production worldwide (Bolourchi, *et al*., 1981).

In the realm of wheat cultivation, biotic factors pose more challenges than abiotic ones. Predominantly, fungal pathogens, particularly rusts, play a major role in wheat damage (Bridsall *et al*.,1985). Rust diseases, caused by the basidiomycetes fungus *Puccinia* manifest as red, brown, and brick-colored lesions on leaves, stems, spikes, glumes, and awns in wheat fields (Singh, *et al*., 2004). Stem rust, a particularly destructive wheat disease, differs from appearing on leaves; it affects the true stem. The spores undergo a color transformation from dark red to black upon maturity, leading to the nomenclature black rust (Pretorious, *et al*., 2000).

Molecular markers have been instrumental in studying Sr genes over an extended period (Sax, 1932). Three markers connected to Sr36: Xstm773-2 (a variant of Xstm773), Xwmc477, and Xgwm319. These co-dominant

markers were validated in 76 cultivars across 12 nations and are easier to score. Using two mapping populations, 'Chinese Spring' x W2691Sr36 and LMPG-6 x Sr36/9*LMPG, the scientists linked these markers to the Sr36 locus (Tsilo *et al*., 2008). An altered segregation promoting the transmission of the Sr36-carrying segment appeared in both cases. Both populations fully linked the markers Xstm773-2 and Xwmc477 to Sr36.The Sr36 gene, derived from *Triticum timopheevi*, exhibits notable resistance against stem rust pathogens (Anderson, 2003). This gene, originating from bread wheat(*Triticum aestivium*.) it has been successfully transferred to durum wheat *(Triticum turgidium*)to enhance tolerance against stem rust. Surprisingly, Pakistani Wheat landraces, which are locally, developed varieties known for their adaptability to biotic and abiotic factors, have not been systematically screened for stem rust resistance genes **(**Kimber and Feldman 2001). These landraces categorized based on their utility in cereals (Zeven, 1998) served as the focus of this study. The main objective of this study is to use molecular marker to detect the Sr36 stem rust resistant gene in Pakistani Wheat Landraces.

MATERIALS AND METHODS

Wheat landrace germplasm was gathered from the USDA National Small Grains Collection (NSGC) in Aberdeen, ID, United States, as outlined in Table 1.

Table 1. Details of Pakistani wheat landraces.

Sr no	Plant ID	Taxon	Name	Country
1	40951	Triticumaestivum	Type no.11	Punjab,Pakistan
2	40953	*Triticumaestivum*	Type no.13	Punjab,Pakistan
3	181087	Triticumaestivum	8639	Sind,Pakistan
4	182079	*Triticumaestivum*	S-3	Sind,Pakistan
5	182084	*Triticumaestivum*	S-10	Sind,Pakistan
6	182086	Triticumaestivum	S-11	Sind,Pakistan
7	182087	Triticumaestivum	S-12	Sind,Pakistan
8	182088	Triticumaestivum	S-13	Sind,Pakistan
9	182089	*Triticumaestivum*	S-14	Sind,Pakistan
10	182090	*Triticumaestivum*	S-16	Sind,Pakistan
11	182091	Triticumaestivum	S-17	Sind,Pakistan
12	182096	Triticumaestivum	S-22	Sind,Pakistan
13	182097	Triticumaestivum	S-23	Sind,Pakistan
14	182098	Triticumaestivum	S-24	Sind,Pakistan
15	182102	Triticumaestivum	S-28	Sind,Pakistan
16	182103	Triticumaestivum	S-29	Sind,Pakistan
17	182105	Triticumaestivum	S-31	Sind,Pakistan
18	182106	Triticumaestivum	S-32	Sind,Pakistan
19	182107	Triticumaestivum	S-33	Sind,Pakistan
20	182109	Triticumaestivum	S-36	Sind,Pakistan
21	182110	Triticumaestivum	S-38	Sind,Pakistan
22	182111	Triticumaestivum	S-39	Sind,Pakistan
23	182115	Triticumaestivum	S-46	Sind,Pakistan
24	182116	Triticumaestivum	S-47	Sind,Pakistan
25	182117	Triticumaestivum	S-48	Sind,Pakistan
26	182120	Triticumaestivum	Bikaner ii	Sind,Pakistan
27	182121	Triticumaestivum	Punjab C217	Sind,Pakistan
28	182122	Triticumaestivum	Punjab C409	Sind,Pakistan
29	182123	Triticumaestivum	Punjab 9D	Sind,Pakistan
30	182124	Triticumaestivum	Tatta	Sind,Pakistan

31	182126	Triticumaestivum	Moro of Sind	Sind,Pakistan
32	189739	Triticumaestivum	S-518	Sind,Pakistan
33	189743	Triticumaestivum	49575	Sind,Pakistan
34	189744	Triticumaestivum	49576	Sind,Pakistan
Sr no	PI	Taxon	Name	Country
35	189753	Triticumaestivum	49586	Punjab,Pakistan
36	189757	Triticumaestivum	1	Pakistan
37	189758	Triticumaestivum	2	Pakistan
38	193383	Triticumaestivum	N/A	Punjab,Pakistan
39	193385	Triticumaestivum	N/A	Punjab,Pakistan
40	193388	Triticumaestivum	N/A	Punjab,Pakistan
41	193389	Triticumaestivum	N/A	Punjab,Pakistan
42	210896	Triticumaestivum	C 217	Punjab,Pakistan
43	210897	Triticumaestivum	C 245	Punjab,Pakistan
44	210898	Triticumaestivum	C 247	Punjab,Pakistan
45	210899	Triticumaestivum	C 248	Punjab,Pakistan
46	210900	Triticumaestivum	C 250	Punjab,Pakistan
47	210901	Triticumaestivum	C 256	Punjab,Pakistan
48	210902	Triticumaestivum	C 258	Punjab,Pakistan
49	210903	Triticumaestivum	C 269	Punjab,Pakistan
50	210904	Triticumaestivum	C 271	Punjab,Pakistan
51	210905	Triticumaestivum	C 273	Punjab,Pakistan
52	210906	Triticumaestivum	C 288	Punjab,Pakistan
53	210907	Triticumaestivum	C 518	Punjab,Pakistan
54	210908	Triticumaestivum	C 591	Punjab,Pakistan
55	210909	Triticumaestivum	BB 14	Punjab,Pakistan
56	210913	Triticumaestivum	T 11	Punjab,Pakistan
57	210914	Triticumaestivum	T 15	Punjab,Pakistan
58	210915	Triticumaestivum	Punjab Type 8A	Punjab,Pakistan
59	210916	Triticumaestivum	Punjab Type 9D	Punjab,Pakistan
60	217544	Triticumaestivum	13940	Punjab,Pakistan
61	217545	Triticumaestivum	C 518	Punjab,Pakistan
62	217546	Triticumaestivum	C 250	Punjab,Pakistan
63	217547	Triticumaestivum	C 217	Punjab,Pakistan
64	218119	Triticumaestivum	14997	Punjab,Pakistan
65	219737	Triticumaestivum	14053	North-west-frontPakistan
66	219744	Triticumaestivum	14086	North-west-frontPakistan
67	219747	Triticumaestivum	14100	North-west-frontPakistan
68	219748	Triticumaestivum	14101	North-west-frontPakistan
69	219749	Triticumaestivum	14103	North-west-frontPakistan
70	219752	Triticumaestivum	14224	North-west-frontPakistan
71	220071	Triticumaestivum	C 217	Punjab,Pakistan
72	220072	Triticumaestivum	C 228	Punjab,Pakistan
73	220073	Triticumaestivum	C 250	Punjab,Pakistan
74	220074	Triticumaestivum	C 271	Punjab,Pakistan
75	220075	Triticumaestivum	C 273	Punjab,Pakistan

76	220076	Triticumaestivum	C 518	Punjab,Pakistan
77	220077	Triticumaestivum	C 591	Punjab,Pakistan
78	250236	Triticumaestivum	K 427	North-west-frontPakistan
Sr no	PI	Taxon	Name	Country
79	250237	Triticumaestivum	K 630	Punjab,Pakistan
80	250411	Triticumaestivum	K 474	azadkashmir Pakistan
81	250412	Triticumaestivum	K 481	azadkashmir Pakistan
82	250413	Triticumaestivum	K 494	Pakistan
83	250414	Triticumaestivum	K 575	Pakistan
84	250584	Triticumaestivum	K 278	Punjab,Pakistan
85	250585	Triticumaestivum	K 289	Punjab,Pakistan
86	250586	Triticumaestivum	K 382	Pakistan

DNA Extraction

Total genomic deoxyribonucleic acid (DNA) from individual genotypes was extracted from young leaves at seedling stage by CTAB (Cetyltrimethyl Ammonium Bromide) according to the standard procedure reported by Doyle (Doyle 1990). For extraction of DNA, five seeds of each wheat genotype were sown in jiffy pots at NARC Islamabad (made from sphagnum peat). After two weeks of germination, 500 grams of young leaves were taken and ground in pestle and mortar with the help of 500ul CTAB (Cetyl Trimethyl Ammonium Bromide), at the appearance of dark green fluid it was transferred into 500ul appendrof tube and centrifuged at 5000rpm for five minutes. The supernatant was dropped, and pellet was transferred into a new appendorf tube and 70ul of isoamyl alcohol was added and kept it for drying at room temperature for an hour.

DNA Quantification

Purity of DNA was checked by using 1.5% Agarose gel under UV light after ethidium bromide staining.PCR amplification PCR reactions were carried out in 25 µl reaction mixture containing 50-100 ng total genomic DNA template, 0.25 mM of each primer, 200 mM of each dATP, dGTP, dCTP, dTTP, 50 mM KCl, 10mMTris, 1.5 mM MgCl2 and 2.5 units of Taq DNA polymerase.The amplification conditions were as follows; initial step of denaturation for 1 minute at 94°C followed by 35 cycles of each consisting of a denaturation step of 1 minute at 94°C, an annealing step of 1 minute with some modification in annealing temperature which was followed as 56 °C, 60 °C and 62 °C for optimizing the different marker, and extension step of 2 minutes at 72°C. Seven minutes were given after the last cycle to the extension step at 72°C to ensure the completion of the primer extension reaction.

Electrophoresis

Electrophoresis was employed to resolve the PCR products by loading 10ul onto 1.2% Agarose gels in 1X TBE buffer, followed by visualization under UV light post-ethidium bromide staining.The gel's visualization involved the utilization of the Gel Documentation (Digecel) system to determine the presence or absence of the gene of interest. For data analysis, information regarding the presence or absence of the marker was gathered through gel visualization and comparison with the known band sizes of markers associated with stem rust resistance genes. Subsequently, this data underwent frequency distribution analysis, and graphs were created accordingly.

Results

Marker Sr36The co-dominant marker Xstm773-2 played a crucial role in identifying the Sr36 gene's presence. This marker specifically generated a 155bp fragment indicative of the Sr36 gene's presence within the landraces. The outcomes derived from the Sr36 marker are outlined in the provided findings. The presence and absence of the Sr36 gene were observed in 36% of Pakistani wheat landraces shown in Figure 1 to Figure 4. Among these, base pair 155 was found to be associated with the presence of the Sr36 gene. Gel electrophoresis displays a molecular marker of 100 base pairs, while the other lanes represent different numbers of landraces. The study's findings indicate that a significant number of these landraces exhibited a positive result for the stem rust resistance gene Sr36.

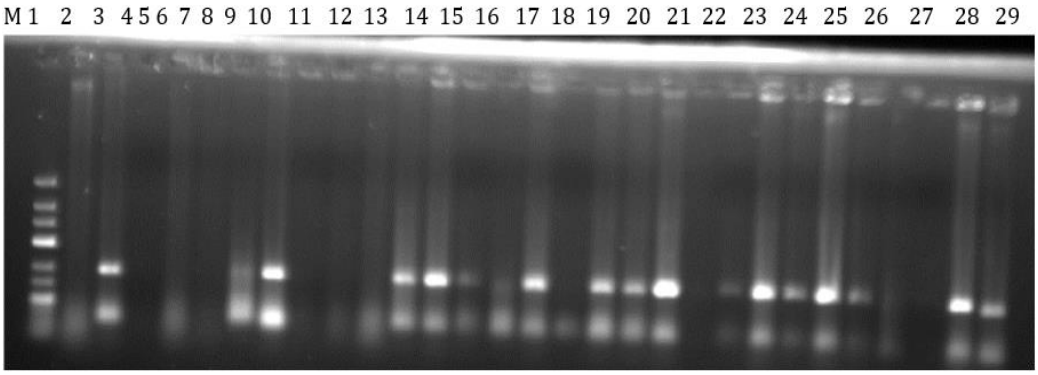

Figure 1. PCR amplification for markers *Xstm773-2* linked to the *Sr36* locus with 155bp.
M=Marker100bp.1.PI.40951.2.PI.40953.3.PI.181087.4.PI.182079.5.PI.182084.6.PI.182086.7.PI.182087.8.PI.182088.9.PI.182089.10.PI.182090.11.PI.182091.12.PI.182096.13.PI.182097.14.PI.182182098.15.PI.182102.16.PI.182103.17.PI.182108.18.PI.182106.19.PI.182107.20.PI.182109.21.PI.\182110.22.PI.182111.23.PI.182115.24.PI.182116.25.PI.182117.26.PI.182120.27.PI.182121.28.PI.182122.29.PI.182123.

The co-dominant marker Xstm773-2 played a crucial role in identifying the Sr36 gene's presence. This marker specifically generated a 155bp fragment indicative of the Sr36 gene's presence within the landraces. The outcomes derived from the Sr36 marker are outlined in Figure 2.

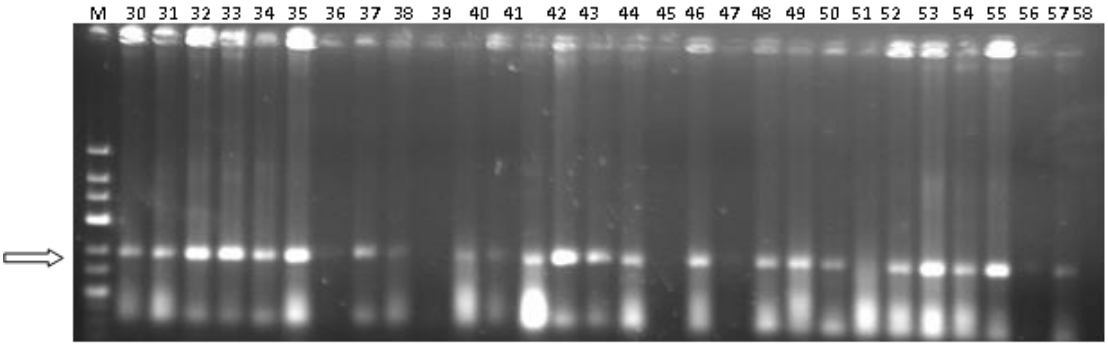

Figure 2. PCR amplification for markers linked to the Sr36.
M=Marker100bp.30.PI.182124.31.PI.182126.32.PI.189739.33.PI.189743.34.PI.189744.35.PI.189753.36.PI.189757.37.PI.189758.38.PI.193383.39.PI.193385.40.PI.193388.41.PI.193389.42.PI.210896.43.PI.210897.44.PI.210898.45.PI.210899.46.PI.210900.47.PI.210901.48.PI.210902.49.PI.210903.50.PI.210904.51.PI.210905.52.PI.210906.53.PI.210907.54.PI.210908.55.PI.210909.56.PI.210.57.PI.210914.58.PI.210915.

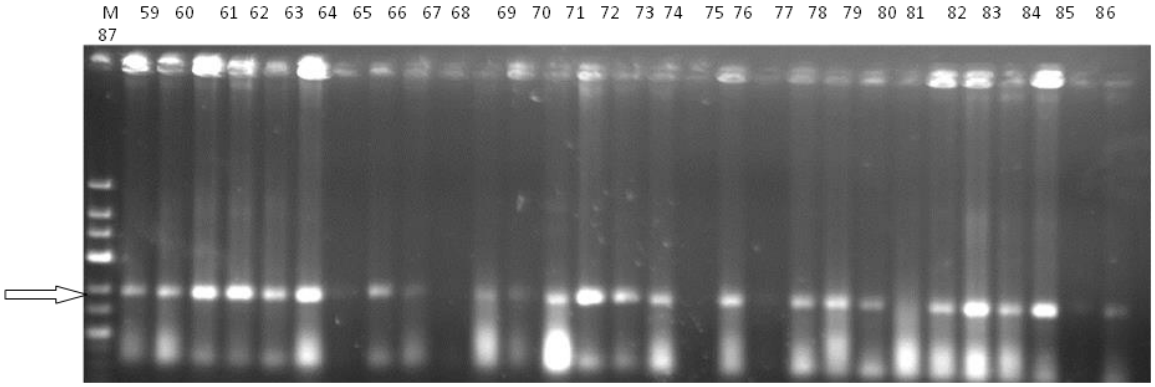

Figure 3. PCR amplification for marker linked to the *Sr36* locus at base pair 155. (Continue…)

M=Marker100bp.59.PI.210916.60.PI.217544.61.PI.217545.62.PI.217546.63.PI.217547.64.PI.218119.65.PI.219737.66.PI.219744.67.PI.219747.68.219748.69.PI.219749.70.PI.219752.71.PI.71.72.PI.220071.73.PI.220073.74.PI.220074.75.PI.220075.76.PI.220076.77.PI.220077.78.250236.79.PI.250237.80.PI.250411.81.PI.250412.82.250413.83.PI.250414.84.PI.250584.85.PI.250585.86.PI.2058.

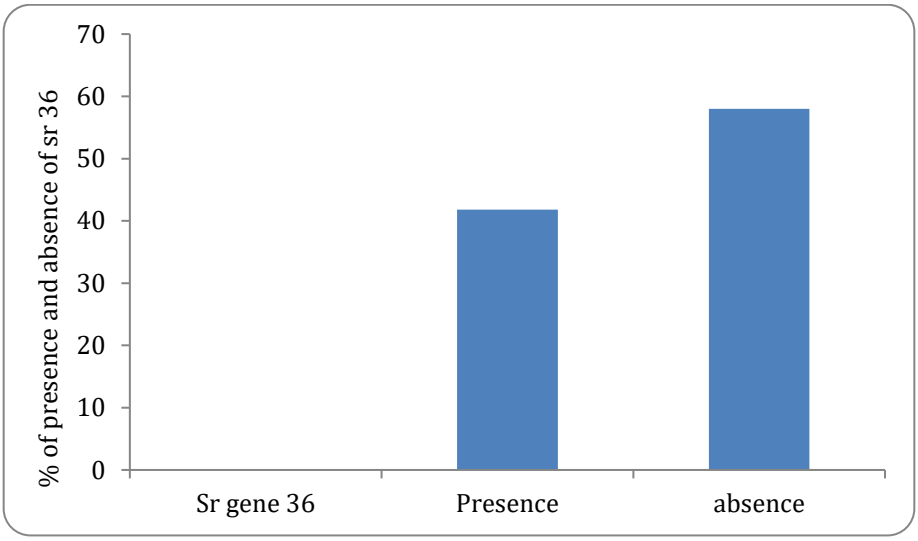

Figure 4. Presence and absence of Sr36 gene in Pakistani Wheat landraces.

DISCUSSION

The Sr36 gene, renowned for its efficacy in conferring stem rust resistance in both bread wheat and durum wheat (McIntosh *et al*., 1995), holds significant promise for molecular breeding initiatives aimed at enhancing stem rust resistance. Detection of this valuable genetic resource within Pakistani wheat landraces presents a compelling opportunity for advancing molecular breeding strategies targeted at stem rust resistance.

The Sr36 gene is a crucial element in the defense against the wheat stem rust pathogen (*Puccinia graminis f. sp. tritici*). In Pakistani wheat landraces, the presence and diversity of Sr36 play a significant role in conferring resistance to stem rust, a major threat to global wheat production. Studies have shown that Sr36 provides a durable and broad-spectrum resistance against multiple races of the stem rust pathogen. The identification and characterization of Sr36 in Pakistani wheat landraces contribute valuable insights into the genetic diversity and evolution of stem rust resistance in local wheat populations(Mirza *et al*., 2010).

It demonstrated the prevalence of Sr36 in a diverse set of wheat varieties, highlighting its importance as a key component of the country's wheat genetic resources (Tsilo *et al*. 2008). Furthermore, the study conducted delved into the allelic variation of Sr36 in wheat germplasm, shedding light on the allelic diversity and its implications for durable resistance (Csosz *et al*. 2001). The conservation and utilization of Sr36 in breeding programs are imperative for developing wheat varieties with sustainable resistance to stem rust in Pakistan.

Our study reveals the remarkable genetic diversity present within Pakistani wheat landraces, primarily attributed to the novel Ug99-resistant Sr36 gene. This finding resonates with Tsilo's seminal work in 2008, where the presence of Sr36 was observed in Red Chinese Spring cultivars. The utilization of the co-dominant microsatellite marker Xstm773-2 facilitated the identification of allelic variations associated with Sr36, evidenced by the presence of a 155 base pair band.

The detection of the Sr36 gene in 36% of the surveyed landraces underscores the rich reservoir of genetic diversity harbored within these traditional wheat varieties. This prevalence not only highlights the potential of Pakistani landraces in contributing to the genetic improvement of wheat but also signifies their importance in breeding programs aimed at combating devastating stem rust pathogens like Ug99(Csosz *et al*. 2001).

However, the presence of landraces lacking amplification of the target gene band in 20% of the surveyed populations raises intriguing questions

regarding the underlying genetic mechanisms. Possible explanations for this observation could include allelic variations in the Sr36 gene, gene loss events, or the presence of alternative resistance mechanisms against stem rust pathogens. Gaining insight into the evolutionary processes of Ug99 resistance in wheat populations will require more research into genetic causes of this diversity.

Our results emphasize how crucial it is to preserve and utilize the genetic variety observed in traditional crop varieties, such as Pakistani wheat landraces. These landraces have tremendous potential for producing resilient wheat cultivars that can tolerate altering disease stresses in addition to function as preserves of important genetic features. Moving forward, decoding the intricate genetic makeup of Ug99 resistance and formulating successful plans for long-term wheat development will need utilizing cutting-edge genomic technologies and carrying out in-depth genetic analysis.

CONCLUSION

The identification of the Sr36 gene in 36% of Pakistani wheat landraces underscores the significant genetic potential these traditional varieties hold for enhancing stem rust resistance. The presence of the Sr36 gene, confirmed through the use of the co-dominant marker Xstm773-2, highlights its crucial role in providing durable and broad-spectrum resistance against multiple races of the stem rust pathogen. This study not only reveals the rich genetic diversity within Pakistani wheat landraces but also emphasizes their importance in breeding programs aimed at combating devastating pathogens like Ug99.The detection of allelic variations and the absence of the Sr36 gene in a subset of landraces suggest the presence of additional resistance mechanisms, warranting further genetic investigation. The conservation and strategic utilization of this genetic diversity are imperative for the development of resilient wheat cultivars. By leveraging advanced genomic technologies and fostering collaborative research efforts, we can enhance our understanding of stem rust resistance and formulate effective breeding strategies. This will ensure the sustainable production of wheat in Pakistan and contribute to global food security.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

FUTURE PERSPECTIVES

Building on the significant findings regarding the presence of the Sr36 gene in Pakistani wheat landraces, several future directions can be outlined to enhance stem rust resistance and wheat breeding strategies. Comprehensive genetic mapping and sequencing should be conducted to understand the genetic mechanisms of the Sr36 gene, identifying allelic variations, gene loss events, or alternative resistance genes. Given that 20% of the surveyed landraces did not show amplification of the Sr36 gene, it is essential to investigate other genetic factors or mechanisms that may confer resistance to stem rust, involving the study of other known resistance genes or discovering new ones through advanced genomic techniques. The identified Sr36-positive landraces should be prioritized in breeding programs to develop new, resistant wheat cultivars using marker-assisted selection (MAS) for efficient incorporation of the Sr36 gene into high-yielding, locally adapted varieties. The rich genetic diversity of Pakistani wheat landraces should be conserved by establishing gene banks and in situ conservation programs, ensuring a genetic reservoir for future breeding efforts. Continuous monitoring of the stem rust pathogen, especially the virulent Ug99 strain, is crucial to track its evolution and spread. Integrating resistance genes like Sr36 requires vigilance against pathogen adaptation to maintain durable resistance. Additionally, utilizing cutting-edge genomic tools, such as CRISPR, will enhance breeding strategies and resistance mechanisms.

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