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FULL-LENGTH RESEARCH ARTICLE

Identification and Genetic Diversity Analysis of *Ascochyta* Species Associated with Blight Complex of Pea in a Northwestern Hill State of India

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Abstract Forty isolates of *Ascochyta* spp. infecting pea collected from different commercial pea growing areas of Himachal Pradesh were characterized into three species—*Ascochyta pinodes*, *A. pisi* and *A. pinodella*—based on symptoms, morphological and cultural traits. The identity of the isolates was confirmed by studying the rDNA sequence patterns. Based on various cultural and morphological trait combinations, 36 isolates were identified as *A. pinodes*, one isolate as *A. pisi* and one isolate as *A. pinodella*. Whereas two isolates, As3 and As14, could not be placed in any of the species as equal number of characters matched with *A. pisi* and *A. pinodes*. However, sequencing of ITS region of these isolates resolved the ambiguity and placed them in *A. pinodes*. This necessitates ITS sequencing as one of the criteria to establish the taxonomic status of *Ascochyta* species infecting pea. RAPD, RAMS, and Rep-PCR analysis with 10, 4 and universal ERIC, and BOX-AIR primers categorized different isolates according to their geographical locations. This study established the predominance of *A. pinodes* in Himachal Pradesh where edible pea is one of the remunerative off-season vegetable crops. This also constitutes first report of genetic variability of *Ascochyta* species infecting pea in India.

Keywords Pea · Blight complex · *Ascochyta* · rDNA · Genetic diversity

Introduction

Pea (*Pisum sativum* L.), an edible legume is grown throughout the world. As a winter season crop in India, pea enjoys the status of an important off-season crop in temperate Himachal Pradesh regions like Kinnaur, Lahaul and Spiti and mountainous locations of Kullu, Mandi, Shimla and Sirmaur districts. The unique geographical diversity of Himachal Pradesh offers an opportunity to produce pea both as a winter crop in low and mid hills, and as summer crop in high hills. It is grown in an area of 16,348 ha with annual production of 1,77,036 metric tonnes [3] and during off-season it sells at a premium in the plains of India. Consequently, pea has emerged as the major off-season vegetable crop of the state.

Full genetic potential of the crop is not realized owing to a variety of biological constraints, and *Ascochyta* blight is one besides others such as powdery mildew. *Ascochyta* blight complex is a severe disease of peas throughout the world and causes huge losses to growers every year [8, 14, 26, 42]. The disease complex is caused by three *Ascochyta* species: *A. pisi* (teleomorph *Didymella pisi*) [12], *A. pinodes* (teleomorph *Mycosphaerella pinodes*) and *Phoma*

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medicaginis var. *pinodella* [25]. However, recently the fourth species *Phoma koolunga* was reported to occur in the south Australia [13]. The principal characteristics used to distinguish the former three species were: (i) the presence of pseudothecia in *M. pinodes*, (ii) size of the conidia (conidia of *P. pinodella* are smaller than those of *A. pisi* or *M. pinodes*), and (iii) carrot red spore masses of *A. pisi* compared with the light buff of *M. pinodes* and *P. pinodella* [25]. The taxonomic position of *Ascochyta* species on the morphological and pathological basis has been controversial for long.

Morphological criteria being influenced by environmental conditions, makes disease diagnosis a cumbersome process [18]. The symptom expression and morpho-cultural methods are highly unstable being environment dependent. Secondly, lack of resistance in pea to *Ascochyta* spp. restricts the detection of variability in this pathogen. Use of molecular markers offers an opportunity to establish the taxonomic status and variability in such pathogens. Recently, molecular approaches using RAPD, AFLP, RFLP, and sequence analysis have been applied in systematic studies of *Ascochyta* species. RAPD fingerprinting methods have been criticized for poor reproducibility [6, 16, 23, 37]. RFLP analysis of rDNA has been applied in population studies of plant pathogens by southern hybridization or restriction digestion of amplified DNA [1, 5, 38]. However, without information on the position of restriction sites, the potential for character homoplasy must be considered when using this type of data for phylogenetic reconstruction [45, 50]. Thus, direct sequence determination provides the most robust data from which phylogenetic reconstruction can proceed with confidence. The most contemporary phylogenetic studies have been based on ITS, mtSSU, mtLSU, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase gene (G3PD), chitin synthase 1 gene (CHS), translation elongation factor 1 alpha gene (EF) and MAT locus sequence analysis, and have supported the organization of *Ascochyta* species into distinct species groups [7, 12, 13, 20, 36].

Information about the dominance of a particular *Ascochyta* species in a particular pea growing region of Himachal Pradesh is lacking. Work on such aspects of the disease has not been undertaken in India. Accurate pathogen identification is most important for diagnosis of the disease and understanding the mechanism of host-pathogen relationship for sustainable disease management strategies. Hence, the objective of this study was to establish the identity of *Ascochyta* specie(s) associated with blight complex of pea using morpho-cultural traits, their predominance in the region and elucidation of molecular diversity among the different species.

Materials and Methods

Collection of Fungus Isolates, Their Purification and Maintenance

Extensive survey of pea growing areas were conducted during June–August 2007–2009 when pea is cultivated as an off-season crop, and 40 isolates of *Ascochyta* spp. causing blight complex were collected to study the population structure and genetic analysis. Each isolate was cultured on potato dextrose agar medium, purified by single spore isolation and maintained for further studies. The symptom development of each isolate was studied on susceptible variety Azad P1 and the characteristic symptoms produced by each isolate were compared according to description given by Lawyer [28] and Sagar [41] (Supplementary Table 1).

Cultural and Morphological Characteristics

The cultural characteristics of the isolates were studied on oat meal agar dispensed (20 ml) into 9 cm diameter Petri plates. Mycelial plugs of 5 mm diameter were cut with the help of cork borer from the margin of an actively growing colony and placed in the center of media plates and incubated at 22 ± 1 °C. Observations were recorded on colony color, zonation and sporulation after 20–25 days of inoculation. Various morphological characters like pycnidiospores (length and breadth), septation of conidia and formation of chlamydospores were recorded. Conidial suspensions prepared from 20 day old sporulating cultures were stained with cotton blue lactophenol followed by microscopic examination of conidial length, breadth, number of septa (range), percent septa, and constriction at septa at 400× magnification using at least 10 pycnidia/plate.

The formation of pseudoperithecia was also studied on oat meal agar slants incubated at 15 ± 1 °C for 25–30 days for formation of perfect stage. Diameter of pseudoperithecia, length and breadth of asci and ascospores were measured as per standard procedure. In order to assign *Ascochyta* isolates into a particular species, consensus speciation was done with traits showing maximum homology.

Molecular Characterization

DNA Fingerprinting

The genetic diversity of *Ascochyta* isolates associated with pea blight was studied by employing random amplified polymorphic DNA (RAPD), rep-PCR and randomly

amplified microsatellites (RAMS). Total genomic DNA of each isolate was extracted using CTAB method [44]. Out of 140 RAPD oligonucleotide primers (Operon and Life Technologies) screened with 5 randomly selected isolates, eight most polymorphic primers (OPA-02, OPA-09, OPA-13, OPD-11, OPQ-13, OPQ-18, S-1466, and S-144) showing consistent banding pattern with two DNA samples of same isolate were used for fingerprinting following the procedure of Padder et al. [34].

Out of eleven RAMS, four most polymorphic primers viz., BA1 (CCA $(CAG)_5$), BA2 $(GTG)_5$, BA8 $(AAC)_5$, and BA9 $(AT)_5$ were used for amplification of 40 isolates following the procedure of Padder et al. [34]. In addition, the test isolates were also amplified by Rep-PCR (with a pair of ERIC and BOX-AIR primers) as described by Mahuku, Riascos [31].

rDNA Analysis

Fifteen isolates of *Ascochyta* identified as *A. pinodes* (13), *A. pisi* (1) and *A. pinodella* (1), and one each from common bean (*Phaseolus vulgaris*), urdbean (*Vigna mungo*) and gram (*Cicer arietinum*) were used for rDNA analysis (Supplementary Table 2). Primers ITS1 and ITS4 [54] were used to amplify approximately 540 bp of ITS1, 5.85 ribosomal gene, and ITS2 region following the conditions of Padder et al. [35]. All PCR assays contained samples of primers without DNA to serve as negative controls. Five µl of the each amplified product was electrophoresed to confirm the successful amplification. The amplified PCR products were resolved by electrophoresis using 1.2 % agarose gel in 0.5X Tris borate EDTA buffer. The gels were stained with ethidium bromide (0.5 µg/ml) for 10 min after electrophoresis followed by distaining under running tap water. The amplicon (3 separate PCR products from each isolate) was lyophilized and sent for custom sequencing (Bioscience India, Pvt. Ltd.). The sequences (forward and reverse) were retrieved from chromatograms using BioEdit version 7.0 [21]. Nucleotide sequences from PCR-amplified fragments (3 amplicons/isolate) were assembled in BioEdit version 7.0 [21] and the consensus sequence was reconfirmed by comparing it with the original sequencing data output. Consensus sequences were aligned using CLUSTAL W [49] option in BioEdit. All the sequences were submitted to gene bank (NCBI) and accession numbers of sequences were obtained (Supplementary Table 2).

Data Analysis

Binary matrices were analyzed by NTYSYS-PC 1.80 and Jaccards coefficient was used to construct dendrogram using SHAN clustered program, selecting the unweighted pair-group arithmetic mean method (UPGMA) in NTSYS-PC V 1.80 (Rolfh 1993). The dendrogram with best fit to similarity matrix based on cophenetic value (COPH) and matrix comparison (MXCOMP) was chosen.

For phylogenetic analysis, ITS regions of *Ascochyta* species infecting pea and other legumes in Himachal Pradesh were compared with 19 other *Ascochyta* sequences from different legumes (Supplementary Table 3) retrieved from NCBI (http://www.ncbi.nlm.nih.gov/nuccore). All nucleotide sequences were aligned using CLUSTAL X 1.8 multiple alignment program [49] and refined manually. The GENEDOC package (www.psc.edu/biomed/genedoc/gdpf.html) was used for formatting the sequences to make them compatible with the desired software. Sequence polymorphism was analyzed, genetic divergence values within and between populations were estimated and a neighbor joining (NJ) tree was constructed for all the haplotypes according to Kimura 2-parameter (K2P) model using PHYLIP ver 3.6 (Felsenstein 1993) in MEGA version 4.1 [48]. The NJ tree was rooted with the rDNA sequences of *Mycosphaerella graminicola*, *M. punctiformis,* and *M. fijiensis* (NCBI GenBank Accession Nos AF181694, AY490760 and AF297225, respectively). Nucleotide diversity (π) and haplotype diversity (h) were estimated using DnaSP 5.0 [29].

Population structure was evaluated using the analysis of molecular variance (AMOVA) model in the ARLEQUIN ver. 3.0 software [17]. Fixation indices (G_{ST}) analogous to F_{ST} [24] were estimated. The analysis used a hierarchical structure in which each country (India, USA, Australia, Israel and Georgia) and each species (*A. pisi*, *A. pinodes*, *A. pinodella*, *P. koolunga*, *A. rabiei* and *Ascochyta* sp.) were considered as populations. The statistical significance of the total and pairwise fixation indices was estimated by comparing the observed distribution with a null distribution, generated by 10,000 permutations of the data matrix. Multiple tests of the same null hypothesis were subjected to table-wide sequential Bonferroni correction to avoid elevated Type I error.

Results and Discussion

Speciation of *Ascochyta* Isolates Based on Symptoms, Cultural and Morphological Characteristics

Species identification criteria (Supplementary Table 1) based on symptoms, cultural characters, conidial size and septation, chlamydospores and formation of pseudothecia were used to identify the *Ascochyta* species associated with blight complex of pea. As per the symptomatology, test isolates were placed in three groups viz., I, II, and III (Table 1). Group I comprised of six isolates viz., As1, As2, As3, As14, As15 and As23 producing circular, light brown

Table 1 Grouping of pea-infecting *Ascochyta* isolates based on symptomatology, cultural and morphological characteristics

Character	Group I (*A. pisi*)	Group II (*A. pinodes*)	Group III (*A. pinodella*)
Symptomatology	As1, As2, As3, As 14, As15, As23	As4, As5, As6, As7, As8, As9, As10, As11, As12, As13, As16, As17, As18, As19, AS20, As21, As22, As24, As25, As26, As27, As28, As29, As30, As31, As32, As33, As34, As35, As36, As37, As38, As39, AS40	–
Colony characters	As2, As3, As4, As16	As5, As6, As7, As8, As9, As10, As11, As12, As13, As14, As15, As17, As18, As19, AS20, As21, As22, As23, As24, As25, As26, As27, As28, As29, As31, As32, As33, As34, As35, As36, As37, As38, As39, As40	As1, As30
Conidial characters	As11, As12, As14, As40	As2, As3, As4, As5, As6, As7, As8, As9, As10, As13, As15, As16, As17, As18, As19, AS20, As21, As22, As23, As24, As25, As26, As27, As28, As29, As30, As31, As32, As33, As34, As35, As36, As37, As38, As39	As1
Chlamydospore formation	As9, As18	As2, As3, As4 , As5, As6, As7, As8, As10, As11, As12, As13, As14, As15, As16, As17, As19, AS20, As21, As22,As23, As24, As25, As26, As27, As28, As32, As31, As33, As34, As35, As36, As37, As38, As39, As40	As1, As29, As30
Pseudoperithecia formation[a]	As1, As2, As3, As4, As5, As6, As7, As8, As9, As11, As12, As13, As14, As15, As16, As17, As18, As19, AS20, As21, As23, As24, As25, As26, As27, As28, As29, As30, As31, As32, As33, As35, As36, As37, As38, As39, As40,		As10, As22, As34

[a] Group I consists of *Ascochyta pisi* and *Ascochyta pinodes*

to tan colored leaf spots measuring 2–6 mm in diameter with a distinct dark margin delineating the border. Isolates of this group induced production of sparse light brown and erumpent pycnidia as minute pinheads. Group II with 34 isolates caused irregular spots measuring 3–15 mm with broad concentric ring pattern in alternate shades of light yellowish brown and grayish purple. Such spots showed no distinct margin like that of isolates in Group I; however, in a few cases, zonation was observed around the spots. Most of the isolates in this group often showed blighted appearance due to coalescing of 2–3 spots. Pycnidia were scattered as small pinheads. Lawyer [28] clearly differentiated the three species with tan colored spots delineated by distinct margin as *A. pisi,* concentric lesions/spots as *A. pinodes*, and the one causing foot rot as *A. pinodella.* These symptoms resemble the description made by various workers [25, 28, 30, 36, 41]. It was interesting to note that of the four isolates placed in group I exhibited two types of leaf spots, i.e., small tan colored (2–6 mm) to large irregular up to 15 mm spots with concentric rings and resembled group II; in addition, these isolates showed marked variation in symptoms under artificial and natural conditions, and are thus categorized as *A. pisi* and *A. pinodella.* Sagar [41] attributed such differences to the environment prevailing during symptom development. Stone [46] and Vaughan [51] considered *A. pinodes* as an ascigerous stage of *A. pisi*, while Jones [25] argued that the two fungi are confused in literature.

All the 40 isolates were divided into three distinct groups on the basis of colony characteristics viz., color, zonation and pattern of pycnidia formation (Table 1). Group I isolates (As2, As3, As4 and As16) formed light colored white colonies possessing few pycnidia without any zonation, whereas Group II accommodating 34 isolates produced brown colonies with concentric ring pattern of alternate dark and brown zonation. Pycnidia were more or less in concentric ring pattern. Two isolates viz., As1 and As30 forming dark gray to black colonies with sparse pycnidia were categorized in group III. Comparison of these characters with other workers revealed that group I, II, and III resembled *A. pisi, A. pinodes,* and *A. pinodella* [28, 30, 33]. The variation in colony color has been attributed to the chlamydospore producing capacity of the three species. Onfroy et al. [33] correlated the gray/black color of *A. pinodella* isolates to profuse chlamydospore production within 15 days as compared to *A. pinodes* forming fewer chlamydospores even after 25–30 days.

The conidial characters (Supplementary Table 4) categorized various *Ascochyta* isolates into three groups viz., I, II, and III accommodating 4, 35, and 1 isolates, respectively (Table 1). On the basis of size of conidia, three groups were distinguished measuring 3–5 × 10–16 µm (54.2 × 14 µm), 3–6.5 × 18–16 µm (4.5 × 12.3 µm), and 2–3 × 3.5–8 µm (4.5 × 12.3 µm) for group I, II, and III, respectively. Onfroy et al. [33] advocated that bicellular conidial production to distinguish pea blight pathogens as average cellular conidia formation was 97.9 ± 2.4 for *A. pinodes,* but only 19.6 ± 6.4 % in *A. pinodella*. The spore dimensions clearly matched group I isolates to *A. pisi,* group II to *A. pinodes* and group III to *A. pinodella* as described by Lawyer (1984). Onfroy et al. [33] observed longer conidia in *A. pinodes* isolates and smaller conidia in *A. pinodella.*

Based on chlamydospore formation, the test isolates were divided into three groups (Table 1). Two isolates As9 and As18 producing no chlamydospores were placed in group I, whereas 35 isolates producing few chlamydospores frequently in about 25-30 day old cultures were placed

in group II. However, in group III, 3 isolates induced profuse chlamydospore formation resulting in dark brown to black colored colonies. Lawyer [28] while distinguishing three *Ascochyta* spp. causing pea blight described chlamydospore formation as a major criterion—with *A. pisi, A. pinodes,* and *A. pinodella*—producing no or few chlamydospores in old cultures and profuse chlamydospores turning the culture medium black, respectively. The present findings are in conformity as group I, II, III resembled *A. pisi, A. pinodes* and *A. pinodella,* respectively. Similar pattern of chlamydospore formation distinguishing the three species has also been reported [25, 30, 33, 41].

Data on pseudoperithecia development on oat meal agar at 15 ± 1 °C revealed that out of 40 isolates only three viz., As10, As22, As34 formed pseudoperithecia and were placed in group III (Table 1). Pseudoperithecia in As10 and As22 were globose, dark brown to black in color having apical ostiole with an average size of 150 × 112.5 μm. Asci were hyaline, bitunicate, cylindrical to sub-clavate measuring 49.69 × 12.19 μm on average (range 41.25–56.25 × 11.25–15 μm). Pseudoperithecia in As34 measured 168.75 × 150 μm with average size of asci being 56.25 × 10.94 μm (range 45–67.25 × 9.37–11.25 μm). The ascostroma was about 131.25 × 112.5 μm. The ascospores were hyaline, bitunicate, two celled, mostly constricted as septum, round and narrower at tips, with guttulate measuring 16.87 × 7.50 μm. Remaining 37 isolates were placed in group I. Efforts to induce perfect stage in *Ascochyta* spp. under artificial conditions were unfruitful and the pseudoperithecial dimensions, size and shape of ascus, and size of ascospores resembled *A. pinodes* [25, 28, 41]. Lawyer [28] stated that principal morphological characteristic distinguishing *A. pinodes* is formation of pseudoperithecia on PDA/Oat meal agar at 16 ± 1 °C. However, Onfroy et al. [33] contested this as a reliable criterion, as perfect stage depends on environmental and/or cultural factors. Bowen et al. [9] succeeded in induction of pseudoperithecia formation in *A. pinodella*, a rare phenomenon otherwise.

Based on symptom type, colony color, and morphological traits it was not possible to distinguish *Ascochyta* species. So identification of test isolates was done using combination of various parameters (Supplementary Table 2). Depending upon matching characters for each species out of 40 isolates, 36 were designated as *A. pinodes*, 1 each of As1 and As2 as *A. pinodella* and *A. pisi*. However, in case of two isolates As3 and As14 (Supplementary Table 2) no inference could be drawn as same cultural characteristics matched both A. *pisi* and *A. pinodes.* The dependability of major differential criteria used for identification of various *Ascochyta* spp. is debatable [10, 18, 39, 47]. Different criteria used in present study resolved the identity in most of the cases and as a consequence, 36 isolates were designated as *A. pinodes* indicating its wide prevalence in Himachal Pradesh. Preliminary studies on symptomatology and pathogen morphology conducted in Himachal Pradesh have shown *A. pisi* as most prevalent in Kullu and Kangra valley of Himachal Pradesh [2]. Chaudhary [11] also identified *A. pisi* as the predominant species causing *Ascochyta* blight and pod spot in three out of four districts of Himachal Pradesh, though possibility of other species was not ruled out. Change in agroclimatic conditions over the years due to global warming could have shifted toward the dominance of *A. pinodes* over *A. pisi*. This was further supported by the absence of *Ascochyta* blight in high altitude areas of Mandi, Chamba, and Kinnaur by Chaudhary [11]. Conversely, areas exhibiting maximum disease incidence previously were found to be disease free in the present study. Similar shift in pathogen populations has also been reported from Canada. Wallen et al. [52] found *A. pinodes* causing pea blight epiphytotic although, formerly *A. pisi* was the predominant species.

Two pathogen isolates could not be differentiated as most of their characters matched both *A. pisi* and *A. pinodes.* These isolates are considered intermediates between two species or misidentified due to lack of dependable differential criteria. Such a case of misidentification is upheld by Faris-Mokaiesh et al. [18] who identified two isolates as *A. pinodes* in the beginning but the PCR–RFLP profiles of IGS region later clustered them in *A. pisi*. They emphasized the need to integrate such molecular tools in the disease diagnosis due to their stability. The probability of these being intermediate species is enhanced by Onfroy et al. [33] who showed two isolates intermediate between *A. pinodes* and *A. pinodella.* The RAPD profiles also separated these two isolates forming a separate clade. However, ITS sequencing of these isolates put them in *A. pinodes* group strengthening the view point of Faris-Mokaiesh et al. [18] to include sequencing of ITS and IGS region as one of the criteria used.

Molecular Characterization

Speciation Based on rDNA Sequence Pattern and Their Phylogenetic Analysis

rDNA segment of 15 isolates of different *Ascochyta* species from pea (Chamba, Kinnaur, and Mandi populations), and one isolate each from common bean, urdbean, and chickpea (Supplementary Table 2) were used to establish the identity of the test isolates determined on morpho-cultural characteristics. rDNA amplification of test isolates with universal primer pairs ITS1 and ITS4 amplified amplicons of 540–550 bp which upon sequencing revealed that sequences of test isolates consisted of 510–545 bp. The obtained sequences were first subjected to BLAST search against *Ascochyta* spp. sequences available online in NCBI GeneBank using blastn program and their identity was

confirmed as *Ascochyta*. The rDNA analysis resolved several ambiguities encountered while determining the taxonomic position of some isolates using morpho-cultural traits. Comparative analysis of sequences of the test isolates infecting pea and reference isolates from gene bank revealed that identity of 13 of the 15 isolates was in confirmation with that of the morphological traits (Supplementary Table 2). Eleven isolates viz., As4, As5, As12, As8, As15, As24, As26, As34, As39, As25 and As16 were more close to *A. pinodes* and 2 isolates As1 and As41 were identical to *A. Pinodella* (Fig. 1).

One isolate As2 misidentified as *A. pisi* based on morphological traits showing more sequence homology with *A.pinodes* was placed in species *A. pinodes* and submitted to the NCBI gene bank. Similarly, isolates As3 and As14 rDNA sequences ascribed the isolates to *A. pinodes* group after blast analysis and were submitted as *A. pinodes* in the NCBI gene bank. Chickpea isolate As44 showing maximum homology to *A. rabiei* sequences was identified as *A. rabiei*. Two other sequences of As42 and As43 isolated from common bean and urd bean were submitted as *Ascochyta* spp.

The use of sequence information from rDNA repeat units is well established in fungal taxonomy for characterizing isolates and resolving taxonomic ambiguities and species definition as this gene cluster occurs within the chromosomes having multiple copies in a single nucleus [19]. These rDNA arrays are homogenized through evolution and the functional nature of these gene blocks minimize mutation within sequences rendering its utility in taxonomy [4]. Hence, nucleotide sequence comparison of ITS region of 18 test *Ascochyta* isolates with 22 other sequences available online in NCBI gene bank established the identity of present isolates as *Ascochyta* spp. The higher sequence homology of As2 (FJ159103) with *A. pinodes* established its identity as *A. pinodes* though it was identified as *A. pisi* on

Fig. 1 Phylogenetic relationship of *Ascochyta* species using the ITS gene nucleotide sequence alignment. Bootstrap values supporting the branches are shown at nodes; Branch lengths are proportional to divergence. *ASP Ascochyta* spp.; *AR Ascochyta rabiei*; *AP Ascochyta pisi; PMP Ascochyta pinodella* (*Phoma medicaginis* var. *pinodella*); *MP Ascochyta pinodes* (*Mycosphaerella pinodes*); *PSP* Phomo spp.; *AUS* Australia; *PK Phoma koolunga*; *IND* India; *ISR* Israel; *USA* United States of America; *GRG* Georgia

morphological basis. Similarly, As3 (FJ188410) was also designated as *A. pinodes* due to its higher sequence homology with *A. pinodes*. Both As2 (FJ 159103) and As3 (FJ188410) were close to *A. pinodes* due to lower genetic distance (0.000–0.012) with *A. pinodes* as compared to *A. pisi* (0.024 with EU383949 and 0.021 with DQ383954). Moreover, these two isolates were grouped in clade I along with *A. pinodes* and *A. pinodella.* Faris-Mokaiesh et al. [18] also confirmed the identity of two isolates MP-007 Fra and MP-014 Fra as *A. pisi*, erroneously identified as *A. pinodes,* in PCR–RFLP analysis of IGS region of rDNA. The true identity of pathogen isolates has also been confirmed with molecular methods in *Beauvaria brongniartii* [32]. Similarly, As44 (FJ032643) isolated from chickpea showed minimum genetic distance of 0.000–0.002 with *A. rabiei* sequences (EU595358, EU573020, DQ383949, and DQ822480) available at NCBI gene bank.

Multiple alignments showed that complete coding regions viz., ITS1, 5.85 ribosomal gene and ITS2 had comparable size with reference isolates from NCBI gene bank with few exceptions. Some of these differences were specific, e.g., changes in ITSI region of As43 (FJ156067) and 5.8S ribosomal RNA gene of As42 (FJ032642), As43 (FJ156067) and As44 (FJ-32643). The size variation within the isolates could be due to genetic changes as reported by DePriest [15] in lichenized-fungi or due to different number of simple sequence repeats [27].

A total of 540–550 bp of the rDNA fragment was successfully sequenced in 15 individuals of *Ascochyta*. In total, four unique haplotypes with 15 variable sites (3.94 %) were identified in 15 samples of *A. pinodes*, and 14 unique haplotypes with 36 variable sites (8.8 %) were identified in 40 samples of various *Ascochyta* species infecting different legumes. Among the polymorphic sites, most were transitions and only a few were transversions. The mean number of nucleotide composition was A = 27.80 %, T = 28.50 %, C = 29.50 %, and G = 14.20 %. Kinnaur and Mandi isolates showed a single haplotype with 0.00 haplotype and nucleotide diversity, whereas in Chamba population, two haplotypes were observed with 0.50 and 0.00567 haplotype and nucleotide diversity, respectively (Table 2). Among the 40 sequences including 18 sequences from Himachal Pradesh, 14 unique haplotypes were detected (Table 2). All the isolates of *A. pinodes* belonged to a single haplotype irrespective of countries; whereas, 4 and 2 haplotypes were observed in *A. pisi* and *A. pinodella*, respectively. *A. pinodes*, *A rabiei*, and *P. koolunga* showed 0.00 haplotype and nucleotide diversity. In case of *A. pisi*, *Ascochyta* species and *A. pinodella* 0.900, 1.00, and 0.40 haplotype diversity and 0.50346, 0.02617, and 0.00455 nucleotide diversity was observed, respectively. AMOVA of clustering at species (Table 3) level showed that the genetic differentiation among species was highly significant ($\Phi_{ST} = 0.834$, $P < 0.001$; 50175 permutations), with 83.38 and 16.62 % of the genetic variability being among species and within species (populations), respectively. Similarly, AMOVA from different countries was highly significant ($\Phi_{ST} = 0.364$, $P < 0.016$; 32453 permutations) with 36.99 and 63.01 % variability being among countries and within countries, respectively (Table 3). High haplotype and low nucleotide diversity was observed at species level primarily because haplotypes were separable by a maximum of 15 nucleotide differences in three populations from Himachal Pradesh and 36 nucleotide differences from the populations of exotic *Ascochyta* species. Such a variation indicates that a recent expansion event(s) eclipsed any previous phylogenetic structure in *Ascochyta* species infecting pea in the region. The present study supports such a phenomenon in Himachal Pradesh as *A. pisi* was dominant over the other species causing the disease up to 1992 [41]. The pathogen has moved hitherto to disease-free areas. The low nucleotide diversity of *A. pinodes* also suggests homogeneity in pathogenic populations in Himachal Pradesh. Analysis of molecular variance (AMOVA) obtained from clustering at species and country level showed maximum variation. Existence of such a variation at species level may be due to different *Ascochyta* species as distinct populations.

In order to study inter-relationship between different *Ascochyta* spp., all the sequences of 18 isolates were compared with other 19 reference sequences of *Ascochyta* available online in NCBI gene bank by molecular evolutionary genetic analysis (MEGA) software version 4.0 and an optimal tree was generated by NJ method, and different taxa were clustered together in a bootstrap test with 1,000 replicates (Fig. 1). The evolutionary distance computed using maximum composite likelihood method (MCLM) were in units of base substitution per site. The optimal tree had branch length of 3.30834065 base substitutions per site and the bootstrap value greater than 50 % for each branch.

It is clear from the dendrogram that all the *Ascochyta* isolates were divided into five clades viz., I–V. In clade I, all the *A. pinodes* and *A. pinodella* isolates (both custom sequenced or available at NCBI gene bank) clustered together indicating that the two species cannot be differentiated on the basis of ITS region. This clade was the largest group accommodating 22 out of the 40 taxa used in the present study. However, it was interesting to note that *Ascochyta* sp. infecting bean (FJ032642) clustered with clade indicating its close relationship with *A. pinodes* and *A. pinodella.*

Further, one isolate As44 identified as *A. rabiei* clustered together in clade III with *A. rabiei* sequences available in the gene bank. Thus *A. rabiei* made a distinct clade. One *A.pisi* sequence clustered together in clade III which also included *Ascochyta* sp. from *Vicia villosa* (DQ383956) and *Pisum elatius* (DQ383955) isolated from common bean

Table 2 Nucleotide and haplotype diversity in *Ascochyta* isolates from Himachal Pradesh and *Ascochyta* species used in present investigation

Haplotypes	Population	Number of sequences	Number of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Himachal Pradesh					
Chm H 1	Chamba	4	2	0.500	0.00567
Chm H 2					
Kin H 1	Kinnuar	5	1	0.000	0.00000
Man H 1	Mandi	5	1	0.000	0.00000
Ascochyta species					
MP H 1	*Ascochyta pinodes*	15	1	0.000	0.00000
PMP H 1 FJ210937	*Ascochyta pinodella*	5	2	0.400	0.00455
PMP H 2 FJ 032641, EU338440, EU338438, EU338439					
AP H 1 EU338442	*Ascochyta pisi*	1	1	0.900	0.50346
AR H 1	*Ascochyta rebiei*	6	1	0.000	0.00000
ASP H 1	*Ascochyta* species	5	5	1.000	0.00000
ASP H 2					
ASP H 3					
ASP H 4					
ASP H 5					
PK H 1	*Phoma koolunga*	4	1	0.000	0.00000

Table 3 Summary of analysis of molecular variance of 6 *Ascochyta* species (*Ascochyta pisi, Mycosphaerella pinodes*, *Phoma medicaginis* var. *pinodella*, *Phoma koolunga*, *Ascochyta rabiei*, and *Ascochyta* species) from different regions of the world (India, Australia, USA, Isreal, and Georgia) evaluated for rDNA sequence analysis

Source of variation	*df*	Sum of squares	Variance component	Percentage of variation	Φ_{ST}	*P value*
Among species	5	124.406	4.38	83.38	0.834	<0.001
Within species	31	27.170	0.88	16.62		
Total	36	151.577	5.26			
Among regions	4	59.872	1.95	36.99	0.364	<0.0013
Within regions	32	103.156	3.33	63.01		
Total	36	163.028	5.28			

indicating their close association with *A. pisi* isolates. All the four isolates of *Phoma koolunga* formed a single clade IV. The isolate As43 infecting *Vigna mungo* was separated and formed the clade V.

The phylogenetic analysis led to the grouping of 37 isolates into five different clades when a consensus tree using bootstrap procedure was drawn. The ITS ribotyping clustered all the *A. pinodes* and *A. pinodella* into a single clade I. This indicated that *A. pinodes* and *A. pinodella* could not be differentiated on the basis of ITS region. There was considerable homogeneity between the isolates because pairwise genetic distance among themselves deviated only up to 0.05 or 0.012 as in As5 (FJ188409). Faris-Mokaiesh et al. [18] reported considerable homogeneity between the two species and could not differentiate them on the basis of PCR–RFLP of ITS and IGS regions of rDNA gene cluster. Fatehi et al. [20] depending on mean genetic distance between *A. pinodella* and *A. pinodes* group sequences and ß-tubulin PCR–RFLP's suggested to reduce two epithets of the two species to synonyms (con-specific), as was observed in the present study. However, there are ambiguities which prevent them from being called as con-specific. First, the differences in symptom expression, cultural and morphological traits point them to be separate species. Fatehi et al. [20] questioned the precision of ITS sequence homology because there is 1–2 % variation

within species and around 5 % variation between species, but no absolute value is considered as evidence of species identity. This means that ITS region does not separate these two species and there is a need to sequence other regions of their genome. In fact, [7, 36] were unable to differentiate these two species on the basis of ITS ribotyping, but could easily differentiate into separate clades on the basis of high mobility group (HMG) domain of MAT gene and glyceraldehyde-3-phosphate dehydrogenase (G3PD) phylogeny.

The third species in the *Ascochyta* blight complex of peas, *A. pisi* formed a separate clade II which also included As42 (FJ032642), an *Ascochyta* spp. isolated from common bean. ITS sequences were clearly heterogenous enough to separate *A. pisi* from other two species. Faris-Mokaiesh et al. [18] on the basis of PCR–RFLP of IGS whereas Barve et al. [7] and Peever et al. [36] on the basis of ITS sequences also differentiated *A. pisi* from *A. pinodes* and *A. pinodella.* Moreover, *A. pisi* showed lower mean/net genetic distance with *A. rabiei* group than *A. pinodes/A. pinodella* group indicating its closeness to *A. rabiei* than two species infecting the same host. Moreover, the branch separating *A. pisi* (clade II) and *A. rabiei* (clade III) from *A. pinodes/A. pinodella* (clade I) clades was long with more than 50 % maximum likelihood bootstrap values establishing its high divergence from clade I species. This implies that *A. pisi* has followed a different evolutionary route than *A. pinodes/A. pinodella* having evolved from a different ancestor. On the other hand, close affinity between *A. pinodella* and *A. pinodes* indicates common ancestory. Fatehi et al. [20] on basis of mitochondrial genomes alluded them to be evolved from a single, less specific ancestor. Based on HMG phylogeny, Barve et al. [7] found that *A. pisi* was closer to *A. rabiei* clade than other two species infecting pea. On the basis of G3PD sequencing, Peever et al. [36] found *A. rabiei* and *A. pisi* to be in the same clade.

Based on phylogenetic analysis, one isolate As44 (FJ032643) infecting chickpea was clustered in clade III having four other *A. rabiei* isolates from NCBI gene bank with pairwise genetic distance of 0.000–0.002, separating it from other three species infecting pea. Similarly, As43 isolated from *Vigna mungo* formed separate clade IV. This means that ITS ribotyping was able to separate the *Ascochyta* spp. on the basis of host specificity.

The genetic distance within individuals of a species was 0.007, 0.005, and 0.001 for *A. pisi*, *A. pinodella,* and *A. pinodella*, respectively. The lower genetic distance within pathogenic populations of a species indicates lower reproductive barrier between them. The lowest genetic distance within individuals of *A. pinodes* indicates its ability for sexual reproduction in nature. In fact, *A. pinodes* being homothallic [40] has no barrier to sexual exchange between individuals. A higher genetic distance within *A. pinodella* on the other hand, indicates to the contrary. In *A. pinodella,* formation of sexual fruiting body (pseudoperithecia) is not usually observed in nature. There is only a single report of teleomorphic stage of *A. pinodella* [9]. On the basis of RFLP data and comparison of crosses made with single ascospore versus pooled ascospores inoculum, they demonstrated that this fungus is heterothallic. The high genetic distance (0.007) within individuals of *A. pisi* indicates that this barrier is stronger than other two. *A. pisi* has no reported teleomorph yet and Peever et al. [36] on the basis of unpublished data on PCR amplification of MAT 1–2 HMG-box region of *A. pisi* isolates indicated that nature of heterothallism is similar to other heterothallic *Ascochyta* spp. This has several implications in Himachal Pradesh. The higher ability of *A. pinodes* populations to undergo sexual reproduction will generate higher diversity within individuals. This means *A. pinodes* has better adaptability to survive under adverse conditions. This can be ascribed to the predominance of *A. pinodes* on pea currently over the earlier dominance of *A. pisi* [11].

One isolate (FJ032642) from common bean clustered together with clade accommodating *A. pinodes* and *A. pinodella*. Their pairwise and mean/net genetic distance with other *Ascochyta* spp. being low indicated their similarity. This implies that either the isolate is *A. pinodes* or *A. pinodella*, or there is a chance change in the nucleotide sequence of the ITS region. The former probability is ruled out because of different traits in the isolate viz., symptoms, colony color, and associated vegetative characteristics as compared to other *Ascochyta* isolates showing genetic segregation of their ITS sequences. However, there is a least likelihood of change in rDNA sequence because of its widespread use in fungal taxonomy. Fatehi and Bridge [19] argued that despite this gene cluster having been derived from single ancestry, the different copies within a replicated nucleus are liable to mutate during crossing over which could be true in the present case. Such changes in gene cluster might include the presence of introns as shown in lichenised fungi [15] or may take the form of sequence differences which preserve the size of particular region as demonstrated in arbuscular mycorrhizal fungi [43] and occurrence of more than one ITS region in filamentous fungi [22].

However, after repeated amplification of nearly 524 bp sized fragment as well as other isolates excludes the possibility that new intron of the amplicon size might have changed. Fatehi and Bridge [19] also found such ITS amplicons with different sequence homology but same size in *Ascochyta* isolates with one major and two minor forms of ITS. The occurrence of more than one amplification products from rRNA gene cluster has also been reported [22, 43]. Harlton et al. [22] suggested that these different ITS regions may be associated with different nuclei where thallus is

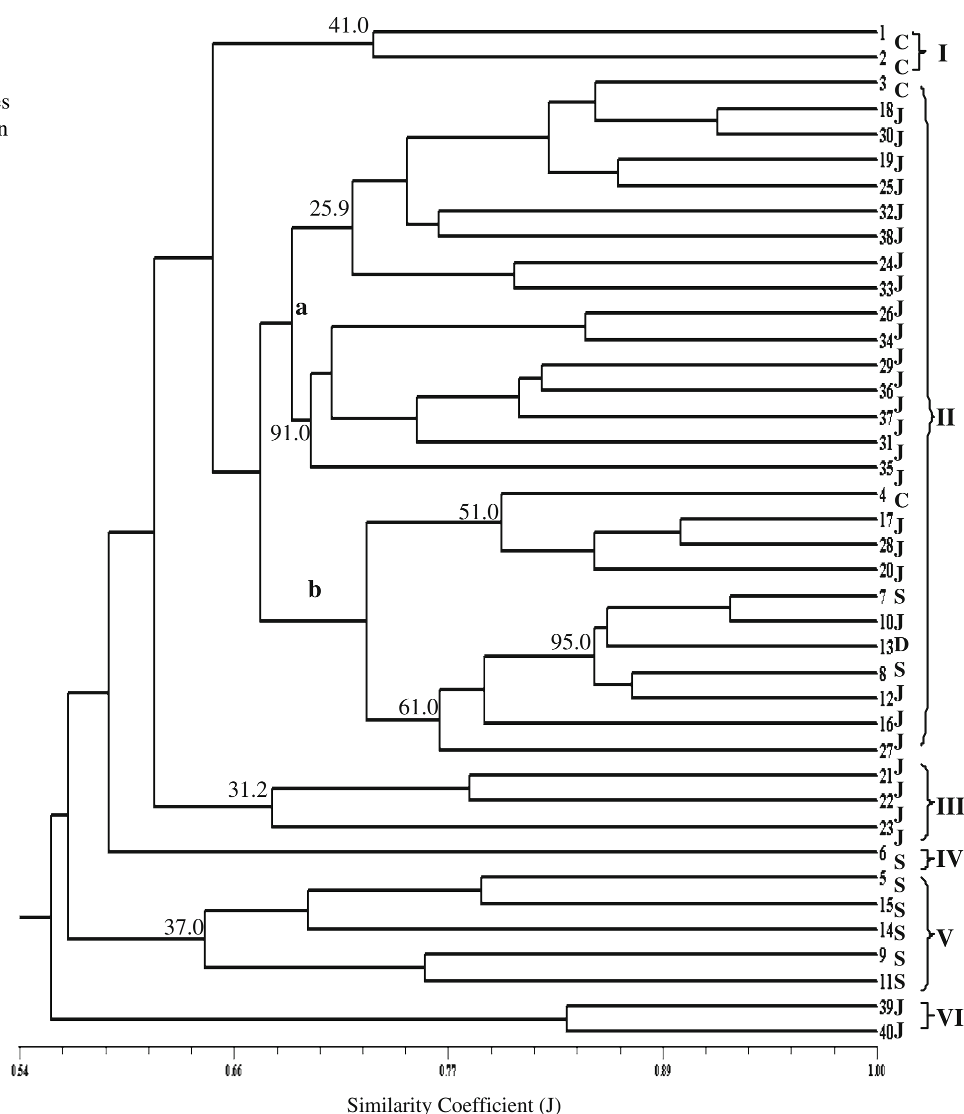

Fig. 2 RAPD dendrogram of 40 *Ascochyta* isolates infecting pea generated by UPGMA based on Jaccard's coefficient. Bootstrap values obtained using WinBoot of more than 25.0 % are shown at nodes

bi-nucleate or multinucleate. The *Ascochyta* cultures used were bi-nucleate and thus different ITS cluster has been amplified, associated with a particular nucleus. The inability to detect other types of ITS clusters might be due to competitive nature of PCR reaction or a case of contamination. The possibility of contamination seems to be remote because cultures were axenic and derived from single conidium.

Genetic Diversity Analysis Using Molecular Markers

RAPD Analysis

Eight 10-mer primers viz., OPA-02, OPA-09, OPA-13, OPD-11, OPQ-13, OPQ-18, S-1466, and S-144 producing consistent polymorphic banding were selected out of 140 for RAPD analysis of 40 isolates of the test fungus. The number of scorable and polymorphic bands ranged from 5 to 8. Among the 40 isolates, all the primers showed 100 % except S-144 showing 71.43 % polymorphism. The overall polymorphism was 96.8 %. The scorable bands subjected to cluster analysis using UPGMA option of NTYSYS-pc package version 1.80 generated a dendrogram categorizing different isolates into six clusters (Fig. 2). Cluster I contained two isolates (As1 and As2) collected from district Chamba. Cluster I showed a similarity of 64.9, 61.6, 59.4, 57.2, and 56.0 % to Cluster II, Cluster III, Cluster IV, Cluster V, and Cluster VI, respectively. Cluster II consisted of 27 isolates with maximum isolates belonging to Mandi district (Jinjhiali). However, Cluster II was more close to cluster I. Cluster II showed a similarity of 61.6, 59.4, 57.2, and 56.0 % with cluster III, IV, V, and VI, respectively. All isolates in cluster III and V belonged to districts of their origin, i.e., Mandi and Kinnaur. Cluster II could be differentiated further into two sub-clusters IIa and IIb accommodating 16 and 11

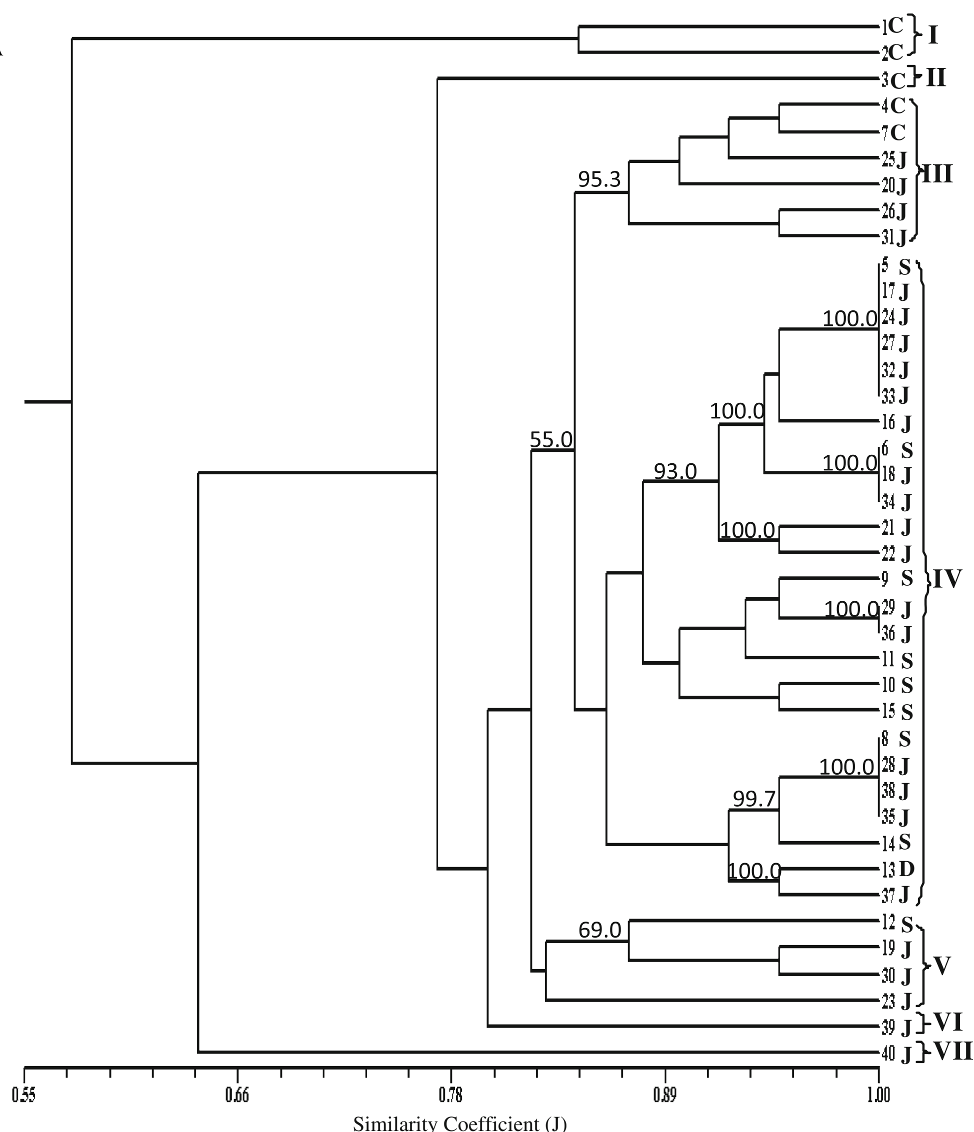

Fig. 3 REP-PCR and RAMS dendrogram generated from UPGMA based on Jaccards coefficient. Bootstrap values obtained using WinBoot of more than 50.0 % are shown at nodes

isolates. Most of the isolates were more or less clustered on the basis of their geographic origin.

RAMS and REP-PCR categorized 40 isolates into seven clusters (Fig. 3). Cluster I contained only two isolates viz., As1 and As2 from Chamba district whereas cluster II, VI, and VII contained only one isolate each, i.e., As3, As39, and As40, respectively. Six isolates viz., As4, As7, As25, As20, As26, and As31 were categorized in cluster III in which all isolates except As4 (Chamba) and As7 (Kinnaur) were from Mandi district. Cluster IV was the largest comprising 25 isolates from all the districts except Chamba. Cluster V comprised four isolates viz., As12, As19, As3, and As2. Isolates As1 and As2 categorized in *A. pisi* and *A. pinodella*, respectively, clustered together in both the dendrograms obtained from RAPD, RAMS, and Rep-PCR analysis. RAPD came out to be a better marker in isolate identification pertaining to their geographical origin. The combined analysis showed less diversity within *A. pinodes* with RAMS and Rep-PCR, whereas RAPD showed maximum diversity.

Due to the inadequacy of overlapping morphological parameters, molecular markers were used to elucidate the variation in *Ascochyta* complex of pea. The RAPD analysis of 40 isolates of *Ascochyta* spp. grouped them into six clusters (I–VI). Almost perfect correlation between RAPD clusters and locations could be established. Such grouping of isolates vis-a-vis their geographical origin has several implications in Indian context where information lacks about genetic pathogen variability. Several workers have employed molecular markers to study this pathogen but were unable to find a correlation in pathogen populations in clustering and their origin [18, 33, 53]. The geographical grouping of the isolates could be attributed to reproductive barriers between the regions. Pathogenic populations have to adapt to the

environmental conditions of a region to sustain by accumulating genes (here novel alleles). If the populations of one region are unable to intermate with the populations of the other regions, movement of genes/alleles across populations is restricted leading to geographic isolation of alleles. This possibility seems plausible in Himachal Pradesh due to hilly terrain thus creating a natural geographical barrier to the pathogen spread. The reproductive isolation is also maintained due to little exchange of seed between the farmers of different regions as they use the seed of previous year crop. If sexual exchange between individuals occurs within an isolated region, sharing of novel alleles will be there between the individuals of a population. Asexual reproduction among such individuals leads to dominance of novel alleles. Since *Ascochyta pinodes* is homothallic, several such sexual exchanges between individuals of a region can occur. Also, asexual reproduction producing millions of pycnidiospores could be responsible for dominance of such novel alleles. Such a reproductive isolation ultimately leads to divergence between the isolated regions and uniformity within a population. These observations are in conformity with Zang et al. [55] who also found direct correlation between clusters and countries of origin in the AFLP analysis of 56 *A. pinodes* isolates of worldwide origin. They grouped isolates of different origin (Australia, New Zealand, and Canada) into separate clades. Further, within isolates from a country separate sub-groups were formed representing different regions.

In conclusion, it is inferred from the present study that *A. pinodes* dominates the *Ascochyta* populations in Himachal Pradesh, where pea is one of the most remunerative off-season cash crop. Different morpho-cultural characteristics categorized various isolates into three major groups with few isolates floating from one group to another on the basis of individual characteristics. However, the combined data resolved the ambiguity and placed majority of isolates in *A. pinodes*. Molecular characterization with RAPD, RAMS, and Rep-PCR grouped isolates according to their geographical origin. Sequencing of ITS region of test isolates and reference gene bank *Ascochyta* spp. revealed identity and ambiguity.

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