Elucidating genetic variability and population structure in *Venturia inaequalis* associated with apple scab diseaseusing SSR markers

Sheikh Mansoor[1], **Nazeer Ahmed**[2], **Vikas Sharma**[1], **Sumira Jan**[3], **Sajad Un Nabi**[3], **Javid I. Mir**[3], **Mudasir A. Mir**[2], **Khalid Z. Masoodi**[2]*

1 Division of Biochemistry, Sher-e-Kashmir University of Agricultural Sciences and Technology Jammu, Jammu and Kashmir, India, **2** Transcriptomics Laboratory, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology (SKUAST-K), Shalimar, Srinagar, Jammu and Kashmir, India, **3** ICAR-Central Institute of Temperate Horticulture, Rangreth, Srinagar, Jammu and Kashmir, India

* masoodikz@skuastkashmir.ac.in

Abstract

Apple scab caused by *Venturia inaequalis* Cooke (Wint.) is one the important diseases of trade and industrial significance in apple. In present study variability studies in pathogen isolates were studied, which is one of the most important factors for devising management studies of scab disease in apple. Genetic diversity of 30 *Venturia inaequalis* isolates from 12 districts of two geographical distinct regions of Jammu and Kashmir was calculated based on the allele frequencies of 28 SSR markers and the internal transcribed spacer (ITS) region of the ribosomal DNA. The ITS based characterized sequences were submitted to NCBI GenBank and accession numbers were sanctioned. Dendrogram showed that all the accessions formed 2 main clusters with various degree of sub clustering within the clusters. Analysis based on SSR study reveals that the heterozygosity ranged from 0.0 and 0.5, with an average value of 0.39. The expected heterozygosis or gene diversity (He) ranged from 0.0 to 0.50 with an average of 0.40. The F_{st} value ranges from 0 to 0.6 with an average of 0.194. Diversity within each population (HS) values ranging from 0.26 to 0.33. Average differentiation among populations (GST) was 0.11 and populations were isolated by significant distance (r 2 = 0.50, P < 0.01). From the AMOVA analysis, 25% of variation was observed among population, 9% among individuals and 66% within individuals observed in the population. Structure analysis grouped isolates into two populations. Principle coordinate analysis explained variation of 36.6% in population 1, 14.30% in population 2 and 13.10% in population 3(Admixture) with 64.07% as overall cumulative percentage of variation. This indicates that extensive short-distance gene flow occurs in Kashmir region that dispersal over longer distances also appears to occur frequently enough to prevent differentiation due to genetic drift. Also it is evident that Jammu and Kashmir most likely has *V. inaequalis* subpopulations linked to diverse climatic conditions of the Jammu region compared to the mountainous inland Kashmir region. The results of present study would help to understand the genetic diversity of *V. inaequalis* from Jammu and Kashmir that would lead in the development of more effective management strategies and development of new resistant cultivars through marker-assisted selection.

Introduction

Apple (*Malus × domestica* Borkh.) is most widely and commercially cultivated species in the
genus Malus throughout temperate regions of world[1]. It is susceptible to number of diseases
incited by fungi, bacteria, viruses, viroids and phytoplasmas[2]. Scab is very severe and among
one of the mainsignificant diseasesall through the world in terms of economic losses in tem-
perate regions with cool and moist climate during spring[3]. It ranks number one disease in
terms of yield loss, which poses potential threat to apple industry[4]. This disease imposes a
severe threat in commercial apple growing regions, due to premature fruit drop and unmar-
ketable diseased fruits and results in losses up to 70%[5] or even complete crop loss is possible
if prophylactic steps are not taken in the orchard for its management[6]. Scab disease is caused
by *Venturia inaequalis* Cooke (Wint.) whichis an ascomycetous heterothallic and hemibiotro-
phicfungus. Earlier, this genus was included in the family *Venturiaceae*, order *Pleosporales*,
according to its "Pleospora-type centrum and bitunicateasci" [7]. on the other hand, recent
molecular phylogenetic analyses of Dothideomycetes, using both nuclear and mitochondrial
gene regions, have indicated that the family Venturiaceae forms a well-supported monophy-
letic group separate from the Pleosporales[8, 9]. Thus, Zhang recently reordered Venturiaceae
into Venturiales. It has a broad geographic dimension and is found in almost all apples grow-
ing areas. The fungus exists in two states i.e., saprophytic (sexual state *Venturia inaequalis*
(Cke) and parasitic (asexual state *Spilocaeapomi* Fr)[3]. It overwinters as pesudothecia in
regions with severe winter, whereas, conidia in dormant buds in regions with moderate winter
[10]. In early spring, when temperature and moisture are suitable, Ascospores start maturing
and are released forcibly in air[11].

One sexual and multiple asexual cycles, of this pathogen annually causes noteworthy varia-
tions in *Venturia inaequalis* population[12]. Recombination takes place by sexual reproduc-
tionwhich ultimately leads to high variation and diversity in fungi and also changes population
genetic structure[13]. In devising the management strategies against the disease, important
factor that is taken into contemplation is variationwithin pathogen population[14]. Detailed
investigations about pathogens variation and population genetic structure in different geo-
graphical regions are required, which reflects the history as well as evolutionary potential of
the pathogen [15] and also give an idea about centers of origin of this pathogen[16]. In wide
range of organisms the ribosomal rRNA genes sequence investigation and the internal tran-
scribed spacer (ITS) region is used as new tool in phylogenetic relationship studies [17]. As
rRNA repeat develop slower as a result it is very handy for studying secluded related organ-
isms[18]. Besides sequence divergence in ITS sequences, polymerase chain reaction (PCR)
amplification length polymorphism in nuclear rDNA due to intron insertion has also been
used to assess the extent of genetic variability within populations [19]. As per reports ITS
based analysis is the best was to identify sub species than rbcL and matK[20]. Keeping in view
the above background information, detailed investigations on the molecular characterization,
genetic diversity and population structure of the *Venturia inaequalis* in numerousapple grow-
ing districts of Jammu and Kashmir was carried out.

Materials and methods

Collection of isolates

Samples were collected from 12 apple growing districts of Jammu and Kashmir India during
the year 2017–18 as shown in Table 1. The locations sites from where samples were collected
are provided in Fig 1. Diseases samples including only apple leaves with scab symptoms were
collected from May to September 2018–19. Most of the cultivars were Red Delicious and

Table 1. Samples collected from two regions of Jammu and Kashmir with sample code, geographic location, latitude, longitude and accession number provided by Genbank from various apple growing areas.

S.No	Sample Code	Location	District	Latitude	Longitude	Accession number
			Kashmir region isolates			
1	M1	Trehgam	Kupwara	34.521˚N	74.184˚E	MK478885.1
2	M2	Bankoot	Bandipora	34.420˚N	74.650˚E	MK359025.1
3	M3	Char-i-sharief	Budgam	33.862˚N	74.766˚E	MK504436.1
4	M4	Syedpora	Shopian	33.72˚N	74.83˚E	MK478887.1
5	M5	Beerva	Budgam	34˚ 001˚N	74.5953˚E	MK359026.1
6	M6	Chadoora	Budgam	33.802˚N	75.100˚E	MK504428.1
7	M7	Batpora	Srinagar	34.936˚N	74.464˚E	MK504429.1
8	M8	Hajin	Bandipora	34.09˚N	74.79˚E	MK359032.1
9	M9	Yaripora	Kulgam	33.7˚N	75.0˚E	MK530205.1
10	M10	Handwara	Kupwara	34.40˚N	74.28˚E	MK359027.1
11	M11	Kakapora	Pulwama	33.88˚N	74.92˚E	MK359028.1
12	M12	Wakura	Ganderbal	34.05˚N	74.47˚E	MK367580.1
13	M13	Gutlibagh	Ganderbal	34.09˚N	74.09˚E	MK504434.1
14	M14	Naidkhai	Bandipora	34.09˚N	74.79˚E	MK359029.1
15	M15	Gantmulla	Baramulla	34.086˚N	74.033˚E	MK504430.1
16	M16	Pinjura	Shopian	33.72˚N	74.83˚E	MK504437.1
17	M17	Pattan	Baramulla	34.85˚N	74.37˚E	MK504431.1
18	M18	Sogam	Budgam	34.020˚N	74.780˚E	MK504435.1
19	M19	Mattan	Anantnag	33.701˚N	75.285˚E	MK359031.1
25	M25	Uri	Baramula	34.080˚N	74.05˚E	MK532035.1
			Jammu region isolates			
20	M20	PranuBaderwah	Doda	32.58˚ N	75.538˚ E.	MK359030.1
21	M21	NalthiBaderwah	Doda	32.93˚N	75.712˚E	MK583539.1
22	M22	Doda	Doda	33.13˚N	75.57˚E	MK504432.1
23	M23	Kishtwar	Kishtwar	33.32˚N	75.77˚E	MK504433.1
24	M24	Tund	Kishtwar	33.32˚N	75.77˚E	MK532037.1
26	M26	Faisal Abad	Kishtwar	33.32˚N	75.77˚E	MK532036.1
27	M27	Padder	Kishtwar	33.13˚N	75.09˚E	MK532034.1
28	M28	Padder	Kishtwar	33.13˚N	75.220˚E	MK532033.1
29	M29	KotiBaderwah	Doda	33.145˚N	75.547˚E	MK532032.1
30	M30	Baderwah	Doda	33.13˚N	75.57˚E	MK532031.1

https://doi.org/10.1371/journal.pone.0224300.t001

Golden Delicious as these are most cultivated apple varieties grown in Jammu & Kashmir. Sampling was carried out from trees having at least two to three scab lesions on leaf. The samples were collected as part of thesis work and due permissions from farmers of the various orchards. The present study did not involve any endangered or protected species of the region.

Isolation, purification and identification of fungal cultures

The fungus from the infected samples was isolated and purified using monoconidial method by streaking out spores on plates containing 2% water agar, pure fungal cultures were obtained by transferring single germinated conidium on potato dextrose agarcontaining antibacterial chloromphenicol (50 μg/ ml) to avoid bacterial contaminations[21]. Total 30 cultures were identified by comparing with available literature[22] and maintained for further studies. The spores were also verified using compound microscope (Olympus) at different resolutions 4x to 40x.
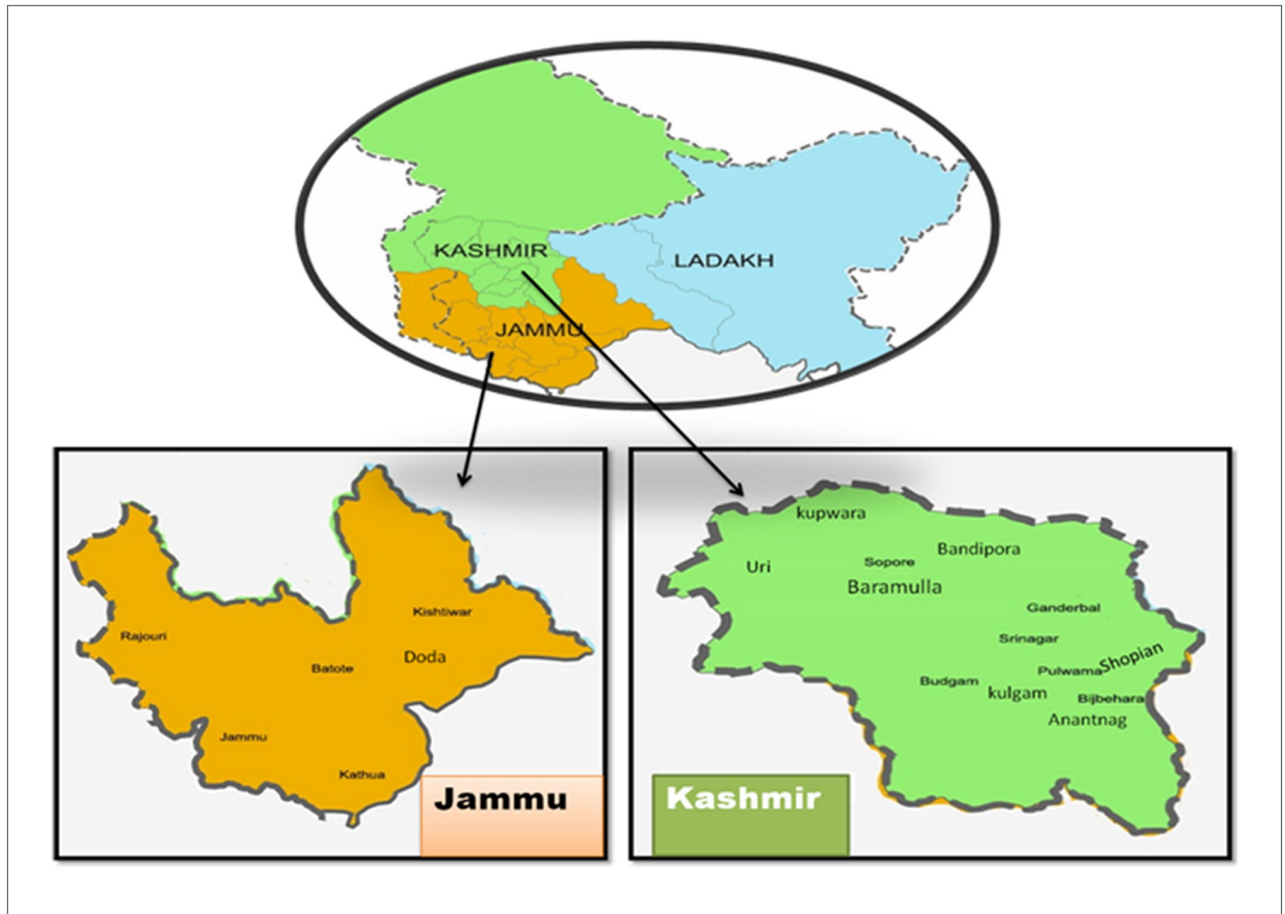
Fig 1. Two apple growing regions of Jammu and Kashmir and areas from where sample collection was carried out.

https://doi.org/10.1371/journal.pone.0224300.g001

DNA extraction

Cultured fungal isolates in 100ml of potato dextrose broth was kept in incubator-shaker at 19˚C for about 25–30 days under continuous dark. The mycelia harvested was blotted dry between the tissue layers and immediately frozen in liquid nitrogen. After freeze-drying, DNA was extracted using Fungal DNA isolation kit (GCC Biotech India Pvt. Ltd). The DNA was quantitatively and qualitatively checked using a Nanodropspectrophotometer (Themoscientific) and was further diluted to a workingconcentration of 30ng/µl and stored at -20˚C for further use.

PCR amplifications

ITS rDNA amplification. All 30 isolates were amplified using polymerase chain reaction (PCR) in a thermal cycler (Takara Japan) using 30ng of genomic DNA in a final volume of 25 µl per reaction. The universal ITS primers with ITS 1 as forward and ITS4 as reverse primerwere used for PCR amplification (White et al., 1991). ThePCR was performed in a 0.2-ml tube containing 0.5µM forward and reverse primer, 200 µM eachdNTP, 1 unit kappa Taqpolymerase and 1ul of genomic DNA in a 10xkappa buffer and 5 mM $MgCl_2$. The PCR was normalized after repetitive cycles till optimal amplification was achieved and consists of 35 cycles involving initial denaturation step at 94˚C for 5 min, followed by 94˚C for 30 s, annealing at

53˚C for 45 s, extension at 72˚C for 1min and final extension at 72˚C for 15min[23]. The PCR products were electrophoresed in 1% agarose gel in 0.5 X Tris-Borate-EDTA buffers (89 mMTris-HCl, 89 mM boric acid, 2.5 mMEDTAand pH 8.5) at 110V. For estimating amplicon size, 100 bp DNA molecular ladder was used (ABgene, UK) and electrophoresis was done for 1 hour[24]. The fragments were observed under UV lamp in gel-documentation (Bio Rad, Gel Doc XR system 170–8170).

SSR amplification. For diversity and structure analysis of selected fungal samples, 28 published SSR primer pairs were used Table 2 [11, 13]Conditions for PCR were initial denaturation at 94˚C for 3 min, followed by 35 cycles of denaturation step at 94˚C for 30 s, 45 s of annealing at 50–60˚C, 1min of extension at 72˚C, and a final extension of 15 min at 72˚C which was performed in a 10 μl final volume containing 2 μl of 10X PCR buffer, 3 mMMgCl2, 0.5mMdNTP, 0.5μl of *Taq* DNA polymerase (kappa), 1μM of each primer and 1μl DNA template [23]. The amplified PCR products were resolved in 2.5% agarose gel at 110 V for 3 h. The bands amplified in different isolates using SSR primers in gel werevisualized using a Gel documentation system (Bio Rad, Gel Doc XR system 170–8170).

Sequencing, nucleotide alignment and phylogenetic analysis. Amplified PCR products were sequenced at Agri Genome Labs (Infopark Road, Kakkanad, Kerala, India). Primers for the sequencing PCR product were the same as for the PCR amplification. The sequences of PCR products were assembled using DNA baser V.4 program to produce complete contig. These were further aligned using CLUSTAL W method of Bio-Edit software and aligned sequences were deposited in NCBI GenBank. A database search of homologous sequences was performed by BLAST analysis at NCBI (http://ncbi.nlm.nih.gov/BLAST). The sequence generated from the present study and reference strain sequences retrieved from GenBank were used to construct phylogeny by neighbour joining method with 1000 replications for each bootstrap value using MEGA 7.0 software version [25]. The other species of *Venturia pirina and nashicola* were also included in phylogeny to separate *Venturia inaequalis* from these species. For validation of results, an out group non-fungal pathogen *Pseudomonas syringae* was selected.

Statistical analysis. Analysis was carried out using POPGENE for gene frequency, allele number, effective allele number, polymorphic loci, gene diversity, Shannon index, gene flow, genetic distance. The GenAlEx version 6.5 for distance-based analysis like AMOVA (Analysis of molecular variance), and PCoA. (Principle coordinate analysis)[26, 27]. The scoring was done as base pair scoring and binary scoring in which bands were scored as '1' (for presence) and '0' (absence) [28]. Index of association rd statistics was applied to examine associations of alleles among different loci [29, 30], which is a comprehensive measure of multilocus linkage disequilibrium[30]. DARwin software version 5.0.158 was used in phylogenetic analysis[31]. Population structureand individual clustering (K) was done by means of Structure software ver. 2.3.4 [32], ΔKmethod[33] was applied to best estimate K, and was computed using Structure Harvester ver. 0.56.3[34, 35].

Results

Morphological identification

Identification based on morphological characters from fungal culture (Fig 2a) revealed that the conidia are single-celled, uninucleate and narrower at one end than the other (Fig 2b). In mass, conidia appear brown or olive, but they are lighter when viewed individually under the microscope. Conidia ranges from 6 to12 μm wide and 12 to 22 μm long and are produced by specialized short hyphae called conidiophore. The characters observed were similar to those described by [22] for *Venturia inaequalis*.

Table 2. List of 28 SSR primers with forward (F) and reverse(R) sequence, allele size and annealing temperature.

Locus	Primer sequence 5′–3′	Allele Size (bp)	Annealing Temp.
Vitc1/2	F:CTTACCTCTCACTTGCTAAC R:GTTCTGACAAGACTGTGTTG	173–241	58
Vitc1/82	F:ACTGTCTCTAGGCGAAAG R:ACTTGGAAGCTCGCTAAG	227–247	58
Vitc1/130	F:GATTGGTGACGCATGTGT R:GCTGGAGATTGCGTAGAC	132–152	58
Vitc2/D	F:GCTCCTTCTGGGTAAGA R:CTCTACATCTCATCCCATC	184–278	58
Viaggt8/1	F:GTGCGGAATATCCAAGTA R:CCAGACTCCTCTACTACAACC	188–196	58
Vica9/152	F:GCACCTGCTCTGTCTATCTC R:AAGGTTCAGGCACTGGAG	167–191	58
Vitcca7/P)	F:GAATACTTCCAAGTGCACAG R:GTGACGCGGATATGGTAC	192–224	58
Vitg11/70	F:GAAGAGGTTGGAGTGGTTG R:GAACCGAATCTGTACAGGAC	184–196	60
Vica9/134	F:ATACAGGAGTGAACAGCAGG R: ATACTGCTCACTTGCGTGC	228–236	60
Vigtg10/95	F: AGGTGTTGCTGTCTTGGAG R: CGATAGTGTCATTTCCAATCC	134–169	58
Vicacg8/42	F: TGTCAGCCACGCTAGAAG R: CACCGGACGAATCATGC	196–232	60
Vigt8/146	F: TGGAGAGAAGAGAAGAGTGG R: GGCAACATCCAATAATCG	128–134	60
Vitc2/16	F: ACATTGACGAAGACGAGC R: TACAATTGAGGCGTGTCC	147–165	58
Viga3/Z	F: ACGCCTCCTTACTTCTTG R: CCCTCGTATTACGTTCTC	87–97	58
Viga7/116	F: GCCTGGTTGTGGATCTGTC R: ATCCTGCTACATCGACCTTC	159–173	60
Vitg9/99	F: CCGTGTCGAGACCTAATATC R: TGGCTCTTCGTAAGTCCTTC	155–167	58
Vica9/X	F: TCGCGCATCACTATCTACAC R: AGACAGGAATGTGGTGGAAG	225–239	58
Vica10/154	F: CCTCCTTCCTATTACTCTCG R: CTGAAGCGAACCTATGTCC	108–172	58
ViaacS10	F: ATTCCAAGCCTTACAACACCC R: TCCACTTCACCCATCGTTC	180–186	58
Vigt10/ε	F: GCAGTGCAGGAATAGTAAGG R: GCTGTGATACCAGAGAACGA	171–173	60
Vitg9/129	F: CTAATTCAACTCGCTGCGTC R: TTTCAGCCAGCTAACCTAGG	267–285	58
1tc1a	F: TCGAGATCCTCAAACTTCCTT R: TTTTAACTGTGCGGCCTG	109–187	54
1tc1b	F: CGATTGGGGATATGAAGACTT R: TTAGTAATCAAATCGCACCCA	149–210	54
1tc1g	F:TCACTCAACAATACAGTTTCTTAG R: TTTCACGGTAGCGATAGGAG	111–185	57
1aac3b	F: AGCGCTAGGTCGTGAAATC R: TTTCTGAAGTGTGTGGGACAT	118–174	55
1aac4b	F: GGTGAGGAGGGAGACGAG R: CATCACGCCCCTATCAAAC	166–177	58
1aac4f	F: CTTGACAGACCACAGCGAC R: CTGACTGAGAGTGGCATCG	96–116	58
1aac4h	F: TCGTTCATCGTTCGTTTTTTCG R:AATAGTGCGTACCCATATATCCA	198–201	56
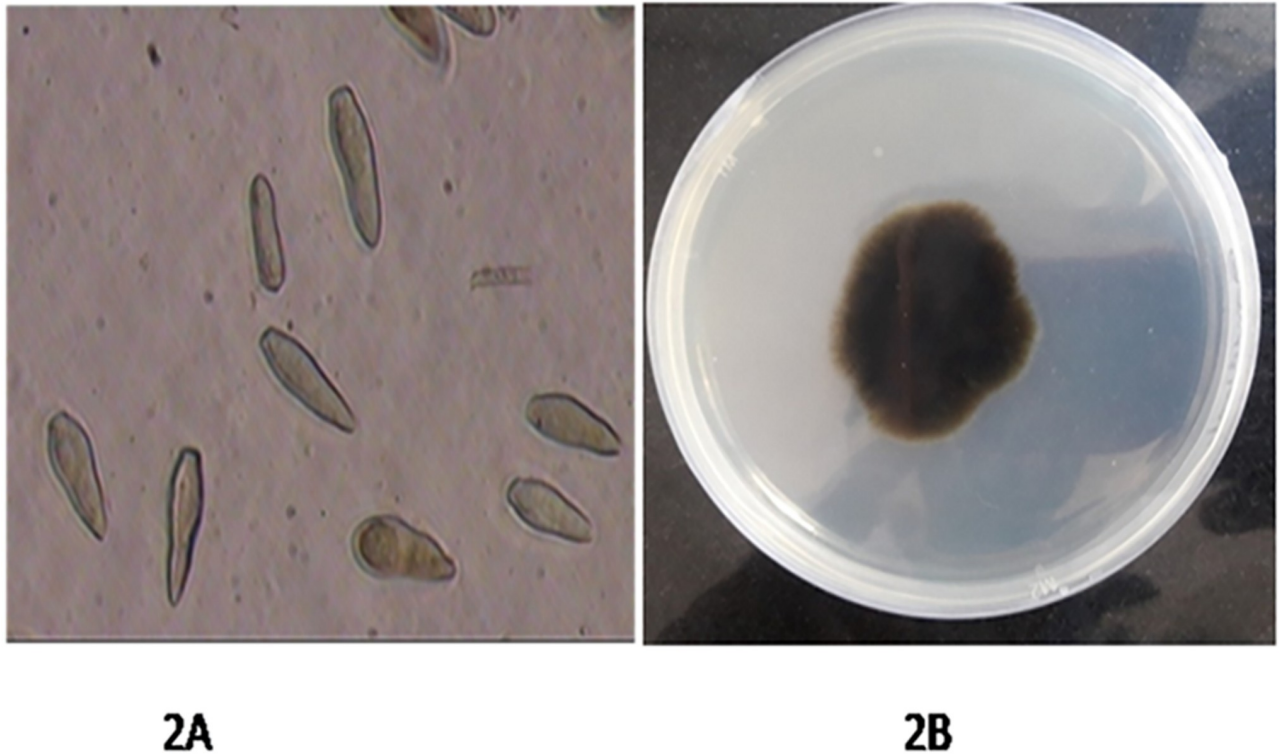
Fig 2. The spores verified under compound microscope (Olympus) at 10x to 40x. 2A. Conidia observed under 40 x resolutions. 2B. Pure culture of *Venturia inaequalis*.

https://doi.org/10.1371/journal.pone.0224300.g002

Molecular characterization

The ITS based primers amplified ~550bp ampliconproducts after sequencing were run for BLASTn and all obtained sequences showed 96%-98% sequence homology with *Venturia inaequalis* GenBank submitted sequences. Sequences were submitted to NCBI GenBank and accession numbers were received (Table 1). Phylogenetic analysis revealed that our isolates clustered along with other submitted *Venturia inaequalis* isolates in GenBank. The sequences of other isolates *Venturia pirina* and *Venturia nashicola* formed separate subclusters. *Pseudomonas syringae* formed a different cluster (outgroup) in phylogeny (Fig 3). During present study the molecular characterization using ITS ribotyping of 30 isolates collected from two regions of J&K showed sequence homology with isolates reported from different regions of worlds particularly Iran (Khe1) and (MG2), South Africa (KELB2), India (Vi22) and Netherland (CBS).

SSR genotyping and genetic diversity analysis

In total 30 *Venturia inaequalis* isolates were genotyped using 28 SSR markers. The results obtained through POPGENE software analysis for genetic diversity parameters are presented in (Table 3). The number of alleles per marker varied from 1 to 15 with an average of 4 per locus detecting the highest number of 15 alleles from Vitc1/2 and Vitc1/130 in the set of 30 isolates. The mean PIC value was found to be 0.073 with minimum value of 0.014 (Vitc1/130) and maximum of 0.195 (Vitg11/70). The average heterozygosity (Ave_Het) ranged between 0.0 and 0.5, with an average of 0.39. The expected heterozygosity or gene diversity (He) ranged from 0.0 to 0.50 with an average of 0.40. The effective number of alleles (Ne) ranged from 1.0
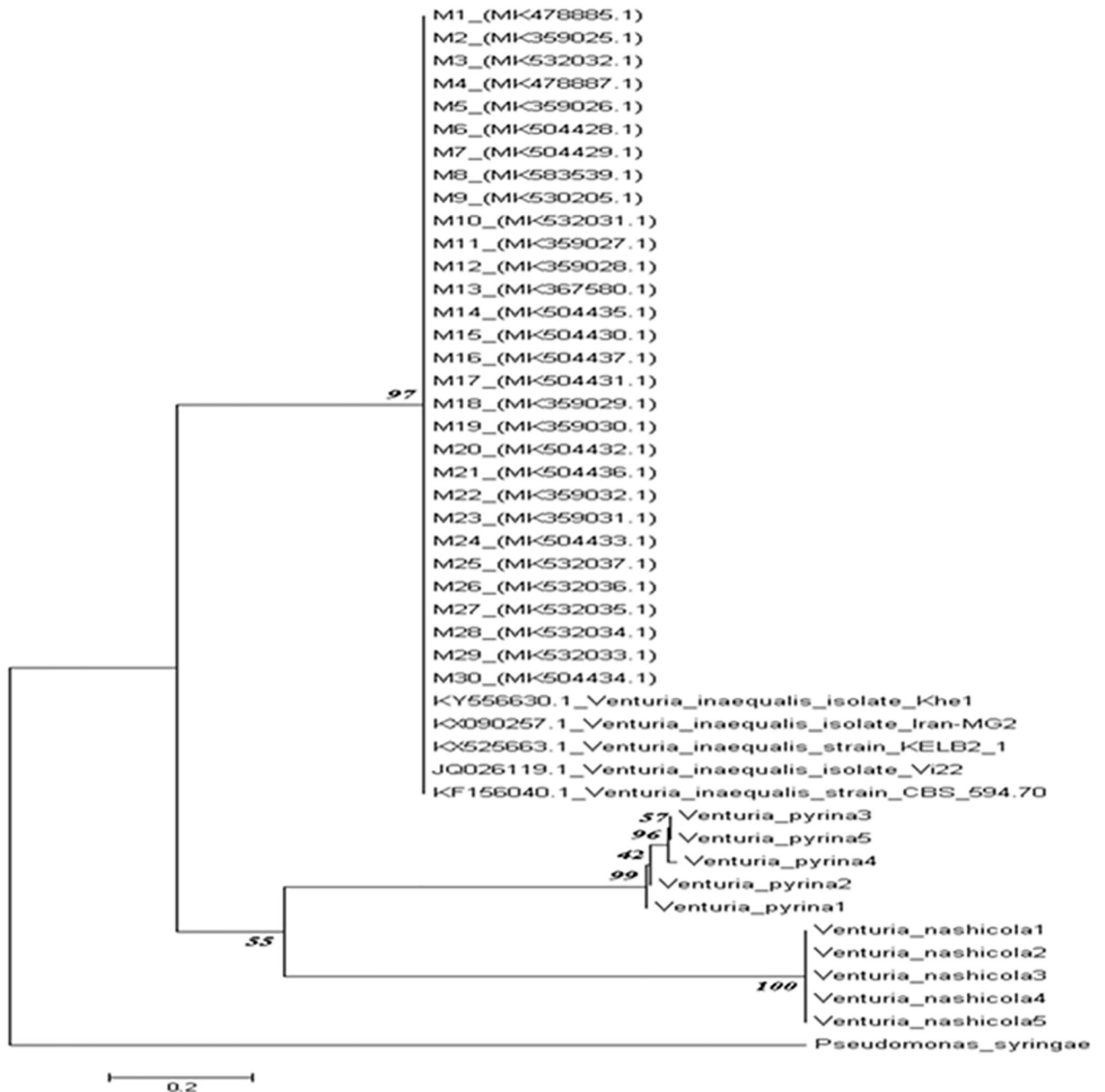
Fig 3. Phylogenetic relationship of *Venturia inaequalis* using the internaltranscribed spacer (ITS) region gene nucleotide sequence alignment with Bootstrap values supporting the branches are shown at nodes; branch lengthsare proportional to divergence.

to 2.0 with an average of 1.74. Shannon index (I) an estimate of diversity ranged from 0 to 0.6 with an average of 0.57. The Fst value ranges from 0 to 0.6 with an average of 0.194.

Cluster analysis

The cluster analysis of 30 isolates revealed a high genotypic diversity within *Venturia inaequalis* populations. Three major clusters I, II, III were obtained using neighbour joining method in Darwin 5.0 software using SSR scoring data. The cluster I accommodated 11 isolates (M20

Table 3. Summary of the genetic analysis of *Venturia inaequalis* isolates with 28 SSR loci.

| Locus	Ne	I	Ob_He	Exp_Ho	Exp_He	Nei	Obs_Hom	Ave_Het	Pic	Fst
Vitc1/2	1.25	0.36	0.23	0.79	0.20	0.20	0.76	0.20	0.20	0.15
Vitc1/82	1.18	0.28	0.16	0.84	0.15	0.15	0.83	0.15	0.15	0.08
Vitc1/130	1.92	0.67	0.66	0.51	0.48	0.48	0.33	0.48	0.10	0.32
Vitc2/D	1.38	0.45	0.33	0.71	0.28	0.27	0.66	0.27	0.25	0.21
Viaggt8/1	1.99	0.69	0.83	0.49	0.50	0.49	0.16	0.49	0.16	0.64
Vica9/152	1.99	0.69	0.86	0.49	0.50	0.49	0.13	0.49	0.32	0.07
Vitcca7/P	2.00	0.69	1.00	0.49	0.50	0.50	0.00	0.50	0.52	0.00
Vitg11/70	1.86	0.65	0.73	0.52	0.47	0.46	0.26	0.46	0.20	0.12
Vica9/134	1.94	0.67	0.76	0.50	0.49	0.48	0.23	0.48	0.16	0.00
Vigtg10/95	1.86	0.65	0.73	0.52	0.47	0.46	0.26	0.46	0.07	0.24
Vicacg8/42	1.99	0.69	0.96	0.49	0.50	0.49	0.03	0.49	0.16	0.00
Vigt8/146	1.94	0.67	0.43	0.50	0.49	0.48	0.56	0.48	0.16	0.00
Vitc2/16	1.98	0.68	0.63	0.49	0.50	0.49	0.36	0.49	0.16	0.15
Viga3/Z	1.14	0.24	0.13	0.87	0.12	0.12	0.86	0.12	0.16	0.00
Viga7/116	1.96	0.68	0.86	0.50	0.49	0.49	0.13	0.49	0.16	0.00
Vitg9/99	1.99	0.69	0.96	0.49	0.50	0.49	0.03	0.49	0.16	0.00
Vica9/X	1.94	0.67	0.83	0.50	0.49	0.48	0.16	0.48	0.16	0.04
Vica10/154	2.00	0.69	1.00	0.49	0.54	0.50	0.00	0.50	0.16	0.00
ViaacS10	1.96	0.68	0.86	0.50	0.48	0.49	0.13	0.49	0.30	0.00
Vigt10/ε	1.99	0.69	0.96	0.49	0.50	0.49	0.03	0.49	0.10	0.00
Vitg9/129	1.64	0.57	0.53	0.60	0.39	0.39	0.46	0.39	0.25	0.00
1tc1a	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.15	0.00
1tc1b	2.00	0.69	1.00	0.49	0.50	0.50	0.00	0.50	0.12	0.00
1tc1g	1.30	0.39	0.26	0.76	0.23	0.23	0.73	0.23	0.10	0.00
1aac3b	1.76	0.62	0.63	0.55	0.44	0.43	0.36	0.43	0.13	0.00
1aac4b	1.86	0.65	0.73	0.52	0.47	0.46	0.26	0.46	0.15	0.00
1aac4f	1.06	0.14	0.06	0.93	0.06	0.06	0.93	0.06	0.10	0.18
1aac4h	1.96	0.68	0.73	0.50	0.49	0.49	0.26	0.49	0.15	0.00
Mean	**1.74**	**0.57**	**0.64**	**0.59**	**0.40**	**0.39**	**0.35**	**0.39**	**0.18**	**0.19**
Standard deviation	**0.34**	**0.19**	**0.31**	**0.15**	**0.15**	**0.15**	**0.31**	**0.15**	**0.08**	**0.03**

(Exp_Ho) Expected homozygosty (Exp_he) heterozygosity were computed using Levene (1949), (Nei) Nei's (1973) Na = Observed number of alleles, Ne = Effective number of alleles [Kimura and Crow (1964)], I = Shannon's Information index [Lewontin (1972)], (Ob_He) observed heterozygosity, (Ob_Ho) homozygosity, Fst = genetic differentiation.

https://doi.org/10.1371/journal.pone.0224300.t003

to M30), cluster IIcontained 18 isolates (M1to M18) and Cluster IIIincluded only 1 isolate (M19). Both The Cluster I and II were further subclustered into two subclusters (Fig 4). The isolates could be grouped into separate clusters on the basis of geographical distribution as shown in Table 1.

Population structure

Structure analysis revealed that isolates of *V. inaequalis* collected from different places Jammu and Kashmir were grouped into two major populations. The assumed values of probable subpopulations (K) were ascertained by choosing higher ΔK value, with respect to the number of clusters inferred by Structure [33]. As per the Evano table output, (S1 Table) the K = 2 was observed to be the best due to high ΔK peak value of 34.6 among the assumed K (Fig 5). Isolates from Jammu region having same latitude in J&K geographical map were grouped as
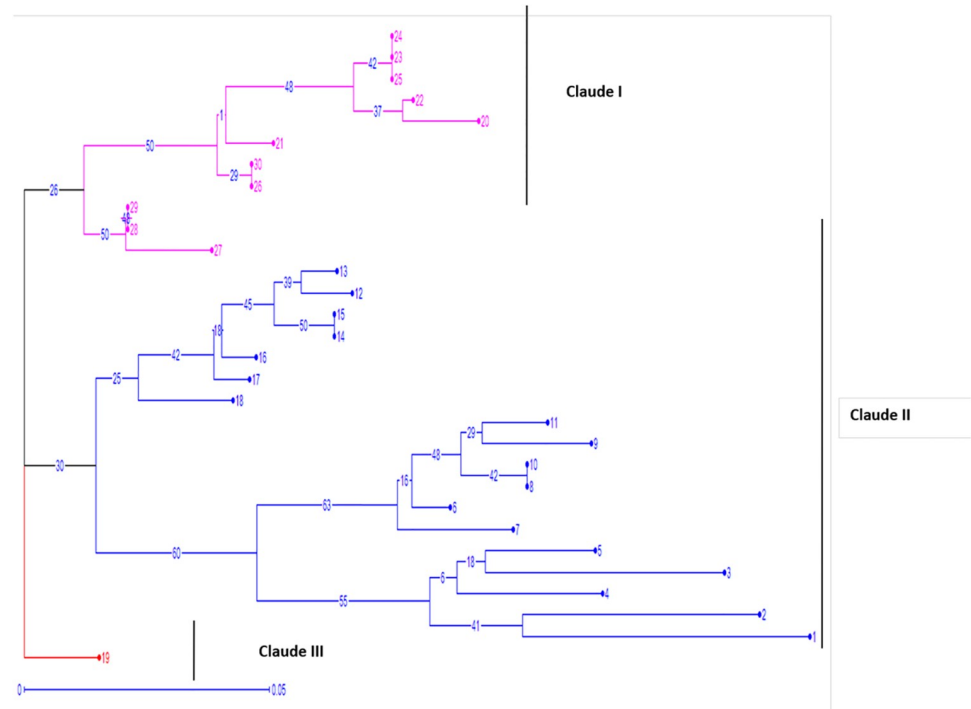
Fig 4. The cluster analysis (dendrogram) of 30 isolates revealing diversity within *Venturia inaequalis* populations. The bootstrap values are given on the nodes.

https://doi.org/10.1371/journal.pone.0224300.g004

subpopulation 1. Similarly, isolates from Kashmir region having same latitude in J&K geographical map were grouped subpopulation 2 with two admixtures (M19 & M27) (Fig 6). Moreover, STRUCTURE analysis grouped 2 individuals (6.6% of the total isolates) with a Q admixture proportion to the second cluster with the probability of 0.2 and 0.8, suggesting a substantial level of gene flow between the two clusters. Population 1 contains isolates from 1–18, while as population 2 comprised of isolates from 20–26, 28, 29 & 30. Two isolates (i.e. 19 &27) fall as admixture minimally.

Analysis of molecular variance (AMOVA)

The two populations along with admixture isolates generated from structure analysis were analyzed for genetic variation among and within populations using AMOVA (Table 4). However, in population 3 sample sizes is less than 5which cannot be considered as a population. From the analysis, 25% of variation was observed among population, 9% among individuals and 66% within individuals observed in the population. Wright's F statistic was estimated to determine deviation of Hardy-Weinberg expectation in the population. The F_{is} for all the 28 marker loci was 0.126, while F_{it} was 0.343 across the clusters. Pair wise F_{st} values showed significant differentiationamong all the pairs of sub-populations ranging from 0.248 to 0.881 suggesting that all the threegroups were significantly different from each other. The F_{st} values and their distribution pattern show clear differentiation of sub populations from each other. This result was also validated by the principal coordinate analysis (PCoA), (Fig 7) where co-ordinate 1 and 2explained variation of 36.6% in population 1, 14.30% in population 2 and 13.10% in population 3 and overall cumulative percentage of variation of 64.07%.
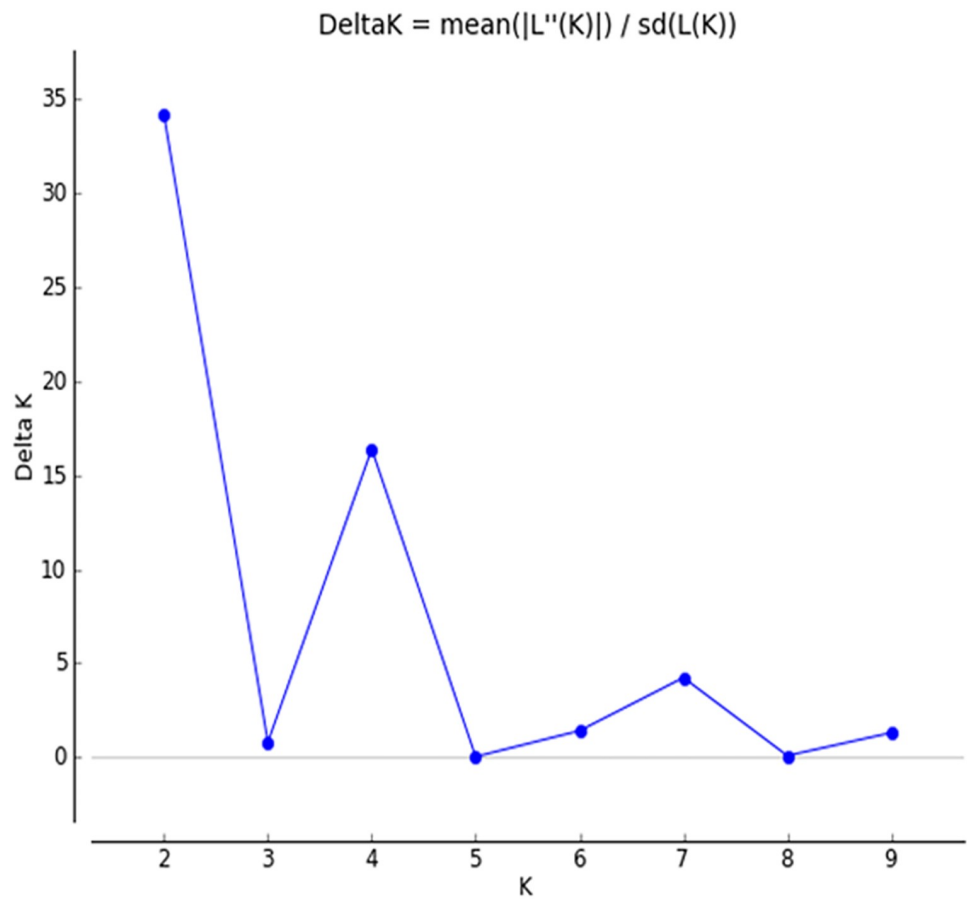
Fig 5. ΔK peak value of 34.6 among the assumed K.

Discussion

Apples are grown in high altitude areas of India particularly in J&K[36]. It is the primary culti-vated crop in J&K because though the climate conditions are distinct (sub-temperate to true
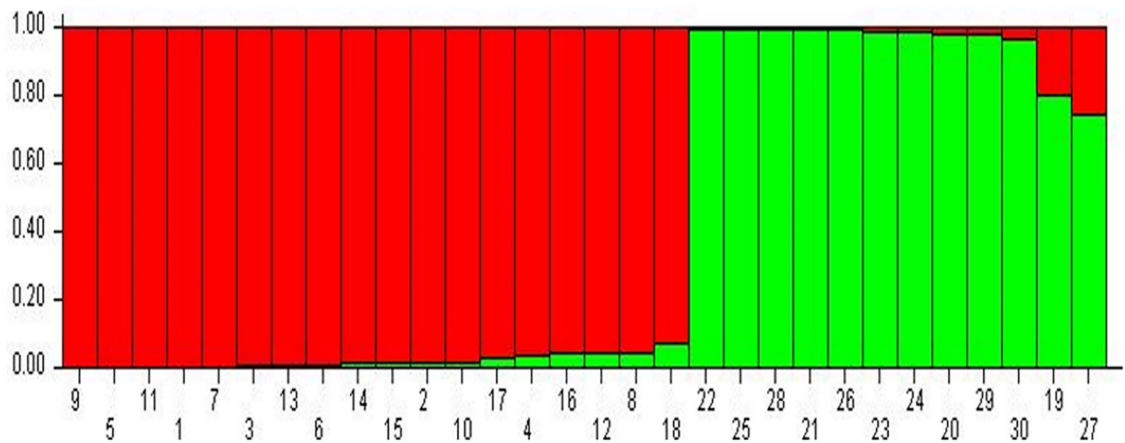

Fig 6. Population distribution using STRUCTURE analysis software, the isolates were grouped into two major populations with a small admixture (19 and 27 only).

Table 4. Genetic variation among and within populations using AMOVA.

Source	Df	SS	MS	Est. Var.	%
Among Pops	2	21.720	10.860	0.554	25%
Among Indiv	27	50.980	1.888	0.211	9%
Within Indiv	30	44.000	1.467	1.467	66%
Total	59	116.700		2.231	100%
F-Statistics	**Value**	**P**			
F_{st}	0.248	0.001			
F_{is}	0.126	0.040			
F_{it}	0.343	0.001			

Df-Degree of freedom, SS Sum of squares, MS Mean sum of squares, Est. Var-Estimated variance

temperate), they are very suitable for the cultivation of apples[37]. This crop suffers huge losses both quantitatively and qualitatively due to frequent epidemics of scab disease [23, 38], which is caused by *V. inaequalis*. The molecular characterization elucidates the genetic diversity among the isolates and for better resistance against any pathogen, the diversity must be known and accordingly resistant varieties can be developed. Hence in present study the molecular characterization of *V. inaequalis* was undertaken in order to provide better management strategies for this disease in the form of resistance and cisgenic breeding approaches.

We used ITS ribotyping of 30 isolates collected from two distinct regions. The noncoding ribosomal DNA ITS sequences doesn't change more rapidly than the coding sequences and
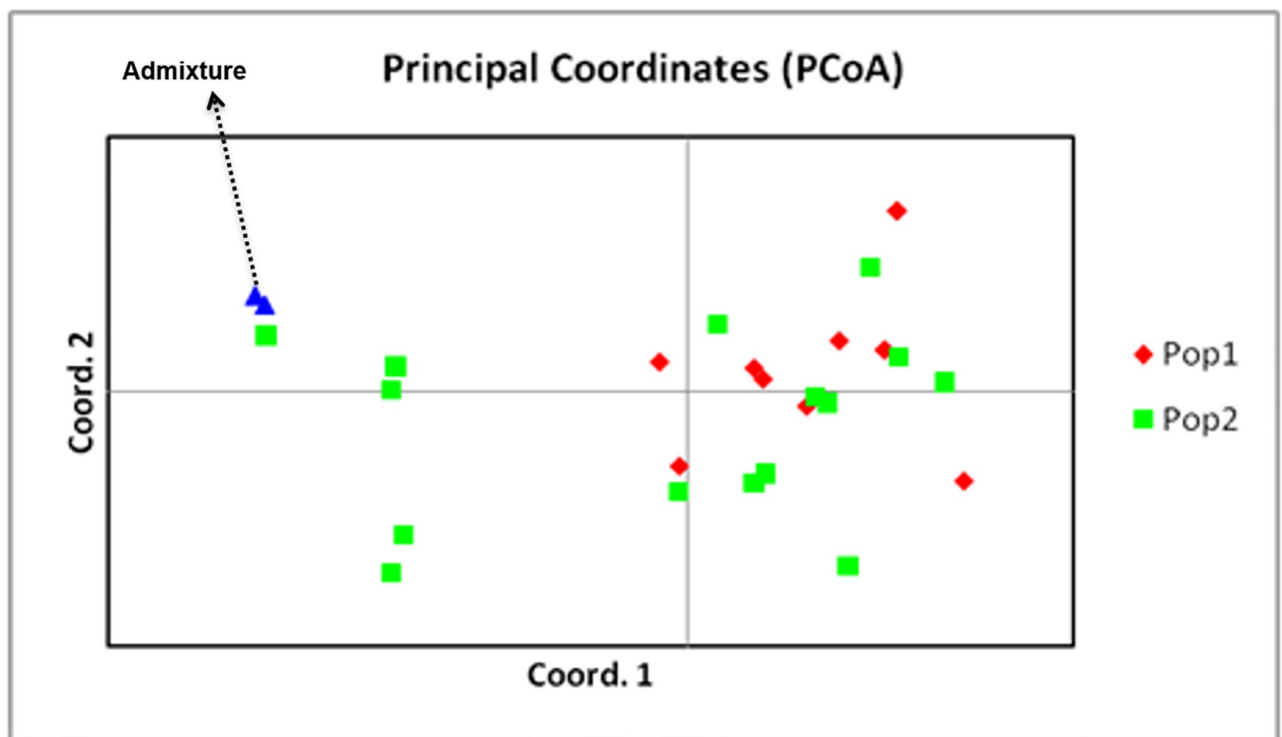

Fig 7. Principal coordinate analysis showing the clustering of two *Venturia inaequalis* populations and admixture population from different apple growing regions in the Jammu and Kashmir with population 1 (36.6%), population 2 (14.30%) and admixture (13.10%).

may diverge between species and populations [18]. AnalyzingITS regions has become one of the primary methods for identification and characterization of a fungal strain or species [39, 40]. We observed sequence homology with isolates reported from different regions of the world particularly Iran, South Africa, Netherlands, and Canada. As expected, after phylogenetic analysis of 45 *Venturia* sequences (35 *V. inaequalis*, 5 *V. pirina*, *and 5 V. nashicola)*, two clades emerged: one with all the *V. inaequalis* sequences and another with the *V. pirina* and *V. nashicola* sequences that further separated into two subclades. Bilal *et al*[21] had similar results. The current study identified taxonomic relationships or differences between 30 *V. inaequalis* isolates, which can help to identify characteristics such as resistance or susceptibility toward a particular anti-fungal agent.

Microsatellites or SSRs are very useful markers for population genetics analysis because of their high specificity, polymorphism, and reproducibility. These are major advantages of using SSR markers over Random Amplification of Polymorphic DNA. The SSR markers used in this study were highly variable and results generally corresponded with previous population genetics studies conducted in Europe. However, in this study the markers1tc1a, 1tc1b, 1tc1g, 1aac3b, 1aac4b, 1aac4f, and 1aac4h showed only one allele compared to eight to ten alleles reported previously[41, 42]. The outcomes of this study along with prior reports[4, 11, 13, 21] confirm the continuation of high variability in *V. inaequalis*.

The distance between the Jammu region and the Kashmir region where we collected samples is approximately 400 km and allowed us to collect *V. inaequalis* isolates from geographically and topologically distinct regions. We observed a high level of diversity, which could be expected due to the climate differences. Xu *et al*[43]also observed remarkable variability between and within *V. inaequalis* isolates obtained from dissimilar apple cultivars in a solo apple orchard. This was credited to assortment pressure applied by diverse cultivars. Another possible explanation of variation in diversity is sexual recombination.

The isolates in this study shared a high percentage of identical alleles, indicating considerable gene flow among all isolates of *V. inaequalis* populations in J&K. Asmost of the apple cultivation area in Jammu and Kashmir is dominated by single cultivar Red Delicious and no resistant variety is under cultivation yet, so there is no selection pressure on pathogen to bring some change, hence this could be the reason having high percentage of identical allelesamong *V. inaequalis* populations. The movement of the *V. inaequalis* from one place to other can be through planting material, high speed winds, but the planting material was one of the most important factors of introduction of *V. inaequalis* to India. This was also observed in the magnitude of migration between the regions. The Kashmir region seems to have the highest migration rate towards it and the lowest away from it. Migration towards the Jammu region was the lowest, indicating that this region is isolated, probably due to warmer winter temperatures. Overall the migration results indicated the possible free movement of the pathogen between the regions. The present study undoubtedly shows that there is high diversity of *V. inaequalis* in the Kashmir valley and reveals that the larger part of variability existed within the individuals.

Structure analyses divided the isolates into two populations (K = 2) with a clear differentiation between the two apple-growing regions. Pathogen distance seems to be the most significant factor in steep gene flow because it explains 50% of the variation among the *V. inaequalis* isolates. Pair-wise F_{st} values, ranging from 0.248 to 0.881 showed noteworthy demarcation between the subpopulations. This signifies that the two groups are notably dissimilar from one another. This outcome is also validated by the principal coordinate analysis, whichdistributed the isolates into two major populations with one admixture (two isolates). The admixture is of smallsize, hence can't be considered as a population.

Conclusion

The genetic variation and population structure of scab causing *V. inaequalis* from different apple growing regions in Jammu and Kashmir shows significant levels of genetic variation within the populations in the similar fashion as observed in other *V. inaequalis* population's studies conducted in Europe and elsewhere. Results indicated that gene flow between regions is occurring and has significant implications for the apple industry if fungicide resistant strains move between regions. Based on ITS sequencing, a database can be maintained to list out the sequence-based isolation of various fungal strains or species. In order to control such menace caused by scab, proper prediction and forecasting systems are need of the hour to prevent apple scab disease well in advance and also understanding the host pathogen interaction, which can provide new insights for effective management of this disease. Cisgenesis can be one of the approaches for introgression of resistance gene through biotechnological intervention under control of its own regulatory sequences from same species or related species which can also maintain the original cultivar characteristics.

Supporting information

S1 Table.
(DOCX)

Author Contributions

Conceptualization: Nazeer Ahmed, Khalid Z. Masoodi.

Data curation: Sheikh Mansoor, Javid I. Mir, Mudasir A. Mir, Khalid Z. Masoodi.

Formal analysis: Sheikh Mansoor, Vikas Sharma, Sumira Jan, Sajad Un Nabi, Javid I. Mir, Mudasir A. Mir, Khalid Z. Masoodi.

Funding acquisition: Nazeer Ahmed, Khalid Z. Masoodi.

Investigation: Javid I. Mir.

Methodology: Sheikh Mansoor.

Project administration: Khalid Z. Masoodi.

Resources: Nazeer Ahmed, Sajad Un Nabi, Javid I. Mir, Khalid Z. Masoodi.

Software: Sheikh Mansoor.

Supervision: Vikas Sharma, Sumira Jan, Sajad Un Nabi, Javid I. Mir, Khalid Z. Masoodi.

Validation: Sheikh Mansoor, Khalid Z. Masoodi.

Visualization: Khalid Z. Masoodi.

Writing – original draft: Sheikh Mansoor.

Writing – review & editing: Sheikh Mansoor, Sajad Un Nabi, Mudasir A. Mir, Khalid Z. Masoodi.

References

1. Ferree DC, Warrington IJ. Apples: botany, production, and uses: CABI; 2003.
2. Nabi SU, Mir JI, Sharma OC, Singh DB, Zaffer S, Sheikh MA, et al. Optimization of tissue and time for rapid serological and molecular detection of Apple stem pitting virus and Apple stem grooving virus in apple'. Phytoparasitica. 2018; 46(5):705–13.

3. Bowen JK, Mesarich CH, Bus VG, Beresford RM, Plummer KM, Templeton MD. Venturia inaequalis: the causal agent of apple scab. Molecular Plant Pathology. 2011; 12(2):105–22. https://doi.org/10.1111/j.1364-3703.2010.00656.x PMID: 21199562

4. Gladieux P, Zhang X-G, Afoufa-Bastien D, Sanhueza R-MV, Sbaghi M, Le Cam B. On the origin and spread of the scab disease of apple: out of central Asia. PLoS One. 2008; 3(1):e1455. https://doi.org/10.1371/journal.pone.0001455 PMID: 18197265

5. MacHardy WE. Apple scab: biology, epidemiology, and management: APS press St. Paul; 1996.

6. Shafi SM. An Overview of Apple Scab, its Cause and Management Strategies. EC Microbiology. 2019; 15:0.1–4.

7. Sivanesan A. The taxonomy and pathology of Venturia species1977.

8. Kodsueb R, Dhanasekaran V, Aptroot A, Lumyong S, McKenzie EH, Hyde KD, et al. The family Pleosporaceae: intergeneric relationships and phylogenetic perspectives based on sequence analyses of partial 28S rDNA. Mycologia. 2006; 98(4):571–83. PMID: 17139850

9. Zhang L. Genetic diversity and temporal dynamics of Venturia inaequalis populations following two apple scab epidemics in Pennsylvania. 2010.

10. Bus VG, Rikkerink EH, Caffier V, Durel C-E, Plummer KM. Revision of the nomenclature of the differential host-pathogen interactions of Venturia inaequalis and Malus. Annual Review of Phytopathology. 2011; 49:391–413. https://doi.org/10.1146/annurev-phyto-072910-095339 PMID: 21599495

11. Tenzer I, degli Ivanissevich S, Morgante M, Gessler C. Identification of microsatellite markers and their application to population genetics of Venturia inaequalis. Phytopathology. 1999; 89(9):748–53. https://doi.org/10.1094/PHYTO.1999.89.9.748 PMID: 18944702

12. Guérin F, Gladieux P, Le Cam B. Origin and colonization history of newly virulent strains of the phytopathogenic fungus Venturia inaequalis. Fungal Genetics and Biology. 2007; 44(4):284–92. https://doi.org/10.1016/j.fgb.2006.10.005 PMID: 17166752

13. Guérin F, Franck P, Loiseau A, Devaux M, Le Cam B. Isolation of 21 new polymorphic microsatellite loci in the phytopathogenic fungus Venturia inaequalis. Molecular Ecology Notes. 2004; 4(2):268–70.

14. Sarkate A, Saini SS, Teotia D, Gaid M, Mir JI, Roy P, et al. Comparative metabolomics of scab-resistant and susceptible apple cell cultures in response to scab fungus elicitor treatment. Scientific reports. 2018; 8(1):17844. https://doi.org/10.1038/s41598-018-36237-y PMID: 30552373

15. McDonald BA. The population genetics of fungi: tools and techniques. Phytopathology. 1997; 87 (4):448–53. https://doi.org/10.1094/PHYTO.1997.87.4.448 PMID: 18945126

16. Stukenbrock EH, McDonald BA. The origins of plant pathogens in agro-ecosystems. Annu Rev Phytopathol. 2008; 46:75–100. https://doi.org/10.1146/annurev.phyto.010708.154114 PMID: 18680424

17. Berbee ML, Taylor JW. Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. Molecular Biology and Evolution. 1992; 9(2):278–84. https://doi.org/10.1093/oxfordjournals.molbev.a040719 PMID: 1560763

18. Liyanage H, McMillan R, Kistler HC. Two genetically distinct populations of Colletotrichum gloeosporioides from citrus. Phytopathology. 1992; 82(11):1371–6.

19. Alahakoon P, Brown A, Sreenivasaprasad S. Cross-infection potential of genetic groups of Colletotrichum gloeosporioides on tropical fruits. Physiological and Molecular Plant Pathology. 1994; 44(2):93–103.

20. Birch JL, Walsh NG, Cantrill DJ, Holmes GD, Murphy DJ. Testing efficacy of distance and tree-based methods for DNA barcoding of grasses (Poaceae tribe Poeae) in Australia. PLoS One. 2017; 12(10): e0186259. https://doi.org/10.1371/journal.pone.0186259 PMID: 29084279

21. Padder B, Shah M, Ahmad M, Sofi T, Ahanger F, Hamid A. Genetic Differentiation among Populations of Venturia inaequalis in. Asian Journal of Plant Pathology. 2011; 5(2):75–83.

22. Winka K, Eriksson OE, Bång Å. Molecular evidence for recognizing the Chaetothyriales. Mycologia. 1998; 90(5):822–30.

23. Ebrahimi L, Fotouhifar K-B, Nikkhah MJ, Naghavi M-R, Baisakh N. Correction: Population Genetic Structure of Apple Scab (Venturia inaequalis (Cooke) G. Winter) in Iran. PLoS One. 2016; 11(11): e0167415. https://doi.org/10.1371/journal.pone.0167415 PMID: 27875584

24. Grimova L, Winkowska L, Konrady M, RYŠÁNEK P. Apple mosaic virus. Phytopathologia Mediterranea. 2016; 55(1):1–19.

25. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution. 2016; 33(7):1870–4. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

26. Peakall R, Smouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes. 2006; 6(1):288–95.

27. Excoffier L, Lischer HE. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular ecology resources. 2010; 10(3):564–7. https://doi.org/10.1111/j.1755-0998.2010.02847.x PMID: 21565059

28. Yeh F. POPGENE 32v. 1.31 Microsoft Window-based Freeware for Population Genetic Analysis. ftp://ftp.microsoft.com/Softlib/MSLFILES/HPGL.EXE.1999.

29. Smith JM, Smith NH, O'Rourke M, Spratt BG. How clonal are bacteria? Proceedings of the National Academy of Sciences. 1993; 90(10):4384–8.

30. Brown A, Feldman M, Nevo E. Multilocus structure of natural populations of Hordeum spontaneum. Genetics. 1980; 96(2):523–36. PMID: 17249067

31. Perrier X, Jacquemoud-Collet J. DARwin software http://DARwin.cirad.fr. DARwin; 2006.

32. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000; 155(2):945–59. PMID: 10835412

33. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology. 2005; 14(8):2611–20. https://doi.org/10.1111/j.1365-294X.2005.02553.x PMID: 15969739

34. Earl DA. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation genetics resources. 2012; 4(2):359–61.

35. Frantz A, Plantegenest M, Mieuzet L, Simon JC. Ecological specialization correlates with genotypic differentiation in sympatric host-populations of the pea aphid. Journal of evolutionary biology. 2006; 19(2):392–401. https://doi.org/10.1111/j.1420-9101.2005.01025.x PMID: 16599915

36. Sheikh MA, Bhat K, Mir J, Mir M, Nabi SU, Bhat HA, et al. Phenotypic and molecular screening for diseases resistance of apple cultivars and selections against apple scab (Venturia inaequalis). IJCS. 2017; 5(4):1107–13.

37. Bhat TA, Lone TA. Potential and Prospects of J&K Economy: Educreation Publishing; 2017.

38. Cooke BM, Jones DG, Kaye B. The epidemiology of plant diseases: Springer; 2006.

39. Monje LD, Quiroga M, Manzoli D, Couri MS, Silvestri L, Venzal JM, et al. Sequence analysis of the internal transcribed spacer 2 (ITS2) from Philornis seguyi (García, 1952) and Philornis torquans (Nielsen, 1913)(Diptera: Muscidae). Systematic parasitology. 2013; 86(1):43–51. https://doi.org/10.1007/s11230-013-9428-5 PMID: 23949648

40. Iwen PC, Hinrichs S, Rupp M. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Medical mycology. 2002; 40(1):87–109. https://doi.org/10.1080/mmy.40.1.87.109 PMID: 11860017

41. Guérin F, Le Cam B. Breakdown of the scab resistance gene Vf in apple leads to a founder effect in populations of the fungal pathogen Venturia inaequalis. Phytopathology. 2004; 94(4):364–9. https://doi.org/10.1094/PHYTO.2004.94.4.364 PMID: 18944112

42. Xu X, Harvey N, Roberts A, Barbara D. Population variation of apple scab (Venturia inaequalis) within mixed orchards in the UK. European journal of plant pathology. 2013; 135(1):97–104.

43. Xu X, Yang J, Thakur V, Roberts A, Barbara DJ. Population variation of apple scab (Venturia inaequalis) isolates from Asia and Europe. Plant Disease. 2008; 92(2):247–52. https://doi.org/10.1094/PDIS-92-2-0247 PMID: 30769384