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Identification of Stem Rust Resistance Genes in Released Wheat Varieties by Linked SSR Markers and Phenotypic Screening

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Abstract: Wheat suffers significant yield losses due to stem rust disease caused by Puccinia graminis Pers. f. sp. tritici Eriks and Henn. Molecular level assessment of existing Sr genes in improved and advanced wheat materials combined with phenotypic screening lays down the basis for effective varietal development against this production constraint. Therefore, this study was carried out: to detect stem rust resistance genes present in Ethiopian bread wheat and durum wheat varieties-using molecular markers; and to determine their effectiveness for the virulent Ethiopian stem rust races including Ug99. Screening of 49 wheat varieties with 11 SSR markers linked to 11 Stem rust resistance genes resulted in the detection of 5 Stem rust resistance genes (*Sr22, Sr25, Sr24, Sr77* and *SrTA10187*) in a subset of 12 varieties. The detected number of genes ranged between 1 and 2 per genotype. Despite amplifying the expected fragment, the markers have also resulted in several off-target amplifications suggesting the need to develop other relatively stable markers specific to the target genes. Field resistance screening at Debre Zeit Research Center resulted in 20 varieties showing good resistance to stem rust of which 2 are durum wheat cultivars and the rest 18 are bread wheat varieties. Recent data in 2022, however, showed only 5 out of the 20 had a resistant reaction while the other even became susceptible. For instance, most of the mega bread wheat cultivars like Ogolcho also were defeated due to the newly emerging race TTKTT. Among the genes detected by molecular markers, only *SrTA10187* seems to be effective against the rust population in the field. Seedling resistances screening gave a range of proportion of Resistant (R) to Susceptible (S) variety varying from 12:36 for TTKTT; 40:8 for TKTTF; 39:9 for TTKSK and 44:4 for TTTTF. Eight varieties (Sulla, Galil, Huluka, Kingbird, Millenium, Obsa, Tate and Ilani) exhibited resistant reaction consistently across the four pathotypes. Nine varieties (Honqollo, Millenium, Kulkulu, Shorima, Hogana, Meraro, Ilani and Galil) identified as resistant at both seedling and Adult plant stage. The genes, *Sr22* in variety Oda and *Sr25* in variety Dinknesh appeared to be effective for TTKTT, TKTTF, TTKSK and TKTTF, TTKSK, TTTTF, respectively. The detected

Stem rust resistance genes in the present study which are effective against the pathotyeps combined with the resistant varieties at seedling and adult plant stage can support the wheat breeding program towards improving the crop.

Keywords: Bread Wheat, Durum Wheat, Gene Detection, Linked Marker, Resistance to Stem Rust, Pathogen Screening

1. Introduction

Wheat is an important staple cereal food crop in Ethiopia providing about 15% of the caloric intake for the country's over 90 million populations [1]. In Ethiopia, wheat is cultivated on over 1.8 million hectares and with an annual production of 4.5 million metric tons. In terms of total grain production, it ranks third after maize and tef and contributes about 15.63% of the grain production in the country [2]. Both bread wheat (*Triticum aestivum* L. Thell) and durum wheat (*Triticum turgidum* L. var. *durum*) are cultivated over a wide range of areas in the country. Demand for more production and productivity of wheat is paralleling the ever-increasing population in the country, thus seeking research intervention for food self-sufficiency, although several biotic stress factors are constraining the wheat industry.

Stem rust also called Black rust caused by *Puccinia graminis* f. sp. *tritici (*Pgt*)* is one of the major cereal diseases that affect wheat production and productivity in Ethiopia [3, 4]. It affects small grain cereals especially wheat, barley and other members of *triticeae* in general [3]. It is highly host specific obligate parasite and changing to virulent races through mutation and sexual recombination. Its life cycle involves both sexual and asexual stages which at large depend both on barberry and wheat for completing its development. Barberry species, the commonly known alternate host and the less common mahonia are necessary to accomplish the sexual cycle, while the asexual cycle occurs on wheat and other grassy alternate hosts [5]. Due to its nature of producing a succession of different types of spores, the pathogen is often known as a polymorphic species with heteroceous and heterothalic life cycle producing five unique spore stages. Among the various spore stages it is the telial stage which is the true diploid stage of the fungus that enables the pathogen to survive cold or dry conditions [6].

In the recent past years, frequent stem rust epidemics have been recorded in different parts of Ethiopia causing great losses [7, 8]. According to Hei *et al*. [9], in 2016, yield losses of 70.70% and 60.00% respectively have been reported in Arsi and Bale zones of Oromia region. In the worst cases, and under severe conditions, the yield loss due to this disease can reach up to total crop loss [10]. Due to stem rust epidemics in Ethiopia that caused a 100 percent yield loss [11], several major cultivars like Digelu harboring SrTmp has been knocked down in 2013 and 2014. The race TKTTF also called the Digelu race has been dominant across the major wheat growing regions of Ethiopia [11]. This race is not only dominant, but also it is different from the Ug99 race (TTKSK) and has become a major threat to wheat production in the country. The emergence of race Ug99 (TTKSK) in 2003 and subsequent outbreaks afterwards threaten wheat production in Ethiopia because they overcome widely used genes that had been effective for many years [12].

Genetic resistance is one of the environmentally friendly options to combat this problem. In view of that, several improved wheat varieties have been released by the national wheat improvement program of Ethiopia. Despite those efforts, the specific stem rust race resistances genes available in those improved varieties are hardly known, and if at all known are defeated by the newly emerging pathotypes. Hence the purpose of this study was to identify reported Sr genes in Ethiopian (bread and durum) wheat varieties through diagnostic/linked molecular markers and evaluate the effectiveness of the varieties in the field and against known virulent stem rust races under greenhouse seedling test.

2. Materials and Methods

2.1. Plant Materials

Forty-nine wheat varieties (43 bread wheat (hexaploid) and 6 durum wheat (tetrapod)) were used for this gene identification study (Table 1). Three to four seeds of each genotype were sown in the greenhouse for DNA extraction and analysis. Most of the varieties originated from CIMMYT and ICARDA with very few from Ethiopia and Kenya. The bread wheat variety seeds were obtained from Kulumsa Agricultural Research Center, while seeds of the durum wheat varieties were obtained from Debre Zeit Agricultural Research Center.

Table 1. *Released bread and durum varieties used for study of detection of Stem rust resistance (Sr) genes.*

No.	Genotype	Pedigree	Source	Type
1	Kakaba	KIRITATI//SERI/RAYON	KARC	Bread wheat
2	Sulla	HAR710/RBC	KARC	Bread wheat
3	Meraro	M/4/HAR 1709/ 3/M//24/E	KARC	Bread wheat
4	K6295-4A	Romany X GB-GAMENYA	KARC	Bread wheat
5	Dinkinesh	CARA/4/CRDN/3/PEL72380/ATR71*2//H567.1	KARC	Bread wheat
6	Dashen	VEE #17, KVZ/BUHO"S"//KAL/BB	KARC	Bread wheat
7	Quai (Gambo)	BABAX/LR42//BABAX*2/3/VIVITSI	KARC	Bread wheat
8	Hawi	CHIL/PRL	KARC	Bread wheat
9	Abola	BOW"S"/BUC"S"	KARC	Bread wheat

No.	Genotype	Pedigree	Source	Type
10	Tussie	COOK/VEE"S"//DOVE"S"/SERI	KARC	Bread wheat
11	Galema	4777(2)//FKN/GB/3/PVN"S"	KARC	Bread wheat
12	Galil	HORK/YAMHILL//KALYANSONA/BLUEBIRD/3/BOBWHITE	KARC	Bread wheat
13	Tossa	ND/VG9144//KAL/BB/3/YACO/4/VEE#5	KARC	Bread wheat
14	Pavon	WORRAKATTA/2*PASTOR	KARC	Bread wheat
15	Sirbo	VS73.600/MRL/3/BOW//YR/TRF	KARC	Bread wheat
16	Ogolcho	WORRAKATTA/2*PASTOR	KARC	Bread wheat
17	Hidase	YANAC/3/PRL/SARA//TSI/VEE#5/4/CROC-1/AE.SQUAROSA(224)//OPATTA	KARC	Bread wheat
18	Menze	MILAN/SHA7	KARC	Bread wheat
19	Biqa	PASTOR//HXL7573/2*BAU/3/WBLL1	KARC	Bread wheat
20	Shorima	UTQUE96/3/PYN/BAU//MILAN	KARC	Bread wheat
21	Alidoro	HK-14-R-251	KARC	Bread wheat
22	KBG-01	(300/SM+501M)/HAR 1709	KARC	Bread wheat
23	ET13A2	UQ 105 SEL. X ENKOY	KARC	Bread wheat
24	Dereselign	CI 8154//2*FR	KARC	Bread wheat
25	Enkoy	(HEBRARD Sel/WIS 245 X SUP51) X FR-FN/Y)2.A	KARC	Bread wheat
26	Bobicho	PEG/PF70354/4/KAL/BB//ALD/3/MRNG	KARC	Bread wheat
27	Huluka	UTQUE96/3/PYN/BAU//MILAN	KARC	Bread wheat
28	Kulkulu	PYN/BAU//MILAN	KARC	Bread wheat
29	Bollo	VEE/LIRA//BOW/3/BCN/4/KAUZ	KARC	Bread wheat
30	Honqollo	NJORO SD-7	KARC	Bread wheat
31	K6290 Bulk	(AF.MAYO X GEM) X ROMANY	KARC	Bread wheat
32	Hoggana	PYN/BAU//MILAN	KARC	Bread wheat
33	Kubsa	ND/VG9144//KAL/BB/3/YACO/4/VEE#5	KARC	Bread wheat
34	Danda'a	KIRITATI//2*PBW65/2*SERI.1B	KARC	Bread wheat
35	Digelu	SHA7/KAUZ	KARC	Bread wheat
36	Tay	ET-12D4/4777(2)//FKN/GB/3/PVN"S"	KARC	Bread wheat
37	Sofumar	LIRA 'S'/TAN"S"	KARC	Bread wheat
38	Kingbird	TAM200/TUI/6PVN//CAR422/ANA/5/BOW/CROW//BUC/PVN/3YR/4/TRAP#1	KARC	Bread wheat
39	Mada-Walabu	TI/3/Fn/Th/Nar 59 *2/4/Bol'S'	KARC	Bread wheat
40	Millenium	ALD/CEP75630//CEP75234/PT7219/3/BUC/BIY/4/	KARC	Bread wheat
41	Lemmu	WAXWING*2/HEILO	KARC	Bread wheat
42	Wane	SOKOLL/EXCALIBUR	KARC	Bread wheat
43	Morocco	-	KARC	Bread wheat
44	Arendato	Landrace	DZARC	Durum wheat
45	Mangudo	MRF_1/STJ2/3/1718/BT24//KARIM,	DZARC	Durum wheat
46	Obsa	ALTAR 84//ALTAR 84/SERI/3/6*ALTAR 84	DZARC	Durum wheat
47	Tate	CD94523 (Selection History)	DZARC	Durum wheat
48	Oda	DZ046881/IMLO//CIT 71/3/RCHI/LD 357//IMLO/4/YEMEN/CIT'S'//PLC'S'/3/TAGANROY	DZARC	Durum wheat
49	Ilani	IMILO/RAHUM//A4#72/3/GERARDO	DZARC	Durum wheat

2.2. Primers and Target Sr Genes

Eleven informative primers linked or diagnostic to reported 11 Stem rust resistance genes (*Sr22, Sr24, Sr25, Sr26, Sr31, Sr33, Sr36, Sr39, Sr52, Sr57 and SrTA10187*) were selected for this study. Their sequences, and all other relevant information associated with them was accessed from MASWHEAT website developed and maintained by University of California, Davis (http://maswheat.ucdavis.edu/protocols/stem_rust/). Some of the genes information was obtained from published articles. The list of the primers and their additional descriptive information is presented in Table 2.

Table 2. *Primer sequences linked to Sr genes and related descriptive information.*

No.	Primer Name	Primer Sequence (5' to 3')	Marker Type	Gene Name	PCR fragment	Reference
1	WMC633	F: ACA CCA GCG GGG ATA TTT GTT AC R: GTG CAC AAG ACA TGA GGT GGA TT	SSR	*Sr22*	117bp	Olson *et al*. (2010) [13]
2	Xbarc71	F: GCG CTT GTT CCT CAC CTG CTC ATA R: GCG TAT ATT CTC TCG TCT TGT TGG TT	SSR	*Sr24*	103 bp and 85 bp suc.107bp	Mago *et al.*(2005) [14]
3	BF145935	F: CTT CAC CTC CAA GGA GTT CCA R: GCG TAC CTA ATC ACC ACC TTG AAG G	EST	*Sr25*	198 & 180 bp in Sr25 lines and 202 & 180 bp in wheat lines without Sr25	Liu *et al.* (2010) [15]
4	*BE518379*	F: AGC CGC GAA ATC TACTTT GA R: TTA AAC GGA CAG AGC CA CG		*Sr26*	303 bp in the absence of Sr26	Liu *et al*.(2010) [15]
5	RYE-NOR	F: CATGTAGCGACTAACTCATC R: CCAGTTTTCCATGTCGC	STS	*Sr31*	400, 600, 700 and 800 - bp	(http://maswheat.ucdavis.edu/protoc-ols/stem_rust/
6	*Xcfd15*	F: CTC CCG TAT GCA GGA AG R: GGC AGG TGT GGT GAT GAT CT	SSR	*Sr33*	179bp	(https://wheat.pw.usda.gov/cgibin/GG3/report.cgi?class=marker;query=*XC

No.	Primer Name	Primer Sequence (5' to 3')	Marker Type	Gene Name	PCR fragment	Reference
						FD15*;name=Xcfd15)
7	*Xgwm319*	F: GGT TGC TGT ACA AGT GTT CAC G R: CGG GTG CTG TGT GTA ATG AC	SSR	*Sr36*	170bp	Tsilo *et al*. (2008) [16]
8	*BE500705*	F: ATC TGT GGC AGT GTG CTC CT R: TCC TGC AAA TGC TTG TCG TT	EST	*Sr39*	166bp in susceptible allele	(http://maswheat.ucdavis.edu/protoc-ols/stem_rust/
9	WMS570	F: TCG CCT TTT ACA GTC GGC R: ATG GGT AGC TGA GAG CCA AA	SSR	*Sr52*	100 - 200 bp	Qi *et al.*(2011)[17]
10	cslv 34	F: GTT GGT TAA GAC TGG TGA TGG R: TGC TTG CTA TTG CTG AAT AGT	STS	*Sr57*	150-bp and 229-bp	(http://maswheat.ucdavis.edu/protoc-ols/stem_rust/
11	cfd49	F: TGA GTT CTT CTG GTG AGG CA R: GAA TCG GTT CAC AAG GGA AA	SSR	*SrTA10187*	196 (in *Ae. tauschii*), 219 (in KS05HW14); 214 (from GrainGene)	Olson *et al.* (2013a) [18]; Olson *et al.*(2013b) [19]

2.3. Molecular Screening

2.3.1. Genomic DNA Extraction

The wheat varieties were sown in the greenhouse at the National Agricultural Biotechnology Research Center (NABRC) on planting trays for raising seedlings. Fully opened second leaf samples approximately 5 cm long were harvested from 2–3 week-old seedlings of each genotype in an Eppendorf tube of 1.5 ml on ice. The tubes with sampled leaf tissue were then immersed in a vessel of liquid nitrogen for approximately 30 seconds and fully ground in to fine powder on TisueLyser II. Genomic DNA was extracted in two replications from the same tissue (to get larger volume) using SDS based DArT protocol with minor modifications. Both ND8000 spectrophotometer and 1% gel electrophoresis were used for quantity and quality assessment of the genomic DNA. For all the samples, the genomic DNA was normalized to 50 ng/µl and that concentration was used for all downstream detection work.

2.3.2. PCR Amplification

Amplification of the target gene regions were done following the PCR setup given for each primer at the MASWHEAT website (http://maswheat.ucdavis.edu/protocols/stem_rust/).
Whenever it did not work however, the thermal cycler program was optimized using gradient PCR. For all the primers, PCR reaction was carried out in a final volume of 13 µl constituted from 0.5 µl of each of the forward and reverse primers, 4 µl of master mix (a ready to go mix of dNTPs, PCR buffer, $MgCl_2$ and Taq DNA Polymerase from sigma Aldrich), 2-3 µl template genomic DNA, and the rest nuclease free water.

2.3.3. Fragment Analysis and Scoring for Presence/Absence of the Target Sr Genes

Fragments obtained from the PCR amplifications were analyzed mostly using horizontal agarose gel electrophoresis with an appropriate size marker (ladder) included. In general, 5 µl of PRC product combined with 2 µl of loading dye-gel red mix making a final volume of 7 µl was loaded on 3% agarose gel in 1xTAE buffer. The gel was run for 2:30 to 3:00 hours at 100 constant voltages, and image capture was carried out with gel documentations system under UV Transilluminator. Fragment analysis was done using the software PyElph 1.4, which takes in to account the size of the marker used during the gel electrophoresis [20]. Decision on the most likely DNA fragment having a size close to the expected fragment linked to the target gene was made by combining the estimated size information and the DNA band on the gel picture. Because fragment sizing and visual observations alone cannot be as precise as sequence information, we generally followed fragment size + or – 3 bp as a general rule to make a decision. That finally laid down the basis to judge if the target gene is present or absent in the tested genotype.

2.4. Phenotypic Screening in Field and Greenhouse

2.4.1. Field Resistance Evaluation at Adult Stage

All the 49 varieties were evaluated for their field resistance against stem rust races prevailing under filed condition at Debre Zeit Agricultural Research Center in 2017. Of these, 36 were further tested in 2022. In the evaluation experiment, each genotype was planted in two rows of one meter length with no replication. Spreader rows of most susceptible varieties such as Morocco, Local Red and Hitosa were planted along the way between blocks of varieties. A starter inoculation was applied on the spreader row with a water suspension of the urediniospores of the stem rust races TTKSK, TRTTF, TKTTF, TTTTF, TTRTF and JRCQC so that uniform infection establishment would be achieved among the varieties. Evaluation of the reaction of the varieties to the disease was carried out following the modified Cobb's scale, which combines the disease severity with host response [21]. Severity was recorded from 0 to 100%, while the host response was recorded using the description of Roelfs *et al*. [22] as I (Immune), R (Resistant), MR (Moderately Resistant), M (Moderate / Intermediate), MS (Moderately Susceptible) and S (Susceptible). If a variety displayed multiple infection responses to stem rust, they were all recorded (example: MRMS, MSS etc). Disease scoring was carried out three times every ten days over the development of the crop, and the last evaluation was used as a basis for deciding the reaction of the varieties. The average coefficient of Infection (ACI) was calculated from the Severity scores and response values. In general ACI values 0-9.7 were considered as Resistant (R) response group; 10 – 20 as Intermediate (I) response group and those with >20 were

classified as Susceptible (S) response group.

2.4.2. Seedling Resistance Evaluation in Greenhouse

The seedling resistance evaluation of all the 49 varieties against four stem rust pathotypes (TTKTT, TKTTF, TTKSK, TTTTF) (Table 3) and a bulk of all the pathotypes was carried out in the greenhouse at Ambo Agricultural Research Center (AARC).

Five seeds of each wheat variety and a susceptible check (MacNair) were planted separately in 5 cm diameter plastic pots filled with growing medium composed of soil, sand and manure in the ratio of 2:1:1, respectively. The spores of each race were suspended in Soltrol 170 (approximately $1x10^5$ spores per 1 ml lightweight mineral oil) and sprayed onto leaves of 7 day-old seedlings (the first leaf is fully expanded and the second leaf is just emerged to grow) of the wheat varieties. Inoculated plants were moistened with fine droplets of distilled water by using atomizer after 30 minutes of inoculation, and seedlings were incubated in the dark for 18 hours at 18°C and 95% relative humidity (RH) in a dew chamber. Thereafter, the seedlings were exposed to fluorescent light for four hours to provide favorable condition for stem rust infection. Seedlings were then allowed to dry their dew for about 2 hours and transferred from dew chamber to glass compartments in the greenhouse, where conditions are regulated at 12 h photoperiod, and a temperature range of 18-25°C and RH of 60-70%. The experiment was arranged in a completely randomized design and repeated three times for each race of the pathogen to exclude the possibility of disease escape. Disease assessment was carried out 14 days after inoculation using the 0 to 4 infection type (IT) scoring scale where infection types "0", ";", ";1"; "1", "1+", "2-", "2", "2+" were regarded as resistant and "3-", "3", "3+", and "4" were considered susceptible [23].

Table 3. *Virulence and Avirulence formula of the Pgt pathotypes used to evaluate the varieties at seedling stage.*

Pathotype	Virulence	Avirulence
TTKTT	*5, 21, 9e, 7b, 11, 6, 8a, 9g, 9b, 30, 17, 9a, 9d, 10, Tmp, 24, 31, 38, McN*	36
TKTTF	*5, 21, 9e, 7b, 6, 8a, 9g, 36, 9b, 30, 17, 9a, 9d, 10, Tmp, 38, McN*	*11, 24, 31*
TTKSK	*5, 21, 9e, 7b, 11, 6, 8a, 9g, 9b, 30, 17, 9a, 9d, 10, 31, 38, McN*	*36, Tmp, 24*
TTTTF	*5, 21, 9e, 7b, 11, 6, 8a, 9g, 36, 9b, 30, 17, 9a, 9d, 10, Tmp, 38, McN*	24, 31

Source: Ambo Plant Protection Research Center, wheat pathology section (Dr. Netsanet Bacha, Personal communication)

2.5. Combined Look at Seedling & Adult Plant Resistance

We examined the resistance pattern among the tested varieties in view of seedling versus adult plant stages. This was done by combining the resistant (R) and Susceptible (S) response groups obtained from the seedling resistance test with the Resistant (R), Intermediate (I) and Susceptible (S) group of adult plant stage which in total makes up six Response groups: RR, RI, RS, SR, SI and SS. For both seedling and adult stage resistance responses, the scores generated from the bulk virulent races test were used.

3. Results and Discussion

3.1. Markers' Effectiveness

The eleven selected markers successfully worked for the detection process across the 49 varieties screened. An example of a gel image showing PCR products ready to go for visual fragment analysis combined with size determination using the software PyElph 1.4 [20] is shown in Figure 1.

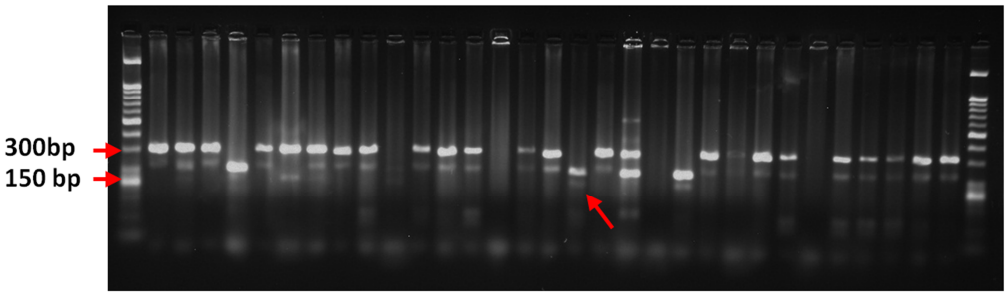

Figure 1. *An Example of Gel image for detection of Sr57 in wheat varieties (1st and last lanes are loaded with DNA Ladder of 25/100 bp while those in between were loaded with PCR products of varieties). The lower faint band in the 18th lane is very close to 150 bp. The expected fragment in resistant varieties is 150 bp, while it is 229 bp in susceptible.*

The highest amplification (100% out of the total varieties) was achieved both by marker WMS570 which is linked to *Sr52* and BE518379 linked to *Sr26* (Figure 2). The lowest amplification (85.7% or 42 of the total varieties), on the other hand, was obtained from marker Sr39#50 which is diagnostic to *Sr39* (Table 4 and Figure 2). However, the respective genes linked to these markers were not detected in any of the varieties. The level of non-amplification cases was so small that it ranged from 0 (for markers WMS570 and BE518379) to 7 for marker *Sr39*#50 (Figure 2).

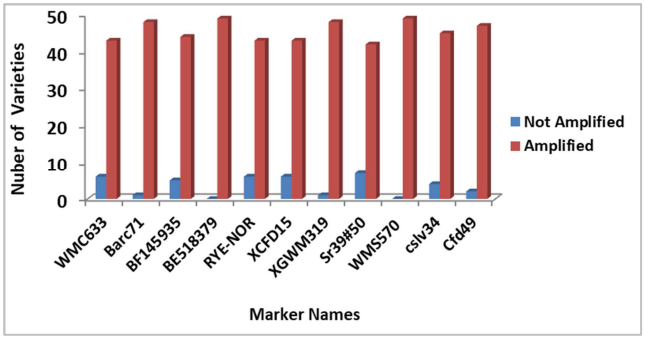

Figure 2. *Level of amplification of the markers across the screened wheat varieties.*

3.2. Detected Genes

Out of the eleven genes targeted in this study, only five were detected (*Sr22, Sr24, Sr25, Sr57* and *SrTA10187)* in a total of 12 varieties (Table 4 and Figure 3). The detected number of genes ranged between 1 and 2 per genotype. Despite amplifying the expected fragment, the markers have also resulted in several off-target amplifications suggesting the need to develop other relatively stable markers specific to the target genes.

Sr22 is among the effective genes against Ug99 and previously mapped on the long arm of chromosome 7A [24]. Of the other additional markers reported for this gene, we only used the closely linked SSR marker to *Sr22* gene WMC633 produced by Olson *et al.* [13] and Olson *et al.* [18]. WMC633 marker can amplify *several* alleles in wheat, *ranging in size from 170 bp to 260 bp.* However 229 bp allele size confers resistance [13]. In our experiment only variety Oda exhibited having the *Sr22* gene.

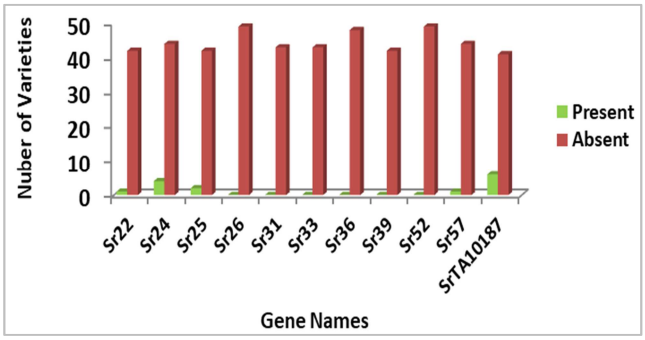

Figure 3. *Level of detection of stem rust resistance genes across screened wheat varieties.*

The gene *Sr24* conffers resistance to most races of stem rust, including the virulent race Ug99 (TTKSK), although now it is ineffective against TTKTT (Table 3). Of the many molecular markers linked to it, XBARC71 is the most distal SSR marker mapped on the long arm of chromosome 3D of wheat [14]. According to Mago *et al.* [14], varieties carrying *Sr24* amplify a pair of diagnostic bands (103 bp and 85 bp), while most of the susceptible lines lacking *Sr24* amplified a 107 bp fragment. In the present study, we were able to detect the gene only in varieties Ogolcho, Biqa, Kulkulu and Sofumar (Table 4).

The *Sr25* gene is also amongst the effective gene against Ug99 and was transferred into wheat from *Thinopyrum ponticum* Bark Worth and Dewey. This gene is known to produce fragment sizes of 198 and 180 bp in *Sr25* lines and 202 and 180 bp in wheat lines without *Sr25* [12]. We used the co-dominant marker BF145935 derived from a wheat EST and were able to detect it in two varieties Dinkinesh and Menze (Table 4).

The *Sr57* gene is the other gene detected in the present study, and it is one of the known multifunctional genes diagnosed by marker Cslv34 selected among the other linked markers. Amplification of fragment size 150 and 229 bp implies the presence and absence of this gene in resistant and susceptible varieties respectively. In our study, it is detected only in the variety Hidase as shown on Table 4.

The last Stem rust resistance gene was SrTA10187 to which the marker Cfd49 is SSR linked. The marker Cfd49 is 1.9 cM from *SrTA10187* on 6DS (chromosome number 6) [19]. It is resistant to races TTKSK and TTKST (both Ug99-related), TTTTF, TPMKC RKQQC and QTHJC. Marker allele size for Cfd49 in hard winter wheat lines is 219 bp [19], whereas Guyomarc'h *et al*. [25] reported (224, 218 bp) in Chinese spring wheat lines. This implies that allele size could be different in different wheat lines. In the present study, varieties K6295-4A, Quai (Gambo), Sirbo, Hidase, Menze, and Honkolo, have shown characteristic diagnostic fragment of SrTA10187 gene (Table 4) confirming the presence of the gene.

As far as the number of genes detected per genotype was concerned, only varieties Hidase and Menze showed prescience of two genes. The rest of the varieties, however, exhibited the presence of only one gene. The most frequently detected gene, on the other hand, was SrTA10187, while Sr25 and Sr57 were the least frequent ones.

Table 4. *Presence/absence matrix of stem rust resistance genes detected by linked molecular markers in bread and durum wheat varieties.*

No.	Variety/cultivar name	*Sr22*	*Sr24*	*Sr25*	*Sr26*	*Sr31*
		WMC633	*XBarc71*	*BF145935*	*BE518379*	*RYE-NOR*
1	Kakaba	-	-	-	-	-
2	Sulla	-	-	-	-	-
3	Meraro	NA	-	-	-	-
4	K6295-4A	-	-	-	-	-
5	Dinkinesh	-	-	+	-	-
6	Dashen	-	-	-	-	-
7	Quai (Gambo)	-	-	-	-	NA
8	Hawi	-	-	NA	-	-

No.	Variety/cultivar name	*Sr22*	*Sr24*	*Sr25*	*Sr26*	*Sr31*
		WMC633	*XBarc71*	*BF145935*	*BE518379*	*RYE-NOR*
9	Abola	-	-	-	-	-
10	Tussie	NA	-	-	-	-
11	Galema	-	-	-	-	-
12	Galil	-	-	-	-	-
13	Tossa	-	-	NA	-	-
14	Pavon	-	-	-	-	-
15	Sirbo	-	-	-	-	-
16	Ogolcho	-	+	-	-	-
17	Hidase	-	-	-	-	-
18	Menze	-	-	+	-	-
19	Biqa	-	+	-	-	-
20	Shorima	-	-	-	-	NA
21	Alidoro	-	-	-	-	-
22	KBG-01	-	-	-	-	-
23	ET13A2	-	-	-	-	-
24	Dereselign	-	-	-	-	-
25	Enkoy	-	-	-	-	-
26	Bobicho	-	-	-	-	-
27	Huluka	-	-	-	-	-
28	Kulkulu	-	+	-	-	-
29	Bollo	NA	-	-	-	-
30	Honqollo	NA	-	-	-	-
31	K6290 Bulk	NA	-	-	-	-
32	Hogana	NA	-	-	-	-
33	Kubsa	-	-	-	-	-
34	Danda'a	-	-	-	-	-
35	Digelu	-	-	-	-	-
36	Tay	-	-	-	-	-
37	Sofumar	-	+	-	-	-
38	Kingbird	-	-	-	-	-
39	Meda-Wolabu	-	-	-	-	-
40	Millenium	-	-	-	-	NA
41	Lemmu	-	-	-	-	-
42	Wane	-	-	-	-	-
43	Morocco	-	-	-	-	-
44	Arendato	-	-	-	-	-
45	Mangudo	-	-	-	-	NA
46	Obsa	-	-	-	-	-
47	Tate	-	NA	NA	-	NA
48	Oda	+	-	NA	-	NA
49	Ilani	-	-	NA	-	-

Table 4. *Continued.*

No.	Variety/cultivar name	*Sr33*	*Sr36*	*Sr39*	*Sr52*	*Sr57*	*SrTA10187*
		XCFD 15	*XGWM319*	*Sr39#50*	*WMS570*	*cslv34*	*Cfd49*
1	Kakaba	-	-	-	-	-	-
2	Sulla	-	NA	-	-	-	-
3	Meraro	-	-	-	-	-	-
4	K6295-4A	-	-	-	-	-	+
5	Dinkinesh	-	-	-	-	-	-
6	Dashen	-	-	-	-	-	-
7	Quai (Gambo)	-	-	-	-	-	+
8	Hawi	-	-	-	-	-	-
9	Abola	-	-	-	-	-	-
10	Tussie	-	-	-	-	NA	-
11	Galema	-	-	-	-	-	-
12	Galil	-	-	-	-	-	-
13	Tossa	-	-	-	-	-	-
14	Pavon	-	-	-	-	NA	-
15	Sirbo	-	-	-	-	-	+
16	Ogolcho	-	-	-	-	-	-
17	Hidase	-	-	-	-	+	+
18	Menze	-	-	-	-	-	+

No.	Variety/cultivar name	*Sr33*	*Sr36*	*Sr39*	*Sr52*	*Sr57*	*SrTA10187*
		XCFD 15	*XGWM319*	*Sr39#50*	*WMS570*	*cslv34*	*Cfd49*
19	Biqa	-	-	-	-	-	-
20	Shorima	NA	-	-	-	NA	-
21	Alidoro	-	-	-	-	-	-
22	KBG-01	-	-	-	-	-	-
23	ET13A2	-	-	-	-	-	-
24	Dereselign	-	-	-	-	-	-
25	Enkoy	-	-	-	-	-	-
26	Bobicho	-	-	-	-	NA	-
27	Huluka	-	-	NA	-	-	-
28	Kulkulu	-	-	NA	-	-	-
29	Bollo	-	-	NA	-	-	-
30	Honqollo	-	-	NA	-	-	+
31	K6290 Bulk	-	-	-	-	-	-
32	Hogana	-	-	-	-	-	-
33	Kubsa	NA	-	-	-	-	NA
34	Danda'a	NA	-	-	-	-	-
35	Digelu	-	-	-	-	-	NA
36	Tay	NA	-	NA	-	-	-
37	Sofumar	NA	-	-	-	-	-
38	Kingbird	-	-	NA	-	-	-
39	Meda-Wolabu	-	-	-	-	-	-
40	Millenium	-	-	-	-	-	-
41	Lemmu	-	-	-	-	-	-
42	Wane	-	-	-	-	-	-
43	Morocco	-	-	-	-	-	-
44	Arendato	-	-	-	-	-	-
45	Mangudo	-	-	-	-	-	-
46	Obsa	-	-	-	-	-	-
47	Tate	NA	-	-	-	-	-
48	Oda	-	-	-	-	-	-
49	Ilani	-	-	NA	-	-	-

Note: The '+' symbol represents the presence of the gene while the '-' symbol indicates the absence of the gene under detection; NA represents "No Amplification"

3.3. Non-Detected Genes

Six of the target genes (*Sr26, Sr31, Sr33, Sr36, Sr39 and Sr52*) were not detected in the present study. Probably, they might be detected upon using a large set of diverse wheat germplasms such as landrace accessions, advanced breeding lines, introductions, and the likes. However, much off-target amplification has been exhibited by the linked markers of these genes. This might be related to existence of allelic variation for markers of the same gene. Such speculation, however, needs separate investigations to see if the variation is associated with the resistance gene markers or not. The gene *Sr31*effective against TKTTF, TTRTF, TKKTF and TTTTF, and *Sr36* which is effective against TTKSK, TKKTF and TTKTT needs a particular attention for current wheat breeding in Ethiopia. Specially, based on the 20 stem rust differentials for race characterization, *Sr36* is the only effective gene against TTKTT, and it might be helpful in the fight against the never sleeping rust races challenging wheat production.

3.4. Filed Resistance at Adult Plant Stage

The field resistance evacuation of the varieties at DZARC (internationally identified hotspot for stem rust) during the 2017 main season resulted in variable responses across the varieties (Table 5). The varieties responding with the reaction value of 0-25 MSMR and having the Average Coefficient of Infection (ACI) 0 - 9.7 are considered as resistant cultivars. Of the evaluated varieties in 2017, about 20 varieties showed good resistance to stem rust. Out of these varieties, 2 of them are durum wheat cultivars and the rest 18 are bread wheat varieties (Table 5). A comparison between the resistant cultivars in 2017 disease data with that of 2022 showed that only 5 out of the 20 had a resistance reaction in 2022, while the others even became susceptible. For instance, most of the mega bread wheat cultivars like Ogolcho have been delisted from production due to the occurrence of virulent stem rust race (TTKTT), which is virulent on *Sr24* and other genes. Among the genes detected by molecular markers, only *SrTA10187* seems to be effective for the rust population in the filed under natural conditions even if it varies from 2017 to 2022. However, the rest of the genes look non-effective particularly when moving from 2017 data to 2022 data depicting that they turn from low ACI to higher values clearly indicating ineffectiveness.

Table 5. *Field stem rust resistance of wheat varieties in field evaluation at Debre Zeit Agricultural Research Center in 2017 and 2022 main seasons.*

No.	Variety/Cultivar name	*Detected genes with linked markers*	Field Resistance Score 2017		Field Resistance Score 2022	
			Final Score	ACI	Final Score	ACI
1	Kakaba	_	40S	24.3	40MSS	34
2	Sulla	_	20MSS	10	_	_
3	Meraro	_	5MS	2.7	30MS	14
4	K62954A	*SrTA10187*	TMS	2.7	_	_
5	Dinknesh	*Sr25*	60S	41.2	80S	53.5
6	Dashen	_	30MSS	13	20M	8
7	Quai (Gambo)	*SrTA10187*	40S	24.3	_	_
8	Hawi	_	30MSS	17	60S	38
9	Abola	_	50S	31.3	60S	50
10	Tussie	_	10MSS	5.8	30MSS	21.5
11	Galema	_	40MSS	17.3	60S	41.25
12	Galil	_	20MSMR	6.7	20MS	12
13	Tossa	_	30MS	10.7	_	_
14	Pavon-76	_	15MSS	5.8	50MSS	45
15	Sirbo	*SrTA10187*	30MSS	10.3	40S	31.25
16	Ogolcho	*Sr24*	TMS	2.7	60S	50
17	Hidase	*sr57, SrTA10187*	30MSS	14.7	70S	48.5
18	Menze	*Sr25, SrTA10187*	20MSS	10	_	_
19	Biqa	*Sr24*	30MSS	20	30MS	18
20	Shorima	_	TMS	2.7	20MS	12
21	Alidoro	_	TMS	2.7	20MS	10
22	KBG-01	_	_	_	_	_
23	ET13A2	_	5MS	2.7	30MSS	27
24	Dereselign	_	40MSS	29.3	50S	35
25	Enkoy	_	0	0	0	0
26	Bobicho	_	40MSS	22.3	70S	48.5
27	Huluka	_	0	0	10MS	6
28	Kulkulu	*Sr24*	5MS	2.7	20MS	10
29	Bollo	_	40S	18.7	_	_
30	Honqollo	*SrTA10187*	TMS	2.7	20M	8
31	K6290Bulk	_	25MSMR	6	20MS	9.5
32	Hogana	_	TMS	2.7	25MS	12
33	Kubsa	_	40S	23.7	50S	35
34	Danda'a	_	15MSS	5.8	50SMS	32.5
35	Digelu	_	40MSS	19.5	60S	40
36	Tay	_	40SMS	22.7	10M	5
37	Sofumar	*Sr24*	30MSS	13.5	30MSS	15.5
38	Kingbird	_	10MS	4	50SMS	32.5
39	Meda-Wolabu	_	25MSS	9.2	30MSS	23.5
40	Millenium	_	10MRMS	3.3	20M	9
41	Lemmu	_	_	_	_	_
42	Wane	_	_	_	_	_
43	Morocco	-	_	_	_	_
44	Arendato	_	30MSS	14.3	20M	9
45	Mangudo	_	_	_	_	_
46	Obsa	_	30MSS	14.7	40S	31.25
47	Tate	_	40MSS	18.4	40S	22.5
48	Oda	*Sr22*	30MSMR	14.7	_	_
49	Ilani	_	20MSMR	9	_	_

Note: The symbol '-' indicates absence of Detected gene and absence of the Varieties in the field evaluation of that specific year

3.5. Seedling Resistance Under Controlled Environment (Greenhouse)

The seedling resistance test conducted on the 49 varieties at Ambo Agricultural Research Center (AARC) under controlled environment in the greenhouse gave high to low infection types (IT) across the four single races pathotypes and the bucked sample (Table 6). The proportion of Resistant (R) to Susceptible (S) variety varied from 12:36 for TTKTT; 40:8 for TKTTF; 39:9 for TTKSK; 44:4 for TTTTF and 35:13 for the bulked sample. Variety Sirbo was missing and no data was generated for it. Eight varieties (Sulla, Gallil, Huluka, Kingbird, Millenium, Obsa, Tate and Ilani) exhibited resistant reaction consistently across the four pathotypes and the bulked sample. Five of these varieties are bread wheat, while three are durum wheat varieties. These varieties can be targeted as potential resistance sources in wheat breeding depending on desirable traits they have. As far as the effectiveness of the detected genes is concerned, *Sr22* in variety Oda and *Sr25* in variety Dinknesh appeared to be effective including for the bulked sample for TTKTT,

TKTTF and TTKSK in the former and TKTTF, TTKSK and TTTTF in the latter. Besides, none of them were consistently effective across the pathotypes as well. Only *SrTA10187* and *Sr22* appeared to be effective for the recently emerging TTKTT; this indicates that this pathotype is aggressively defeating many of the genes and may result in an epidemics in due time. Hence, research strategies should focus on a quick identification of new resistance sources, and development of resistant varieties for quick deployment in the production.

Table 6. *Seedling resistance score (Infection Type (IT)) of tested wheat varieties as resulted from screening against four Pgt Pathotypes in the year 2019.*

No.	Variety/Cultivar	*Detected genes with linked markers*	TTKTT		TKTTF		TTKSK		TTTTF		BULK	
			IT	RES	IT	RES	IT	RES	IT	RES	IT	RES
1	Kakaba	_	3-	S	3-	S	3-	S	2	R	3-	S
2	Sulla	_	;2-	R	;1	R	;	R	;1	R	2-	R
3	Meraro	_	3	S	3-	S	;	R	3-	S	2+	R
4	K62954A	*SrTA10187*	;1	R	3	S	3-	S	;1	R	3-	S
5	Dinknesh	*Sr25*	3	S	;	R	;	R	;1	R	;1	R
6	Dashen	_	3	S	;1	R	;1	R	;1	R	;1+	R
7	Quai (Gambo)	*SrTA10187*	2+3-	S	;1	R	3-	S	2	R	2-	R
8	Hawi	_	3-	S	;1+	R	;3-	S	2-	R	2+	R
9	Abola	_	3-	S	0	R	;1	R	;1	R	2+	R
10	Tussie	_	3-	S	;1	R	;	R	;	R	3-	S
11	Galema	_	3-	S	;	R	;2	R	;1	R	2+	R
12	Galil	_	2	R	;	R	;	R	;	R	2+	R
13	Tossa	_	3-	S	;1+	R	;1	R	2+	R	2	R
14	Pavon-76	_	3-	S	;	R	;	R	;1+	R	2+	R
15	Sirbo	*SrTA10187*	-	-	-	-	-	-	-	-	-	-
16	Ogolcho	*Sr24*	3-	S	0	R	;	R	;1	R	;1	R
17	Hidase	*sr57, SrTA10187*	3-	S	3-	S	;2	R	;2-	R	2+	R
18	Menze	*Sr25, SrTA10187*	3-	S	3	S	3-	S	3-	S	3-	S
19	Biqa	*Sr24*	3-	S	0	R	;1+	R	;2	R	;2+	R
20	Shorima	_	3-	S	;1	R	;	R	;1+	R	;1	R
21	Alidoro	_	;1+	R	0	R	;2	R	;1	R	3-	S
22	KBG-01	_	3-	S	3	S	3-	S	2+	R	2-	R
23	ET13A2	_	3-	S	;	R	;	R	0	R	3-	S
24	Dereselign	_	3-	S	1	R	;	R	0	R	3-	S
25	Enkoy	_	2	R	1	R	;1	R	;1+	R	3-	S
26	Bobicho	_	3-	S	0	R	;2	R	1+	R	3-	S
27	Huluka	_	2+	R	;1	R	;	R	;1	R	;1	R
28	Kulkulu	*Sr24*	3-	S	;1	R	;1	R	;1	R	;1	R
29	Bollo	_	3	S	0	R	3-	S	2+	R	3-	S
30	Honqollo	*SrTA10187*	3	S	;1	R	;	R	;1	R	2+	R
31	K6290Bulk	_	3-	S	3	S	3-	S	2+	R	3-	S
32	Hogana	_	3	S	;1+	R	;	R	;1	R	2-	R
33	Kubsa	_	3-	S	2	R	;2	R	3	S	2	R
34	Danda'a	_	3-	S	;1	R	;1	R	;1	R	3	S
35	Digelu	_	3-	S	0	R	3-	S	2+	R	3-	S
36	Tay	_	3-	S	;1	R	;1	R	;1	R	2	R
37	Sofumar	*Sr24*	3-	S	;1	R	;	R	;1	R	;1	R
38	Kingbird	_	2+	R	;1	R	;	R	2	R	;2	R
39	Meda-Wolabu	_	3-	S	;	R	;2+	R	2+	R	2+	R
40	Millenium	_	2+	R	;1	R	;	R	;1	R	;1	R
41	Lemmu	_	3-	S	;1	R	;	R	;1	R	;1+	R
42	Wane	_	3-	S	;1	R	;1	R	;1	R	;1+	R
43	Morocco	-	3-	S	2-	R	;2	R	2-	R	;2-	R
44	Arendato	_	3-	S	3-	S	;1	R	2+	R	2	R
45	Mangudo	_	3-	S	;	R	;1	R	;1	R	;2-	R
46	Obsa	_	;1+	R	;	R	;1	R	;1	R	2+	R
47	Tate	_	;1 2+	R	1	R	2-	R	2+	R	2-	R
48	Oda	*Sr22*	2+	R	;	R	2+	R	3-	S	;1+	R
49	Ilani	_	1	R	;	R	;1	R	;1	R	;1+	R
50	MacNair	_	3+	S	3	S	3+	S	3+	S	3-	S

Note: The symbol '-' indicates absence of Detected gene and absence of the Varieties in the seedling test for that specific stem rust race

3.6. Seedling Resistance Versus Adult Plant Resistance

Seedling resistance also called monogenic or major genic resistance is the resistance that is controlled by a single gene and usually expressed as presence or absence. Adult plant resistance on the other hand, is polygenic and known to be controlled by many genes of minor effect which collectively provide durable resistance. Besides, they are expressed

mostly at the adult plant stage. Combining investigation of the resistant response groups between seedling and adult plant stages resulted in various proportions across the six Response groups: RR (20.9%), RI (25.6%), RS (23.3%), SR (9.3%), SI (11.6%) and SS (9.3%) (Figure 4).

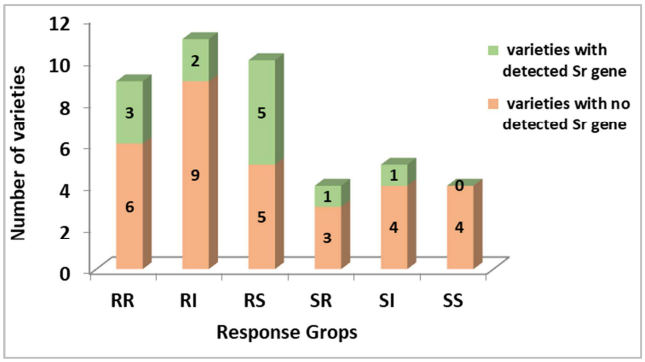

Figure 4. *Combined response of wheat varieties in Seedling and Adult plant stage. (R: Resistance, I: intermediate and S: Susceptible).*

Obviously, the RI response group is the one which had the highest proportion of the tested varieties with the SI & SS groups attained the least. In the global view, in terms of number of varieties, seedling resistance has outnumbered adult stage resistance in the ratio of 30:13 where 10 varieties are commonly resistant in both under response group RR. This is reflection of the current wheat improvement program in our research system which at large dwells on hunting monogenic rust resistance genes than the relatively durable polygenic adult plant resistance.

Apart from the phenotypic seedling and adult stage resistance, some of the varieties in the various response groups were found to harbor the known and already reported Sr genes. Accordingly, RS had the highest number of (5) Sr genes while SS has none. This is interesting because those varieties having phenotypic resistance but don't have any of the Sr genes means potentially, they have novel resistance genes. For instance, 9 of the varieties in response group RI are resistant in seedling stage and intermediate response in adult stage but none of them have the known Sr genes. Such varieties can be targeted as good parental lines to start breeding for rust resistance. In general, varieties in response groups RR and RI can be regarded as a good source of resistance for further wheat improvement.

4. Conclusions

For most of the reported stem rust resistance genes, the linked/diagnostic markers have enabled successful detection process with the given PCR setup. However, detected resistance genes have not been sequenced and investigated in the present study. Therefore future detection activities should be coupled with fragment sequencing and checking. The effectiveness of the linked/diagnostic markers for the detection process is good; however, most of them have resulted in many off-target amplifications. Therefore, as fragment based diagnosis is subject for non-specificity over time, a more stable method of diagnosis such as Kompetitive Allele Specific PCR (KASP) based SNP assay should be sought for and used in similar future research works.

Although most of the genes detected here are already known to be defeated by the virulent races, the varieties containing them can be recipient parents for marker assisted introgression of other undefeated resistance genes for Ethiopian races.

Coupling the molecular detection with phenotypic screening under field natural pathogen population and against known pathotypes under controlled greenhouse conditions is a very relevant method to maximize reliability of results. That is mainly because it provides a way of cross-checking results at the foreground obtained from phenotyping with that of results at the background obtained from the genotyping at molecular level.

A combined look at both seedling and adult plant stage resistance should be sought in the research towards achieving durably resistant cultivars. Such approach, in some cases reveals novel sources of resistance which complement each other and lays down the basis for durable rust resistance.

Conflict of Interest

The authors have declared no conflict of interest about this work.

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