Population differentiation of wheat leaf rust fungus *Puccinia triticina* in South Asia

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Leaf or brown rust caused by *Puccinia triticina* (*Pt*) is one of the most important diseases of wheat. Among the rusts, it is the most ubiquitous in all the wheat-growing regions and causes considerable yield loss. Microsatellite marker-based genotyping and virulence-based phenotyping of 48 pathotypes of *Pt* was performed. The pathotypes exhibit low virulence frequencies for Indian leaf rust differentials *Lr24, Lr9, Lr10, Lr19, Lr28* and *Lr9*. Using avirulence/virulence formula six major clusters of pathotypes were observed, revealing high degree of phenotypic variation. Molecular analysis performed using SSR markers showed high genetic diversity among the pathotypes, and grouped them in seven major clusters. The percentage of polymorphic loci ranged from 17.95 to 84.62, Nei's gene diversity from 0.07 to 0.32 and Shannon's information index from 0.11 to 0.47. Analysis of molecular variance revealed significantly high genetic variation within *Pt* population. Mantel's *Z* test proved low positive correlation ($r = 0.28$) between virulence and molecular diversity, suggesting independent nature of the duo. These findings offer valuable information for framing suitable disease management strategies through appropriate region-specific gene deployment and improve the understanding of the population biology and evolution of *Pt* in the Indian subcontinent.

Keywords: Genetic differentiation, leaf rust, microsatellites, *Puccinia triticina*, virulence phenotype.

WHEAT, the most important cereal as a protein source and next to rice as a source of calories for majority of the population in developing countries, occupies about 225 m ha area worldwide[1]. It fulfils 21% of the food calorie and 20% of the protein requirements of over 4.5 billion people living in more than 90 developing countries[2]. The predictable demand for wheat is estimated to increase by 60% by 2050 in developing countries; on the other hand, wheat production is expected to reduce by 20–30% due to climate change-induced biotic and abiotic stresses[3]. Among the fungal diseases, rusts are the most detrimental to wheat worldwide. Brown (leaf) rust (*Puccinia triticina* Eriks.) (*Pt*) probably causes more damage than any other rust of wheat[4] and is the most widely distributed disease in India[5]. Like stripe and stem rusts of wheat, leaf rust is preferably controlled by genetic resistance besides using fungicides and agronomic practices. However, changes in the pathogen population brought about by mutation, somatic recombination and through selection of virulent types on the major (*R*) genes deployed on larger areas result in the evolution of new pathotypes of the pathogen. Thus, the emergence of new pathotypes and shift in virulence patterns, render the resistant wheat varieties susceptible. For effective management of leaf rust of wheat, identification of suitable resistance sources and appropriate gene deployment strategies based on racial pattern of a particular region are necessary.

The degree and distribution of phenotypic and genotypic variation within and among the pathogen populations are important for understanding their population biology. Genetic structure can be used to deduce the impact of different forces that influence the evolution of a pathogen population. This in turn provides a better understanding of the evolutionary pattern that may allow prediction of the potential for pathogen populations to evolve in agricultural ecosystems[6]. Higher genetic diversity in the pathogen population within a small area advocates the probability of rapid adaptation by the pathogen to changing host or environmental factors. On the other hand, higher genetic similarity among pathogen populations between widely separated regions could be the result of substantial long-distance dispersal of the pathogen or gene flow. Subsequently it poses a risk in the deployment of disease resistance genes arrayed to local pathogen populations; as exotic pathogens, having different virulence genes may overcome resistance in local host cultivars[7]. In spite of differential varieties, discrimination and changes in populations of pathogenic fungi have been detected using different molecular markers like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR)[8–10]. Microsatellites (SSRs) are tandemly repeated DNA sequence units of 1–6 bp. They have abundant and random distribution throughout the genome. The SSR markers are highly polymorphic, multi-allelic, co-dominant,

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PCR-based, reproducible and lead to high genetic diversity due to their higher mutation rate than other regions of the DNA[11]. Recently, use of microsatellites has been encouraged because of several advantages associated with them, viz. the genetic markers are inherited in a Mendelian fashion as co-dominant markers. Moreover, extensive distribution throughout the genome of an organism and frequent polymorphism rates make SSRs one of the most widely accepted genetic markers for population genetics studies[12]. Despite several benefits, there are few drawbacks of using SSR markers such as their high mutation rate, and that the marker site may not be conserved between two species. These shortcomings could lead to misleading interpretation for population structure studies. To overcome such gaps, some new markers such as the single-nucleotide polymorphism (SNP) markers, which can detect variation in a single nucleotide that occurs at a specific position in the genome, are promising for future research on population structure. On the presumption that knowledge of the population biology of *Pt* could eventually contribute to the development of more durable disease management strategies, the present study was conducted with the hypothesis that molecular polymorphism in the South Asian collection of *Pt* populations is dependent on their differential virulence and geographical distribution. Genotypic variation among leaf rust pathotypes from South Asia was explored using microsatellites.

Materials and methods

Pathogen isolation, purification and multiplication

For genetic differentiation studies, 2980 *Pt* isolates were collected from different parts of South Asia. Indian wheat leaf rust differential set[13] (Table 1) categorized these isolates in 48 pathotypes (Table 2), which are being maintained at the national repository of wheat rust pathotypes at the Regional Station of ICAR-Indian Institute of Wheat and Barley Research (erstwhile Directorate of Wheat Research), Shimla, Himachal Pradesh, India. Isolation, purification and multiplication of pathotypes were done on susceptible wheat cv. Agra Local; except for pathotypes 16 and 16-1, which were multiplied on Khapli, a Dicoccum wheat. The sterilized mixture of fine loam and farmyard manure (3 : 1) was used for growing plants. Five grams of nitrogen (N) : phosphorus (P) : potassium (K) (12 : 32 : 16) fertilizer mixture per 5 kg of soil was used as nutrient supplement. The seedlings were raised in plastic pots (12.5 cm diameter) inside spore-proof chambers (indoors) at 22 ± 2°C and 50–70% relative humidity along with 12 h daylight. When seedlings were 10 days old, 15 ml of maleic hydrazide acid (2.5%) was added to the soil in each pot before inoculation to reduce the ageing of seedlings and encourage sporulation. One-week-old seedlings were atomized with uredosporic inoculum suspension in non-phytotoxic isoparaffinic mineral oil Soltrol 170 (Chevron Phillips Chemical Company, USA) and incubated in saturated humidity chambers for 48 h. Subsequently, these plants were transferred to the greenhouse where temperature of 20 ± 2°C and relative humidity of 50–70% was maintained. Inoculum collected after 15 days of inoculation was used to test the purity of the pathotypes. Purity of the pathotypes and avirulence/virulence structure were confirmed after examining the characteristic infection type of the isolates (pathotype) on each of the entries of the differential set. After ensuring the purity of all the pathotypes, they were mass multiplied on susceptible host as mentioned earlier. During the whole multiplication procedure, pots inoculated with different pathotypes were kept in separate cabins for ensuring pathotype purity. After 15 days of inoculation, the uredospores were collected and dried in a desiccator at 4°C for three days and stored at –20°C till further use.

Virulence diversity

Virulence diversity of the pathotypes was determined using the Indian leaf rust differential sets for pathotype nomenclature (Table 1), consisting of 9, 9 and 7 entries in subsets '0', 'A' and 'B' respectively[13,14]. Uredospores from all the pathotypes were inoculated on 10-day-old seedlings of the differential set. After inoculation, seedlings were placed in incubation chambers for 48 h at >80% relative humidity and then transferred to a greenhouse and kept at 20 ± 2°C for 15 days. Based on the presence of necrosis and chlorosis, and the intensity of sporulation, infection type (IT) of each pathotype was scored individually on each entry. IT score based on a 0–4 rating scale was followed[15]. On standard evaluation scale infection types 0–2 (immune to moderate uredia with necrosis and/or chlorosis) were considered low infection types or avirulent, and 3–4 (moderate to large uredia without chlorosis or necrosis) were considered high infection types or virulent.

Molecular analysis of Pt

DNA extraction: Forty-eight *Pt* pathotypes used in the virulence analysis were studied for molecular genotyping.

Table 1. Set of differentials for the identification of pathotypes of *Pt* on wheat in India

Set 0	Set A	Set B
IWP 94 (*Lr23+*)	*Lr14a*	Loros (*Lr2c*)
Kharchia Mutant (*Lr9*)	*Lr24*	Webster (*Lr2a*)
Raj 3765 (*Lr13+10+*)	*Lr18*	Democrat (*Lr3*)
PBW 343 (*Lr26*)	*Lr13*	Thew (*Lr20*)
UP 2338 (*Lr26+34+*)	*Lr17*	Malakoff (*Lr1*)
K 8804 (*Lr26+23+*)	*Lr15*	Benno (*Lr26*)
Raj 1555	*Lr10*	HP1633 (*Lr9+*)
HD 2189 (*Lr13+34+*)	*Lr19*	
Agra Local	*Lr28*	

Table 2. Leaf rust pathotypes of wheat (*Puccinia triticina*; *Pt*) studied for virulence and molecular diversity

Pathotype[¥]	Year of first detection/collection	Place of collection	First detected on (host)	Susceptible *Lr* genes/lines
10	1931	Punjab	Local	Malakoff
11	1947	Multai, Madhya Pradesh	A090	*20*
12	1966	Thordi, Gujarat	Local	*3*
12-1	1983	Gwalior, Madhya Pradesh	Local	*26*
12-2	1979	Hansi, Haryana	Local	*23*
12-3	1989	Dharwad, Karnataka	Bijaga Yellow	*15*, *26*
12-4	1990	Pusa, Bihar	Agra Local	*3*, *10*, *20*
12-5	2005	Hubli, Karnataka	Local Red	*23*, *26*
12-6	2006	Dharwad, Karnataka	KH65	*20*, *26*
12-7	2008	Arabhavi, Karnataka	HP1912	*10*, *23*, *26*
12-8	2008	Pantnagar, Uttarakhand	HD2204	*15*, *20*, *26*
16	1959*	Nilgiris, Tamil Nadu	Khapli	Agra local**
16-1	2004	Belgaum, Karnataka	Dicoccum	Agra local, Raj1555
17	1957	Nilgiris, Tamil Nadu	Mal-4	Malakoff, 15, 20
20	1935	Hisar, Haryana	UP Local	Malakoff, 20
63	1931	Shimla, Himachal Pradesh	Local	*20*
77	1954	Pusa, Bihar	Mal 4	*3*, *15*
77-1	1985	Nilgiris, Tamil Nadu	Burgas-2	20, 26
77-2	1984	Nilgiris, Tamil Nadu	PAU Wheat	20, 23
77-3	1989	Nilgiris, Tamil Nadu	*Lr15*	26
77-4	1989	Nilgiris, Tamil Nadu	Lal Bahadur	23
77-5	1992	Nilgiris, Tamil Nadu	Crossing material	23, 26
77-6	1997	Nilgiris, Tamil Nadu	C306	23, 26
77-7	1998	Nilgiris, Tamil Nadu	*Lr9*	9, 23, 26
77-8	2004	Arabhavi, Karnataka	Off type in Kh. mutant	*19*
77-9	2008	Belgaum, Karnataka	HI1511	*23*, *26/2***
77-10	2008	Nilgiris, Tamil Nadu	HW5212	23, 26,28
77-11	2009	Ugar, Karnataka	HD2932	*23*, *20/26***
77-12	2013	Wellington, Tamil Nadu	Local	23, 26/2a, 2c, 20**
77 A	1974	Dharwad, Karnataka	CC62	*10*, *20*
77A-1	1976	Haryana	HD 2009	*20***
104	1973	Janakpur, Nepal	Local	1, 3
104-1	1985	Borkheda, Maharashtra	Kalyansona	20, 23
104-2	1991	Malan, Himachal Pradesh	Off type in Transec	*23*, *26/20***
104-3	1993	Naval Parasi Sunwal, Nepal	local wheat &Lr20	*20*, *23*, *26*
104-4	2010	Arki, Himachal Pradesh	HS240	*HS240/Democrat***
104A	1975	Patna, Bihar	HD1981	*20*
104B	1980	Hansi, Haryana	C306	*23*
106	1935	Haldwani, Uttarakhand	Local	*2c*, *20*
107	1935	Khanewal, Punjab	PB8A	*15*
107-1	1988	Dharwad, Karnataka	Local	*15*, *26*
108	1935	Banaras, Uttar Pradesh	Pusa 4	Malakoff, *20*
108-1	1989	Deorighat, Himachal Pradesh	Sonalika	Malakoff, *3*, *15*, *20*
162	1957	Gwalior, Madhya Pradesh	NP720	*10*
162-1	2002	Akola, Maharashtra	Agra Local	*10*, *20*, *26*
162-2	2005	Kanpur, Uttar Pradesh	Agra Local	*10*, *26*
162-3	2006	Ahmadnagar, Gujarat	GW1	*10***
162A	1961	Nilgiris, Tamil Nadu	Local	*10*, *20*

[¥]Based on old system (well-known globally) of Indian wheat rust nomenclature; names based on the new system and their North American notation are presented in Table S1, see Supplementary material online. *Replaced in 2000; **Avirulent.

Uredospores were harvested from infected leaves 15 days post-inoculation, dried and kept at –20°C. Total genomic DNA from dried uredospores (~50 mg) was extracted with cetyltrimethylammonium bromide (CTAB) as described by Kiran *et al.*[16]. The purity of genomic DNA and quantification of template DNA for PCR were measured in duplicate using NanoDrop 2000® UV-Vis Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) and stored at –20°C till further use.

SSR analysis: *Pt*-specific SSR primers (Table 3) were designed from the genome sequences of *Pt* in our previous study[16] using Primer3 software and synthesized from Agile Life Science Technologies India Pvt Ltd, Sant Nagar, Garhi, New Delhi. All the PCR reactions were carried out in 20 µl volume containing 25 ng of template DNA, 200 µM each of the four dNTPs, 1× PCR buffer (10 mM *Tris* pH 9.0, 50 mM KCl), 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Himedia Laboratory Pvt Ltd, India) and

Table 3. Markers used in genetic diversity study of leaf rust pathotypes

Primer code	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	AT* (°C)	Number of amplified alleles	Allele size (kb)
SSR-P GT-42	GGGGTGAGTTTCTGTATTGA	CAGAGATCATCGAGGAAAAC	50.4	02	0.08–0.3
SSR-P AG-40	CTTTCTTACCCCCACAACTAC	CTCTCTCTCTCTCTCTCTCTC	53	01	0.12
SSR-P CT-36	ACTCTCAAACTCACTCCCTCT	GACTACACCATTTCAAACCAA	51.9	01	0.13
SSR-P AC-32	ACAAAACAAACAGATCCACTG	ACGTATTTGGTCTTCTTCTCC	51	01	0.13
SSR-P TC-32	TAGAATTCTTGGTAGGACGAG	CGGTCAGAGTGTCTGTCAATA	50	02	0.12–0.3
SSR-P CAA-60	AACTGCGAGGACAACTTTC	CGTCTGCTGAGTTTCTGTATT	50	01	0.13
SSR-P AGA-48	CAAACGAAGCAAACTAGAAGA	TGTTGTTGTTGTTGTTGTTGT	50	01	0.12
SSR-P GGT-45	GCTGCTTGATGGAGGATG	AACAGCTTCAGCGACCTC	51	02	0.13–0.3
SSR-P GTT-45	GATGAGGTTGTTGAAGGAGA	ACCAGAACCAACAAAACAAC	49.6	02	0.14–0.4
SSR-P CAC-45	GAAGACCATCCTCACGACT	TTCTTCTTGTTGGTTTTTCTG	51	02	0.13–0.3
SSR-P CAAC-44	AGCGTAGAGTCAGTCAGTCAG	GCTAATAAGGAGATTGGGTTG	51	02	0.1–0.25
SSR-P TATC-40	AAGCGTGATCAAGTAGGTTTA	GATGGACAAGTAGAGAGATGG	50.4	01	0.1–0.3
SSR-P TCCG-36	TTTTTCTAGATCCACCAACC	TACGAACAGGAGTCCCTCA	50.4	03	0.15–1.0
SSR-P AGCC-32	GGGAAAGAAAAACACATCCT	GTCTCTTCGCTGATCTGG	50	01	0.15
SSR-P TGGA-32	GCATTTGTTTTTGTTGATTG	AGACACCTCCCCTTAAAAAC	48	02	0.12–0.2
SSR-P TATTG-60	TCAAACAACTTCATCCTGAAC	ATGTGATATCTTTTGGATTGG	48	10	0.15–0.5
SSR-P TCTTT-50	GGGTTTATATGGTGGGTGT	GTTGAGTGGGTGAGATGAGTA	48	11	0.15–0.5
SSR-P TAGCG-40	GCTAACGCTATGCAAAATAGA	CAGTTCAGTACCCACCAGTTA	48	02	0.12–0.35
SSR-P CCCGT-35	TTTTTGAAGGGCTTGTAGTG	AAAGGGACAGTTATGGGATAG	48	03	0.09–0.7
SSR-P GTGGA-35	TGTTTGGGAGTGTATGTGTG	GCCGAGTACCACTACCACTA	20	04	0.09–1.0
SSR-P TGAGGA-48	GTATCGGATGTTGTTGTGAAG	CTACCAAGTCTATCCGTCCTC	57.9	05	0.08–1.0
SSR-P ACAAAC-48	ATACATTTTGGTTACCCACCT	TGTGTTTGTTTGTGTTTGTGT	48.9	01	0.1–1.3
SSR-P CCAGAA-48F	GAAGAACTCGATCCCAGAA	CTGGTTTGTTGTTGTTGTTG	49.6	04	0.15–0.9
SSR-P CCGCAC-60	TTTTGGCTGAAGTTCTGAAT	GTTGTTGAGTTGAAGGACAAG	50.9	02	0.14–0.35
SSR-P GCTGTT-60F	GATGAGCAGCATGAGGAG	CACCAGAACAACATACTCCAT	51.9	03	0.15–0.25

*AT, Annealing temperature.

10 pmol of both forward and reverse primers. The reaction programs were set at 94°C for 2 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at a primer annealing temperature and 1 min at 72°C, with a final extension at 72°C for 10 min in a thermal cycler (Boeco Thermal Cycler TC-PRO, Hamburg, Germany). On completion of amplification, the amplified DNA was analysed on 3% Super MT4 agarose gel (Life Technologies India Pvt Ltd, Pitampura, Delhi, India) in 1× TBE buffer at 65–70 V for 2–3 h. DNA fragments were visualized under UV light and photographed using Gel Documentation System (Bio-Rad Laboratories, Inc., Hercules, California, USA).

Data analysis: Virulence frequency was determined as the percentage of the pathotypes virulent for a specific gene or entry of the differential set from the total pathotypes under study. The virulence and avirulence ITs of each isolate on the differential genotypes were assigned with binary codes of 1 and 0 respectively. The presence or absence of individual, distinct and reproducible bands was scored as '1' for presence and '0' for absence. Binary data were used to calculate Jaccard similarity coefficient. Cluster analysis was performed using NTSYSpc version 2.0 (ref. 17) and dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA).

Genetic diversity parameters, including the observed number of alleles (Na), effective number of alleles (Ne), percentage of polymorphic loci (Pp), Nei's gene diversity (*h*) and Shannon's index (I) were calculated to estimate the level of genetic variation using POPGENE version 1.31 (ref. 18). Analysis of molecular variance (AMOVA) was performed using ARLEQUIN version 3.0 (ref. 19) to examine differences among and within *Pt* populations. STRUCTURE 2.3.4 software[20] was used to analyse population structure using a burn-in period of 10,000 and 100,000 Markov chain Monte Carlo (MCMC) replications[20]. Mantel's *Z* test in zt version 1.1 (ref. 21) was used to test correlation between molecular genotypes and virulence phenotypes.

Results

Virulence diversity and cluster analysis

Avirulence/virulence patterns based on data from the Indian leaf rust differentials were determined separately for all the pathotypes (Table 4). Virulence frequency of 48 South Asian *Pt* pathotypes varied from 0.00 (for *Lr24*) to 95.8% (for Agra Local; Figure 1). The pathotypes displayed low virulence frequencies (less than 4%) for Kharchia Mutant (*Lr9*+), Raj1555, *Lr19*, *Lr28* and HP1633. The virulence frequency of pathotypes was moderate (20–43%) to IWP 94, Raj 3765, PBW 343, UP 2338, K 8804, HD 2189, *Lr15*, *Lr10* and Benno (*Lr26*), and high (45–95%) to Agra Local, *Lr14a*, *Lr18*, *Lr13*,

Table 4. Avirulence/virulence pattern of leaf rust pathotypes on *Lr* genes

Pathotype	Avirulence/virulence pattern
10	*Lr3, 9, 10, 15, 17a, 17b, 19, 21, 23, 24, 25, 26, 27, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr1, 2a, 2b, 2c, 11, 12, 13, 14a, 16, 18, 22a, 22b, 30, 33, 34, 35, 37, 38, 40, 44, 48, 49*
11	*Lr1, 2a, 2b, 2c, 3, 9, 10, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 19, 21, 22a, 22b, 23, 24, 25, 26, 28, 29, 30, 32, 33, 34, 36,37, 38, 39, 40, 42, 43, 44, 45, 47, 48, 49/Lr11, 20, 27+31, 35*
12	*Lr1, 2a, 9, 14b, 15, 17a, 17b, 18, 19, 20, 23, 24, 25, 26, 28, 29, 32, 36, 38, 39, 42, 43, 45, 47/Lr2b, 2c, 3, 10, 11, 12, 13, 14a, 14ab, 16, 21, 22a, 22b, 27, 30, 33, 34, 35, 37, 40, 44, 48, 49*
12-1	*Lr1, 2a, 9, 10, 11, 15, 17b, 19, 20, 23, 24, 25, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr2b, 2c, 3, 12, 13, 14a, 14b, 14ab, 16, 17a, 18, 21, 22a, 22b, 26, 27, 30, 33, 34, 35, 37, 38, 40, 44, 48, 49*
12-2	*Lr1, 2a, 9, 10, 13, 15, 17a, 17b, 18, 19, 20, 24, 25, 26, 28, 29, 32, 36, 39, 40, 42, 43, 45, 47/Lr2b, 2c, 3, 11, 12, 14a, 14b, 14ab, 16, 21, 22a, 22b, 23, 27+31, 30, 33, 34, 35, 37, 38, 44, 48, 49*
12-3	*Lr1, 2a, 9, 19, 20, 23, 24, 25, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr2b, 2c, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17a , 17b, 18, 21, 22a, 22b, 26, 27, 30, 33, 34, 35, 37, 38, 40, 44, 48,49*
12-4	*Lr1, 2a, 9, 15, 19, 23, 24, 25, 26, 28, 29, 32, 39, 42, 43, 45, 47/Lr2b, 2c, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 27, 30, 33, 34, 35, 36, 37, 38, 40, 44, 48, 49*
12-5	*Lr1, 2a, 9, 10, 13, 15, 19, 24, 25, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr2b, 2c, 3, 11, 12, 14a, 14b, 14ab, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 26, 27+31, 30, 33, 34, 35, 37, 38, 40, 44, 46, 48,49*
12-6	*Lr1, 2a, 9, 10, 11, 14b, 14ab, 15, 17a, 17b, 18, 19, 23, 24, 25, 27+31, 28, 29, 32, 36, 38, 39, 42, 43, 45, 47/ Lr2b, 2c, 3, 12, 13, 14a, 16, 20, 21, 22a, 22b, 30, 33, 34, 35, 37, 40, 44, 46, 48, 49*
12-7	*Lr1, 2a, 9, 13, 15, 19, 24, 25, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr2b, 2c, 3, 10, 11, 12, 14a, 14b, 14ab, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 26, 27+31, 30, 33, 34, 35, 37, 38, 40, 44, 46, 48, 49*
12-8	*Lr1, 2a, 9, 10, 13, 19, 23, 24, 25, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr2b, 2c, 3, 11, 12, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 26, 27+31, 30, 33, 34, 35, 37, 38, 40, 44, 46, 48, 49*
16	*Lr1, 2a, 2b, 2c, 3, 9, 10, 11, 13, 14, 14b, 14ab, 15, 16, 17, 18, 19, 20, 21, 23, 24, 25, 26, 27, 28, 29, 30, 32, 34, 36, 37, 38, 39, 42, 44, 45, 47, 48/Lr12, 22a, 22b, 33, 35, 49*
16-1	*Lr1, 2a, 2b, 3, 9, 10, 11, 13, 14b, 14ab, 15, 16, 17, 19, 21, 24, 25, 26, 27, 29, 30, 32, 34, 36, 37, 38, 39, 42, 44, 47, 48/Lr2c, 12, 14a, 18, 20, 22a, 22b, 23, 33, 35, 49*
17	*Lr3, 9, 10, 19, 23, 24, 25, 26, 27+31, 28, 29, 36, 42, 43, 45, 47/Lr1, 11, 12, 13, 14a, 15, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 34*
20	*Lr3, 9, 10, 15, 16, 17a, 19, 23, 24, 25, 26, 27+31, 28, 29, 36, 42, 43, 45, 47/Lr1, 2a, 2b, 2c, 11, 12, 13, 14a, 17b, 18, 20, 21, 22a, 22b, 33, 34, 37, 38, 40, 44, 48, 49*
63	*Lr1, 2a, 2b, 2c, 3, 9, 10, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 19, 21, 22a, 22b, 23, 24, 25, 26, 28, 29, 30, 32, 33, 34, 35, 36, 37, 38, 39, 40, 42, 43, 44, 45, 47, 48, 49/Lr11, 20, 27+31, 35*
77	*Lr9, 10, 19, 23, 24, 25, 26, 27+31, 28, 29, 32, 36, 39, 42, 43, 45/Lr1, 2a, 2b, 2c, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17, 18, 20, 21, 22a, 22b, 30, 33, 35, 37, 38, 44, 48, 49*
77-1	*Lr9, 17, 17a, 17b, 19, 23, 24, 25, 27+31, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr1, 2a, 2b, 2c, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 18, 20, 21, 22a, 22b, 26, 30, 33, 35, 37, 38, 44, 48, 49*
77-2	*Lr9, 19, 24, 25, 26, 28, 29, 32, 36, 39, 42, 43, 44, 45, 47/Lr1, 2a, 2b, 2c, 3,10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 27+31, 30, 33, 34, 35, 37, 38, 40, 48,49*
77-3	*Lr9, 19, 20, 23, 24, 25, 27+31, 28, 29,32, 36, 39, 42, 43, 45, 47/Lr1,2a,2b, 2c,3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17, 17a, 17b, 18, 21, 22a, 22b, 26, 30, 33, 35, 37, 38, 44, 48, 49*
77-4	*Lr9, 19, 20, 24, 25, 26, 28, 29, 32, 36, 39, 45, 47/Lr1, 2a, 2b, 2c, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17, 18, 21, 22a, 22b, 23, 27+31, 30, 33, 34, 35, 36, 37, 38, 40, 44, 48, 49*
77-5	*Lr9, 19, 24, 25, 28, 29, 32, 39, 42, 43, 45, 47/Lr1,2a, 2b, 2c, 3,10, 11, 12,13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 26, 27, 30, 33, 34, 35, 36, 37, 38, 40, 44,48, 49*
77-6	*Lr9, 18, 19, 20, 24, 25, 28, 29, 32, 39, 40, 42, 45, 47/Lr1, 2a, 2b, 2c, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 21, 22a, 22b, 23, 26, 27+31, 30, 33, 34, 35, 36, 37, 38, 43, 44, 48, 49*
77-7	*Lr18, 19, 24, 25, 28, 29, 32, 39, 40, 42, 45, 47/Lr1, 2a, 2b, 2c, 3, 9, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 20, 21, 22a, 22b, 23, 26, 27+31, 30, 33, 34, 35, 36, 37, 38, 43, 44, 48, 49*
77-8	*Lr9, 23, 24, 25, 26, 27+31, 28, 29, 32, 36, 39, 45, 47/Lr1 2a, 2b, 2c, 3a, 10, 11, 13, 14a, 14b, 14ab, 15, 16, 17, 18, 19, 20, 21, 22a, 22b, 30, 33, 35, 37, 38, 44, 48, 49*
77-9	*Lr2a, 2b, 2c, 9, 19, 24, 25, 28, 32, 39, 42, 45, 47/Lr1, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 26, 27+31, 30, 33, 34, 35, 36, 37, 38, 44, 46, 48, 49*

(*Contd*)

Table 4. (*Contd*)

Pathotype	Avirulence/virulence pattern
77-10	*Lr2a, 2b, 2c,9, 19, 24, 25, 32, 39, 42, 45, 47/Lr1, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 26, 28, 27+31, 30, 33, 34, 35, 36, 37, 38, 44, 46, 48, 49*
77-11	*Lr2a, 2b, 2c, 9, 19, 24, 25, 26, 28, 29, 32, 36, 39, 42, 45, 47, 57/Lr1, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 27+31, 30, 33, 34, 35, 37, 38, 44, 46, 48, 49, 51*
77-12	*Lr2a, 2b, 2c, 9, 19, 20, 24, 25, 28, 32, 39, 42, 45, 47/Lr1, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 26, 27+31, 30, 33, 34, 35, 36, 37, 38, 44, 46, 48, 49*
77-A	*Lr9, 17, 19, 23, 24, 25, 26, 27+31, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr1, 2a, 2b, 2c,3, 10, 11, 12,13,14a, 14b, 14ab,15, 16, 18,20, 21, 22a, 22b, 30, 33, 35, 37, 38, 44, 48, 49*
77A-1	*Lr9, 17,19, 20,23,24, 25, 26, 27+31, 28, 29, 32, 36,39, 42, 43, 45, 47/Lr1, 2a, 2b,2c, 3, 10, 11, 12, 13, 14a,14b, 14ab, 15, 16, 18,21, 22a, 22b,30,33, 35, 37, 38, 44, 48, 49*
104	*Lr2a, 9, 10, 13, 15, 19, 20, 23, 24, 25, 26, 27, 28, 29, 32, 39, 42, 43, 45, 47/Lr1, 2b, 2c, 3, 11, 12, 14a, 14b, 14ab, 16, 17a, 17b, 18, 21, 22a, 22b, 30, 33, 34, 35, 36, 37, 38, 40, 44, 48, 49*
104-1	*Lr2a, 9, 15, 19, 24, 25, 26, 28, 29, 32, 39, 42, 43, 45, 47/Lr1, 2b, 2c, 3, 10, 11, 12, 13, 14a, 14b, 14ab, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 27, 30, 33, 34, 35, 36, 37, 38, 40, 44, 48, 49*
104-2	*Lr9, 10, 13, 15, 19, 20, 24, 25, 28, 29, 32, 36, 39, 42, 43, 45, 47 /Lr1,2a, 2b, 2c, 3, 11,12, 14a, 14b,14ab, 16, 17a, 17b, 18, 21, 22a, 22b, 23, 26, 27+31, 30, 33, 34, 35, 37, 38, 40, 44, 48,49*
104-3	*Lr9, 10, 13, 15, 17a, 19, 24, 25, 27+31, 28, 29, 32, 36, 39, 42, 43, 45, 47/Lr1, 2a, 2b, 2c, 3, 11, 12, 14a, 14b, 14ab, 16, 17b, 18, 20, 21, 22a, 22b, 23, 26, 30, 33, 34, 35, 37, 38, 40, 44, 48, 49*
104-4	*Lr2a, 3, 9, 15, 19, 24, 25, 28, 32, 39, 42, 43, 45, 47/Lr1, 2b, 2c, 10, 11, 12, 13, 14a, 14b, 14ab, 16, 17a, 17b, 18, 20, 21, 22a, 22b, 23, 26, 27+31, 29, 30, 33, 34, 35, 36, 37, 38, 40, 44, 46, 48, 49, 51, 57*
104A	*Lr2a, 9, 10, 15, 19, 21, 23, 24, 25, 26, 27, 28, 29, 32, 39, 42, 43, 45, 47/Lr1, 2b, 2c, 3, 11, 12, 13, 14a, 14b, 14ab, 16, 17a, 17b, 18, 20, 22a, 22b, 30, 33, 34, 35, 36, 37, 38, 39, 40, 44, 48, 49*
104B	*Lr2a, 9, 15, 19, 20, 24, 25, 26, 28, 29, 32, 39, 42, 43, 45, 47/Lr1, 2b, 2c, 3, 10, 11,12, 13, 14a, 14b, 14ab, 16, 17a, 17b, 18, 21, 22a, 22b, 23, 27, 30, 33, 34, 35, 36, 37, 38, 40, 44, 48,49*
106	*Lr1, 2a, 2b, 3, 9, 10, 11, 12, 13, 14a, 14b, 14ab, 15, 16, 17, 18, 19, 21, 22a, 22b, 23, 24, 25, 26, 27+31, 30, 33, 37, 38, 39, 44, 47, 48/Lr2c, 20, 35*
107	*Lr1, 3, 9, 10, 19, 20, 23, 24, 25, 26, 27+31, 28, 29, 30, 32, 36, 38, 39, 40, 42, 43, 45,46, 47/Lr2a, 2b, 2c, 11, 12, 13, 14a, 18, 15, 21, 22a, 22b, 33, 34, 35, 37, 38, 40*
107-1	*Lr1, 3, 9, 10, 19, 20, 23, 24, 25, 27+31, 28, 29, 30, 32, 36, 38, 39, 40, 42, 43, 45,46, 47/Lr2a, 2b, 2c, 11, 12, 13, 14a, 18, 15, 21, 22a, 22b, 26, 33, 34, 35, 37, 38, 40*
108	*Lr3, 9, 10, 15, 17a, 17b, 19, 23, 24, 25, 26, 27+31, 28, 29, 32, 47 /Lr1, 2a, 2b, 2c, 11, 12, 13, 14a, 16, 18, 20, 21,22a, 22b, 33, 34, 35, 36, 37, 38, 44, 48, 49*
108-1	*Lr3, 9, 10, 17a, 17b, 19, 23, 24, 25, 26, 27+31, 29, 39, 42, 45, 47/Lr1, 2a, 2b, 2c, 11, 12, 13, 14a, 15,16, 18, 20, 21, 22a, 22b, 33, 34, 35, 36, 37, 38, 44, 48, 49*
162	*Lr1, 9, 13, 14ab, 15, 19, 20, 21, 23, 24, 25, 26, 27+31, 28, 29, 32, 36, 38, 39, 40, 42, 43, 45, 46, 47/Lr2a, 2b, 2c, 3, 10, 11, 12, 14a, 14b, 16, 17a, 17b, 18, 22a, 22b, 30, 33, 34, 35, 37, 44, 48, 49*
162-1	*Lr1, 9, 13, 14ab, 15, 19, 21, 23, 24, 25, 27+31, 28, 29, 32, 36, 38, 39, 40, 42, 43, 45,46, 47/Lr2a, 2b, 2c, 3,10, 11, 12, 14a, 14b, 16, 17a, 17b, 18, 20, 22a, 22b, 26, 30, 33, 34, 35, 37, 44, 48, 49*
162-2	*Lr1, 9, 13, 14ab, 15, 19, 20, 21, 23, 24, 25, 27+31, 28, 29, 32, 36, 38, 39, 40, 42, 43, 45,46, 47/Lr2a, 2b, 2c, 3,10, 11, 12, 14a, 14b, 16, 17a, 17b, 18, 22a, 22b, 26, 30, 33, 34, 35, 37, 44, 48, 49*
162-3	*Lr1, 9, 10, 13, 14ab, 15, 19, 20, 21, 23, 24, 25,26, 27+31, 28, 29, 32, 36, 38, 39, 40, 42, 43, 45,46, 4/ Lr2a, 2b, 2c, 3, 11, 12, 14a, 14b, 16, 17a,17b, 18, 22a, 22b, 30, 33, 34, 35, 37, 44, 48, 49*
162A	*Lr1, 9, 13, 14ab, 15, 19, 21, 23, 24, 25, 26, 27+31, 28, 29, 32, 36, 38, 39, 40, 42, 43, 45,46 , 47/Lr2a, 2b, 2c, 3, 10, 11, 12, 14a, 14b, 16, 17a, 17b, 18, 20, 22a, 22b, 30, 33, 34, 35, 37, 44, 48, 49*

Lr17a, Loros (*Lr2c*), Webster, Democrat, Thew (*Lr20*) and Malakoff (*Lr1*). None of the pathotypes was virulent to *Lr24*.

Virulence data generated six major clusters of pathotypes showing 48–66% similarity within a cluster (Figure 2). Clusters B, C and D were further divided into subclusters. Clusters E and F had only one pathotype each, i.e. 16-1 and 16 respectively. These two pathotypes have unique character of their virulence on Raj1555 and avirulence on Agra Local, which separates them from rest of the pathotypes. Pathotype 16-1, showing virulence to leaf rust resistance genes *Lr14a*, *Lr18*, *Lr2c* (Loros) and *Lr20* (Thew) differs from pathotype 16, which is avirulent to these genes. Pathotypes 77-1, 77-2, 77-3, 77-4, 77-8, 77A and 77A-1, virulent on *Lr14a*, *Lr13*, *Lr17a*, *Lr15*, *Lr10*, *Lr2c*, Webster, Democrat and Malakoff (*Lr1*) and

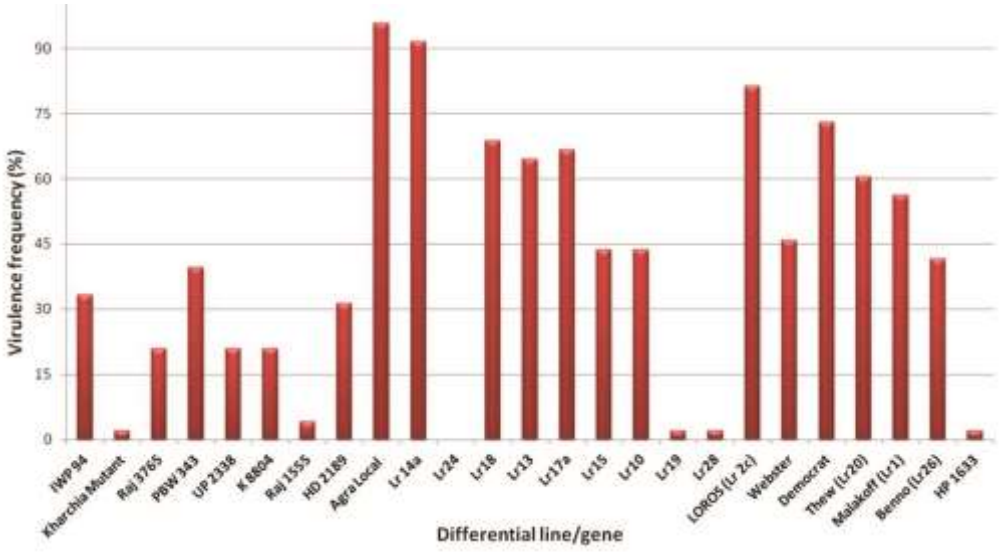

Figure 1. Virulence frequency (%) of leaf rust pathotypes on Indian leaf rust differentials.

avirulent on *Lr24*, *Lr28* and HP 1633, shared more than 75% virulence similarity. Pathotypes 11 and 63 displayed 100% similarity.

Population genetic variation

The percentage of polymorphic loci recorded in the *Pt* populations from 12 states was 97 (Table 5). Isolates collected from Karnataka had the highest percentage of polymorphic loci (84) followed by Tamil Nadu (82), while the isolates from Uttarakhand had the lowest percentage (17) followed by Punjab (20). The observed alleles per locus were highest for Karnataka (1.85) and lowest for Uttarakhand (1.18). However, the effective number of alleles was highest for pathotypes collected from Maharashtra (1.8) followed by Tamil Nadu (1.56). The average effective number of alleles was 1.59 with Uttarakhand (1.12), Punjab (1.15) and Uttar Pradesh (1.18) having lesser effective number of alleles. Nei's genetic diversity for the whole *Pt* population was 0.34, with Tamil Nadu (0.32) and Uttarakhand (0.07), having the highest and lowest values respectively. Highest Shannon's information index (SII) was observed among the pathotypes from Tamil Nadu (0.47), whereas lowest SII was for the pathotypes from Uttarakhand (0.11). The overall total variability, variability within population, diversity among populations and fixation index were found to be 0.33, 0.17, 0.52 and 0.13 respectively. AMOVA ($P < 0.001$) confirmed 87.43% genetic variation within the *Pt* population, whereas the variation among populations was 12.57% (Table 6).

Molecular genotyping

Among the primers (Table 3) used, SSR-PTCCG-36, SSR-PTGGA-32, SSR-PTATTG-60, SSR-PTCTTT-50, SSR-PCCCGT-35, SSR-PGTGGA-35, SSR-PTGAGGA-48, SSR-PCCAGAA-48 and SSR-PGCTGTT-60 were polymorphic. These nine primers amplified 45 alleles with an average of five alleles per primer. Primers SSR-P TCTTT-50 and SR-PTGGA-32 amplified highest (11) and lowest (2) number of alleles respectively. Interestingly, the allelic pattern with primer SSR-PTATTG-60 was more or less similar among the pathotypes of virulence-based groups (Table S1, see Supplementary Material online). Primer SSR-PTCTTT-50 amplified maximum alleles followed by SSR-PTATTG-60 and SSR-PTGAGGA-48 (Table 3).

The overall molecular marker data revealed poor genotypic similarity among pathotypes. The maximum genotypic similarity (89%) was observed between pathotypes 12-3 and 12-7. This was followed by 87% genotypic similarity among pathotypes 12-3, 12-6 and 12-7. Genotypic cluster analysis generated seven major clusters (Figure 3). Clusters A and E had single pathotype 16 and 104-2 respectively. The results of STRUCTURE 2.3.4 analyses performed on *Pt* population dataset indicated two distinct subpopulations (K) (Figure 4). The subpopulations S1, S2 and M contained 15, 26 and 7 pathotypes each of *Pt* respectively (Table S2, see Supplementary Material online). The genetic distance as fixation index (F_{ST}) within S1 and S2 subpopulations was 0.3275 and 0.3086 respectively.

Mantel's Z test

Correlation analyses using the Mantel's *Z* test revealed positive but weak association between molecular and virulence data. There was positive ($r = 0.28$) and statistically significant ($P = 0.0007$) correlation between molecular and virulence data. The phylogenetic trees did not indicate such relationship between SSR genotypes and virulence phenotypes.

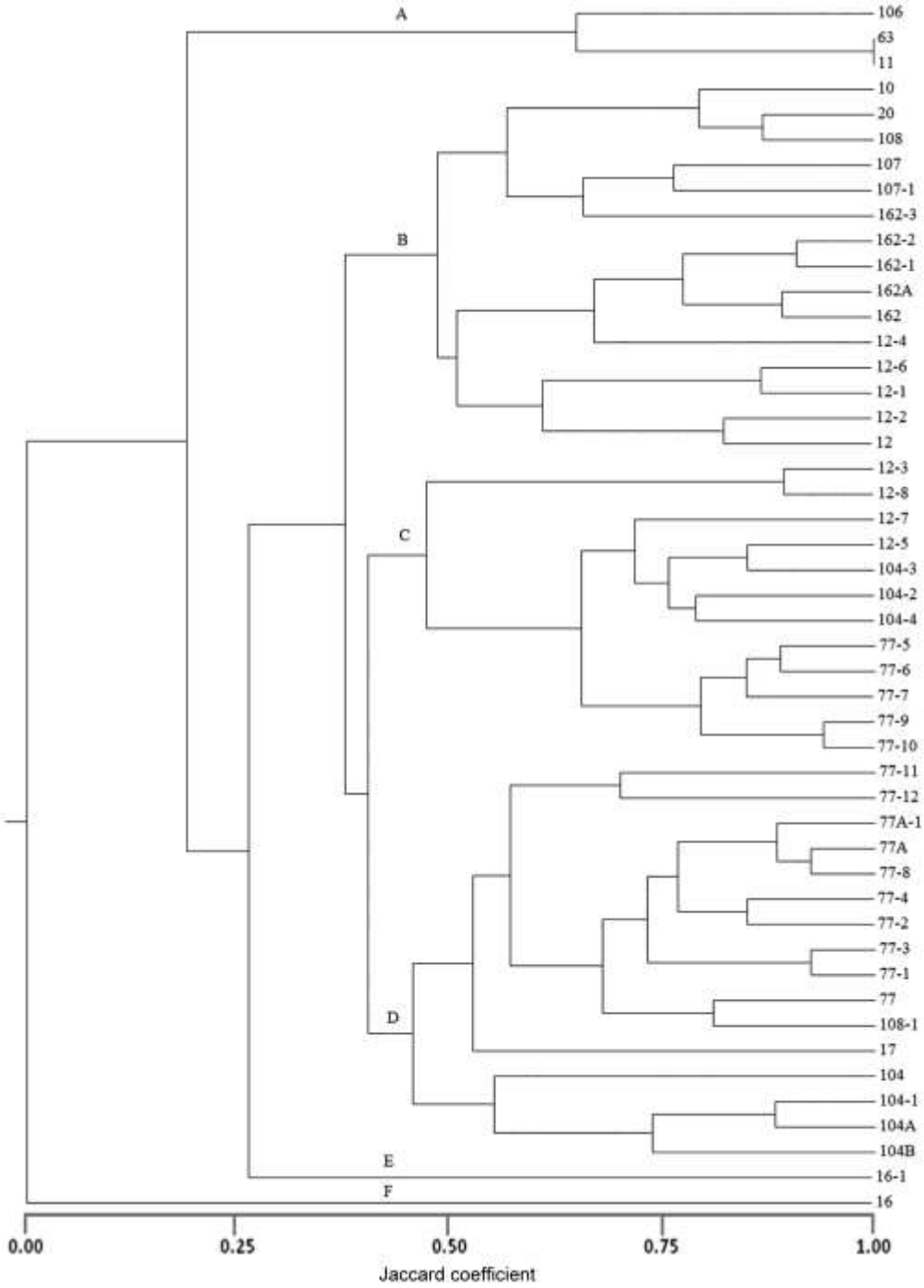

Figure 2. Dendrogram of *Puccinia triticina* pathotypes based on their virulence on 25 leaf rust differentials. Alphabets at the base of the branch indicate the name of the major group.

Discussion

This study was undertaken to distinguish the phenotypic and genotypic variations in *Pt* populations from South Asia. The result suggest that populations of *Pt* are highly variable and majority of genetic variation was distributed within population (Table 6). Virulence study revealed that none of the pathotypes was virulent on *Lr24*. Although several wheat varieties carrying effective leaf rust resistance gene *Lr24* (linked to *Sr24*) occupy relatively larger proportion of cultivated varieties in South Asia, wheat breeders need to diversify leaf rust resistance as the presence of *Lr24* in many varieties poses a potential threat of boom and bust cycle due to emergence of *Lr24* virulence in South Asia. Moreover, there is possibility of entry of *Lr24* virulence from neighbouring countries[22].

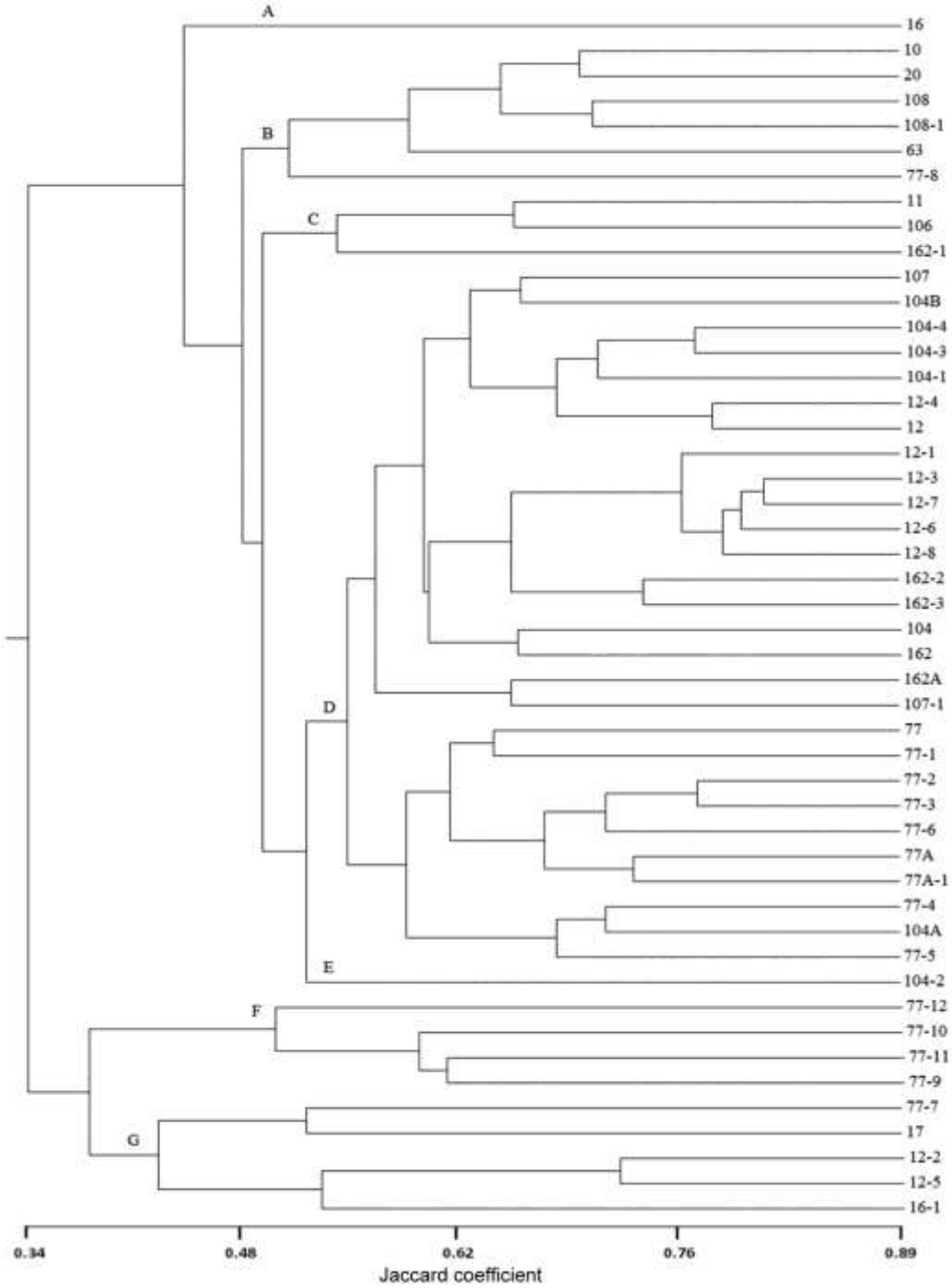

Figure 3. Dendrogram of *Pt* pathotypes based on molecular marker data. Alphabets at the base of the branch indicate the name of the major group.

Virulence frequency for *Lr14a*, *Lr18*, *Lr13*, *Lr17a*, Loros (*Lr2c*), Webster, Democrat, Thew (*Lr20*) and Malakoff (*Lr1*) was higher. Therefore, discouraging cultivation of cultivars carrying these genes would definitely affect the survival of the pathotypes which are virulent on these genes/entries. The virulence-based clustering suggests that the pathotypes were highly variable in their virulence to Indian leaf rust differentials. The pathotypes belonging to similar virulence groups (group 77 and 12), to some extent, were also grouped in virulence-based phylogenetic tree (Figure 2). Pathotypes 16 and 16-1 form two separate major groups in the virulence-based phylogenetic tree, which is obvious from their different avirulence/virulence nature from rest of the pathotypes. In spite of lacking a sexual recombination cycle in the Indian subcontinent[23], the high genetic diversity of the leaf rust pathogen

Table 5. Analysis of divergence of genetic variation in *Pt* populations

Population	Number of samples collected	Pathotypes identified	Na ± SD	Ne ± SD	*h* ± SD	*I* ± SD	Pp (%)
Madhya Pradesh	238	03	1.61 ± 0.49	1.43 ± 0.39	0.24 ± 0.20	0.36 ± 0.29	61.54
Gujarat	253	02	1.41 ± 0.49	1.29 ± 0.35	0.17 ± 0.20	0.24 ± 0.30	41.03
Haryana	138	04	1.44 ± 0.50	1.34 ± 0.42	0.18 ± 0.22	0.27 ± 0.31	43.59
Karnataka	437	10	1.85 ± 0.36	1.55 ± 0.38	0.31 ± 0.18	0.46 ± 0.25	84.62
Bihar	246	03	1.28 ± 0.45	1.24 ± 0.40	0.12 ± 0.21	0.18 ± 0.29	28.21
Uttarakhand	155	02	1.18 ± 0.38	1.12 ± 0.27	0.07 ± 0.16	0.11 ± 0.23	17.95
Himachal Pradesh	315	04	1.44 ± 0.50	1.24 ± 0.33	0.15 ± 0.18	0.22 ± 0.26	43.59
Tamil Nadu	419	12	1.82 ± 0.39	1.56 ± 0.35	0.32 ± 0.17	0.47 ± 0.24	82.05
Nepal	148	02	1.28 ± 0.45	1.20 ± 0.32	0.11 ± 0.18	0.17 ± 0.27	28.21
Maharashtra	362	02	1.25 ± 0.44	1.8 ± 0.31	0.16 ± 0.18	0.15 ± 0.26	25.64
Punjab	145	02	1.20 ± 0.40	1.15 ± 0.28	0.08 ± 0.16	0.12 ± 0.25	20.51
Uttar Pradesh	124	02	1.26 ± 0.44	1.18 ± 0.31	0.11 ± 0.18	0.15 ± 0.26	25.64
Total	2980	48	1.97 ± 0.16	1.59 ± 0.33	0.34 ± 0.15	0.50 ± 0.19	97.44

Na, Observed number of alleles; SD, Standard deviation; Ne, Effective number of alleles; *h*, Nei's gene diversity; I, Shannon's Information index; Pp, Percentage of polymorphic loci.

Table 6. Analysis of molecular variance (AMOVA) among and within the populations of *Pt* pathotypes

		Observed partition*	
Source of variation	Degree of freedom	Variance	Variation (%)
Among populations	11	0.860	12.57
Within populations	36	5.985	87.43
Total	47	6.846	100

**P* value = 0.05.

to a certain extent, may be explained by phenomena like introduction of exotic and genetically distinct pathotypes, recurrent mutation, etc. that are reported to be responsible for variability in wheat rust pathotypes[24].

Molecular marker data-based clustering revealed high variability among the pathotypes. A total of seven major clusters were observed. Molecular markers-based polymorphism was relatively higher compared to virulence-based polymorphism. The genotypic clustering pattern of pathotypes was quite random compared to virulence-based clustering, as there was no uniform grouping among the pathotypes belonging to one particular geographical region or virulence-based groups. This could be justified through the highly migratory nature of *Pt* and virulence-independent nature of microsatellite markers used in the study. Clustering based on virulence and molecular analysis was not fully correlated to each other. This is quite understandable from the fact that pathogenicity or virulence in the pathogen is not at all related to the molecular markers like RAPD, SSRs or others, unless they are designed from a part of the genome of the pathogen which decides the pathogenicity or virulence[25,26]. Primers SSR-PTATTG-60 and SSR-PTCTTT-50 supported the virulence-based clustering as they displayed almost similar allelic patterns among the pathotypes of virulence-based groups. This fact can be supported by assuming that these primers may have amplified parts of the genome having sequences responsible for pathogen virulence[26], but we cannot be completely assured unless further functional studies of these markers are undertaken. Pathotypes 12-1, 12-3, 12-6, 12-7 and 12-8 shared more than 80% similarity. Likewise pathotypes 77-2, 77-3, 77-6, 77A and 77A-1 shared more than 70% similarity, which indicated that they might have evolved from genetically similar ancestors[27]. The effective number of alleles, Nei's gene diversity and SII were highest for pathotypes from Tamil Nadu. Green bridges or cultivation of wheat crop round the year may be helping the mutated isolates in their survival in the higher hills of Tamil Nadu and thereby diversifying *Pt* populations. Moreover, it is advocated that Nilgiri and Palni hills in Tamil Nadu, which are the primary foci as the source of brown rust pathotypes for Tamil Nadu and Karnataka[23], receive high UV intensity sunlight. The higher UV intensity in these hills might be contributing towards faster evolution of rust pathogens through mutation. Very short duration of congenial environment for wheat leaf rust development in the North Indian states followed by harsh summers do not allow the mutated isolates of *Pt* to adopt such conditions and survive until the next crop season, which could be a reason for lesser Nei's gene diversity in this study for *Pt* populations from Uttarakhand (0.07) and Punjab (0.08).

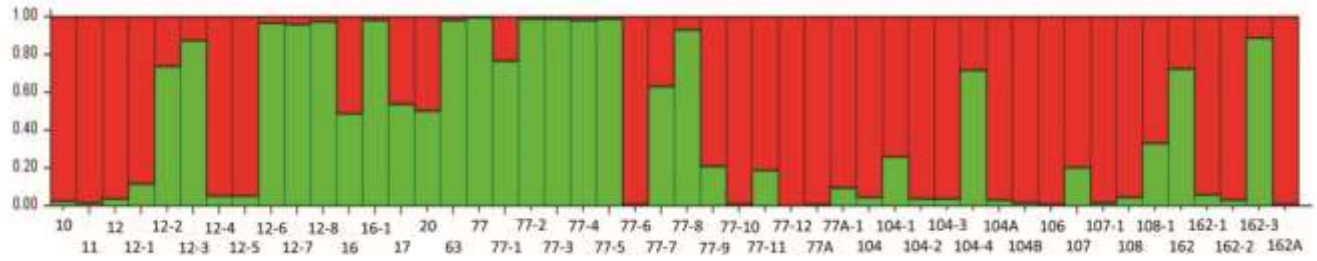

Figure 4. Structure analysis of 48 South Asian *Pt* pathotypes. Pathotypes indicated in green and red colours indicate two different allelic patterns among them. Pathotypes sharing more than 70% allelic frequency in each category were grouped into a particular subpopulation group, while sharing less than 70% were considered as admixture population.

AMOVA revealed 87.43% variation within the *Pt* populations from different states. Such variation indicates that the populations have the potential to evolve relatively quickly to changing climate and resistance pattern. However, the genetic variation among the *Pt* populations was a mere 12.57%, suggesting similarity between molecular genotypes from distant areas. Uredospores of *Pt* as other rust pathogens are wind-dispersed, and circumstantial evidence of migration over hundreds or thousands of miles has been reported[28]. This migratory nature of the pathogen may be responsible for the limited genetic variation between two distant populations observed in this study. Similar findings are also reported by Hovmoller *et al.*[29], where they observed similarity between virulence patterns and AFLP data among the *Pt* populations from United Kingdom, France, Germany and Denmark. STRUCTURE program differentiated *Pt* populations into three subpopulations – S1, S2 and M. More than 50% of the pathotypes were grouped in subpopulation S2. The F_{ST} value for subpopulations S1 and S2 was more than 0.25, which belongs to very great genetic differentiation category[30] and corroborates with high genotypic and virulence variations, and diverse geographic distribution of the *Pt* pathogen subpopulations. Using STRUCTURE version 2, similar significant variation in *Pt* populations from Central Asia and the Caucasus was also observed, which separated these populations from the North American isolates of *Pt* (ref. 31).

Mantel's *Z* test suggests that molecular diversity is not fully correlated to the virulence diversity among *Pt* pathotypes. This was quite obvious as such correlation would have been observed if we had used virulence-specific primers; however, here microsatellites were used, which shows virulence or pathogenicity-independent DNA polymorphism. Chen *et al.*[32] justified this fact by reporting that molecular polymorphism was independent of pathogenicity and whole genome of the pathogen evolves at a much faster rate than genes governing pathogenicity in yellow rust of wheat (*Puccinia striiformis*).

In conclusion, the South Asian collection of *Pt* was highly variable for virulence phenotypes and SSR genotypes. It was placed in six virulence phenotype and seven SSR genotype based groups. The high diversity among the pathotypes might be the result of evolution and mutation in the pathogens along with the long-term cultivation of wheat varieties in different wheat-growing zones of the country. Moreover, the introduction of new virulence from neighbouring countries may be another factor contributing to the high pathotype diversity. The virulence and molecular-based variability studies of wheat leaf rust pathotypes from different South Asian countries might provide versatile information on the origin and further movement of new virulences among these countries. The finding of this study would provide a reference for wheat leaf rust resistance breeding, better understanding of *Pt* population dynamics, preliminary idea for designing breeding strategies at the regional level, scientific awareness of deploying available resistance sources for disease management and assist in tracking variation in *Pt* populations over time and space.

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

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