

Original Research Article

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Phylogenetic Relationship of *Venturia carpophila*, the Causal Agent of Almond Scab from Kashmir Valley as Inferred by ITS nr DNA

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ABSTRACT

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The fungus *Venturia carpophila* causes scab in almond. In order to gain insight, an *in vitro* culture of the fungus has been established and its identity confirmed by its nr DNA. Internal transcribed spacer-ribosomal DNA (ITS-rDNA); the fungal molecular marker was used for molecular analysis. The target region of rDNA (ITS1-5.8S-ITS2) of this species was amplified using universal fungal primers (ITS1 and ITS4). The sequencing of amplified products and their subsequent Basic Local Alignment Search Tool analysis confirmed the identification of species by comparing the sequence of the species with respective species sequences present in Gen Bank. Phylogenetic analysis also confirmed the identification of fungi belongs to *Venturiaceae* family having 100% similarity to other *V. carpophila* species.

Introduction

Almond scab caused by the fungus *Venturia carpophila* (Fisher) (Fisher, 1961) (anamorph, *Fusicladium carpophillum*) is the most common fungal disease on almonds in Kashmir valley. In India almond is mainly grown in Jammu and Kashmir and Himachal

Pradesh with commercial cultivation confined to the state of Jammu and Kashmir, occupying an area of 5710 ha with an annual production of 13109 metric tonnes (Anonymous, 2018). Almond scab is posing serious problem to the orchardist of Kashmir valley from last few years, in which the severity of disease on leaves and twigs ranges

from 24.30 and 26.50 percent respectively (Kacho *et al.*, 2017). Symptoms of scab occurs on shoots, leaves and fruits, first on twigs in the first week of May and later on leaves in the second week of May and in severe cases on fruits. Lesions on twigs, are circular to oval with brown centres and slightly raised purple margins, while leaves have small, indistinct, somewhat circular, greenish yellow blotches undersurface. The lesions later enlarge same reaching 10 mm or more in diameter. With the production of spores, they take an olivaceous appearance and eventually brownish black. On fruit, spots were dark grey to black sooty appearance and coalesced into large dark blotches (Fig. 1). The disease affects fruits and also leads to premature leaf fall resulting in low productivity and poor fruit quality. Management of almond scab is done with dormant treatments of liquid lime sulfur or with copper-agricultural oil mixtures to delay and reduce sporulation of twig lesions (Forster *et al.*, 2009) and within season treatments to prevent new infections. Before the introduction of the quinone outside inhibitor (QoI) fungicides azoxystrobin, trifloxystrobin, and pyraclostrobin, multi-site mode of action fungicides such as captan, ziram, maneb, or wet table sulfur were applied during the petal fall period during leaf emergence and commonly prior to spring rains to manage the disease. Additionally, the single-site mode-of-action methyl benzimidazole carbamate fungicides (e.g., benomyl and thiophanatemethyl) were also used. Resistance against these latter fungicides developed in the pathogen populations in different regions (Ogawa and English, 1991). A number of pathogens from *Venturiaceae* family has been reported on prunus species causing scab like symptoms such as *F. pomi*, *V. carpophila*, *F. obducens* and *F. cerasi* (Schubert *et al.*, 2003). In India, the pathogen was first reported to be causal agent of almond scabin 2017 by (Kacho *et al.*,

2017) from Kashmir valley. However sexual state of *V. carpophila* has been reported in Australia on apricot trees in 1961 (Fisher, 1961), providing clues about pathogen has capacity to undergo genetic recombination. Based on dominant marker system like RAPD and UP-PCR markers, genetic diversity and divergence within closely related species has clearly show difference between *V. effuse* and *V. carpophila* and also indicated difference between isolates of *V. carpophila* of peach and almond (Chen *et al.*, 2014). However, the result of dominant markers is un reliable because of poor reproducibility, artefactual variation and limitations in understanding of population genetics (Novelo *et al.*, 2010). Some molecular analysis has been used to understand the phylogenetic relationships between *V. carpophila* and other *Venturia* species, by (Schnabel *et al.*, 1999), however this is less useful for taxonomic interpretation because of *Cladosporium caryigenum* was taken as out group, which is true anamorph of *Venturiaceae*. In Kashmir valley no phylogenetic analysis has been carried out on *V. carpophila* and preset study objective was to mine and characterize phylogenetic relationships of *V. carpophila* using ITS nrDNA with other species of *Venturiaceae* family.

Materials and Methods

Sample collection and fungal isolation

Samples were collected from leaves and fruits of almond in 2015 from Kashmir valley. They were first dried with absorbent paper in the laboratory for isolation of the pathogen and stored at -4 °C for further processing. The mono conidial isolations were carried out on water agar in Petri plates as described by (Xu *et al.*, 2008). A small fragment of an infected leaf disc was added to a little amount of distilled water and agitated thoroughly to release conidia. The conidial suspensions

were diluted to $8 \times 10^3 \text{ ml}^{-1}$ conidia and 200 μl of the suspension pipetted onto the water agar plates and spread evenly. These plates were incubated at $20 \pm 1^\circ\text{C}$ for 24 h. The individual germinated spores were excised using a cork borer or scalpel blade under a compound microscope and transferred to Petri dishes containing MEA under aseptic conditions. The plates were incubated and maintained at $20 \pm 1^\circ\text{C}$. At least 40 isolates from different plant species were collected and maintained for further studies.

DNA extraction, PCR, sequencing

Total genomic DNA of fungal isolates was extracted using modified CTAB (Cetyltrimethyl ammonium bromide) method (Murray and Thompson, 1980). The ITS nrDNA internal transcribed spacer (ITS) with primers ITS-1 and ITS-4 was amplified and sequenced with primers (White *et al.*, 1990). Comparisons to other nrDNA sequences were conducted with BLAST2.2.24 queries (National Center for Biotechnology Information, National Institute of Health, Bethesda, Maryland). Representative sequence was deposited in GenBank.

Sequence alignment and phylogenetic analysis

Sequence generated were analysed with other sequences obtained from GenBank (Table 1). A BLAST query was performed to find possible sister groups of the sequenced taxon, and closely related sister groups are included in the phylogenetic analysis (Table 1). A multiple alignment was conducted in MEGA v. 6.02 (Tamura *et al.*, 2013) and analyses were performed. Prior to phylogenetic analysis, ambiguous sequences at the start and the end were deleted and gaps manually adjusted to optimize alignment. ITS rDNA dataset was analysed in this study. Best-fit model of nucleotide evolution (T92+G) was

selected by Akaike information criterion (AIC) (Posada and Buckley, 2004) in MrModel test 2.3. Bootstrap analysis with 1000 replicates was used to test the statistical support of the branches. The nucleotide sequences reported in this paper were deposited in GenBank and accession number was obtained as MK482360.

Results and Discussion

During the survey of Kashmir valley, scab like disease on almond which was previously reported by (Kacho *et al.*, 2017) as *C. carpophilum* on the morphological basis was collected. Since the pathogen is reported as anamorph on almond. However, its sexual stage has been reported on apricot by Fisher in 1961 from Australia. This pathogen is difficult to culture because of slow growth rate on culture media which can be masked by other pathogens. The mycelial colony on MEA was dark green turning black after 30-35 days of incubation in pure culture (Fig. 2). Number of isolates was cultured and one isolate were sequenced to find its phylogenetic relationship with other closely related species.

Molecular characterization of *V. carpophila*

To elucidate the relationships between our isolates and other related *Venturia* species, we carried out an analysis of ITS sequences in which new sequence data of *V. carpophila* was used for present analysis together with other additional sequences obtained from GenBank at NCBI. The accession number of present isolate is MK482360. The GenBank sequences with highest identity to ITS1-5.8 rDNA-ITS2 from *V. carpophila* were identified using BLASTN analysis as shown in (Table 1). The sequence of *V. carpophila* shows 91.22-100% identity with other *Venturia* species including *V. carpophila*. The phylogenetic tree was generated from the

ITS-5.8 rDNA-ITS2 of *V. carpophila* isolate with other species of *Venturiaceae*, using neighbor-joining method of the MEGA v. 6.02 (Tamura *et al.*, 2013). The *V. carpophila* isolate clustered with another *V. carpophila* forming a well-supported (100% bootstrap) distinct clade (Fig. 3). The isolates of other species like *C. caryigenum*, *V. martianoffiana* and *V. populina* form distinct clade with 100%, 98%, and 97% of the bootstrap value

in the family of *Venturiaceae*, provides supporting data of the taxonomic position of almond scab fungus (Fig. 3). The observed distances between *V. carpophila* and other *Venturia* species were in a similar range to that observed within the genus *Venturia*, indicating that the sexual phase of *V. carpophila* places this species within the *Venturia* genus.

Table.1 Percentage identity between the ITS1-5.8S rDNA-ITS4 sequences of *Venturia carpophila* and other *Venturia* species

Accession No.	Organism	% identity
KX261879	<i>V. carpophila</i>	100
KX261877	<i>V. carpophila</i>	100
KX261873	<i>V. carpophila</i>	100
KX261875	<i>V. carpophila</i>	100
AF065850	<i>C. caryigenum</i>	92.17
AF065851	<i>C. caryigenum</i>	92.14
KU985131	<i>V. martianoffiana</i>	91.38
KU985132	<i>V. martianoffiana</i>	91.80
AY173018	<i>V. populina</i>	91.97
KF793783	<i>V. populina</i>	91.92
AF065840	<i>V. pyrina</i>	91.67
AF065841	<i>V. pyrina</i>	91.54
AF065845	<i>V. nashicola</i>	91.97
GU086319	<i>V. nashicola</i>	91.85
KX815322	<i>F. pyracanthae</i>	91.42
AF065838	<i>V. inaequalis</i>	91.61
AF531078	<i>V. inaequalis</i>	91.54

Fig.1 Symptoms of *V. carpophila* on Almond (a) fruits (b) Leaves (c) twigs



Fig.2 Colony of *V. carpophila* growing on Malt extract agar

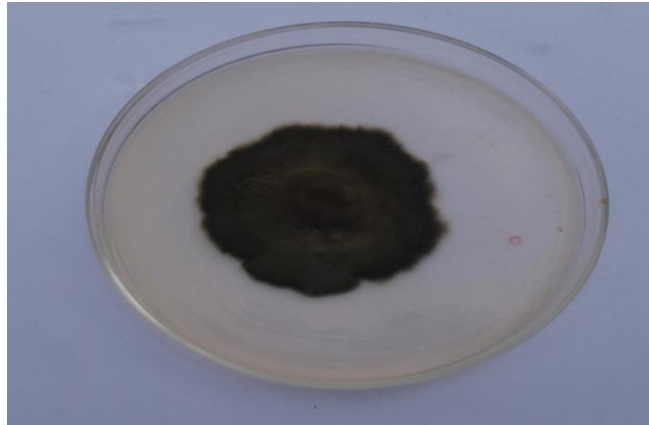
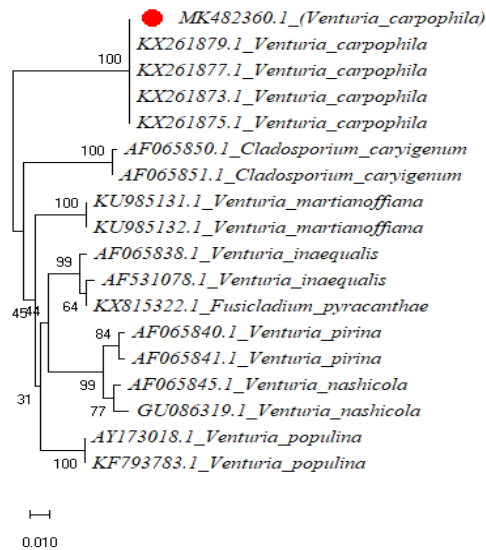


Fig.3 Phylogenetic tree from the ITS1-5.8S rDNA-ITS4 sequences: the tree was constructed using the neighbour-joining method. Bootstrap values (1000 replicates) are indicated above the nodes. Branch lengths are proportional to the genetic distance between the taxa



To study the plant-pathogen interaction a reliable classification of the pathogens is required. Therefore, phylogenetic classification of an organism is based on previous information of model organisms or related taxon's (Taylor, 1995). In mycology, phylogenetic classification based exclusively on morphology is conflicting. This is also true for Deuteromycetes which makes it difficult to compare related sexual and asexual pathogens (Bruns *et al.*, 1991) and can be

overcome with the use of molecular markers. At present, the most reliable sequencing used for this purpose is DNA encoding the small ribosomal subunit (Valente *et al.*, 1999). In our study, the phylogeny of the almond scab fungus based on ITS1-5.8 rDNA-ITS2 region was in consistent with the phylogeny based on the ITS region and are in conformity with (Beck *et al.*, 2005; Braun *et al.*, 2003; Schubert *et al.*, 2003; Schubert and Braun 2002), they put rDNA ITS data of some

cladosporioid *Venturia* anamorphs in a more comprehensive context of *Cladosporium*-like fungi and concluded that these are true members of the *Venturiaceae*. Schubert *et al.*, (2003) described and discussed the taxonomic value of these features for *Venturia* anamorphs in detail, listed morphologically intermediate species and concluded that a separation based on conidial formation and proliferation of the conidiogenous cells is not tenable. As far as possible, anamorphs should reflect monophyletic holomorphic taxa (Reynolds 1993). However, such reassessments and re-evaluations have to be made for all groups of ascomycetes individually. The treatments of cercosporoid *Mycosphaerella* anamorphs (Crous *et al.*, 2000, Crous, *et al.*, 2001, Crous and Braun 2003) and *Venturia* anamorphs (Schubert *et al.*, 2003) are first examples. Moreover, the use of variable sequences like ITS1-5.8S rDNA-ITS2 to confirm the phylogeny of *V. carpophila*, established that this species belongs to the *Venturia* genus. This is valuable information for studying the interaction between *V. carpophila* and almond trees since previous information known from species of the *Venturia* genus can be applied.

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