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RESEARCH PAPER

Molecular characterization of Pakistani wheat genotypes for leaf rust resistance

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Key Message: This research study was carried out to find out the major leaf rust resistance genes in indigenous lines of wheat in Pakistan using DNA markers, and it was successful in identifying resistant genes in all of the studied lines.

ABSTRACT: Brown rust caused by *Puccinia triticinia* is an individual of the absolute negative and detrimental wheat disease. Issue of leaf rust and yield losses can easily be reduced by emerging new cultivars of wheat by following advanced approaches and techniques. Marker assisted selection (MAS) is one of the prominent ways to get the goals quickly and accurately. Information regarding the presence of leaf rust gene is almost zero in Pakistani accessions. Therefore, the present study was designed to find out the major leaf rust resistance genes in indigenous lines using DNA markers. A total of seven markers including three inter simple sequences repeats (ISSR) and four simple sequences repeats (SSR) were used for reorganization of resistant genes in 30 advance lines. Markers *Xgwm314*, *Xgwm159*, *CsLV-34*, *UBC659*, *UBC-818* and *UBC-873* showed the presence of resistant genes. Genetic variability was observed for leaf rust genes in the advance lines of Pakistan. *Xgwm314* (*Lr34*) showed its presence in 26% of the used lines. Similarly, marker *Xgwm159* (*Lr60*) was found to be present in 30% of wheat lines. *CsLV34* was present in 13% of the wheat lines. ISSR markers were able to detect resistant genes in all of the studied lines. Moreover, this study was successful in identifying rust resistant lines and these results will help in designing and hybridization of new programs by breeders.

Keywords: Marker assisted selection, DNA markers, Rust resistant genes, wheat genotypes

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is commonly grown food crop internationally. It is cultivated on about one sixth of the total land in the world. In Pakistan, average yield during the year 2017 was reported to be 2973 kg/ha with overall production of 26.7 million tones (Food and Agriculture Organization [FAO], 2019). Out of all biotic diseases, rust is one of the main threats to crop production all over the world. Brown rust caused by *Puccinia triticinia* is a major factor in declined growth all over the world especially in Asia (Duveiller et al., 2007). Brown rust in bread wheat and rye is one of the lethal fungal diseases that affects yield drastically (Duveiller et al., 2007). North and South America and most of the regions of Mexico have been badly affected by this disease giving heavy losses in term of yield. Prominence of leaf rust is critical in winter wheat rather than spring wheat, causing 1-20% loss in yield as the leaves infected by this disease become weak and unable to withstand the stress. The fungus is an obligate parasite

capable of producing infectious urediniospores by depleting all the nutrients, as long as infected plant and leaf tissue remains alive (Bolton et al., 2008).

One of the main methods to counter rust is spraying i.e. chemical control that is costly, and a lot of risks are involved especially to environment (Agriculture Victoria Government, 2019). One of the active approaches to minimize wheat losses is the identification of resistance genes and to incorporate them through different breeding programs to make varieties rust resistant (Ellis et al., 2014) .Conventional breeding can have some limitations which involves various environmental issues, linkage genes and low genetic base for particular trait (Tester & Langridge, 2010). All of these issues can be resolved to more extent by recent advancement known as marker assisted selection (MAS) in molecular genetics by identifying and integrating genes of interest (Xu and Crouch, 2008).

Up till now, scientists have been successful in developing molecular marker systems for tagging genes for various traits. All important classes like RAPD ($S336_{775}$, $S3_{450}$) (Nsabiyera et al., 2016), RFLP (Sr26#43 for the shortest 6Ae#1 chromosome segment) (Dundas et al., 2015), AFLP (Sr26 was identified by DNA fragment 303-bp (Jawhar et al., 2016), the most important SSR (gwm429, barc07, barc55 (Nsabiyera et al., 2016) and ISSR (HBS10, HBS11 and 844A (Shapana et al., 2018) come in this system. Closely linked markers to race-specific rust resistance genes have been identified in wheat (Lagudah, 2011). These molecular markers of resistance genes offer new prospects for efficiently selecting desirable resistant genes through marker assisted selection (MAS). Molecular markers also enable the combination, selection and sketching of resistant genes in wheat breeding through MAS (Bartos et al., 2002). The present study was designed to characterize 30 advanced lines of wheat for leaf rust resistance using SSR and ISSR markers.

MATERIALS AND METHODS

Plant material

The study was designed for 30 advanced lines of wheat including NUWYT lines of 2008-09 (Table 1). Seeds were kindly provided by Wheat Research Programme, NARC, Islamabad, Pakistan.

DNA extraction

DNA was extracted from seven day old seedlings of 30 wheat lines (dry mature seed) (Table 2) using the procedure described by Kang et al. (1998). Healthy seeds (5 to 6) were crushed by tissue lyzer 2 apparatus and then they were transferred to micro centrifuge tubes (1.5 µl). Then, 600 µl of 2 percent CTAB was added to DNA solution and incubated at 65 ^{0}C for 30 min. Then centrifugation was performed at 12000 rpm for 12 min. Supernatant was then transferred to new tube by adding chloroform: isoamyl alcohol and inverted gently 4-5 times followed by centrifugation. Chilled iso-propanol was added afterwards and again incubated for 10 min at 4 ºC. After centrifugation pellet was removed and washed with 75% ethanol and oven dried for 30 min. The pellet was re-suspended in 100 µl of nuclease free water.

Lambda standard preparation

Standard contained (i) Lambda DNA, (ii) 1x loading dye and (iii) ddH_2O. Standard solution of 500*u*l with 30 ng/µl concentration was prepared by using the following standard formula:

$C_1V_1 = C_2V_2$

Where 'C_1' is stock concentration of Lambda standard (341 ng/µl); 'V_1' is the volume of Lambda stock required; 'C_2' is the desired concentration (30ng/µl); and 'V_2' is the total volume of the standard solution to be made (500 µl).

Lambda stock volume = (30*500)/341 = 44 µl

Similarly, volume of the 6x loading dye was calculated and the rest of volume was made by ddH_2O.

Gel electrophoresis for DNA quantification

DNA quantification was done by using 1% agarose gel. 5 µl of Lambda DNA standard and 4 µl of DNA samples with 1 µl 6X loading dye was loaded onto the gel. The gel was allowed to run for about 40 min at 100 volts. Visualization of gel was done in gel documentation system for scoring of bands. Samples concentration (DNA) was done by relating the strength of the bands with those of Lambda DNA standard (Table 2). DNA samples were diluted to about 2.0 ng/µl for PCR analysis.

PCR analysis

Polymerase chain reaction markers reported in Lagudah et al. (2006) was used to determine the presence/absence of *Lr-34/Yr18* gene cluster. Polymerase chain reaction was done in a reaction mixture of 20 µl. The reaction mixture contained 1× PCR buffer with $(NH_4)_2SO_4$, 3.0 mM $MgCl_2$, 0.2 mM dNTPs mix, 10 pmol (pico moles) each of the reverse and forward primers of all the primers used (Table 3-4), one unit of Taq DNA polymerase (Ferments, Life Sciences) and DNA template of 10-20 ng. PCR amplification was carried out in an automated Applied Bio-systems Thermal Cycler (Verity 96 well) at 94 ºC for 5 min, followed by 35cycles each consisting of three steps; one step of denaturation at 94ºC for 40 sec, one step of annealing at 57 ºC for 30 sec and an extension step for 40 sec at 72 ºC. At the end, 10 min were given to final extension 72 ºC. Amplified products were electrophoresed on 1.5% agarose gel stained with ethidium bromide. Gel was read in gel documentation system for scoring of bands (Table 3).

RESULTS AND DISCUSSION

Amplification of genomic DNA was done in 30 advanced breeding lines of Pakistani wheat. SSR marker *Xgwm159* was used to distinguish the presence of *Lr60* gene whose presence is indicated by the amplification of a 200 bp fragment. Out of 31 advanced lines (with check), only six lines showed the absence while all other were positive but11, 15 18 and 25 wheat line showed dim bands of 200-bp fragment specifying the presence of *Lr60* gene (Table 5). Bar graph (Fig 1a-b) shows that more bands frequency were observed by ISSR primers in both of the cases, while SSR markers were unable to detect the genes in many of the 31 accessions, moreover the missing bands area is mostly contributed by SSR primers (Fig. 2). Similarly, primer ISSR UBC-818 showed its presence in all of the accessions, while ISSR-873 was present in all accessions except accession 8. Similarly, ISSR-659 also showed bands in all accessions except 23 and 28. Comparing ISSR with SSR it is clear that former performed better than later as their band frequency was 0.37, 0.81, and 0.81 as compared to 0.97, 1and 0.94 (Fig 1a).

Microsatellite markers *Xgwm314* were used to detect the presence/absence of *Yr26* gene, which amplified a 150-bp fragment in 25 wheat lines (Table 5) (Fig. 3-4). All advance wheat lines were checked by *CSLV34-F* and *csLV34-R* primers (*Lr34/Yr18*) yielded a PCR product of 150 bp amplification (Fig. 3-4). Their size was approximately same with that of positive control in almost 14 of the advanced breeding lines. These results clearly show that these advance lines hold genes of *Lr34/Yr18* which belongs to leaf and stripe rust. All other lines studied had a PCR fragment of 229 bp similar in size to that of the negative control AVS suggesting that these lines possessed the susceptible allele at *Lr34/Yr18* locus. No amplification was observed for AVS, SD13 and SD1.

Lr34 is located on chromosome 7DS (Dyck, 1987) and is an adult plant leaf rust resistance gene first described by Dyck (1977). *Lr34* gene is tightly linked with *Yr18*, a yellow rust resistance gene (Lagudah et al., 2006), and is also completely linked with leaf tip necrosis (Singh, 1992). The adult plant resistance conferred by *Lr34* is due to reduction in haustorium formation in early stage of infection, reduced colony size and a longer latency period, in association with relatively little or no host plant cell necrosis (Rubiales & Niks, 1995). Singh et al. (2005) reported that an unknown electro-dense substance (cell wall apposition) is accumulated in the cell walls of *Lr34* carrying wheat genotypes on the site of infection that hinders the penetration of haustorium tube. The use of *Lr34/Yr18* along with some other minor genes to be incorporated in varieties can help farmer to get near to complete resistance for stripe and leaf rust infection (Singh et al., 2000). In an environment of rust pores development, the lines which hold *Lr34* gene had 40% lethality, while lines which had *Lr34* along with some other minor genes (2-3 only) showed almost 1-5% lethality as compare to nearly 100% leaf rust lethality for susceptible lines (Singh et al., 2004). Similarly, *Yr18*, in combination with 3-4 additional minor genes confer adequate level of resistance to stripe rust (Singh et al., 2000).Whereas ISSR showed its presence in all of the accessions except 27.

Results of the present study demonstrated that a reasonably large number of advanced breeding lines included in NUWYT of Pakistani carried the leaf and yellow rust resistance gene complex Lr34/Yr18. Previously, most adapted wheat varieties have become susceptible to leaf and yellow rusts. So approval of these lines as commercial varieties will increase the genetic diversity in terms of rust resistance genes. This will ensure the improved wheat production and will help in reduction of global wheat loss along with other rust resistance genes in varieties. The DNA markers used in this study can greatly facilitate the incorporation of these two tightly linked genes. It is strongly recommended that the advanced breeding lines included in the NUWYT should be studied more for the presence of major rust resistance genes using the molecular markers. This will ensure the release of wheat varieties with improved rust resistance genes in future.

Table 1 Parentage of entries for National Uniform Wheat Yield Trials (NUWYT) advanced wheat lines (2008-09)

No.	Line/Variety	Parentage/Pedigree	Institute
RF-1	DN-62	SW89.5181/Kauz CMSS93B00824S-24Y-010M-010Y-010M-9Y-0M-0HTY	ARI, D. I. Khan
RF-2	V-04178	SH88/90A-204//MH97	WRI/AARI, Faisalabad
RF-3	SM-07018	Shalimar-88/Atilla//MH97	NIFA, Peshawar
RF-4	22-03	Snb(s')//Kea(s')/Snb(s')	NIA, Tandojam
RF-5	B-07/Bkhtwr	LFN/1158.57//Prl/3/Hahn/4/Kauz CMBW 89Y1044-0t0PM-8Y-010M-020B-0NPL-0T0Y	WRI, Sakand
RF-6	V-05082	Chenab2000/INQ.91	WRI/AARI, Faisalabad
RF-7	PR-90	CNDO/R143//Ente/Mexi-213/… CMSS93Bo1824M-040Y-73Y-010M-010Y-010M-10Y-0M	CCRI, Pirsabak
RF-8	ZAS70	Inqalab 91*2/Tukuru CGSS99B00015F-099Y-099M-099Y-099M-31Y-0B	UAP, Biotechnology
RF-9	33010	KT/Bage//Fnu/3/CH-86 BR.4457-1B-5B-3B-0B	RARI, Bahawalpur
RF-11	AUP-4008	Gen*2//Buc/Flk/3/Buchin CMSS96M03098S-12M-010SY-010M-010SY-3M-0Y	UAP, PBG
RF-12	NIA-8/7	SHA4/Weaver//Skauz*2/SRMA	NIA, Tandojam
SD-1	V-05066	Amsel/Attila//INQ.91/Pew'S'	WRI/AARI, Faisalabad
SD-3	CT-03457	Attila*2/Yaco CGSS96B00134F-099B-028Y-099M-4Y-0B	NIFA, Peshawar
SD-4	66284	INQ-91/CB-271	RARI, B.Pur
SD-9	SD-4085/3	Sarsabz/Sunco*2	NIA, Tandojam
SD-5	NR-356	Oasis/Skauz//4*BC/3/2*Pastor CMSS00Y01881T-050M-030Y-030M-030WGY-33M-0Y-01D	NARC, Islamabad
SD-6	9268	7012/PBW-222	UAF, PBG
SD-7	V-05BT006	Maya/Mon'S//Hork/Fsd85 Biotech-0R4-1R1-2R7-3RK-0R	WRI/Biotech, Faisalabad
SD-8	V-04022	INQ.91/3/Crow/Nac//Bow'S'	WRI/AARI, Faisalabad
SD-10	NR-358	PFAU/Weaver*2//Kiritati CGSS01B00076T-099Y-099M-099B-75Y-0B-01D	NARC, Islamabad
SD-11	PR-98	CMH84.3379/CMH78.578//Milan CMSS93Y006285-7Y-010Y-010M-010Y-10M-0Y-3KBY-0KBY	CCRI, Pirsabak
SD-12	NR-360	PFAU/Seri.1B//AMAD/3/Waxwing CGSS02Y00153S-099M-099Y-099M-46Y-0B-01D	NARC, Islamabad
SD-13	SN-151	Kambara-1 CGSS9500016F-099Y-099B-099Y-099B-15Y-0B-0SY	ARS, S Naurang, Bannu
SD-14	04FJS35	PASTOR//HXL7573/2*BAU CMSS97M00306S-0P5M-095Y-90M-010Y	BARS, Fateh Jang
SD-15	AZRC-2008-1	Tracha's//CMH76-252/Pvn's ICW93-0065-6AP-0L-3AP-0L-1AP-0AP	AZRC, Quetta
SD-16	PR-99	Hamam-4/Star"S"/Liz 0F-0K-2F-0K	CCRI, Pirsabak
SD-17	KT-4	ALTAR84/AE.SQUARROSA219//SER CMBW91Y00892S-8Y-11KBY-2KBY-010M-9Y-3M-0Y-0SY	BARS, Kohat
SD-18	V-05003	Karvan2/4/Burgus/Sort12-13//Kal/BB/3/Pak81	WRI, AARI, Faisalabad
SD-19	NRL-0320	FRET 2 CGSS96Y00146T-099B-099Y-099B-16Y-0B-0SY	NIFA, Peshawar

Table 2 Plant material used for characterization of Pakistani wheat genotypes for leaf rust resistance

Line	DNA concentration (ng/µl)	Line	DNA concentration (ng/µl)
RF-1	150	SD-5	80
RF-2	150	SD-6	25
RF-3	150	SD-7	100
RF-4	40	SD-8	25
RF-5	200	SD-10	75
RF-6	200	SD-11	25
RF-7	200	SD-12	25
RF-8	75	SD-13	10
RF-9	40	SD-14	10
RF-11	40	SD-15	15
RF-12	75	SD-16	150
SD-1	50	SD-17	10
SD-3	40	SD-18	10
SD-4	40	SD-19	15
SD-9	25	Positive control	30

Table 3 Marker type along with gene linkage used for characterization

S. No.	Primer name	Linkage	Marker type
1	UBC-659	*Lr34*	ISSR
	UBC-818	*Lr34*	ISSR
2	UBC-873	*Lr34*	ISSR
	Xgwm-159	*Lr34*	SSR
3	Xgwm-314	*Lr34*	SSR
	CSLV34	*Lr34/Yr18*	SSR (Gene specific)

Table 4 Primer pair and their sequences used for detecting gene cluster

S. No.	Primer	5`- 3` sequence	Product size	PIC value
1	UBC-659	GTCACGTCCT	520-1550 bp	0.902
2	UBC-818	CACACACACACACACAG	200-1400 bp	0.889
3	UBC-873	GACAGACAGACAGACA	250-1000 bp	0.896
4	Xgwm-159	5' GGGCCAACACTGGAACAC 3' 5'GCAGAAGCTTGTTGGTAGGC 3'	197 bp & 200 bp	0.927
5	Xgwm-314	5'AGGAGCTCCTCTGTGCCAC3' 5'TTCGGGACTCTCTTCCCTG3'	174 bp & 165 bp	0.927
6	CSLV34	5' AACTTTCCCACCACCACCGCGG 3' 5' ACCGTCGCCTTCCACTTTCCCC 3'	+150 bp & -229 bp	0.984

Table 5 Characterization of 30 Pakistani advanced wheat lines for Lr60, Lr34/Yr18 locus

S. No.	Advanced Line	ISSR-UBC-818	ISSR-UBC-873	ISSR-UBC-659	GWM-314	XGWM-159	*CSLV-34*
1	RF-1	+	+	+	+	-	+
2	RF-6	+	+	+	+	-	+
3	RF-7	+	+	+	+	-	+
4	RF-8	+	+	+	+	-	+
5	RF-11	+	+	+	+	+	+
6	SD-3	+	+	+	+	-	+
7	SD-6	+	+	+	+	+	+
8	SD-9	+	-	+	+	+	+
9	RF-3	+	+	+	-	+	+
10	RF-4	+	+	+	+	+	-
11	RF-12	+	+	+	+	+	+
12	SD-1	+	+	+	+	+	+
13	SD-4	+	+	+	+	+	+
14	SD-5	+	+	+	+	+	+
15	SD-8	+	+	+	+	+	-
16	SD-10	+	+	+	+	-	-
17	SD-13	+	+	+	+	+	-
18	SD-16	+	+	+	+	+	-
19	SD-11	+	+	+	-	+	-
20	SD-14	+	+	+	-	+	-
21	SD-18	+	+	+	-	+	-
22	SD-19	+	+	+	+	+	-
23	SD-17	+	+	-	+	+	-
24	SD-12	+	+	+	-	+	-
25	SD-20	+	+	+	+	+	-
26	RF-5	+	+	+	+	+	-
27	SD-7	+	+	+	+	+	-
28	RF-2	+	+	-	+	+	-
29	RF-9	+	+	+	-	+	-
30	SD-15	+	+	+	+	+	-
31	Positive control	+	+	+	+	+	+

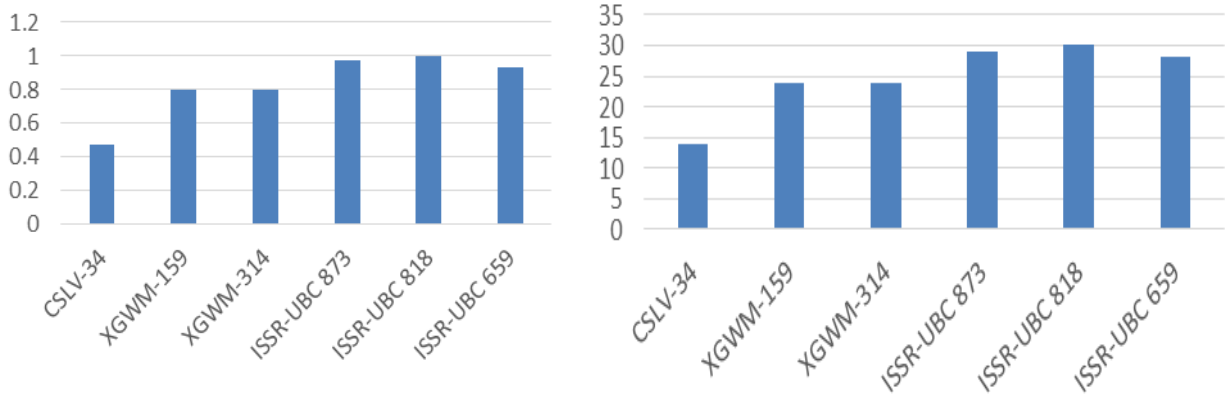

Fig. 1 (a) Bar graph showing average band frequency and (b) total number of bands observed for each SSR and ISSR markers in advanced wheat lines

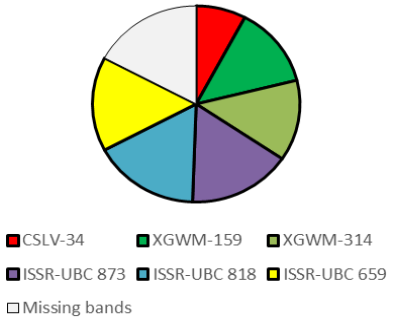

Fig. 2 Pie chart showing comparison of ISSR and SSR marker for advanced wheat lines

CONCLUSIONS AND RECOMMENDATIONS

Results of the present study confirmed that ISSR markers gave probable and reproducible results as compared to SSR markers. Our study specifies that ISSR markers are better for revealing genetic diversity in wheat germplasm. Furthermore advanced wheat lines were having important rust resistant genes. It is recommended that these advanced breeding lines should be used for breeding programs aimed at rust resistance which will ultimately help breeders to develop wheat varieties by pyramiding leaf rust resistance genes to overcome yield losses in future.

Author Contribution Statement: Sania Begum, Armghan Shahzad, Muhammad Fayyaz and Ghulam Muhammad Ali planned the experiment, guided the students for research and provided the materials used for this research study. Sahir Hameed Khattak, Saima Noor, Syed Zaheer uddin, Zabih ullah and Farhatullah executed the experiment and wrote the manuscript. Wajya Ajmal contributed in the analysis of data.

Conflict of Interest: The authors declare that they have no conflict of interest.

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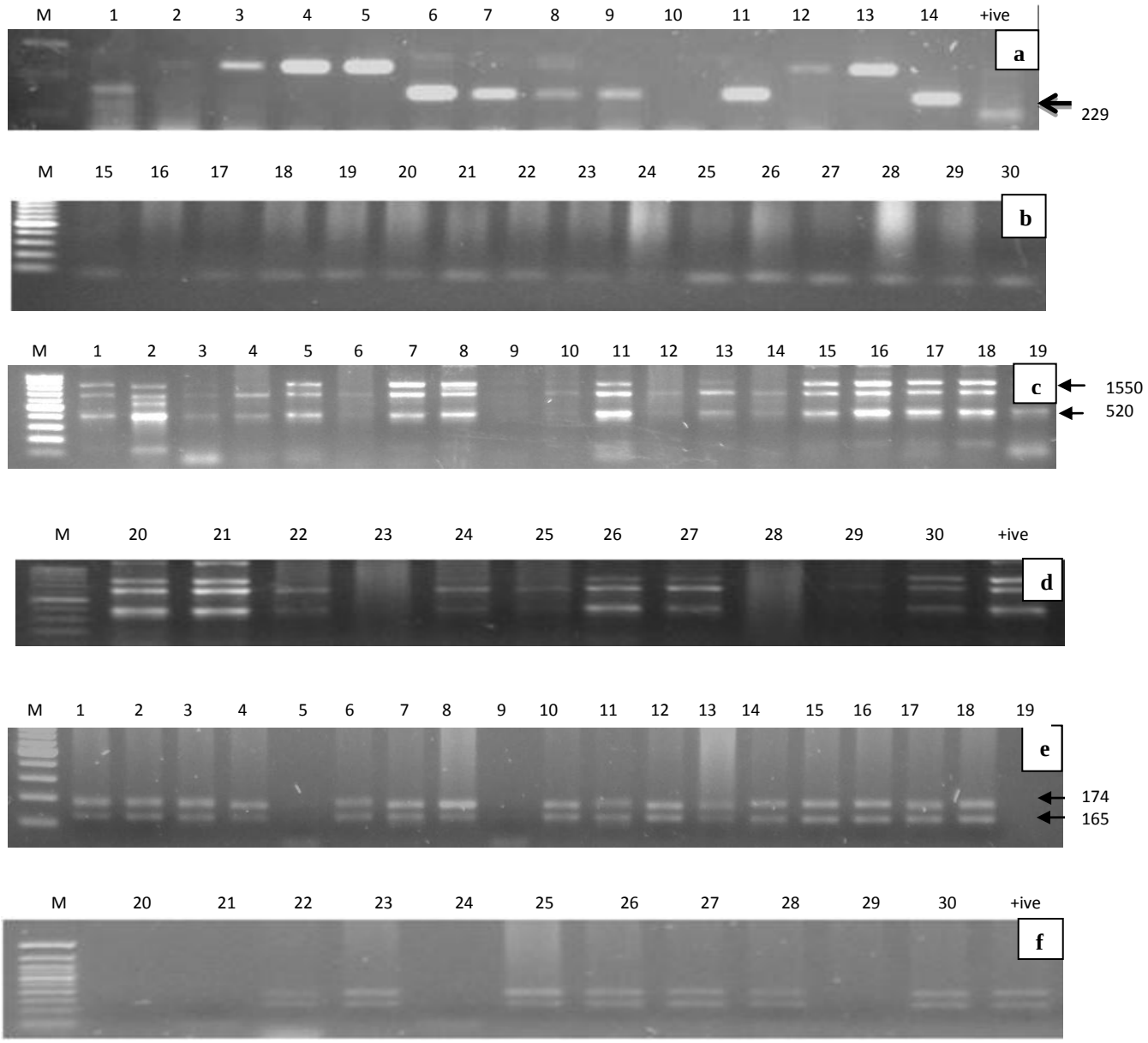

Fig. 3 a. Microsatellite *CSLV-34* marker with 100 bp ladder for 1-15 accession; **b.** Microsatellite *CSLV-34* marker with 100 bp ladder for 15-30 accession; **c.** Microsatellite ISSR *UBC-659* marker with 100 bp ladder for 1-19 accession; **d.** Microsatellite ISSR *UBC-659* marker with 100 bp ladder for 20-31 accession; **e.** Microsatellite *XGWM-314* marker with 100 bp ladder for 1-19 accession; **f.** Microsatellite *XGWM-314* marker with 100 bp ladder for 20-30 accession for detection of rust resistant genes in 32 advanced wheat lines.

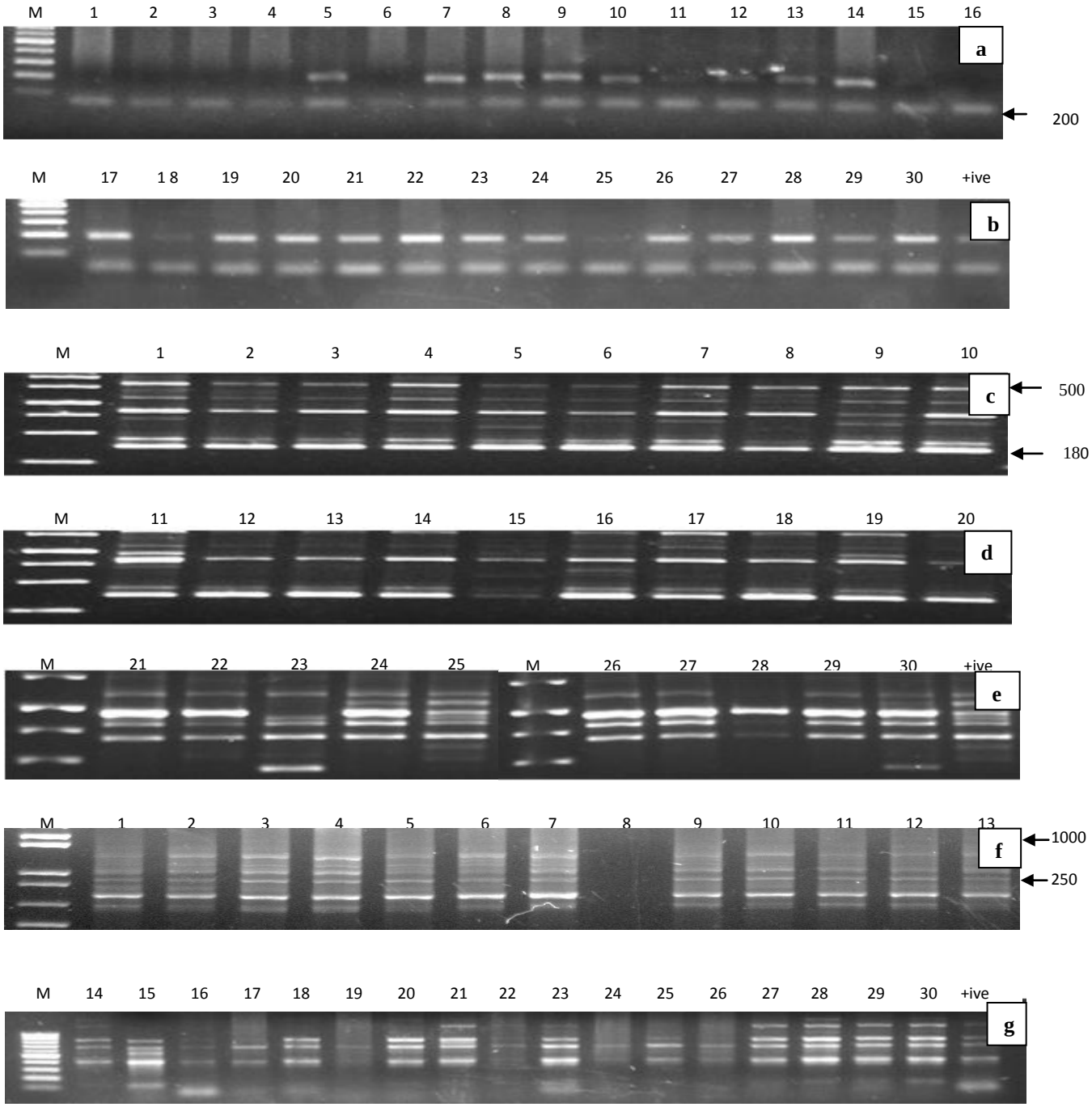

Fig. 4 a. Microsatellite *XGWM-159* marker 100 bp ladder for 1-16 accession; **b.** Microsatellite *XGWM-159* marker 100 bp ladder for 17-31 accession; **c.** Microsatellite *ISSR-UBC-873*marker 100 bp ladder for 1-13 accession; **d.** Microsatellite *ISSR-UBC-873*marker 100 bp ladder for 14-31 accession; **e.** Microsatellite *ISSR- UBC-818* marker 100 bp ladder for 1-10 accession; **f.** Microsatellite *ISSR- UBC-818* marker 100 bp ladder for 11-20 accession; **g.** Microsatellite *ISSR- UBC-818* marker 100 bp ladder for 21-31 accession.