DNA barcoding of *Venturia* species infecting various host plants in Kashmir

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(2015-585-D)

Division of Plant Pathology

Faculty of Agriculture

Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

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Thesis

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Dedicate
this thesis
to my beloved
Parents,
Advisor
And
Inspiring Minds

Sher-e-Kashmir
University of Agricultural Sciences and Technology of Kashmir
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Certificate – I

This is to certify that the thesis entitled, **"DNA barcoding of *Venturia* species infecting various host plants in Kashmir"** submitted in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Plant Pathology**, to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** is a record of bonafide research work carried out by **Mr. Mohammad Saleem Dar (Regd. No. 2015-585-D)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or information received during the course of investigation has duly been acknowledged.

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We, the members of the Advisory Committee of **Mr. Mohammad Saleem Dar (Regd. No. 2015-585-D)**, a candidate for the degree of **Doctor of Philosophy in Plant Pathology** have gone through the manuscript of the thesis entitled, **"DNA barcoding of *Venturia* species infecting various host plants in Kashmir"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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Certificate – III

This is to certify that the thesis entitled, **"DNA barcoding of *Venturia* species infecting various host plants in Kashmir"** submitted by **Mr. Mohammad Saleem Dar (Regd. No. 2015-585-D)** to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Plant Pathology** was examined and approved by the Advisory Committee and External Examiner on …….

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ABSTRACT

Venturia is one of the important ascomycetous fungal genus that invades a large variety of host plants worldwide causing adverse effect on the quantity and quality of the produce. Therefore, prediction and monitoring of origin, introduction pathways and spread, population biology and phylogenetics of the pathogen populations are required for its effective management strategies. The present investigation on "DNA barcoding of *Venturia* species infecting various host plants in Kashmir" was carried out to ascertain the phylogenetic relationships among *Venturia* species using multigenic approach. Fifty-five isolates of *Venturia* species from different hosts *viz*., apple, almond, crataegus, cotoneaster, pear, peach, and poplar grown in Srinagar, Pulwama and Anantnag districts were isolated, purified and maintained on PDA. The phylogenetic analysis based on ITS sequences led to grouping of *Venturia* species in two main clades. Clade I composed of all isolates of *V. carpophila, V. crataegi, C. humile, V. populina* and *V. pyrina*, while clade as II comprised of *V. inaequalis* isolates. Therefore, *Venturia* species proved to be the monophyletic group based on ITS region, and isolates with different host origin couldn't be differentiated. The phylogenetic analysis based on β-tubulin grouped them into six different clades *viz*., Clade I composed of *V. inaequalis* isolates, Clade II accommodated *V. carpophila*, Clade III was comprised of *V. crataegi*, Clade IV contained *V. pyrina*, and Clade V and VI accommodated *V. populina* isolates. While the phylogenetic analysis based on EF1α gene sequence grouped them into five different clades *viz*., Clade I comprised of *V. carpophila* isolates, Clade II of *V. crataegi*, Clade III of *V. populina*, Clade IV of *V. pyrina* and Clade V of *V. inaequalis*. This also lead to little splitting on the basis of host origin. Multigene phylogenetic analysis based

concatenated data set using rDNA-ITS, β-tubulin and EFlα gene sequences together grouped them into nine clades. Clade I comprised of *V. crataegi* isolates, Clade II of *V. populina*, Clade III of *V. pyrina*, Clade IV of *V. inaequalis* and Clade V to Clade IX comprised of *V. carpophila* isolates, which reflected close co-evolution between *Venturia* and the respective hosts, and species boundaries within the genus also existed. The observed level of polymorphism was very high based on the number of haplotypes (2-11) and the haplotype diversity (0.60-1.00), indicating that *Venturia* species has maintained high level of genetic diversity compared to their nucleotide diversities in terms of nucleotide differences in sequences (π) and Watterson's (θ) which varied from 0.001 to 0.087 and 0.001 to 0.108, respectively. Most of the populations showed high level of genetic diversity and random association among alleles, corresponding to high level recombination events (1-21) and segregating polymorphism (1-346). The pattern of genetic divergence showed association of heterogeneity with genomic and life cycle changes of *Venturia* species. Genetic differentiation parameters *viz*., pair wise fixation index (F_{ST}) and gene flow (Nm) ranging from 0.134 to 0.904 and 0.01 to 1.61 respectively indicated low levels of gene flow and high genetic differentiation, however, corresponding Kst and Snn values revealed existence of reproductive barriers and non-random matting populations. Analysis of molecular variation revealed that the variations were distributed among populations than within population which is supported by corresponding high F_{ST} values (0.71-0.93) indicating the presence of separate regional populations due to strong genetic differentiation among *Venturia* species. Clustering analysis based on Bayesian algorithm of STRUCTURE programme portioned the data set into distinct clusters that corresponded to five genetic populations indicating each *Venturia* species as distinct population. The distinction between these clusters was also visible in the results of Principal component analysis, where first and second coordinate representing 24.75-68.37 and 10.84 -25.04 per cent respectively, of total variation indicated a high genetic diversity, sexual reproduction and population expansion in *Venturia* species. For molecular based detection of *Venturia* species using SSR markers, out of 37 primers designed for *Venturia* species, only three primer pairs Vi1, Pr3 and C4 were found specific to *V. inaequalis*, *V. pyrina*, *V. carpophila,* respectively. These species-specific markers were highly sensitive and reliable for early detection of pathogen even before symptoms appear.

Key Words: *Venturia* species, DNA barcoding, genetic diversity, divergence, differentiation, population genetics, species-specific markers, early detection

Signature of Student
Dated: _____

Signature of Major Advisor
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INTRODUCTION

Fruit production in India is a commercial venture owing to its high remunerative returns per unit area. The importance of fruits for human nutrition and health has increased the per capita fruit consumption. The Pome and stone fruits represent 22 per cent of the global fruit production (Anonymous, 2017). India is the second largest producer of various fruits after China with a production of 88.8 million tonnes over an area of 6.3 million hectares with the productivity of 13.9 metric tonnes per hectare, accounting to 12 per cent of the total world production of fruits (Anonymous, 2016). In India, pome and stone fruits production is restricted to J and K, Himachal Pradesh, Uttarakhand, Punjab, and Arunachal Pradesh. Commercial plants belonging to Rosaceae family are important and extensively cultivated worldwide. The family includes species belonging to tribe Pyreae such as bearberry cotoneaster (Cotoneaster), pear (*Pyrus* species), chokeberry (Aronia), apple (*Malus* species), flowering quince (Chaenomeles), firethorn (Pyracantha), hawthorn (Crataegus), medlar (Mespilus), stranvaesia (Photinia), service tree (Sorbus) and shadbush (Amelanchier), and tribe amygdaleae such as peach (*Prunus persica*), cherry (*P. avium)*, plum (*P. domestica*), apricot (*P. armeniaca*), almond (*P. dulcis*) blackthorn (*P. spinosa*); cherry accolade *(P. accolade*); cherry laurel *(P. laurocerasus*); cherry plum (*P. cerasifera*), Chinese plum (*P. mume*), Fuji cherry (*P. incisa*), Higan cherry (*P. subhirtella*) and Japanese cherry (*P. serrulata*, (Ruiz *et al.*, 2017). The state of Jammu and Kashmir offers tremendous scope for cultivation of fruit trees such as apple, pear, peach, plum, apricot, almond and cherry (Bandey and Sharma, 2010). Poplar and willow based agroforestry system in Kashmir has been integrated traditionally with orchards to protect them from wind damage besides providing fuel, fodder and timber (Qasir and Khan, 2010). About 6 lakh families, comprising of 30 lakh people are directly or indirectly associated with horticulture in Jammu and Kashmir (Bandey and Sharma, 2010). The area in the state under

fruit cultivation is 3.8 lakh hectares which accounts for 18.83 lakh tonnes of fruit production, with district Baramulla leading in apples, district Budgam leading in pear and almond production and district Anantnag leading in walnut, peach and plum production (Anonymous, 2015).

Comparing to international standards, the productivity is still low, owing to many factors of which biotic and abiotic factors play a major role. The spread of transboundary plant pests in recent years can cause significant losses to farmers and threaten food security (Ruiz *et al.*, 2017). Crop losses due to plant diseases affect food security, availability, production, utilization, reserves and import either directly or indirectly through trade, policies and societies (Zadoks., 2008). Various economically important diseases on different host plant species are scab, powdery mildew, root rot, collar rot, Alternaria leaf blotch, cankers, fire blight, bacterial spot etc. Among these, scab caused by *Venturia* species is globally important disease as majority of commercially grown crops belonging to Rosaceae family are highly susceptible to this pathogen (Beck *et al.*, 2005; Gessler *et al.*, 2006). Severe infections of scab can lead in reduced flower bud formation in fruit crops and ultimately weaken the tree (Verma and Sharma., 1999); in poplar clones and willows, it leads to spring defoliation (Anselmi, 2009).

The genus *Venturia* belongs to family Venturiaceae, order Venturiales, sub-class Pleosporomycetidae, class Dothidiomycetes, sub-division Pezizomycotina and phylum Asomycota with 193 species names listed in the *Index Fungorum* estimated to be comprised of 57 species (Kirk *et al.*, 2008). The *Venturia* species are distributed in northern temperate regions of the world as one of the notorious plant pathogens on a large variety of host plants with haplontic life cycle (Zhang *et al.*, 2015). The various species of *Venturia*, are well known for their adverse effects in causing characteristic leaf spots, necrosis and scab as well as leaf and fruit deformations of members of at least 52 angiospermous plant genera (Hashemi *et al.*, 2014). The *Venturia* has *Fusicladium* (Bonord), *Pollaccia* (Baldacci and Ciferri) or *Spilocaea* (Fries) as anamorphic genera (Zhang *et al.*,

2011). The species of *Venturia* causing scab on a variety of hosts are *Fusicladium pomi*, *F. pyrorum* and *F. asperatum* on *Malus* species, *F. obducens*, *F. cerasi*, *F. carpophilum*, *F. pomi* and *Fusicladium*-state of *Apiosporina morbosa* on *Prunus* species, *F. ahmadii*, *F. pomi*, *F. nashicola* and *F. pyrorum* on *Pyrus* species, *F. pyrorum* and *F. pomi* on *Eriobotrya* species, *F. pomi* on *Pyracantha* species, *F. oleagineum* on olive, *F. martianoffianum*, *F. romellianum*, *F. subsessile*, *F. mandshuricum*, *F. elegans*, *F. radiosum* var. *populi-albae*, *F. radiosum* var. *radiosum*, *F. radiosum* var. *lethiferum* and *Cladosporium humile* on *Populus* species and *F. catenosporum* and *F. saliciperdum* on *Salix* species (Schubert *et al.*, 2003). These genera are distinguished based on the proliferation mode of conidiogeous cells and conidial formation in which *Fusicladium* has sympodial proliferation, *Pollaccia* has monoblastic with determinate to percurrent conidiogenous cells with few inconspicuous annellations and *Spilocaea* has percurrent proliferation and numerous conspicuous annellations (Schubert *et al.*, 2003). However, separation of these genera is not tenable due to uniform conidiogenous cells, loci and morphologically intermediate species (Braun *et al.*, 2002). Most of the anamorph states of *Venturia* have been classified as *Fusicladium*. This name was proposed to designate the asexual stage of *Venturia* spp. (Braun *et al.*, 2002), and used in the monograph written by Schubert *et al.* (2003). Very recently, the International Commission on the Taxonomy of Fungi has proposed the use of *Venturia* instead of *Fusicladium* for the species with only anamorph stage, following the guidelines of the Amsterdam Declaration on Fungal Nomenclature (Gonzalez-Dominguez *et al.*, 2017).

Since the late nineteenth century, apple scab has been thoroughly investigated, and information pertaining to biology, genetics, epidemiology and control of the disease has been published and reviewed by Machardy (1996) and Bowen *et al.* (2011). In contrast to this, little work has been conducted on *Venturia* spp. affecting different host plants, as depicted by difference in research efforts and number of publications. However, this might be due to little

investments and less specialized management measures directed towards non-apple crops, and the common use of information developed for *V. inaequalis* for managing the other fruit scabs. The internal transcribed spacer (ITS) region is commonly used for specific identification and phylogenetic analysis, and has been declare as DNA barcoding region in fungi (Schoch *et al*., 2012). All copies of barcoding region within the genome should be constant and identical, and intraspecific variation lower than interspecific variation in taxonomic and phylogenetic studies. The β-tubulin and elongation factor 1 alpha (EF1α) genes are also becoming prevalent in fungal identification and phylogenetic reconstructions at species level (Froslev *et al*., 2005; Rodriguez-Estrada *et al*., 2010; Zhao *et al*., 2012). Authentic fungal identification depends on sequence homogeneity within the individual and species (He e*t al*., 2017). Although, Venturaceae family has relatively high cryptic species-level diversity (Braun *et al*., 2002; Le Cam *et al*., 2002 and Schubert *et al*., 2003), and high levels of cryptic diversity has also been found in other families like *Graphidaceae* and *Lobariaceae* of Ascomycota (Moncada *et al*., 2014; Sohrabi *et al*., 2014). The DNA barcoding approach for rapid and accurate identification is commonly implementated in taxonomically challenging Venturiaceae family containing a number of cryptic species. It leads to the discovery of new cryptic species with putative cosmopolitan and widely disjunct distributions (Zhang *et al*., 2011). In Venturaceae, a few nominal species with a wide distribution pattern has been critically investigated using molecular sequence data (Zhao *et al*., 2012). Despite progress in accurate species identification in *Venturia* genus, morphology based identification remains challenging because of the difficulties in discerning suitable diagnostic morphological characteristics (Zhang *et al*., 2016). Therefore, this study provides a valuable model system to test the potential of DNA barcoding for the accurate identification and phylogentic studies. DNA barcoding were initiated to study the phylogenetic connection and to provide evidence for re-assessment of the generic taxonomy of *Venturia* species by Schnabel *et al.* (1999). The analyses were restricted to species that occurred on *Rosaceous* hosts, and were later

supplemented by Kasanen *et al.* (2001) who examined *V. ditricha, V. populina* and *V. tremulae*. Morphologically *F. eriobotryae* is similar to *V. inaequalis* or *V. pyrina*, and *Cladosporium humile* refers to either *F. martianoffianum or F. romellianum* (Schubert *et al.*, 2003). The above morphological similarity is also evident from *V. saliciperda* on *Salix*, but clustering with *Fusicladium* of *Populus* based on internal transcribed spacer (ITS) phylogeny, thereby indicating an ability of switching of between host genera and colonizing new hosts (Beck *et al.*, 2005). Thus, defining the limits of species is of great interest to evaluate diversity of the pathogen and predict the potential spread of genes involved in pathogenicity (Raabe and Gardner, 1972; Le Cam *et al.*, 2002). In these studies, *Venturia* species and their anamorphs formed a monophyletic clade composed of several small sub-clades. Most of the phylogenetic analyses are based on the ITS region, the most commonly used locus for fungal taxa delimitation and identification (Nilsson *et al.*, 2008). However, the results from this region cannot be considered conclusive for distinguishing closely related fungal species (Kiss, 2012; Schoch *et al.*, 2012), and none of the single-locus genealogies show a clear partitioning of isolates from each host plant into separate clusters (Gladieux *et al.*, 2010). To better understand the phylogenetic relationships, β-tubulin and elongation factor genes are used and serve as ideal molecular phylogenetic markers to deduce the evolutionary history and genetic variation of morphologically similar species (Barnes *et al.*, 2004). Multigene analysis of *V. nashicola* and *V. pirina* formed a distinct evolutionary lineage, thus providing more reliable and qualitative estimation of the inter-specific phylogenetic relationships (Zhao *et al.*, 2012). However, intra-specific relationships were not revealed among isolates of different pathological races in *V. nashicola*. For closely related taxa, a number of analytical approaches takes into account the pattern of genetic variation within and between taxa, in order to estimate parameters in a coalescent framework and provide important insights into the history of divergence (Becquet and Przeworski, 2007). A species identification criterion based on phylogenetic concordance of multiple unlinked genes (Taylor *et al.*, 2000) provides an

indication of lack of genetic exchange among taxa, which appears more widely applicable and discriminating species recognition criteria in fungi (Giraud *et al.*, 2008). To better understand the phylogenetic relationships at all taxonomic levels, sequences from more than one gene such as single copy genes, protein coding genes and mitochondrial DNA sequences have been explored in recent years (Merwe *et al.*, 2007) and a clear framework provided at different taxonomic levels by comparative analyses of the evolutionary histories of different lineages (Cao *et al.*, 1994). In Kashmir diminutive work has however, been carried out on phylogenetics of *V. inaequalis* (Padder *et al.*, 2013), despite several species of *Venturia* having been so far reported from Kashmir. Most of reports are available on epidemiology, disease management, development of resistant cultivars (Farooqi and Dalal., 2003) and distribution of apple scab race flora and identification of resistance sources against *V. inaequalis* in Kashmir valley (Dar *et al.*, 2015).

Therefore, an alternative method for phylogenetics and classical identification must be developed**.** Keeping in view these facts, the present investigation is focused to collect various *Venturia* species from different hosts prevalent in Kashmir valley for characterization based on ITS regions, β-tubulin and elongation factor 1α (EF1α) for development of DNA barcode(s) with following objectives:

- to ascertain the phylogenetic relationships in *Venturia* species using multigenic approach; and
- to develop PCR based species-specific markers

Chapter – 2

REVIEW OF LITERATURE

2.1 Historical background and economic importance of the *Venturia* species

The genus *Venturia* belongs to phylum Ascomycota and class Dothideomycetes (Schoch *et al.*, 2009). It has been traditionally included in the family *Venturiaceae* (genus introduced by Muller and von Arx, 1950), order *Pleosporales*, based on its "Pleospora-type centrum and bitunicate asci" (Sivanesan, 1977). However, phylogenetic analyses, using both nuclear and mitochondrial gene regions, indicated that the family *Venturiaceae* forms a well-supported monophyletic group separate from the *Pleosporales* (Kodsueb *et al.*, 2006; Kruys *et al.*,2006; Zhang *et al.*, 2011), and thus necessitating re-ordering *Venturiaceae* into *Venturiales* ord. nov. (Zhang *et al.*, 2011). The genus *Venturia* Sacc. currently contains more than 250 species pathogenic on a wide range of hosts which belong to families such as *Acaraceae*, *Betulaceae*, *Cornaceae*, *Oleaceae*, *Rosaceae*, and *Salicaceae* (Sivanesan, 1977; Gautam, 2014). Most of the *Venturia* species are host-specific and occur only in one or a few host species resulted from host-fungus coevolution (Schnabel *et al.*, 1999; Beck *et al.*, 2005). In 2003, *Fusicladosporium* was proposed as a new genus which included *F. effusum*, *F. carpophillum* and *F. humile*, teleomorph *V. acerina* based on morphological differences (Partridge and Morgan-Jones, 2003). However, this new taxon seems unjustified due to the existence of two older generic names for *Venturia* anamorphs (Hormocladium and Ramalia) and ITS phylogenetic analysis demonstrated that the erection of *Fusicladosporium* results in a polyphyletic genus (Schubert *et al.*, 2003; Beck *et al.*, 2005). Thus, *Fusicladosporium* is currently considered a synonym of Fusicladium (Schubert *et al.*, 2003; Scherm *et al.*, 2008; Seyran *et al.*, 2009; Lalancette *et al.*, 2012). Braun *et al.* (2002) suggested that the separation of these anamorphic genera is not tenable since the conidiogenesis and structure of the conidiogenous loci were uniform. Molecular

examinations clearly showed that *Venturia* is a monophyletic clade that could not be separated into sub-clades relevant to these anamorphic genera (Beck *et al.*, 2005), and it was proposed that the genera could to be merged in one anamorphic genus. Hence, the name *Fusicladium* was conserved against other names (Braun, 2005). Since the asexual and sexual morphs of the same genus must have one name with preference for *Venturia* over *Fusicladium* or *Pollaccia* (Rossman *et al.*, 2015), being the more widely known. Recently, International Commission on the Taxonomy of Fungi has proposed the use of *Venturia* instead of *Fusicladium* for the species with only anamorph stage following the guidelines of the "Amsterdam Declaration on Fungal Nomenclature" (Gonzalez-Dominguez *et al.*, 2017). *Venturia* including *V. rosea* De Not. and *V. dianthi* De Not. was first described by De Notaris (1844). Subsequently according to Zhang *et al.* (2016), Cesati and De Notaris (1863) described two more species of this genus, *V. dickiei* (Berk. & Broome) Ces. & De Not. and *V. eres* (Berk. & Broome) Ces. & De Not. Saccardo (1882) amended the description of *Venturia* excluding both *V. rosea* and *V. dianthi* and retaining *V. dickiei* and *V. eres*. *Venturia* Sacc. description was widely accepted and neotypified by *V. inaequalis* (Korf, 1956; Sivanesan, 1977). In the monograph of Sivanesan (1977), herbarium specimens of 65 species were studied including type materials of 31 species; however, 193 names comprising of 57 species were listed in the *Index Fungorum* (Kirk *et al.*, 2008). Specimens of 35 *Venturiaceous* species were studied by Barr (1967) from North America of which 12 species were based on type material; however, species boundaries were difficult to define due to lack of type studies (Zhang *et al.*, 2011).

Subsequently according to Zhang *et al.* (2016), the diagnostic characteristics of *Venturia* species were; habitat biotrophic or hemibiotrophic on dicotyledonous leaves: ascomata small-sized, solitary, scattered or gregarious, initially immersed, becoming erumpent, globose to subglobose, wall black, papillate, ostiolate; peridium thin, composed of a few rows of pigmented cells of *textura angularis*; hamathecium rare, usually evanescent in mature ascomata; asci

8-spored (rarely 4-spored), bitunicate, oblong to obclavate with a short, thick pedicel or pedicel lacking, with an inconspicuous ocular chamber; ascospores obliquely uniseriate and partially overlapping to biseriate, especially at the base, ellipsoidal, with broadly rounded ends, pale brown, 1-septate, slightly constricted at the septum, the upper cell shorter than the lower one, smooth-walled. *Fusicladium*, *Pollaccia* or *Spilocaea* are the anamorphs (Zhang *et al.*, 2011). These genera were distinguished based on the proliferation mode of conidiogeous cells and conidial formation in which *Fusicladium* has sympodial proliferation, *Pollaccia* has monoblastic with determinate to percurrent conidiogenous cells with few inconspicuous annellations, and *Spilocaea* has percurrent proliferation and numerous conspicuous annellations (Schubert *et al.*, 2003).

Apple scab is a major problem in apple growing regions worldwide, particularly in temperate regions with cool and moist weather. The first report of apple scab infection was reported by Fries (1819) from Sweden. The geographical region where the scab occurred first time is unknown but the oldest record of apple scab is depicted in a painting (1600AD) by Michel Angelo Caravaggio, "The Supper at Emmaus", displayed at the National Gallery in London, England. In "Still Life and Apple and a Pomegranate" by Gustave Courbet painted in 1871-72 displayed in the Mesdag Museum Den Haag, apples were depicted with several clearly painted black spots (MacHardy *et al.*, 2001). The fungus *V. inaequalis* (Cke.) Wint. (anamorph *Spilocea pomi* Fr.), is restricted to the genus *Malus* and prevalent in all the apple growing regions of world. At the end of the nineteenth century, scab became increasingly a major problem for apple growers with heavy epidemics and serious damage (Aderhold, 1899). In India, its occurrence was first time reported by Nath (1935). In 1973, the disease appeared in epidemic form from Kashmir valley and severely damaged the Ambri cultivar (Joshi *et al.*, 1975; Verma and Sharma, 2003). There were numerous reports about the worldwide distribution of apple scab (MacHardy, 1996; Schnabel *et al.*, 1999; Rossi *et al.*, 2007; Gladieux *et al.*, 2008, 2010b; Xu *et al.*, 2009; Bowen *et al.*, 2011; Li *et al.*,

2011; Padder *et al.*, 2013), with the exception of West Australia, where the disease was eradicated (McKirdy *et al.*, 2001). *Venturia inaequalis* probably emerged in Central Asia, the center of apple origin (Tenzer and Gessler, 1999; Gladieux *et al.*, 2008; Gladieux *et al.*, 2010a; Xu *et al.,* 2008; Xu *et al.,* 2013), and followed its host's expansion into Europe and other regions (Gladieux *et al.*, 2008). Overall, *V. inaequalis* appears to be a model invasive plant pathogen with a broad geographic distribution and well-established populations (Gladieux *et al.*, 2010a).

Venturia pyrina has a worldwide distribution that is closely associated with the distribution of its host, European pear '*Pyrus communis*' (Ogawa and English, 1991), and considered as an economically important disease in commercial orchards in most of the pear growing areas worldwide (Lattore *et al.*, 1985; Shabi, 1990; Bakker, 1999; Pierantoni *et al.*, 2007; Chevalier *et al.*, 2008; Spotts and Castagnoli, 2010; Bouvier *et al.*, 2011). The disease has been recorded for the first time from Belgium in 1832 (Libert, 1832). However its first authentic report by Fuckle in 1869 as *Fusicladium pirinum* (Lib.) Fuck. and Anderhold described its perfect stage as *Venturia pyrina* Aderh. In India, it was first recorded by Joshi on *Pyrus pashia*, in 1951 as *Fusicladium dendriticum* (Chona *et al.*, 1956). In Kashmir, its occurrence was reported in 1984 (Putto and Chaudhary, 1984). In contrast, *V. nashicola* as scab pathogen on Japanese pear (*Pyrus pyrifolia*) based on morphological and pathological characteristics was first described by Tanaka and Yamamoto in 1964; Ishii and Suzaki., 1994. *Fusicladium ahmadii* was reported to cause infection on *Pyrus pashia* and has been reported for the first time from Pakistan (Ellis, 1976; Schubert *et al.,* 2003). The fungus has subsequently also been reported from Himachal Pradesh, India (Gautam, 2014). Pear scab is also a serious problem in organic orchards causing severe crop losses (Timmermans *et al.*, 2010; Sugar and Hilton, 2011).

Venturia carpophila (Fisher) (syn. *Fusicladosporium carpophilum* Partridge and Morgan Jones, *Cladosporium carpophilum* Thum,

Megacladosporium carpophilum Thum. (Vienn.-Bourg), *Fusicladium carpophilum* Thum. (Oudem.), *Cladosporium americanum* (Greene) is an important fungal pathogen in the family *Venturiaceae* responsible for scab in peach (*Prunus persica*), apricot (*P. armeniaca*), almond (*P. dulcis*), and plum (*P. domestica*) (Chen *et al.*, 2017). The *Venturia carpophila* was first time reported from Klostemenberg, Austraila by Thumen in 1877 on peach as *Cladosporium carpophilum*. The disease has been reported to occur in other parts of the world (Pammel, 1892; Anderson, 1956). Infection of almonds by *V. carpophila* in California is widespread (Ogawa and English 1991; Strand 1999), resulting in huge yield losses and requiring therefore, application of fungicides to manage the disease (Adaskaveg *et al.*, 2013; Luo *et al.*, 2013). Infection of peach fruit by *V. carpophila* can result in fruit downgrading and rejection if the infection is severe (USDA, 2004). The sexual state of *V. carpophila* was only reported in Australia on apricot trees in 1961 (Fisher, 1961), demonstrating that the pathogen has the capacity for genetic recombination. Fisher (1961) reported differences in the host range of the pathogens infecting different species of stone fruit. Schubert *et al.* (2003) subsequently reported four species of *Fusicladium*, (Fr.) Lind, *V. carpophila*, *F. obducens* Pat., and *F. cerasi* (Rabenh.) Erikss. that infect various *Prunus* species. In India, it was reported first time from Kullu valley on peach (Khosla *et al.*, 2009). Later this disease was first time reported from Kashmir in 2014 as scab of almond caused by *Cladosporium carphophilum* (Kacho *et al.*, 2017).

A number of species of *Venturia* are recognized on Poplar in Europe, Asia, and North America (Morelet, 1985; Wu and Sutton, 1995; Newcombe, 1996). Some of them cause leaf and shoot blight (Morelet, 1983). The parasitic species of *Venturia* kill leaf and shoot tissue upon which they reproduce asexually (anamorph- *Pollaccia*). It overwinters in the sexual state (*Venturia*) on host tissue after its necrosis. Shoot diseases cause economic losses through deaths and visible damage which result in lost sales from nurseries. On aspen (*Populus tremula*), a

common and destructive shoot blight was reported to be caused by an ascomycete fungus *Venturia macularis* (syn. *V. tremulae*; anamorph *Pollaccia radiosa*; (Weisgerber, 1968; Dance, 1959). The shoots of poplar varieties (sect. *Tacamahaca*) were frequently killed by *Venturia populina* (anamorph: *P. elegans;* Dance, 1961). The life cycles of the two pathogens and the disease symptoms caused by them were similar, which sometimes resulted in wrong identifications (Dance, 1961). *V. populina* was originally described in Europe as a parasite of young leaves and shoots of *P. nigra* (Vuillemin, 1889). *V. mandshurica* was described on *P. simonii* in 1993 (Morelet, 1993), no other species of *Venturia* was thought to occur on *Populus* sects. cottonwood, black poplar (*Aigeiro*s) and balsam poplar (*Tacamahaca)*, till *V. inopina* as a new species was identified (Newcombe, 2003). In the monograph on *Fusicladium sensu lato*, eight *Fusicladium* species *viz*., *F. elegans* (*Venturia populina)*, *F. mandshuricum* (*Venturia mandshurica*), *F. martianoffianum, F. radiosum var radiosum* (*V. tremulae var. tremulae*), *F. radiosum var. lethiferum* (*V. tremulae var. grandidentatae*), *F. radiosum var. populi-albae* (*V. tremulae var. populi-albae*), *F. romellianum* (*V. borealis*) and *F. subsessile* had been reported on poplar (Schubert *et al.*, 2003). In India, Koul *et al*. (1989) reported first time poplar scab caused by *F. radiosum* from Kashmir. Singh *et al.*, 1983 and Rehil *et al.*, 1988 reported leaf spot as a severe disease of poplar. *Cladosporium humile* causing scab on *Populus* spp. was also reported by Beig and Khan (1999); however, very less literature is available on poplar scab due to non recognization of severe consequences of scab on poplar host. Accordingly, Schubert *et al.*, (2003) reported that the *F. crataegi* is the only cosmopolitan fungus that parasitizes *Crataegus* members, and for the first time, it was reported from Poland on *Crataegus coccinnae* in 2006 (Michalska and Połec, 2006). On cotoneaster plant, *Fusicladium pomi* (Schubert *et al.*, 2003) has been reported; *V. tomentosae* has also been reported from Switzerland. But no molecular work has been carried out on these pathogens to validate their taxonomic positions.

2.2 Molecular examinations and phylogeny of *Venturi*a species

Phylogenetic and population genetic methods were used to identify the species or populations and also their mating systems (clonal or recombining) and population structure (large recombining populations or sub-division into distinct genetically isolated populations) based on multilocus datasets of DNA variation (Taylor *et al.*, 1999). A species identification criterion based on phylogenetic concordance of multiple unlinked genes (Taylor *et al.*, 2000) is used to indicate a lack of genetic exchange among taxa. It appears more widely applicable and discriminating species recognition criteria than others in fungi (Giraud *et al.*, 2008). For closely related taxa, a number of analytical approaches are taken into account; as for example the patterns of genetic variation within and between the taxa in order to estimate parameters in a coalescent framework and provide important insights into the history of divergence (Becquet and Przeworski, 2007). For fungi, application of such approaches is still limited. However, a combination of coalescent analysis was used by Stukenbrock *et al*. (2007) to indicate the pathogen populations of *Mycosphaerella graminicola* on wheat emerging from populations on uncultivated grasses, due to the lack of current gene flow between populations and rapid speciation associated with a host-shift from wild to domesticated. Schnabel *et al.* (1999) published for the first time molecular analysis based on ITS and rDNA gene of *Venturia* species on various hosts belonging to *Rosaceae* family in which different *Venturia* species were placed into three distinct groups. First group comprised of *V. inaequalis*, the second group of *V. pyrina* and *V. nashicola* and the third group of *V. cerasi*, *V. carpophila*, and *V. asperata*. The described intron and ITS1 alleles in *V. inaequalis* provide the genetic markers for sub-dividing *V. inaequalis* populations but the cladogram published had limited taxon sampling and have less useful for phylogenetic analysis and taxonomic interpretations. The reason was that *C. caryigenum* taken to serve as outgroup is now known to be a true anamorph of the *Venturiaceae* as confirmed by molecular results and reflected by its morphology

(Schubert *et al.*, 2003). There are controversies regarding the existence of host range of various *Venturia* spp. on fruit trees. Monograph published by Sivanesan in 1977 on *Venturia* species, listed 14 species of *Rosaceae* as hosts of *V. inaequalis*, belonging to the five genera such as *Cotoneaster*, *Malus*, *Pyracantha*, *Pyrus* and *Sorbus*. Later, Schubert *et al.* (2003) included 7 more genera by adding *Amelanchier*, *Aronia*, *Docynia*, *Eriobotrya*, *Heteromeles*, *Kageneckia* and *Prunus*. However, the author did not cite literature on ability of *V. inaequalis* to infect these hosts, and some of these host-pathogen interactions have been refuted (Le Cam *et al.*, 2002; Chevalier *et al.*, 2004; Sánchez-Torres *et al.*, 2009). Le Cam *et al.* (2002) studied genetic relationships of *V. inaequalis* isolates from *Malus* species and *Spilocaea pyracanthae* isolates from *Pyracantha* species responsible for causing scab using sequence analysis of the complete internal transcribed spacer (ITS) region (ITS1-5.8S to ITS2), and revealed similarity between these two putative species, which were considered as two *formae speciales* belonging to *V. inaequalis*. Sanchez-Torres *et al.* (2009) performed further molecular analyses (a phylogenetic analysis of the G3PD gene, a microsatellite-primed PCR analysis, and RAPD fingerprinting) and pathogenicity tests for *F. eriobotryae* and *V. inaequalis*, and ultimately concluded that the loquat scab fungus is a distinct species from *V. inaequalis*. Gladieux *et al.* (2010), while studying the patterns of multilocus DNA sequence variation within and between the populations of *V. inaequalis* causing scab on apple, pyracantha and loquat, provided insight into the history of divergence using six nuclear loci *viz.*, act (actin gene), Ef1a (elongation factor gene), ypt1 (gene coding for the GTP-binding protein ypt1), cpcp (gene coding for a hypothetical protein conserved among fungi), scq5 (RAPD locus) and ITS1 (internal transcribed spacer of ribosomal DNA), in globally distributed samples, in which isolates from different hosts were differentiated but did not form diagnosable distinct phylogenetic species and supported a scenario of recent divergence in allopatry with groups from loquat and pyracantha being the most recently differentiated. In Kashmir valley, Padder *et al.* (2013) investigated 71 isolates of *V. inaequalis* on International Differential Hosts of apple, and analysed

them by random amplified polymorphic satellites (RAMS), PCR-RFLP and sequencing of rDNA for elucidating variability and phylogenetics. The 24 sequences of rDNA in *V. inaequalis* led to their grouping into four different clades, in which clade I clustered together all sequences of *V. pirini* and *V. naschicola* (thereby indicating that the two species could not be differentiated on the basis of ITS region) and clade II, III, IV accommodated all sequences of *V. inaequalis* indicating high level of diversity in *V. inaequalis*.

Venturia species with *Pollaccia* anamorphs on Salicaceae family hosts examined using morphology and genetic markers, revealed that its ascospores were similar to that of *V. populina* but the conidia were closer to *P. radiosa* anamorph of *V. macularis* (Kasanen *et al.,* 2001). If only the morphology of ascospores was used as criteria, it would have been identified as *V. populina*; however, analysis of 49 RAMS markers and variation within the ITS gene sequences as well as a single marker locus derived from RAMS fingerprints suggested that the *Venturia* is a previously undescribed species. *V. populina* was thought to cause spring defoliation of three species in sect. *Aigeiros* and nine species in Tacamahaca in Europe and North America. However *V. inopina* was confirmed based as a new species on ITS sequencing (Newcombe, 2003) and caused sporulating leaf lesions on *P. trichocarpa* and its hybrids but non-sporulating lesions on *P. nigra* cv. *italica*. Phylogenetic relationships of the anamorphic genus *Fusicladium*, *Pollaccia* and *Spilocaea* inferred by ITS DNA sequences revealed that *Pollaccia* and *Spilocaea* did not form monophyletic groups but were intermingled between *Fusicladium* species constituting a morphologically variable genus thus providing support for the monophyly of the anamorphic genus *Fusicladium*. The geographic origin of species did not affect the phylogenetic groupings as evident from the patterns of *F. radiosum* or *F. mandshuricum* and *V. saliciperda* collected from different geographic locations (Beck *et al.*, 2005).

The species name of *V. pyrina*, was used in Japan for pear scab until *V. nashicola* was first described by Tanaka and Yamamoto (1964) as the pathogen of scab on Japanese pear. Based on rDNA nucleotide sequence *V. nashicola* was found to be very close to *V. pyrina* and both were genetically distinct from the *Venturia* spp. found on other fruit trees (Schnabel *et al.*, 1999). Recently, Ishii and Yanase (2000) re-examined the relationship between these two putative fungal species and concluded that *V. nashicola* was a species distinct from *V. pyrina*. Their results based on pathogenicity tests and mating experiments clearly demonstrated the existence of host specificity and sexual barrier. Both *V. nashicola* and *V. pyrina* exhibited differential pathogenicity on pear cultivars and several pathological races have been categorized. In *V. pyrina*, Shabi *et al.* (1973) first designated five races specific to different cultivars of European pear and the wild Syrian pear species *P. syriaca* Boiss. In *V. nashicola*, Ishii *et al.* (2002) reported three pathological races (races 1, 2 and 3) to have differential pathogenicity on the Japanese pear cultivar 'Kousui' and one Asian pear strain 'Mamenashi12'. Zhao *et al.* (2012) used multigene phylogenetic analysis of *Venturia* species isolated from pear and revealed two phylogenetic groups that corroborated with the earlier taxonomic separation of *V. nashicola* from *V. pyrina*; as two distinct evolutionary lineages were found in *V. pyrina*, indicating that the evolution of pathological races in *V. nashicola* might have occurred recently compared with *V. pyrina* based on nucleotide sequences of rDNA-ITS, partial β-tubulin and elongation factor 1α genes. However, intraspecific relationships were not revealed among isolates of different pathological races in *V. nashicola*. Previous research showed that the intraspecific isozyme variation and sequences of endopolygalacturonase genes were related with pathogenicity variation in *V. nashicola* (Ishii and Suzaki, 1994; Isshiki *et al.*, 2000; Katoh *et al.*, 2008). Besides genes encoding pathogenicity factors such as endopolygalacturonase and exopolygalacturonase, the application of rDNA-IGS regions, RAPD and simple sequence repeat (SSR) markers have been proved to be helpful to reveal the

relationships of genetic divergence with pathogenicity variation (Guerin *et al.*, 2004; Lievens *et al.*, 2009; Sanchez-Torres *et al.*, 2009).

Different molecular approaches have been used to understand the phylogenetic relationships between *V. carpophila* and other *Venturia* species (Schnabel *et al.*, 1999; Beck *et al.*, 2005), and to gain insight into resistance in *V. carpophila* to quinone outside inhibitor (QoI) fungicides (Luo *et al.*, 2013). However, the molecular tools to study genetic variability among various populations of *Fusicladium* species from different hosts and geographic locations using 10 RAPD and 5 UP-PCR markers by Chen *et al.* (2014) revealed high node value (100 %) differentiating all the isolates of *V. carpophila* from *F. effusum* and a moderate node value (68 %) differentiating peach and almond isolates of *V. carpophila*, thereby indicating divergence between the *V. carpophila* populations of almond and peach and various levels of genetic diversity within the two populations.

All these analyses were largely based on the ITS region, which is the most widely used region for species identification. However, results from this region cannot be considered conclusive for distinguishing closely related fungal species (Kiss, 2012; Schoch *et al.*, 2012). As far as possible, anamorphs should reflect monophyletic holomorphic taxa (Reynolds, 1993). However, such re-assessments and re-evaluations have to be made individually for all groups of ascomycetes (Schubert *et al.*, 2003).

Chapter -3

MATERIALS AND METHODS

The present study entitled "DNA barcoding of *Venturia* species infecting various host plants in Kashmir" was carried out in the Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K) Shalimar, Srinagar during 2015-2019. The materials used and the methods adopted to achieve the objectives of the present study are described as under:-

3.1 Collection, isolation, and maintenance of fungal culture

3.1.1 Collection of diseased samples

The infected leaves, fruits and twigs showing typical disease symptoms (Plate 1) from different plant species, such as apple, pear, almond peach, crataegus, cotoneaster and poplar were collected from different locations of Srinagar, Pulwama and Anantnag, and brought to the laboratory for isolation of the fungus.

3.1.2 Isolation of fungus

3.1.2.1 Composition and preparation of culture media

For preparation of potato dextrose agar (PDA) medium, the peeled potatoes (200 g) were cut into small pieces and boiled in one litre of water for 20 min. The potato extract collected by filtering through double layered muslin cloth was added with dextrose (20 g), agar agar powder (15 g) and water, boiled till agar agar dissolved and the final volume made one litre with distilled water. For preparation of potato dextrose broth, no agar agar was added to the potato extract. Likewise, water agar medium (2%) was prepared by adding dextrose (20 g), agar agar powder (20 g) to one litre of distilled water. The prepared media were poured into Erlenmeyer flasks (250 ml) and test tubes (18 × 150 mm), followed by plugging with non-absorbent cotton and sterilized by autoclaving at 15 lbs psi

(A)

(B)

(C)

(D)

Plate 1: **Disease symptoms of scab on Apple (A), Pear (B), Poplar (C), Peach (D)**

(E)

(F)

(G)

Plate 1: **Disease symptoms of scab on (E) Almond, (F) Crataegus, (G) Cotoneaster**

(1.05 kg/sq.cm) for 20 min. After autoclaving, the slants containing approximately 10 ml PDA were placed in tilted position (45º angle) for solidification. The flasks containing media were allowed to cool (luke warm), poured (15-20 ml) into Petri plates under aseptic conditions, allowed to solidify and further used for isolation and purification of pathogen cultures.

3.1.2.2 Mono-conidial isolation and maintenance of fungal cultures

The mono-conidial isolations were carried out on water agar as described by Xu *et al.*, (2008). A small fragment of an infected leaf disc was added to a little amount of sterilized water and agitated thoroughly to release conidia. The conidial suspensions were diluted to 8×10^3 conidia ml^{-1} and 200 µl of the suspension pipetted onto plates containing water agar medium and spread evenly. These plates were incubated at 20±1ºC for 24 h. The individual germinated spores were excised using a cork borer under a compound microscope and transferred to Petri plates containing PDA medium under aseptic conditions. 55 isolates of pathogens from different host species were collected and maintained for further studies. For identification, morphological characters exihibited by the *Venturia* species on culture were studied. The identity was confirmed by sequencing of ITS region of the pathogen using ITS1 and ITS4 primer.

3.1.2.3 Morpho-cultural characteristics of the fungal isolates

Colony characteristics such as growth type, type of margins and colony colour of *Venturia* species were assessed on potato dextrose agar medium (PDA). Approximately, 20 ml of sterilized media were poured in Petri plates and then allowed to solidify. A 5 mm diameter disc cut with the help of sterilized cork borer from 20-25 days old cultures of different isolates were inoculated at the center of each Petri plate. The inoculated Petri plates were incubated at 20±1ºC and observations in terms of colony characteristics such as colony colour, texture, margins and shape were recorded after 30 days of incubation.

Spore (conidia) characteristics such as shape, size, colour and septation of *Venturia* species were assessed on PDA medium. The hyphae colour, size, shape and sepetation were also assessed. The spore measurements were recorded under the microscope previously calibrated using ocular and stage micrometers.

3.2 Molecular studies of *Venturia* isolates

3.2.1 Genomic DNA extraction

For the extraction of genomic DNA, 55 fungal cultures were grown in conical flasks containing 150 ml potato dextrose broth inoculated with 2 mycelial discs of 8 mm diameter from 15-day-old cultures and incubated at 20±1^{o}C for 60 days. The mycelium was harvested by filtration through sterile cheese cloth; agar plugs were removed, and the resultant mycelium dried under aseptic conditions, and stored at -80^{o}C for further studies.

Total genomic DNA of the fungal isolates was extracted using modified CTAB (Cetyl trimethyl ammonium bromide) method (Murray and Thompson, 1980). The dried mycelium from each isolate was ground into fine powder by constant crushing in liquid nitrogen using pre-chilled autoclaved mortar and pestle. About 40-50 mg of fine powdered tissue was transferred into 1.5 ml polypropylene centrifuge tube containing about 700 µl pre-heated (65°C) 2X CTAB extraction buffer [100 mM TrisHCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% CTAB, 2% PVP and 0.5% β-mercaptoethanol (added just before use)]. The powder was suspended in the buffer by inverting and rotating the tubes properly and mixing occasionally while maintaining at 65°C for one and half hours in water bath. To each tube, 700 µl of chilled chloroform-isoamyl alcohol (24:1) (v/v) was added. The contents in microfuge tubes were mixed thoroughly and centrifuged at 10,000 rpm for 15 minutes at 4^{o}C. The supernatant was transferred to a clean sterile 1.5 ml polypropylene centrifuge tube using large bore tip. The chloroform-isoamyl alcohol step was repeated. The aqueous phase was then transferred to new tubes and equal volume of pre-chilled isopropyl alcohol

(approximately 600-700 µl) added and the tubes inverted gently several times and kept at -20^{o}C overnight. The microfuge tubes were centrifuged at 10,000 rpm for 10 minutes at 4^{o}C and supernatant decanted. The DNA pellet was washed thrice with 70 per cent ethanol, dried and dissolved in 200 µl of Tris EDTA (10 mM Tris HCl and 1 mM EDTA, pH 8.0). RNase @ 10 µl/ml (MBI, Fermentas, CA, USA) was added and the emulsion incubated for 2 hours at 37^{o}C and DNA stored at -80^{o}C for further use.

3.2.2 Assessment of quality and quantity of DNA

Quality and quantity of the extracted DNA was checked by Agarose gel electrophoresis. In this, 1 g of agarose was dissolved in 100 ml of 0.5X Tris acetate EDTA (TAE) electrophoresis buffer. The mixture was heated till agarose was dissolved completely i.e. when solution became transparent and clear. It was cooled down to 60°C with constant stirring. Ethidium bromide was added to a final concentration of 0.5 µg/ml of buffer. The agarose solution was then poured into an already prepared gel mould with combs and was left for 20-30 min for solidification. DNA samples for loading were prepared by adding 2 µl loading dye (6X) (0.25% w/v bromophenol blue, 50% glycerol in sterile water) to 8 µl DNA such that the final concentration of loading dye was 1X. The 5 µl DNA samples were loaded into wells with the help of micropipette. Along with the DNA samples, marker of known concentration was also loaded. The gel was run for about one hr, visualized and photographed using gel documentation system (Alfa Imager EC, Protein Simple, USA). The intensity of fluorescence of each sample was compared with that of a standard marker (uncut Lambda DNA), and the DNA concentration of each sample ascertained. The quality of DNA samples was judged based on whether DNA formed a single high molecular weight band (good quality) or a smear (degraded/poor quality). The DNA of all the isolates was diluted to approx. 20 ng/µl by adding double distilled sterile water, and used for genotyping.

3.2.3 Selection of DNA barcode markers

Genomic DNA of 55 isolates of *Venturia* species amplified with three sets of primer pairs for species identification, phylogenetic analysis and taxonomic studies. The (ITS1-5.8S-ITS2) region of rDNA of *Venturia* was amplified using the primers ITS1 and ITS4 (Gardes and Bruns, 1993; White *et al.*, 1990). For the partial β-tubulin gene, a pair of PCR primers V1 and V2 were used to amplify this gene (Koenraadt *et al.*, 1992), and to amplify elongation factor-1α gene, a set of primers ELF1 and ELF2 were used (Gladieux *et al.*, 2010). The sequences of these three primer pairs are given in Table 1.

Table 1: rDNA-ITS, β-tubulin, EF1α primers used for PCR analysis of *Venturia* species, their sequences and annealing temperatures

S. No.	Primer	Sequence	Annealing Temperature
1	ITS1	5′TCCGTAGGTGAACCTGCG3′	
	ITS4	5′TCCTCCGCTTATTTGATATGC3′	54ºC
2	V1	5′GAGGAATTCCCAGACCGTATGATG3′	
	V2	5′GCTGGATCCTATTCTTTGGGTCGAACAT3′	50ºC
3	ELF1	5′CGAGAAGTTCGAGAAGGT3′	
	ELF2	5′CCCAATGACGGTGACATAG3′	58ºC

3.2.4 Polymerase Chain Reaction (PCR) amplification

PCR amplification was performed in 0.2 ml PCR tubes in a T-Gradient Whatman Biometra thermal cycler using 40 ng of genomic DNA in a final volume of 25 µl per reaction. The stock and final concentration of different components used in PCR amplification is given in Table 2.

Table 2: Stock and final concentration of various components of reaction mixture used in PCR for rDNA-ITS, β-tubulin and EF1α analysis

Component	Stock concentration	Volume (µl)	Final concentration
Sterile distilled water	--	14.8	--
PCR buffer*	10X	2.5	1.0 X
$MgCl_2$	25 mM	1.5	1.5 mM
dNTPs	2.5 mM	2.0	0.2 mM
Primer forward	10 pmol	1.0	0.4 pmol
Primer reverse	10 pmol	1.0	0.4 pmol
Taq Polymerase	5 U/µl	0.2	1.0 Unit
DNA template	20ng	2.0	40.0 ng
Total		**25.0**	

*10X PCR buffer: 10mM Tris HCl, pH 8.3, 50mM KCl, 0.08% (v/v) Nonidet P40.

The reaction mixture in PCR tubes was vortexed in microfuge (Thermo Scientific, Thermo Electron Corporation). PCR amplifications were performed in 96 wells of thermal cycler, programmed for initial denaturation at 94ºC for 5 min. followed by 35 cycles with denaturation at 94ºC for 1 minute, annealing at different temperatures ranging from 50ºC to 58ºC (Table 3.1) for 1 minute and extension at 72ºC for 1 minute with a final extension of 10 minutes at 72ºC.

3.2.5 Visualization of PCR products

To 25 µl of the amplified product, 4 µl of 6X loading dye was added so as to make the final concentration of loading buffer in reaction samples to 1X. PCR products were resolved by electrophoresis on a 1 per cent (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide in TAE buffer [40 mM Tris-HCl, 20 mM

sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4], and along with 100bp ladder for measuring the size of PCR product on agarose gel, and then visualized using gel documentation system (Alfa Imager EC, Protein Simple, USA) and photographed.

3.2.6 Sequencing of different isolates of *Venturia* species

After PCR amplification using rDNA-ITS, β-tubulin, EF1α primer in 50µl reaction mixture, 5 µl of PCR product of each isolate was electrophoresed to ensure successful amplification, and the remaining 45 µl PCR product of 55 isolates of *Venturia* species were lyophilized and sent for custom sequencing (Xcelaris India, Pvt. Ltd, Ahmadabad).

3.3 Data Analysis

3.3.1 Data generated by rDNA-ITS, β-tubulin and EF1α primers

In this analysis, isolates belonging to different hosts, such as apple, pear, poplar, almond, peach, crataegus and cotoneaster were considered as populations irrespective of geographical origin. After custom sequencing of *Venturia* species isolated from seven hosts for three genes *viz.*, rDNA-ITS, β-tubulin and EF1α, the sequences from each species were checked for quality, and compared with available sequences of data base at National Centre for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov/nuccore) using programme BLASTn. All the sequences were submitted to gene bank (NCBI) for Accession numbers (Table 3).

Table 3: List of sequences from *Venturia* species submitted to gene bank and their accession number

Venturia gene / Host	rDNA-ITS	β-tubulin	EF1α
Apple	MK751634-MK751644	MK506302-MK506312	MK578311-MK578321
Pear	MK478449-MK478465	MK506287-MK506296	MK578300-MK578310
Almond	MK463637-MK463645	MK506278-MK506286	MK578332-MK578338
Poplar	MK573003-MK573008 MK575493-MK575495	MK593456-MK593461	MK578343-MK578348
Peach	MK472057-MK472062	MK506273-MK506277	MK578339-MK578342
Crataegus	MK478372-MK478376	MK506270-MK506272	MK578327-MK578331
Cotoneaster	MK478020-MK478024	MK506297-MK506301	MK578322-MK578326

3.3.2 Phylogenetic analysis

For phylogenetic analysis, the sequences were aligned manually using Bioedit ver.7.09 (Hall, 1999) to remove ambiguous base and primer sequences, and the consensus sequences were reconfirmed by comparing with the original data output. Multiple alignments were performed using CLUSTAL W option in Bioedit ver.7.09 (Hall, 1999). To make comparison of sequences with the results from former research, sequence pertaining to ITS, β-tubulin, EF1α from *Venturia* species were compared with other sequences of different *Venturia* species which were retrieved from National Centre for Biotechnological Information (NCBI). All nucleotide sequences were aligned using CLUSTAL X 1.8 multiple alignment programme (Thompson *et al.,* 1997), and refined manually. MEGA ver. 6.02 (Tamura *et al.*, 2013) was used for neighbor joining (NJ) and maximum parsimony (MP) analyses. In this study, separate sequence data and combined data sets of rDNA-ITS, partial β-tubulin and EF1α genes were analyzed separately using NJ, MP. Script implemented in MEGA programme was used to calculate the best fit evolutionary model of DNA substation for various data sets. The best fit model was T92+G for rDNA-ITS, K2+G for partial β-tubulin and EF1α and HKY+G for combined data sets. Bootstrap confidence values were calculated for 1000 pseudo-replicates. Sequence polymorphism was analysed; genetic divergence values within and between populations were estimated and neighbour joining (NJ) tree was constructed for all the haplotypes according to Kimura 2-parameter (K2P) model using MEGA ver. 6.02 (Tamura *et al.*, 2013) after bootstrap analysis utilizing 1,000 re-samples of the data. Maximum parsimony trees were inferred with MEGA ver. 6.02 (Tamura *et al.*, 2013) using 100 random stepwise – addition heuristic searches and tree bisection-reconnection branch swapping (TBR), with a maximum of 3 trees retained. Bootstrapping was performed with heuristic search (1000 replicates; stepwise addition; TBR branch swapping).

3.3.3 DNA polymorphism analysis

3.3.3.1 Polymorphism, neutrality and recombination

Standard population genetic analysis was performed using DnaSP 5.0 (Libardo and Rozas, 2009). For each locus and populations, the polymorphism was estimated in terms of number of segregating sites (S), number of haplotypes (h), haplotypic diversity (Hd), two estimators of population genetic diversity (π) which is measure of average number of nucleotide differences between pair of sequences and θ, based on number of polymorphic sites. DnaSP 5.0 (Libardo and Rozas, 2009) were used to calculate minimum number of recombination events (Rm) and Tajima's (D) and Ramos-Onsis and Rozas(R2) were used to test departure from the neutral model of evolution. Significance was obtained from 10,000 coalescent simulations in DnaSP.

3.3.3.2 Divergence, differentiation and population genetic structure

Divergence among various groups was calculated as the average pair-wise number of nucleotide differences per site 'Dxy' (Nei, 1987). Differentiation between groups was tested using two statistics, K_{ST} (Hudson *et al.*, 1992) and Snn (Hudson, 2000), because of their ability to have the highest power to detect differentiation under simple models of population structure and to measure different aspects of differentiation (Hudson, 2000). Differentiation among groups was also estimated from an Analysis of Molecular Variance (AMOVA) implemented in Arlequin 3.11 (Excoffier *et al.*, 2005). The AMOVA framework incorporates haplotype divergence into a matrix of squared-distances among all pairs of haplotypes used to produce estimates of variance components and designated 'F_{ST} statistics' (Excoffier *et al.*, 2005). F_{ST} values between pairs of groups of isolates were also calculated. In this analysis, isolates belonging to hosts-apple, pear, poplar, almond, crataegus, peach and cotoneaster-were considered as populations and isolates belonging to particular species irrespective of their geographical origin were also considered as population. The statistical

significance of the total and pair-wise fixation indices was estimated by comparing the observed distribution with the null distribution generated by 1023 permutations of data matrix.

Two types of clustering methods were used to assess the genetic structure in *Venturia* species isolates from seven hosts using Genalex (Peakall and Smouse, 2006) and Arlequin 3.00 (Excoffier *et al.*, 2005). Principal Coordinate Analysis (PCoA) using Genalex (Peakall and Smouse, 2006) was conducted to construct plots of the most significant axes for grouping pattern verification. First, a matrix of Kimura's two-parameter distances among sequences concatenated across loci was constructed (Kimura, 1980) using MEGA ver. 6.02 (Tamura *et al.*, 2013), treating missing data and gaps using the 'pair-wise deletion' option. The resulting distance matrix was submitted to a Principal Coordinate Analysis (PCoA) as implemented in GenALEx (Peakall and Smouse, 2006). The spatial genetic structure was analyzed using Bayesian clustering program STRUCTURE 2.2 (Pritchard *et al.*, 2000) with pseudo-assembled data sets (fasta file) used as input in Genalex software making data set compatible to STRUCTURE program. To estimate the number of clusters (K), 10 independent runs of K = 1–12 were carried out without any prior information of origin (location or host) of individual sample. For each run, a burn-in period of 50,000 iterations was used followed by a run length of 5,00,000 iterations and a model with correlated allele frequencies and admixture among populations. The number of populations that represented the observed data under the model implemented was determined by maximizing estimated ln likelihood of the data for different values of K, and Δ_K index which is based on the rate of change in the ln likelihood of the data between successive Ks (Evanno *et al.*, 2005).

3.4 Development of PCR-based Molecular Markers

3.4.1 Primer Designing

The whole genome sequences of three *Venturia* species (*V. inaequalis*, *V. pyrina* and *V. carpophila*) were downloaded in fasta format (Table 4) from NCBI. The primers were efficiently designed through standalone primer3 software (Rozen and Skaletsky, 2000). The primers were designed with a product size ranging from 140 to 400 bp, primer length of 19-25 bp with optimal length of 22 bp, annealing temperature between 55 to 65°C with optimal melting temperature (Tm) of 60°C; the other parameters were set as default as the shorter lengths are considered less variable. The longer PCR products are difficult to replicate by polymerase enzyme and therefore shorter product size amplicons (Tautz, 1989) were developed. In total, 37 primer pairs were designed comprising of 12 for *V. inaequalis*, 12 for *V. pyrina* and 13 for *V. carpophila* (Table 5).

3.4.2 *In silico* PCR validation of designed primers

To get the information about the species specificity of each marker designed, all the 37-primer pairs were used for amplification of *Venturia* spp. through a standalone electronic PCR (ePCR) module. These 37 primers were used as input file and three *Venturia* genomes remained as the subject to get information weather these primers were able to amplify the genomic region across the *Venturia* genomes. The parameters used for the ePCR module were: primer nucleotide mismatch of not more than 2bp, 0bp gap, 200bp margin, and 150-1000bp product size.

3.4.3 PCR based validation of designed primers

After ePCR validation, all the 37 primers were selected for experimental validation on 35 representative isolates of *Venturia* species from different hosts (Table 6). Polymerase chain reaction (PCR) was performed in 25 µl reaction containing 40ng of genomic DNA, 1x PCR buffer, 1.5mM MgCl2, 0.2µM dNTPs, 0.2 µM of each primer and 1U *Taq* polymerase (Fermentas, CA, USA) with the

Table 4: Overview of three *Venturia* species genomes

Species	Genome size (Mbp)	G+C content (%)	Total number of sequence/scaffolds	Scaffold examined	Total genome covered by examined scaffolds (Mbp)	Reference
V. inaequalis	55.12	38.10	1012	11	3.24	Deng *et al.* (2017)
V. pyrina	41.17	47.30	364	11	4.75	Cooke *et al.* (2014)
V. carpophila	36.91	47.40	657	12	6.40	Chen *et al.* (2017)

Table 5: List of primers designed from three Venturia genomes along with their sequences, annealing temperature, number of alleles, product size and scaffold positions

Primer Name		Sequences (5'--------3')	Annealing temperature	No. of alleles	PCR product size (bp)	Scaffold No./Sequence position
Vi1	F	GCCCTCAGGCTACACAACTC	53	1	250	>scaffold_1005 88884
	R	TGGTGAGGTCGTTGGTAGGT				
Vi2	F	CGAGGAATGGTGATGATGGT	50	1	300	>scaffold_904 62386
	R	CAATTCTTTGCGCATCTCTAAC				
Vi3	F	AGTAGGAGGCGAGGAAGGAG	53	1	375	>scaffold_356 3934
	R	GCCGCCGCTATTATAAGTTT				
Vi4	F	TCGAGTCTAAGTCGTCCCGTA	53	1	0	>scaffold_985 24883
	R	CCCGTATTAGCGCGATGTAT				
Vi5	F	AAACCGAGCCCAATTATTCA	50	1	0	>scaffold_811 168626
	R	GAACCGGTGGAGATGATACC				
Vi6	F	TGATGATCCCAAACGTGATG	52	1	335	>scaffold_180 372749
	R	TGGCTGTGTTCTCATCTTGG				
Vi7	F	GGAGGGCGTACTGTAATGGA	56	2	223	>scaffold_841 150130[-
	R	TACTGTCGGTTCGGCTTCTT				
Vi8	F	TCCGGATAATGTGGATGGTT	53	2	115- 179	>scaffold_857, 135017
	R	ATGGAATCGGCTTTTGTGAG				
Vi9	F	ACCACAACCAACGTTCACAA	54	1	226-247	>scaffold_580 845187
	R	CGACGATGACGAAGGAGAAT				
Vi10	F	TTTATAGGCAAAGCGGGAGTT	56	1	139	>scaffold_207 175
	R	TCCCTCTTATATCCCCTCCCTA				
Vi11	F	CCACATCCACATCCTCCTCT	55	2	129-130	>scaffold_417 97356
	R	GACCTGGGAGCATTGAAGAA				

Vi12	F	CCACATCCACATCCTCCTCT	54	2	139-140	>scaffold_417 97453
	R	GACAGCGAGGGAAAGATCAC				
Pr2	F	GAGGGCGGGACGTAGTTAGT	53	1	0	>scaffold_142 102199
	R	TTGCAAGCCACTATGATGGA				
Pr4	F	AAATCGCGGCAATAGAAAGA	53	1	300-400	>scaffold_42 238636
	R	CTTGAACGAGGTCGGGATAG				
Pr3	F	CGAATCCACAACACCACTTG	50	1	200	>scaffold_1 548965
	R	TAGAAACCTTCCCGAAACCA				
Pr5	F	AGCGGGGAGATAGAAGGAAC	54	1	342-344	>scaffold_8 141
	R	GATCGAGGTTTCGTTGCAGT				
Pr6	F	AAACGTCTTGGCCTCCTTTT	52	1	372	>scaffold_64 70656
	R	AAAACGCACTGTTCCCAAAC				
Pr7	F	CAATAATGGCACAGCAAACTC	56	2	373-374	>scaffold_122 67425
	R	TGTTCATGACAGGTTTCTACTCG				
Pr8	F	CCCTTTTGAACAAACAAGGAGT	54	3	338-340	>scaffold_30 22397
	R	AGCATTGTTAATGGGAGTGC				
Pr9	F	ATGGGTGGTCCAGGTGTTTA	54	3	329-333	>scaffold_7 109793
	R	AGGGATGAGAGCTCCGTTTT				
Pr10	F	GATCCAGGAGTGTCGCAAGT	55	1	353-356	>scaffold_42 64289
	R	CAGACGACGTTGAAGTGGAA				
Pr11	F	AACCAAGCACACCACACAAA	54	1	286-292	>scaffold_108 63847
	R	CGACGCGATCCTCTTCTCTA				
Pr12	F	GTCGTAGGTTCCTCGCAAGA	56	1	124-123	>scaffold_151 29769
	R	CGTTTTCCAATCTCGGACCT				
Pr13	F	ATACATCCCCTGCTGCTGTT	54	1	168-174	>scaffold_24 296783
	R	CCAGCCCAACCCTACTACAA				
C1	F	GGTGGAATCGTGCTTCTCAT	50	1	331-332	>MECS01000001.1 14568
	R	AAGGAGGCTAACGAAAATCCA				

C2	F	ATCACGAACGATCCAAAAGG	53	2	300	>MECS01000062.1 194725
	R	GTGAGGGAGTGAGGGAGTGA				
C3	F	TCATCCTCGTCCTCATCCTC	53	1	350	>MECS01000013.1 696951
	R	GCCAGATTCTTGATGGTCGT				
C4	F	GTGATGGTCTTGGCTCTGGT	54	1	300	>MECS01000119.1 101418
	R	GCATGATCCCTACCCTCTCA				
C5	F	TTCTCGATGGTGCAGGTATG	50	1	195-196	>MECS01000007.1 164438
	R	TTGGTTGTACCGCAATGTGT				
C6	F	AAAAGGTAGGTGGCGAGGTT	54	1	153-165	>MECS01000035.1 42833
	R	TGAGGGAACCCAAAGTATGC				
C7	F	CCCGATATACTTACGCGCTAT	55	1	191-192	>MECS01000084.1 11578
	R	GTCCTATTCCGCGAGCTTA				
C8	F	CTCGTGCTGGAGGAGTATGC	58	2	224-229	>MECS01000037.1 87228
	R	CTACCGGAACACCGAGTCAT				
C9	F	GGATCTGTGAAGAGGCGAAG	55	2	287-288	>MECS01000010.1 1013854
	R	TCCCACTCTCAACATCACCA				
C10	F	AGGCGGGTTGTATTCACTTG	56	1	336-337	>MECS01000010.1 640057
	R	TGCCACCCTCTCTCTCATCT				
C11	F	ACCGAGTAACCGGCTAAAGT	55	2	302-303	>MECS01000018.1 1965
	R	AAGGCGCTGGTCTATTTAAGG				
C12	F	GTCAAAAGCCCTCACTCAGC	53	2	138-139	>MECS01000029.1 215145
	R	ACGTCAGGTTGAAAGGATGG				
C13	F	GCTCGACTATACGGCTTCCT	53	2	265-278	>MECS01000121.1 44338
	R	TATTCGTACCTTGGCCTTCG				

PCR programme: initial denaturation at 94°C for 3 min. followed by 30 cycles with denaturation at 94°C for 30 sec., annealing at different temperatures for different primers (Table 4) for 30 sec. and extension at 72°C for 30 sec., with the final extension at 72°C for 5 min. The amplified products were electrophoretically separated on 1.5 per cent agarose gel and stained with ethidium bromide. The product sizes were determined by comparing the band size with 100 and 20 bp DNA ladders and visualized using gel documentation system (Alfa Imager EC, Protein Simple, USA) and photographed.

Table 6: List of host species and their corresponding number of fungal isolates used for screening designed markers

Host	No. of fungal isolates used
Apple	5
Pear	5
Almond	5
Poplar	5
Crataegus	5
Peach	5
Cotoneaster	5

3.4.4 *In planta* validation of designed markers

Apple scab, being the disease of national importance (Gupta, 1990), species-specific marker were tested for *in planta* detection of apple scab pathogen. For *in planta* validation, apple leaves (5 samples) infected with apple scab were collected and DNA was extracted as described by Murray and Thompson (1980). Five leaves without scab lesions served as control. The extracted DNA was amplified using *V. inaequalis* species-specific primer as described in previous section.

3.4.5 Multiplexing

To analyse the species specificity of developed primers, DNA (200 ng) from five *Venturia* species was pooled. Further, two multiplex primes (forward and reverse) of 10µM concentration were prepared from the primer stocks. DNA from individual *Venturia* spp. along with pooled DNA was PCR amplified using simplex and multiplex primers as described in previous section.

3.4.6 Real time PCR (qPCR validation)

Ten-fold dilutions of DNA were prepared to generate standard curves for *V. inaequalis*, *V. pyrina* and *V. carpophila*. Each dilution (100, 10, 1, 0.1, 0.01, 0.001, 0.0001ng) was used as a template for qPCR with two technical replicates. qPCR was set in a total volume of 12.5µl reaction mixture containing 6µl of FASTSYBR Green PCR mixture (Fermentas, Thermo Fisher Scientific, US), 0.5µl each of forward and reverse primers (50 µM), 1µl template DNA and 4.5µl of nuclease free water. A fast qPCR was carried out on a Qiagen 5plex real time PCR system (Qiagen, Hilden, Germany) with a temperature regime of 40 cycles at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. A melt curve program of 65-95°C was carried out using a cycle of 65°C for 15 s followed by a slow temperature increase to 95°C at the rate of 0.2°C/s to check the specificity of the PCR product. Primer efficiencies were determined with standard curves generated from 10 fold dilutions of DNA as template. Amplification efficiencies, amplification factor and coefficient of determination for each primer pair were calculated.

Chapter – 4

EXPERIMENTAL FINDINGS

4.1 Collection, isolation, purification and maintenance of the pathogen

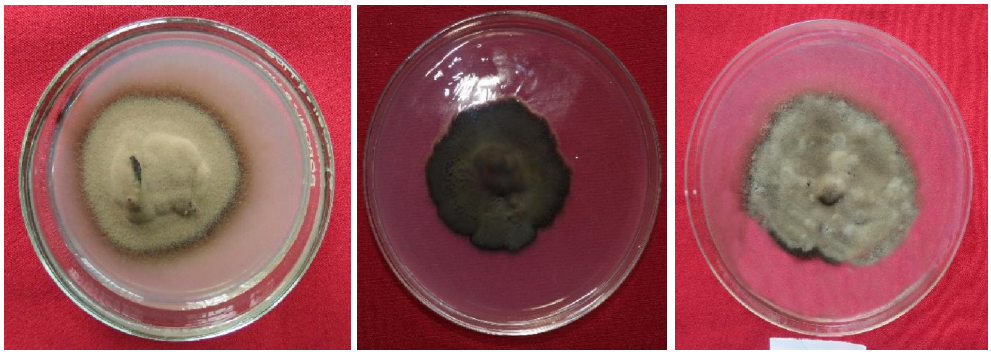
Survey of various locations *viz*., Srinagar, Pulwama and Anantnag, were carried out during the year 2015 for collection of samples (leaves, fruits and twigs) infected with scab disease. Samples showing typical symptoms of scab disease on different hosts *viz*., apple, pear, almond, poplar, peach, crataegus and cotoneaster from various locations were collected and brought to laboratory for isolation of the pathogen on potato dextrose agar (PDA) medium using standard pathological procedures. Purification of 55 cultures was done following single spore technique (Xu *et al.*, 2008). These cultures were maintained on PDA for further studies.

4.1.1 Morpho-cultural characterization

The fungal cultures identified on the basis of studies conducted on its morphological characters in culture and compared using standard literature (Schubert *et al.*, 2003), and identity was confirmed by sequencing of rDNA-ITS, EF1α and β-tubulin genes of the fungus. These sequences were submitted to Genbank of NCBI for Accession number.

Various isolates of different *Venturia* species isolated from different hosts species were cultured on potato dextrose agar medium and didn't show any significant variations in their colony characteristics (Plate 2). *Venturia* species showed flat, erumpent to fluffy cottony type of growth and colony margins in most of the isolates were irregular or uneven. Colony colour also varied from blackish centre surrounded by greyish region in *V. populina*, greyish black to dark black colony in *V. inaequalis*, rough blackish in *V. carpophila*, velvety greyish black in *C. humile*, mousy black in *V. pyrina*, greyish white to grey in *V. crataegi*. These characteristics varied in different isolates belonging to different species

Poplar **Cotoneaster** **Almond**



Pear **Apple** **Crataegus**

Poplar **Peach**

Plate-2: Colonies of *Venturia* species isolated from different host plants

4.1.1.1 Microscopic characteristic

V. inaequalis. The fungus produced septate, interwoven compact, light olivaceous to brown mycelium with a width of 4-10 µm. The conidia were solitary, light pale to light olivaceous in colour, obipyriform, straight, without septa but occasionally one septate, measuring about 16.23- 32.94 × 6.84-11.72 µm with an average of 27.94x 7.84 µm (Plate3a).

V. pyrina. The fungus produced septate, compact stromatic, light brown to dark brown coloured mycelium with a width of 3-8 µm. The conidia were solitary, light yellow to olivaceous brown in colour, fusiform to pyriform, straight to slightly curved, without septa but occasionally one septate, measuring about 15.96-36.92×6.76- 9.40 µm with an average of 28.57×7.66 µm (Plate3b).

V. populina. The fungus produced septate, swollen, sub-hyaline to light yellowish mycelium, with a width of 3-7 µm. The conidia were solitary, sub-hyaline to light olivaceous in colour, fusiform to ellipsoidal, straight, rarely curved having 0 to 2 septa measuring about 15.97- 33.46 × 8.12-12.96 µm with an average of 27.76 × 9.35 µm (Plate3c).

V. crataegi. The fungus produced septate, branched interwoven, light pale to olivaceous brown mycelium with a width of 3-5 µm. The conidia were solitary, light yellow to olivaceous brown in colour, fusiform to obclavate having 0 to 1 septa with slightly constricted at the septum measuring about 11. 47-19.18 × 4.71-6.90 µm with an average of 15. 64 × 5.76 µm (Plate3d).

V. carpophila. The fungus produced septate, interwoven, branched, dark brownish mycelium with a width of 4-6µm. The conidia were mostly simple, sub-hyaline to light brown, cylindrical to fusiform, sometimes ovate, straight, having 0 to 1 septa with slightly constricted at septum measuring 11.67-22.50 × 4.45-7.96 µm with an average of 18.30 × 6.46 µm (Plate 3e).

C. humile. The fungus produced septate, tubular, light pale to olivaceous colour mycelium with a width of 2-6 µm. The conidia were catenate, sub-hyaline to light

olivaceous green in colour, cylindrical to fusiform, straight or slightly curved having scar on the ends, 0 to 1 septa, measuring about 15.47-27.46 × 4.43-9.53 µm with an average of 20.12 × 7.71 µm (Plate3f).

4.2 Characterisation of rDNA-ITS, β-tubulin, EF1α genes using PCR

Isolation of genomic DNA using CTAB method (Murray and Thompson, 1980) was adopted to extract DNA from 50 days old culture of 55 isolates of *Venturia* species. The quality of DNA was checked in agarose gel electrophoresis. The genomic DNA of the isolates was seen as single unshared band which indicated good quality of DNA. Dilutions were made by TrisEDTA buffer to maintain approximately 20-25 ng/µl concentration of DNA which was further used for PCR amplification. The fragments of rDNA-ITS, β-tubulin and EF1α genes of *Venturia* species were amplified successfully using PCR based gene specific markers from all the samples (Plate -4). ITS regions of rDNA of sixty-four isolates were amplified using ITS1 and ITS4 primer pairs yielding 650 base pair product, whereas EFα1 gene was amplified using primer pairs ELF-1 and ELF-2 amplifying approximately 500 base pair product. However, β-tubulin gene was amplified using primer pairs V1 and V2 in PCR amplifying nearly 450 base pair product. The size of amplified product was measured on the basis of 100bp ladder.The amplified products of each isolate for 3 genes were separately sent for custom sequencing. The amplified products were custom sequenced from Xcelaris India, Pvt. Ltd, Ahmadabad. The sequences were retrieved from the chromatograms of each forward and reverse primers (Plate 5) and subjected for quality check using phread score above 30 indicating high quality sequences. Sequences were aligned using DNA baser software to form consensus DNA sequence/Contig from forward and reverse sequence for each isolate. In the same manner, 162 aligned sequences were obtained from all the three genes.

4.2.1 Sequence analysis of ITS, EFα1 and β-tubulin genes

The information on sequence of three genes were analysed through

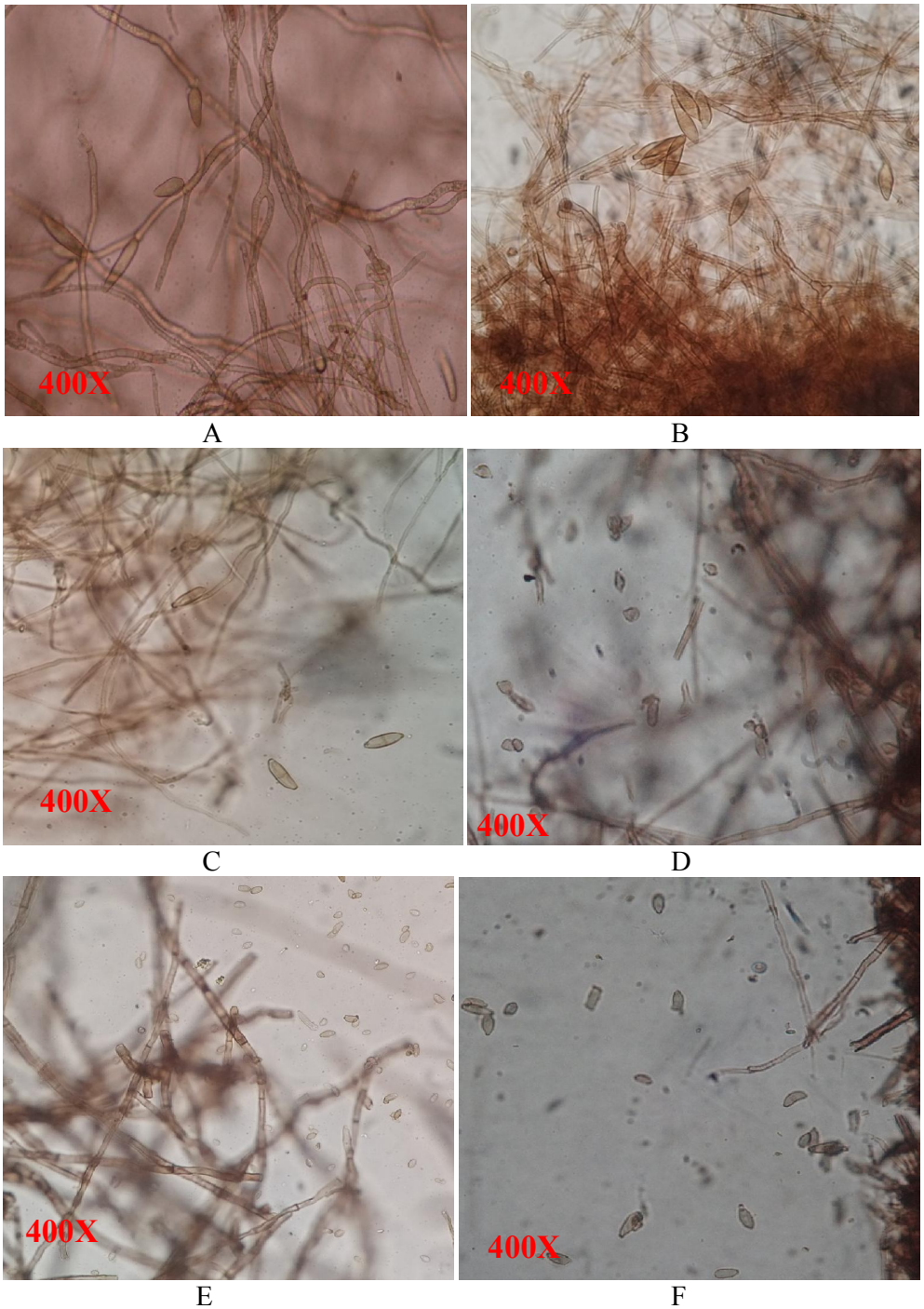


Plate 3: **Conidia and mycelium of *Venturia* species; *V. inaequalis* (A), *V. pyrina* (B), *V. populina* (C), *V. crataegi* (D), *V. carpophila* (E), *Cladosporium humile* (F)**

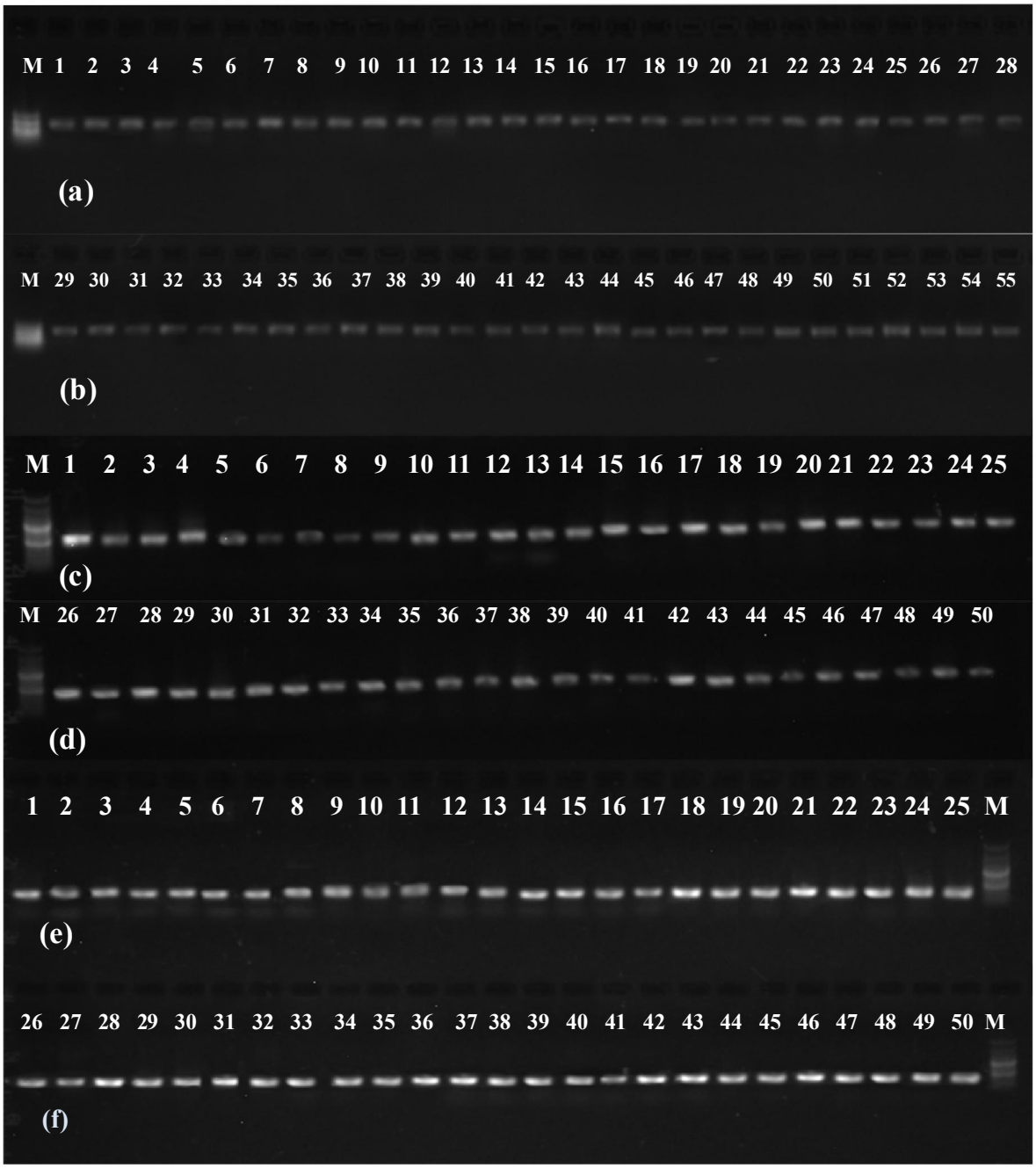
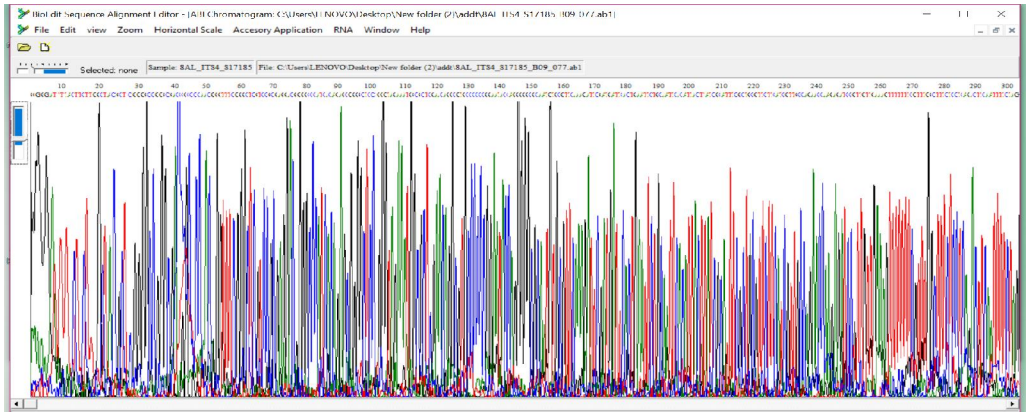
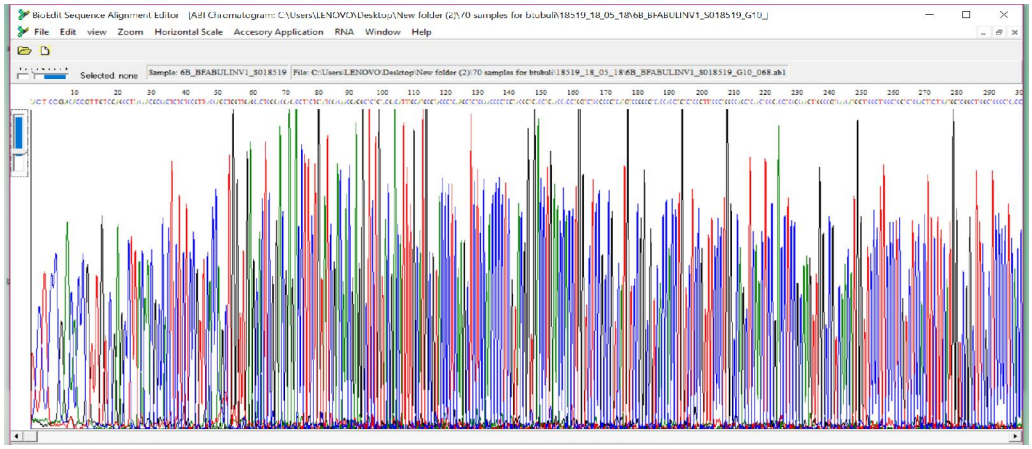


Plate 4: **DNA amplification profile of *Venturia* species with ITS (a and b), EF1α (c and d) and β-tubulin (e and f) primers**

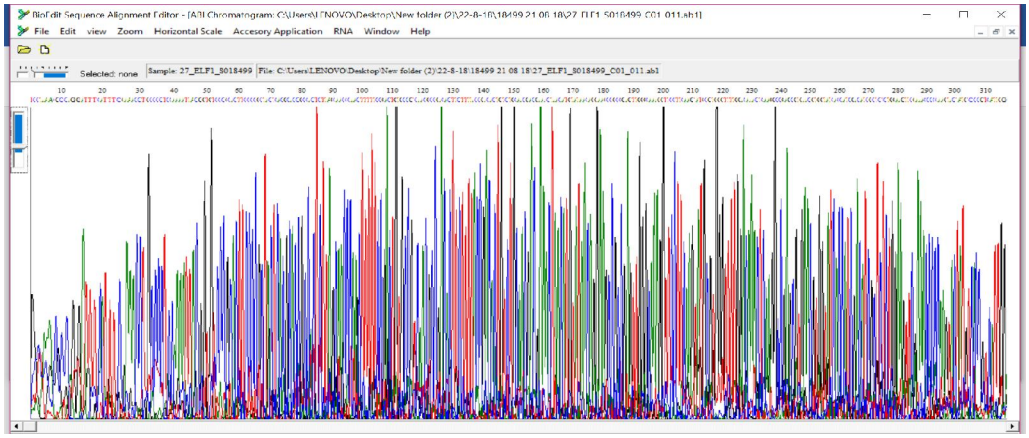
M= 100bp ladder, ITS = 650bp product size (a and b), EF1α = 500bp product size (c and d) and β-tubulin = 450bp product size (e and f)



(a)



(b)



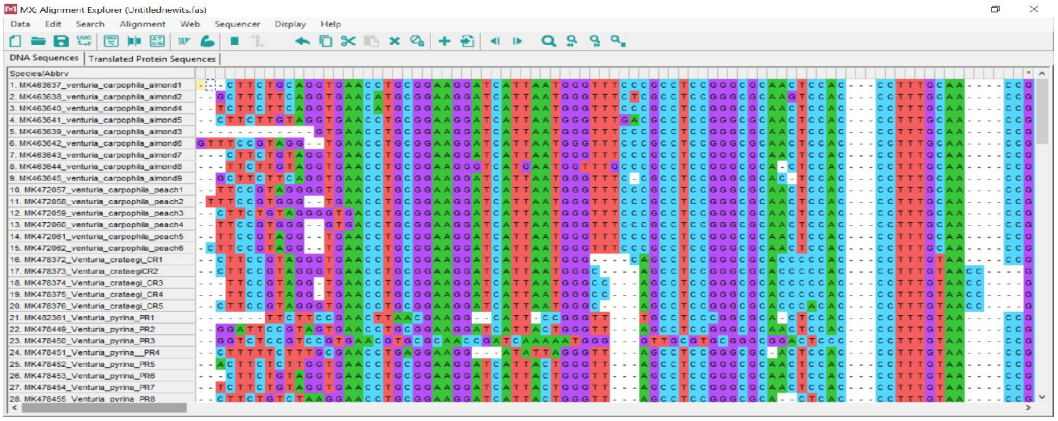
(c)

Plate 5: **Chromatograms based on (a) ITS, (b) β-tubulin and (c) EF1α genes of selected *Venturia* spp. isolates**

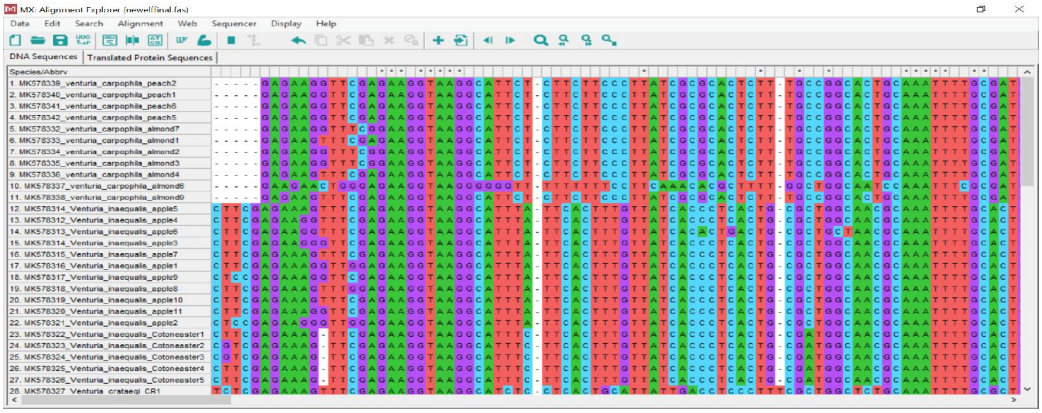
BLASTn programme of NCBI to compare them with the available sequences of database at NCBI (http://www.nlm.nih.gov/nuccore), which indicated that the sequences showed maximum similarity with their respective sequences of *Venturia* species available at NCBI. One hundred sixty-two sequences were submitted to GenBank using NCBI submission tool Banklt and Accession Numbers obtained (Table 3). Sequence analysis of rDNA-ITS region of *V. carpophila, V. crataegi, V. inaequalis, V. pyrina, V. populina* and *C. humile* isolates representing different geographical regions and host species, revealed that the final alignment of sequences was 570bp after excluding primer sequences, out of which a total of 108 variable sites were detected having 14 sites as singletons and 94 sites as parsimony informative sites. For β-tubulin, there were 450 bp in aligned sequences with a variable site of 83, in which 21sites were singletons and 62 sites were parsimony informative characters. The alignment of partial EF1α gene sequences was 460 bp after excluding ambiguities and the total variable sites were 119, in which 23 represented singletons and 96 were parsimony informative characters. The total length of concatenated sequences for the three loci was 1530 bp, in which the total number of variable sites was 93 across all the three loci with 28 singletons and 65 parsimony informative sites.

4.2.2 Phylogenetic analysis

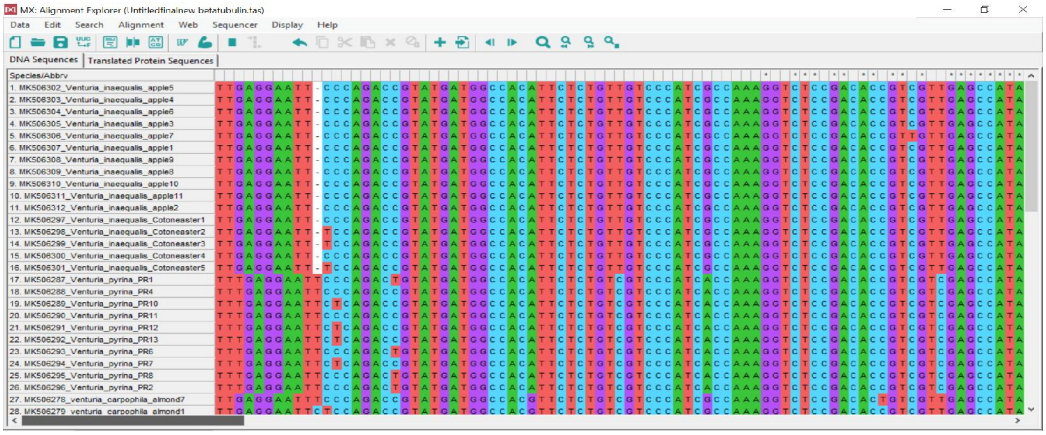
The sequence data of the three genes *viz*., rDNA-ITS, β-tubulin and EF1α, for 162 isolates of test *Venturia* species, *V. carpophila, V. crataegi, V. inaequalis, V. pyrina, V. populina and C. humile*, have been used for phylogenetic analysis along with the known sequences retrieved from GenBank at NCBI (National Centre of Biotechnology Information). Separate sequence data sets of each gene as well as combined data set of ITS, β-tubulin and EFlα genes were generated by Sequence Matrix software. Sequences were aligned through CLUSTAL W multiple alignment programme (Plate 6). Phylogenetic analysis was carried out at individual and combined gene levels using MEGA 6.02 and four the cladograms were generated based on these data sets. The analysis provides the first molecular



(a)



(b)



(c)

Plate 6: **Sequence alignment of selected *Venturia* spp. isolates through CLUSTAL W multiple alignment programme using software MEGA, (a) ITS, (b) EFα1 and (c) β-tubulin gene sequences**

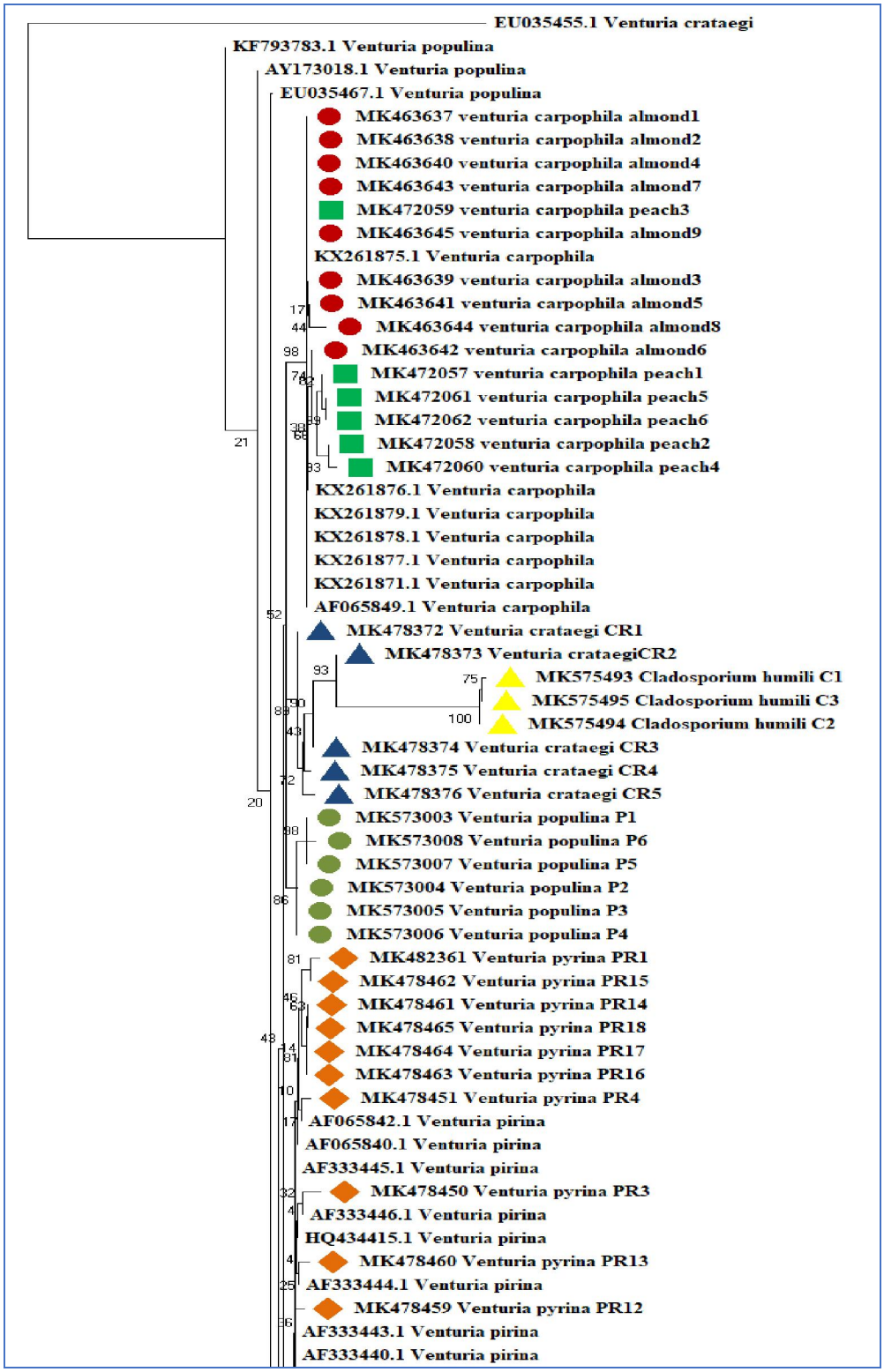
results for *Venturia* species and the corresponding probable phylogenetic relationships.

4.2.2.1 rDNA-ITS based phylogeny

An optimal tree was constructed with neighbour-joining method implemented in molecular evolutionary genetic analysis (MEGA) programme with Kimura-2-parameter substitution model, different taxa were clustered together in a bootstrap test with 1000 replicates (Fig.1). In this phylogenetic tree, *Venturia* species were grouped into 2 main clades and 4 independent lineages, and formed a monophyletic clade of Venturiaceae family. Clade first was composed of two subclades, in which sub clade I was composed of further several sub-clades accommodating *V. carpophila* isolates from almond and peach, *V. crataegi*, *C. humile* and *V. populina* isolates from crataegus and poplar together, while four independent lineages contained single isolates of *V. crataegi* and *V. populina* respectively. The subclade II clustered all *V. pyrina* isolates together (custom sequenced and available at NCBI GenBank). Clade second grouped all *V. inaequalis* isolate from apple and cotoneaster together.

4.2.2.2 β-tubulin gene phylogeny

The phylogenetic tree based on β-tubulin gene phylogeny has grouped all the isolates of *Venturia* species collected from different hosts into six main clades (Fig. 2). The clade I supported the monophyly of all *V. inaequalis* isolates (with high support of Bootstrap value; BS=99 collected from apple and cotoneaster without partitioning them on the basis of host origin. *V. carpophila* isolates collected from peach and almond were grouped and scattered in clade II without their host origin. Clade III accommodated all isolates of *V. crataegi* collected from crataegus host. Clade IV was a monophyletic group of *V. pyrina* isolates with high bootstrap support (BS= 99). It contained all the isolates including test sequences and those available at NCBI GenBank). Clade V contained the *V. populina* isolates that were retrieved from GenBank NCBI, while as Clade VI


EU035455.1 Venturia crataegi
KF793783.1 Venturia populina
AY173018.1 Venturia populina
EU035467.1 Venturia populina
MK463637 venturia carpophila almond1
MK463638 venturia carpophila almond2
MK463640 venturia carpophila almond4
MK463643 venturia carpophila almond7
MK472059 venturia carpophila peach3
MK463645 venturia carpophila almond9
KX261875.1 Venturia carpophila
MK463639 venturia carpophila almond3
MK463641 venturia carpophila almond5
MK463644 venturia carpophila almond8
MK463642 venturia carpophila almond6
MK472057 venturia carpophila peach1
MK472061 venturia carpophila peach5
MK472062 venturia carpophila peach6
MK472058 venturia carpophila peach2
MK472060 venturia carpophila peach4
KX261876.1 Venturia carpophila
KX261879.1 Venturia carpophila
KX261878.1 Venturia carpophila
KX261877.1 Venturia carpophila
KX261871.1 Venturia carpophila
AF065849.1 Venturia carpophila
MK478372 Venturia crataegi CR1
MK478373 Venturia crataegiCR2
MK575493 Cladosporium humili C1
MK575495 Cladosporium humili C3
MK575494 Cladosporium humili C2
MK478374 Venturia crataegi CR3
MK478375 Venturia crataegi CR4
MK478376 Venturia crataegi CR5
MK573003 Venturia populina P1
MK573008 Venturia populina P6
MK573007 Venturia populina P5
MK573004 Venturia populina P2
MK573005 Venturia populina P3
MK573006 Venturia populina P4
MK482361 Venturia pyrina PR1
MK478462 Venturia pyrina PR15
MK478461 Venturia pyrina PR14
MK478465 Venturia pyrina PR18
MK478464 Venturia pyrina PR17
MK478463 Venturia pyrina PR16
MK478451 Venturia pyrina PR4
AF065842.1 Venturia pirina
AF065840.1 Venturia pirina
AF333445.1 Venturia pirina
MK478450 Venturia pyrina PR3
AF333446.1 Venturia pirina
HQ434415.1 Venturia pirina
MK478460 Venturia pyrina PR13
AF333444.1 Venturia pirina
MK478459 Venturia pyrina PR12
AF333443.1 Venturia pirina
AF333440.1 Venturia pirina

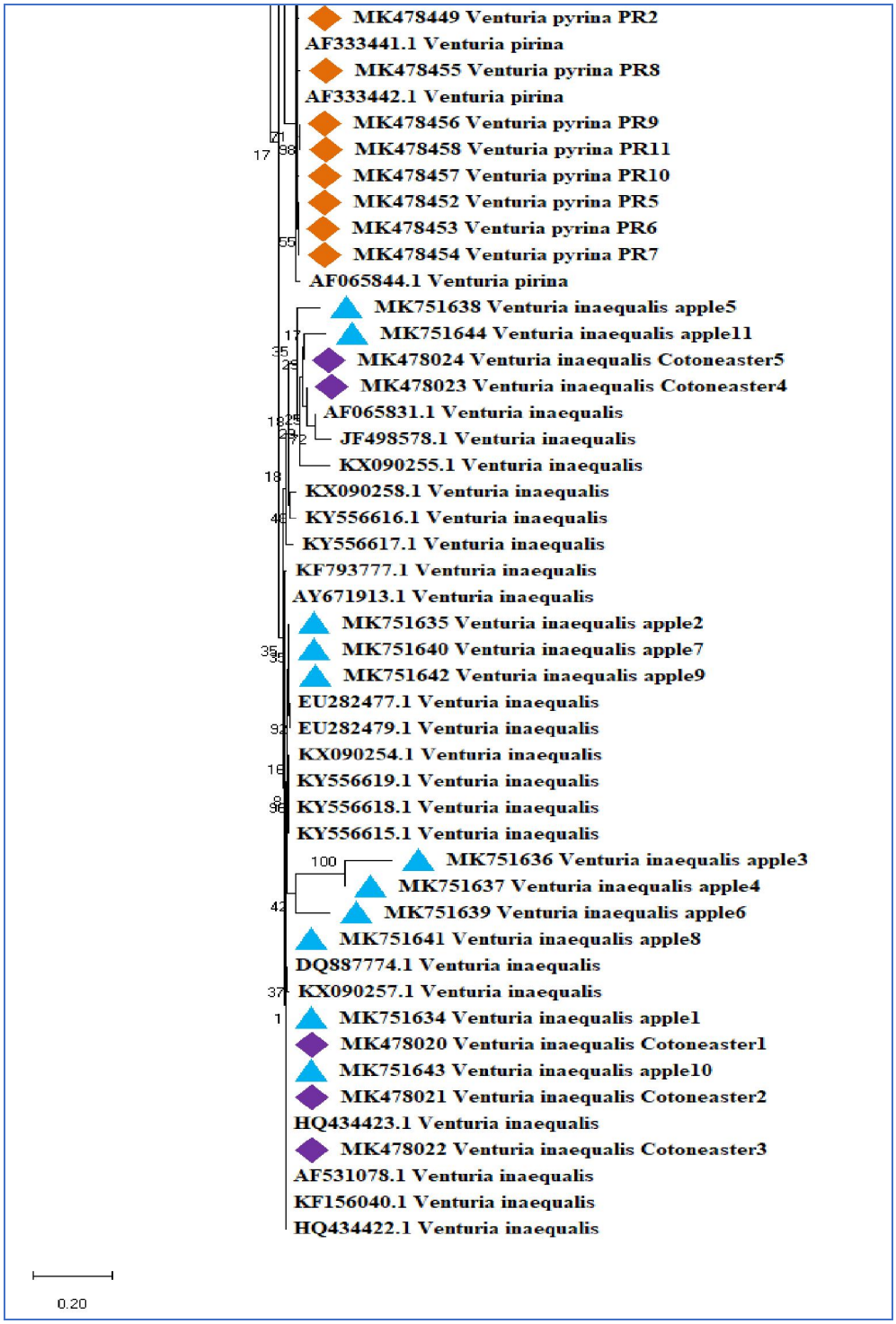


Fig. 1: **Phylogenetic relationship among *Venturia* species based on Internal transcribed spacer (ITS) region**

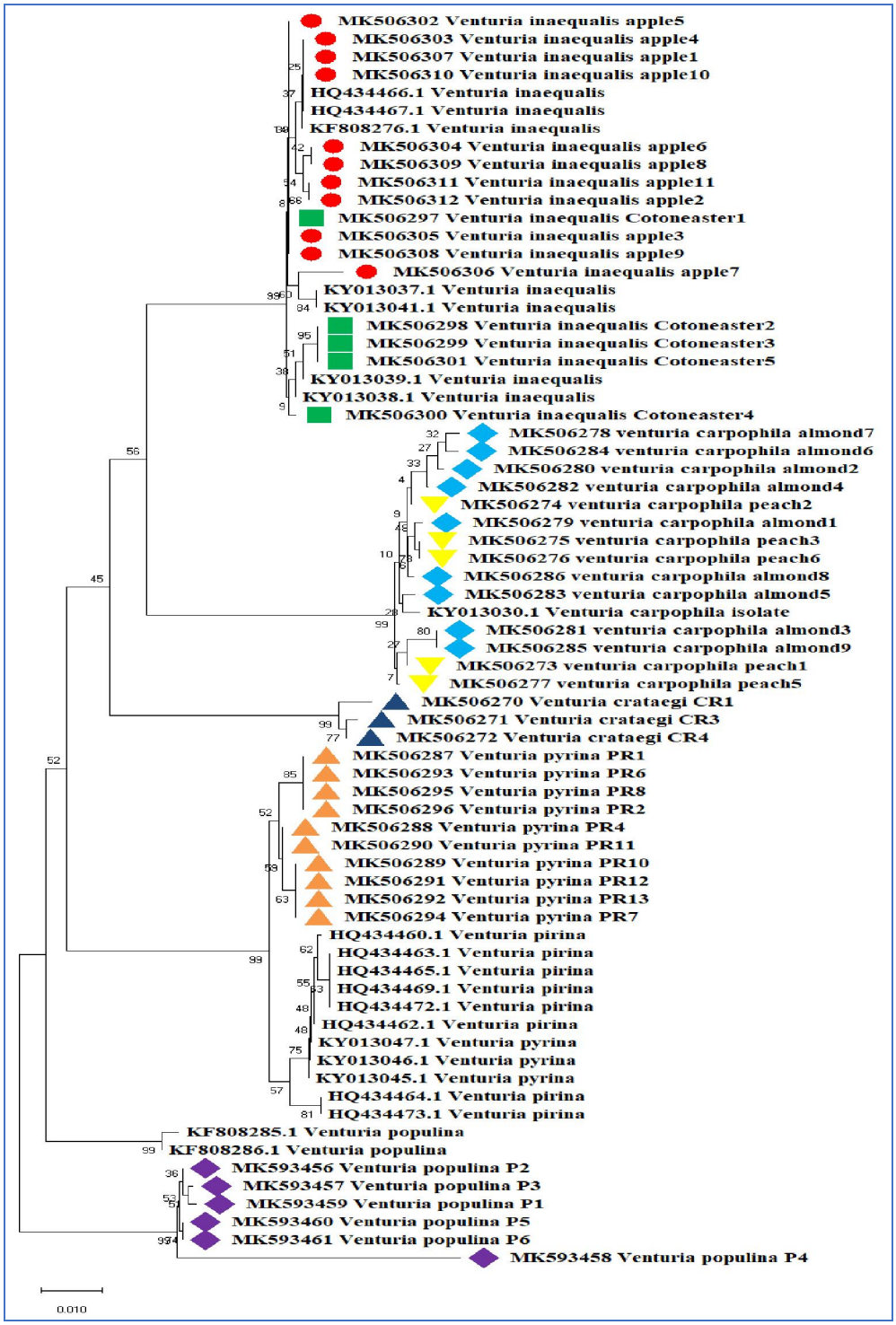


Fig. 2: **Phylogenetic relationship among *Venturia* species based on β-tubulin gene**

composed of all the isolates from *V. populina* with high boot strap value (BS=99), in which P4 isolate formed a diverged lineage which might be a new race. Perusal of the phylogeny based on β-tubulin indicated that there is wide-spread inter-specific variation that leads to specific recognition and grouping of these *Venturia* species into many clusters and this could be used as a barcode in identification and phylogenetic studies of *Venturia* species.

4.2.2.3 Elongation factor gene phylogeny

The genealogy based on EF1α gene region of *Venturia* species isolates collected from different hosts was best described by unrooted neighbour joining phylogenetic tree. The cladogram displayed five well supported major clades and the relationships among the isolates of same species collected from different hosts were topologically well defined (Fig. 3). Clade I encompassed all isolates of *V. carpophila* from almond and peach and has shown a little separation with respect to their host origin. Clade II was a monophyletic group of *V. crataegi* isolates collected from crataegus host with a well-supported bootstrap value (BS=100). Clade III accommodated all the sequences of *V. populina* isolates both custom sequenced and the available at NCBI GenBank. Clade IV was formed by the *V. pyrina* isolates as monophyletic group with high support of bootstrap value (BS=88). Clade V contained all the isolates of *V. inaequalis* collected from apple and cotoneaster and formed well-supported two sub-clades. The first sub-clade clustered all the isolates which originated from cotoneaster while second the sub-clade clustered all the isolates which originated from apple, there by leading to dichotomy of *V. inaequalis* with respect to their origin host.

Overall, single gene phylogenetic analysis did support the monophyly of these *Venturia* species but the phylogenetic differences of *Venturia* species from different host were not supported by rDNA-ITS phylogeny. However, β-tubulin and EF1α based phylogeny lead to separation of *Venturia* species with respect to their host but clear discrimination was not observed. The tree discrepancy obtained from different phylogenetic markers was due to different evolutionary

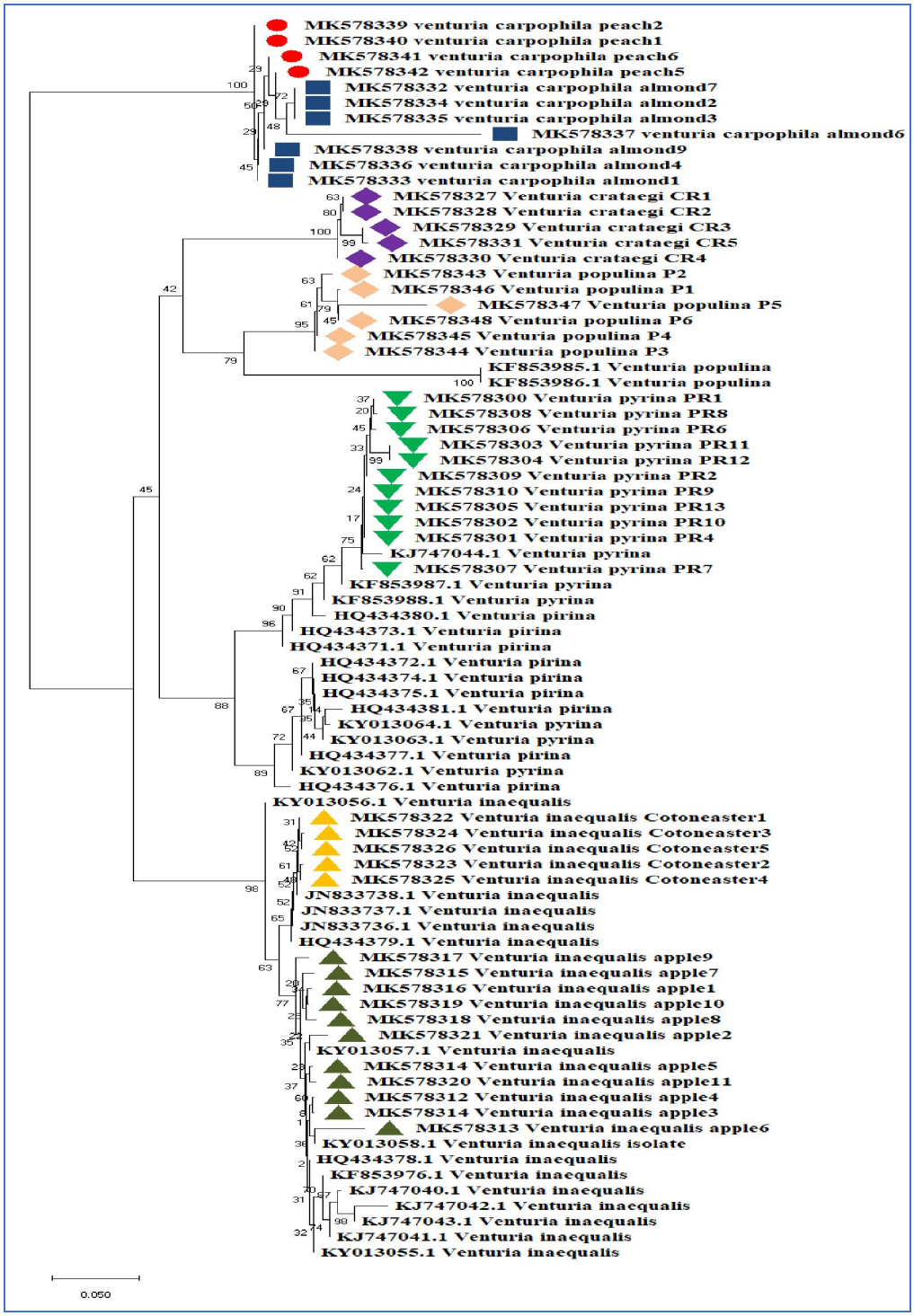


Fig. 3: **Phylogenetic relationship among *Venturia* species based on elongation factor (EF1α) gene**

histories of gene lineages. Because of the accumulation of mutations in the tested loci and statistical approaches, different tree topologies may be produced. In comparison with previous phylogenetic research, in the current study it was highlighted that studies on genetic relationships might decrease the incongruence of results and avoid the influence of topology discrepancy using more diverse isolates (Lievens *et al*., 2009). Although multiple gene sequences evolve with different substitution patterns, concatenating all gene sequences yields a super-gene alignment with more accurate tree (Gadagkar *et al*., 2005). In the present study, we conducted a multigene phylogenetic analysis using not only rDNA-ITS regions but also β-tubulin and EF-1α gene sequence data. Multiple gene phylogenies based on concatenating sequence data set of three genes of different isolates resulted in a tree topology with a high reliability and relationship in *Venturia* species. Our results provided the first comprehensive phylogenetic study of *Venturia* species collected from various host, cultivars and locations.

4.2.2.4 Multiple-gene phylogeny using concatenated data set based on rDNA-ITS, β-tubulin and EF1α gene sequences

A phylogenetic tree was constructed from the combined rDNA-ITS, β-tubulin and EF1α gene sequence data using maximum-parsimony method as implemented in MEGA programme. The topologies of trees representing all the three gene regions were best described by a single unrooted most-parsimonious tree with well supported bootstrap values (Fig. 4). The combined alignment leads the grouping of these *Venturia* species into 9 clades, in which clade I consisted of all the isolates of *V. crataegi* collected from crataegus host while clade II grouped all the *V. populina* isolates collected from poplar. Clade III comprised of all *V. pyrina* isolates from pear host. In case of clade IV, bootstrap values supported for dichotomy of *V. inaequalis* collected from cotoneaster and apple, thereby clearly separating *V. inaequalis* on the basis of host origin. The sub-clade of clade IV comprises of isolates originating from cotoneaster, while the sub-clade second grouped together the isolates from apple. Clade V to IX were comprised of scattered and dispersed isolates of *V. carpophila* having different host origins such

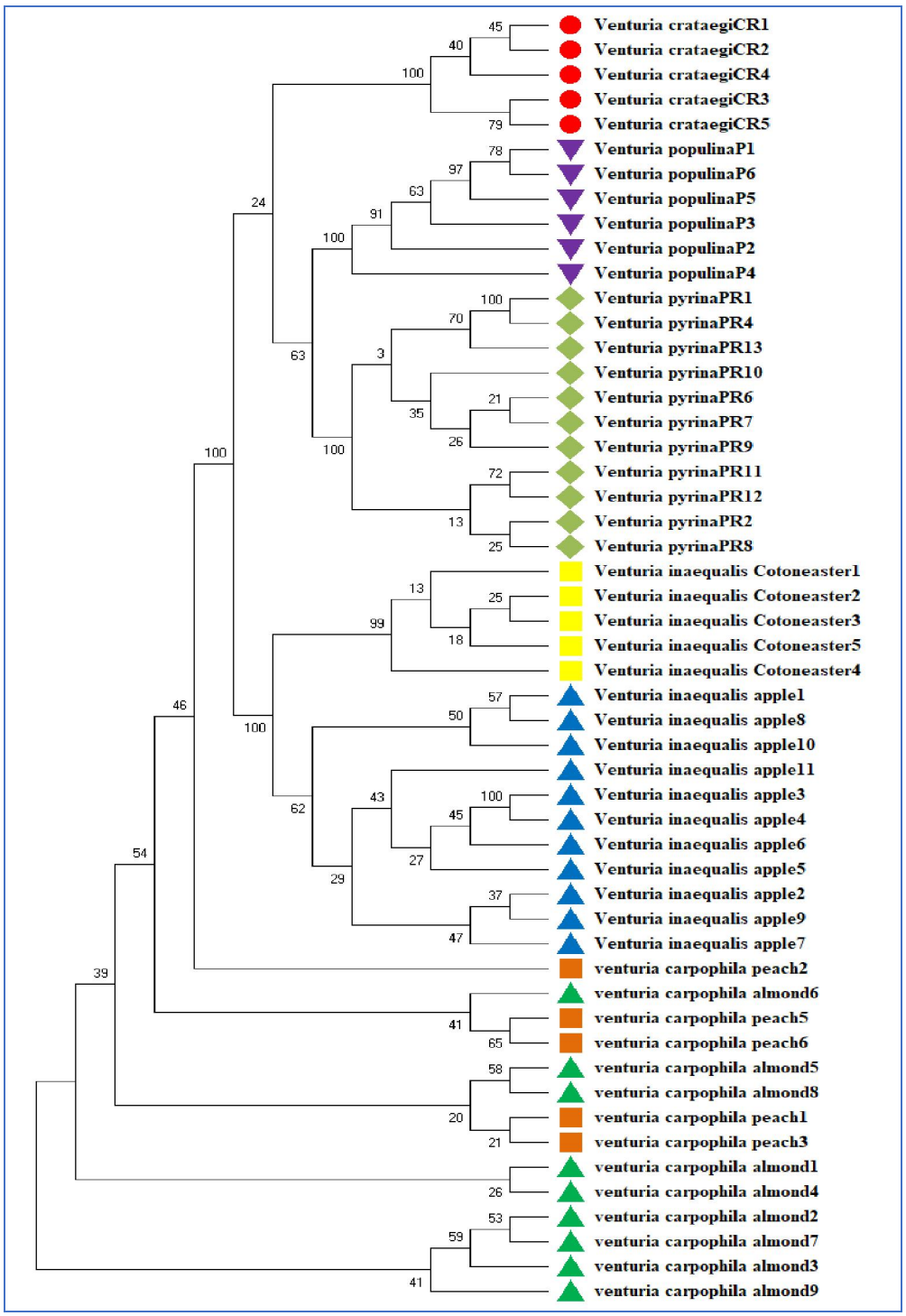


Fig. 4: Phylogenetic relationship among *Venturia* species based concatenated data set

as peach and almond with weakly supported bootstrap values for different lineages.

4.3 DNA polymorphism, Neutral evolution tests and recombination survey of *Venturia* spp. based on their host origin

Estimates of nucleotide variation for each locus and the concatenated data set are summarised in Table 7 and 8. The number of samples at each locus ranged between 3-18, while for concatenated data set the sample size ranged from 5 to 11. The sequence size utilized for the analysis of each locus ranged from 193 to 553 bp. For ITS locus, the maximum size of sequence was found in the isolates from poplar (*C. humile*) with 553 bp, while the lowest sequence size was found in the isolates from pear with 193bp. For β-tubulin, the highest sequence size was found in apple and cotoneaster isolates with 439 bp, while the lowest was observed in crataegus isolates with sequence size of 438 bp. Similarly, EF1α gene exhibited highest sequence size with 454 bp in the isolates from pear and crataegus. The lowest sequence size with 328 bp was observed in the isolates from almond and peach. For concatenated data set (Table 8) the highest sequences size with 1256 bp was found in the isolates from apple, while the lowest sequence size with 590bp was recorded for the isolates from peach. On an overall basis the sequences were therefore polymorphic in nature.

The number of haplotypes for each group of isolates collected from different hosts ranged from 2 to 11(Table 7). For locus ITS, the highest number of haplotypes (10) were found in the isolates from apple, while the lowest number of haplotypes (3) were found in the isolates (*V. populina* and *C. humile*) from poplar. Similarly, at β-tubulin locus, the highest number of haplotypes (8) were found in the isolates from almond and the lowest number of haplotypes (3) were found in the isolates from cotoneaster, pear, crataegus and poplar. Likewise at EF1α locus, the highest number of haplotypes (8) were found in the isolates from apple and the lowest number of haplotypes (2) were recorded in the isolates in peach. Based on concatenated data set (Table 8), the highest number of haplotypes (8) were

associated with apple isolates, and the lowest number of haplotypes (5) were found in peach and crataegus isolates, thereby indicating that the pathogen recombines sexually more often which leads to less shared haplotypes. The haplotype diversity for each group of isolates collected from different hosts ranged from 0.60-1.00 (Table 7). The highest haplotype diversities (1.00) were found in crataegus and poplar (*C. humile*) isolates at ITS locus, in crataegus isolates at β-tubulin locus, and in apple and cotoneaster isolates at EF1α locus. The lowest haplotype diversities (0.73 and 0.60) were found in poplar (*V. populina*) isolates at ITS and β-tubulin loci, respectively. Similarly, the lowest haplotype diversity (0.66) was found in peach isolates at EF1α locus. For concatenated data, set haplotype diversity ranged from 0.97 to 1.00 (Table 8). Over all, haplotype diversity indicates that *Venturia* species has maintained high level of genetic diversity. Estimates of intra-isolate nucleotide diversities in terms of nucleotide differences in sequences (π) and watterson's (θ) varied from 0.001 to 0.087 (π) and 0.001 to 0.108 (θ), respectively (Table 7). In case of locus ITS, higher estimates of intra-isolate nucleotide diversity (π and θ) were found in apple isolates with a value of 0.087 and 0.108, and lower in poplar (*V. populina*) isolates having values of 0.013 and 0.014, respectively. Similarly, in case of locus β-tubulin, higher estimates of intra-isolate nucleotide diversity (π and θ) were found in poplar (*V. populina*) isolates with values of 0.015 and 0.020, respectively, while the lowest intra-isolate nucleotide diversity was associated with pear isolates with values of 0.003 and 0.002, respectively. Likewise, in case of EF1α locus, higher estimates of intra-isolate nucleotide diversity (π and θ) were found in almond isolates (0.030 and 0.037, respectively) and lower estimates were found in case of peach isolates with a value of 0.001. For concatenated data set (Table 8), intra-isolate nucleotide diversity (π and θ) was the lowest (0.008 and 0.007) for cotoneaster isolates and the highest (0.108 and 0.138) for poplar isolates.

Tests of neutral evolution, Tajima's D test and Ramos-Onsins and Rozas' R_2 test were performed in each group of isolates collected from different host

plants. Tajima's D test for each group of isolates showed evidence of nonneutral evolution at all loci which ranged from 1.83 to -1.829 (Table 7). Isolates collected from apple, almond and poplar (*V. populina*) showed significant negative value for Tajima's D test at all tested loci which ranges from -0.265 to -1.829. In case of isolates collected from pear and crataegus, at least one locus i.e., ITS showed negative values for Tajima's D test (-0.130 to -1.185), while two other loci i.e., β-tubulin and EF1α, shows positive value (0.820 to 1.83) for Tajima's D test. Similarly, isolates collected from cotoneaster and peach showed positive value for Tajima's D test (0.699 to 1.632). In case of concatenated data set (Table 8), Tajima's D test showed negative value (-0.328 to -1.490) for the isolates collected from apple, pear, almond and poplar, while positive values (0.028 to 0.988) were obtained for isolates collected from cotoneaster, peach and crataegus. Overall, the results show consistency in negative values of Tajima's D test across loci.

Test of neutral evolution based on R_2 statistics across all test loci has shown a deviation from neutral expectation which ranged from 0.020 to 0.350 (Table 7). In case of loci ITS and β-tubulin, R_2 statistics has the highest values (0.289 and 0.350) for the isolates of poplar (*V. populina*), respectively, and the lowest values (0.020) for the isolates of cotoneaster at ITS locus and (0.204) for almond isolates at β-tubulin locus. Similarly, in case of locus EF1α, R_2 statistics has the highest value (0.333) for the isolates of peach and the lowest value (0.141) for apple isolates. In case of concatenated data set (Table 8), the R_2 statistics has also shown deviation from neutral expectation and has the highest value of R_2 statistics (0.332) for the isolates collected from poplar and lowest value (0.112) for the isolates collected from cotoneaster.

The recombination events based on each locus ranged from 0 to 11 (Table 7). Recombination events were rare at locus ITS for the isolates collected from almond and poplar (*C. humile* and *V. populina*). Similarly, at locus β-tubulin, recombination events were rare for the isolates collected from cotoneaster, peach, pear, crataegus and poplar (*V. populina*), while at locus EF1α, recombination

Table 7: Polymorphism survey and tests for neutral evolutions form individually on three loci for populations of *Venturia* species collected from seven host

Group	locus	n	pb	S^c	h	Hd	π	θ	D	R_2	R_m
Apple	ITS	11	392	124	10	0.98	0.087	0.108	-0.911	0.092	11
	b-tub	11	439	6	5	0.85	0.004	0.004	-0.515	0.210	1
	Elf1α	11	428	29	11	1.00	0.019	0.023	-0.667	0.141	7
Cotoneaster	ITS	5	237	11	4	0.90	0.024	0.022	0.708	0.020	1
	b-tub	5	439	4	3	0.70	0.005	0.004	0.957	0.246	0
	Elf1α	5	423	3	5	1.00	0.004	0.003	1.572	0.300	1
Almond	ITS	9	501	62	6	0.83	0.029	0.045	-1.829	0.209	0
	b-tub	9	440	8	8	0.97	0.008	0.006	-1.029	0.204	3
	Elf1α	7	382	35	4	0.81	0.030	0.037	-1.070	0.321	0
Peach	ITS	6	237	22	5	0.93	0.049	0.040	1.322	0.211	3
	b-tub	5	440	3	4	0.90	0.003	0.003	0.699	0.240	0
	Elf1α	4	382	1	2	0.66	0.001	0.001	1.632	0.333	0
Pear	ITS	18	193	19	9	0.79	0.019	0.028	-1.185	0.135	4
	b-tub	10	441	3	3	0.71	0.003	0.002	1.83	0.266	0
	Elf1α	11	454	8	7	0.83	0.007	0.006	0.820	0.196	2
Crataegus	ITS	5	237	14	5	1.00	0.027	0.028	-0.130	0.221	1
	b-tub	3	438	4	3	1.00	0.006	0.006	Nd	0.311	0
	Elf1α	5	454	8	4	0.90	0.010	0.008	1.394	0.234	0
Poplar (*V. Populina*)	ITS	6	235	8	3	0.73	0.013	0.014	-0.510	0.289	0
	b-tub	6	442	20	3	0.60	0.015	0.020	-1.492	0.350	0
	Elf1α	6	435	24	5	0.93	0.023	0.024	-0.265	0.271	1
Poplar (C. Humile)	ITS	3	553	13	3	1.00	0.016	0.015	Nd	0.044	0

Sample size (n). Total number of sites in alignments(pb), Number of segregating sites(s).Number of haplotypes(h), Haplotypic diversity(Hd), Average number of nucleotide differences per site(π), Watterson's estimate (ϑ), Tajima's (D), Ramos-Onsins and Rozas' (R_2), Minimum number of recombination events (Rm), Not determined (Nd)

Table 8: **Polymorphism survey and tests for neutral evolutions form the concatenated locus for populations of *Venturia* species collected from seven hosts**

Host	n	pb	S	h	Hd	π	θ	D	R_2	R_m
Apple	11	1256	159	11	1.00	0.035	0.043	-0.862	0.140	21
Cotoneaster	5	1100	18	5	1.00	0.008	0.007	0.988	0.112	4
Almond	9	741	70	8	0.97	0.019	0.027	-1.490	0.186	3
Peach	5	5	25	5	1.00	0.018	0.017	0.028	0.117	1
Pear	11	991	102	10	0.98	0.032	0.035	-0.328	0.137	7
Crataegus	5	691	22	5	1.00	0.016	0.015	0.449	0.211	2
Poplar	6	1091	346	6	1.00	0.108	0.138	-1.442	0.332	1

Sample size (n). Total number of sites in alignments(pb), Number of segregating sites(s).Number of haplotypes(h), Haplotypic diversity(Hd), Average number of nucleotide differences per site(π), Watterson's estimate of the population scaled mutation rate, expressed per site (θ), Tajima's (D), Ramos-Onsins and Rozas' (R_2), Minimum number of recombination events (Rm)

events were rare for the isolates collected from almond, peach and crataegus. However, at locus ITS, maximum number of recombination events (Rm=11) were estimated for the isolates collected from apple, while minimum number of recombination events (Rm=1) were estimated for isolates collected from cotoneaster and crataegus. Similarly, at β-tubulin, locus the maximum (Rm =3) and the minimum number (Rm=1) of recombination events were estimated for the isolates collected from almond and apple, respectively. While in case of EF1α locus, the maximum (Rm=7) and the minimum (Rm=3) number of recombination events were estimated for the isolates collected from apple and cotoneaster, respectively. For concatenated data set (Table 8), the maximum number of recombination events (Rm =21) were estimated for the isolates collected from apple and the minimum (Rm=1) such number were estimated for the isolates collected from peach and poplar, respectively. The number of segregation sites measured for the isolates collected from different hosts based on each locus ranged from 1 to 124 (Table 7). At locus ITS, the maximum (S=124) and the minimum number (S=8) of segregating sites were measured for the isolates collected from apple and poplar (*V. populina*), respectively. While in case locus β-tubulin, the maximum number of segregating sites (S=20) was shown by the isolates collected from poplar (*V. populina*) and the minimum (S=3) such number were measured in case of isolates collected from peach and pear. Similarly, at Elf-1α locus, the maximum (S=35) and the minimum (S=1) number of segregating sites were measured for the isolates collected from almond and peach. Likewise, for concatenated data set (Table 8), segregating sites were also studied and the maximum (S=346`) and the minimum (S=18) number of segregating sites were measured for the isolates of poplar and cotoneaster, respectively.

4.3.2 Divergence, differentiation and gene flow in *Venturia* spp. based on their host origin

4.3.2.1 Divergence

In order to know the emergence/origin of *Venturia* spp. from different lineages, nucleotide divergence was studied over the concatenated data set of three loci *viz*., rDNA-ITS, β-tubulin, EF1α (Table 9) which was found to range from 0.011to 0.165. The lowest values of nucleotide divergence (0.011 and 0.032) were found between the isolates originated from almond and peach, followed by those of apple and cotoneaster, respectively. The highest values of nucleotide divergence (0.165 and 0.163) were found between the isolates originated from peach and poplar, followed by those originated from almond and poplar, respectively. The overall results suggest that the recently diverged species have lowest values of divergence, the higher values being accounted for ancient divergence however; the diversification is still weak.

Since genetic data provides a meaning for identifying the reproductive isolation, to define the biological species in a cryptically outcrossing or clonal species, fixed difference and shared polymorphism were studied, which were found to range from 0 to 86 and 0 to12, respectively, between the isolates of *Venturia* spp. collected from different hosts (Table 9). The highest number of fixed differences (86 and 84) were found between the isolates originated from apple and poplar, followed by those originated from cotoneaster and pear hosts, respectively. While minimum number of fixed differences (13) were found between the isolates originated from apple and cotoneaster hosts. However, no such fixed differences were found between the isolates originated from almond and peach hosts. Similarly, the highest shared polymorphism (12) was found between isolates originated from cotoneaster and peach, while minimum number of shared polymorphism (1) were found between isolates originated from apple and pear, followed by isolates from pear and crataegus. However, no such shared polymorphism was found between those originated from pear and poplar, followed by isolates originated from almond and crataegus. Therefore, it is clear from the results that there are different levels of fixation of polymorphism and shared polymorphism among the isolates collected from different hosts that

Table 9: Divergence and differentiation and gene flow between *Venturia* species collected from different host based on concatenated data set of ITS, EF1α, β-tubulin genes

Hosts	Dxy	Sf	Ss	Kst	Snn	Fst	Nm
Apple/Cotoneaster	0.032	13	3(0.016)	0.284	1.00	0.521	0.23
Apple/Pear	0.135	79	1(0.106)	0.652	1.00	0.782	0.07
Apple/Almond	0.095	49	9(0.068)	0.554	1.00	0.716	0.10
Apple/Peach	0.097	43	2(0.076)	0.583	1.00	0.783	0.07
Apple/Crataegus	0.146	65	4(0.116)	0.607	1.00	0.795	0.06
Apple/Poplar	0.158	86	8(0.088)	0.430	1.00	0.557	0.20
Cotoneaster/Pear	0.150	84	2(0.134)	0.754	1.00	0.891	0.03
Cotoneaster/Almond	0.077	45	3(0.070)	0.823	1.00	0.904	0.03
Cotoneaster/Peach	0.079	41	12(0.065)	0.709	1.00	0.814	0.06
Cotoneaster/Crataegus	0.134	75	2(0.120)	0.821	1.00	0.892	0.03
Cotoneaster/Poplar	0.146	88	2(0.087)	0.436	1.00	0.599	0.17
Pear/Almond	0.125	41	11(0.091)	0.585	1.00	0.733	0.09
Pear/Peach	0.158	22	3(0.113)	0.505	1.00	0.714	0.01
Pear/Crataegus	0.146	83	1(0.127)	0.742	1.00	0.871	0.04
Pear/Poplar	0.132	71	0(0.112)	0.727	1.00	0.846	0.05
Almond/Peach	0.011	0	3(0.001)	0.074	0.750^{ns}	0.134	1.61
Almond/Crataegus	0.094	15	0(0.075)	0.697	1.00	0.797	0.06
Almond/Poplar	0.163	49	2(0.075)	0.353	1.00	0.464	0.29
Peach/Crataegus	0.114	14	2(0.076)	0.530	1.00	0.669	0.12
Peach/Poplar	0.165	46	2(0.074)	0.291	1.00	0.447	0.31
Crataegus/Poplar	0.117	64	4(0.100)	0.761	1.00	0.856	0.04

Divergence was measured between groups (Dxy), Number of fixed differences between groups.(Sf), The number of shared polymorphisms (Ss), A weighted measure of the ratio of the average pairwise differences within groups to the total average pairwise differences (Hudson *et al.*, 1992)(Kst), The proportion of nearest neighbours in sequence space that are found in the same group (Hudson, 2000)(Snn), Wright's pair wise genetic differentiation (Fst), Significance levels for Kst, Snn and Fst were assessed using permutation tests with 1000 permutations and were significant at $P < 0.05$, Gene flow(Nm), ``Non-significant (ns)

resulted in long history of reproductive isolation and following secondary contact between *Venturia* species.

4.3.2.2 Differentiation and gene flow

Four different statistical parameters were used to know as to how much differentiation existed between the isolates of *Venturia* species collected from different hosts. A pair-wise fixation index (F_{ST}) and gene flow (Nm) estimated (Table 10) was found to range from 0.134 to 0.904 and 0.01 to 1.61, respectively. The highest pair-wise fixation index (F_{ST} = 0.904) and minimum gene flow (Nm = 0.01) were found between the isolates collected from cotoneaster and almond, and those from pear and peach hosts, respectively. The lowest pair wise fixation index (F_{ST} = 0.134) and the highest gene flow (Nm = 1.61) were found between isolates collected from almond and peach hosts. Similarly, in case of isolates collected from apple and cotoneaster, moderate pair wise fixation index (F_{ST} = 0.521) and gene flow (Nm = 0.23) were observed. The highest value of F_{ST} corresponds to significant genetic differentiation the between isolates collect from different hosts, while the lowest value of F_{ST} indicate low genetic differentiation, meaning thereby that the population are homogenous and the corresponding gene flow indicate migration of individuals from one population to another population. Kst and Snn are other two measures of differentiation, in which the lowest value of Kst (0.074) was observed between the isolates collected from almond and peach hosts and the highest (0.823) Kst was found between those collected from cotoneaster and almond hosts. Similarly, isolates collected from apple and cotoneaster exhibited Kst value of 0.284. These results indicate that genetic differentiation exists in terms of reproductive barriers between these groups of isolates which ranges from low to high. Similarly, all groups of isolates collected from different hosts showed highest value for Snn (1.00) except for the group of isolates collected from almond and peach which showed lowest non-significant value for Snn (0.75). The results thereby suggest that high value of Snn corresponds to populations which are significantly differentiated and the lowest

values indicate that the two populations are part of same random matting or panmictic population.

4.3.3 Variability between *Venturia* species clustered at species level

Clustering of *Venturia* spp. isolates on species level, collected from different hosts grouped them into six populations *viz., V. carpophila, V. crataegi, V. inaequalis, V. pyrina, V. populina and C. humile*. Polymorphism survey of these *Venturia* spp. based on ITS sequence data set revealed that the number of sequences were 15, 5, 16, 18, 6, 3, and number of haplotypes were 9, 5, 10, 9, 3, and 3 in *V. carpophila, V. crataegi, V. inaequalis, V. pyrina, V. populina, C. humile* populations, respectively (Table 10). Some haplotypes were shared between the populations. Haplotype diversity in populations ranged from 0.73 to 1.0, thereby indicating a high level of genetic diversity between *Venturia* spp. Nucleotide diversity was low and varied from 0.01 to 0.07, owing to conserved regions within ITS sequences. The survey of *Venturia* spp. based on β-tubulin sequence data revealed that the number of sequences were 14, 3, 16, 10 and 6, and the number of haplotypes were 12, 3, 7, 3 and 3, in *V. carpophila, V. crataegi, V. inaequalis, V. pyrina, V. populina* populations, respectively (Table 11). Some haplotypes were shared between populations. Haplotype diversity in populations ranged from 0.60 to 1.0, thereby indicating a high level of genetic diversity in *Venturia* spp. Nucleotide diversity was also less and varied from 0.003 to 0.015, owing to highly conserved regions within β-tubulin sequences. Similarly, polymorphism survey (Table 12) based on EF1α sequence data of *Venturia* spp. revealed that the number of sequences were 11, 5, 16, 11 and 6, and the number of haplotypes were 6, 4, 15, 7 and 5 in *V. carpophila, V. crataegi, V. inaequalis, V. pyrina, V. populina* populations, respectively. Some haplotypes were shared between populations. Haplotype diversity in populations ranged from 0.87 to 0.93, thereby indicating a high level of genetic diversity among these *Venturia* spp. at EF1α locus. The nucleotide diversity was very less varying from 0.007 to 0.02.

Table 10: Polymorphism survey of *Venturia* species based on ITS sequence data

Venturia species	No. of sequence	No. of haplotypes	Haplotype diversity	Nucleotide diversity
V. carpophila	15	9	0.84±0.08	0.02±0.006
V. crataegi	5	5	1.00±0.12	0.02±0.008
V. inaequalis	16	10	0.90±0.09	0.07±0.02
V. pyrina	18	9	0.79±0.09	0.01±0.006
V. populina	6	3	0.73±0.15	0.01±0.005
C. humile	3	3	1±0.27	0.01±0.004

Table 11: Polymorphism survey of *Venturia* species based on β-tubulin sequence data

Venturia species	No. of sequence	No. of haplotypes	Haplotype diversity	Nucleotide diversity
V. carpophila	14	12	0.97±0.03	0.007±0.0007
V. crataegi	3	3	1.00±0.27	0.006±0.002
V. inaequalis	16	7	0.88±0.04	0.005±0.001
V. pyrina	10	3	0.71±0.08	0.003±0.0004
V. populina	6	3	0.60±0.21	0.015±0.009

Table 12: Polymorphism survey of *Venturia* species based on EF1α sequence data

Venturia species	No. of sequence	No. of haplotypes	Haplotype diversity	Nucleotide diversity
V. carpophila	11	6	0.89±0.06	0.02±0.01
V. crataegi	5	4	0.90±0.16	0.01±0.002
V. inaequalis	16	15	0.92±0.02	0.02±0.003
V. pyrina	11	7	0.87±0.08	0.007±0.001
V. populina	6	5	0.93±0.12	0.02±0.008

4.4 Genetic Variation among *Venturia* populations

4.4.1 Analysis of molecular variance (AMOVA)

A hierarchical analysis of molecular variance (AMOVA) was performed based on ITS, β-tubulin, EF1α and concatenated data set to describe the distribution of genetic variation among and within populations defined by populations of *Venturia* species from various hosts. The results are shown in Table 13, 14, 15 and 16. The results revealed that maximum percentage of variation was distributed among populations (77.12, 87.10, 92.63 and 90.68 %) than within populations (22.88, 12.90, 7.37, and 9.32 %) with F_{ST} values ranging from 0.71 to 0.93 based on ITS, β-tubulin, EF1α and concatenated data set respectively. These results strongly indicate the presence of the separate regional populations of *Venturia* species on various hosts.

4.4.2. Pair-wise fixation index

To know how much differentiation existed among the various *Venturia* populations, pair-wise fixation index (F_{ST}) values were estimated (Table 17, 18, 19 and 20). Most of the pair-wise fixation index values based on ITS data set were high (0.20 to 0.97) among the populations of *Venturia* spp. grouped according to species level. Similarly, pair-wise fixation index values based on β-tubulin data set were very high (0.66 to 0.93). The pair-wise fixation index values based on EF1α data set ranged between (0.87 to 0.97) among *Venturia* populations. The pair-wise Fixation index based on concatenated data set between *Venturia* populations was also high (0.79 to 0.96). The high genetic differentiation values reveal a strong genetic differentiation among *Venturia* spp.

Table 13: Analysis of molecular variance (AMOVA) for testing homogeneity based on ITS data set of *Venturia* species

Source of variation	Degrees of freedom	Variance component	Percentage of variation	F_{ST}	P-value
Among population	7	10.39	77.12	0.77	<0.05
Within population	55	3.08	22.88		

Table 14: Analysis of molecular variance (AMOVA) for testing homogeneity based on β-tubulin data set of *Venturia* species

Source of variation	Degrees of freedom	Variance component	Percentage of variation	F_{ST}	P-value
Among population	6	12.15	87.10	0.71	<0.05
Within populations	42	1.80	12.90		

Table 15: Analysis of molecular variance (AMOVA) for testing homogeneity based on EF1α data set of *Venturia* species

Source of variation	Degrees of freedom	Variance component	Percentage of variation	F_{ST}	P-value
Among population	6	21.48	92.63	0.93	<0.05
Within population	42	1.70	7.37		

Table 16: Analysis of molecular variance (AMOVA) for testing homogeneity based on concatenated data set

Source of variation	Degree of freedom	Variance component	Percentage of variation	F_{ST}	P-value
Among population	6	40.87	90.68	0.90	<0.05
Within population	45	4.19	9.32		

Table 17: Pair-wise genetic differentiation between *Venturia* species based on ITS sequence data

***Venturia* species**	***V. carpophila***	***V. crataegi***	***V. inaequalis***	***V. pyrina***	***V. populina***	***C. humile***
V. carpophila	0.00					
V. crataegi	0.65	0.00				
V. inaequalis	0.56	0.20	0.00			
V. pyrina	0.82	0.78	0.53	0.00		
V. populina	0.78	0.73	0.79	0.82	0.00	
C. humile	0.93	0.92	0.79	0.96	0.97	0.00

All are significant P<0.05

Table 18: Pair-wise genetic differentiation between *Venturia* species based on β-tubulin sequence data

***Venturia* species**	***V. carpophila***	***V. crataegi***	***V. inaequalis***	***V. pyrina***	***V. populina***
V. carpophila	0.00				
V. crataegi	0.91	0.00			
V. inaequalis	0.90	0.91	0.00		
V. pyrina	0.91	0.93	0.91	0.00	
V. populina	0.81	0.66	0.81	0.77	0.00

All are significant P<0.05

Table 19: Pair-wise genetic differentiation between *Venturia* species based on EF1α sequence data

***Venturia* species**	***V. carpophila***	***V. crataegi***	***V. inaequalis***	***V. pyrina***	***V. populina***
V. carpophila	0.00				
V. crataegi	0.90	0.00			
V. inaequalis	0.88	0.87	0.00		
V. pyrina	0.94	0.97	0.91	0.00	
V. populina	0.87	0.89	0.87	0.95	0.00

All are significant P<0.05

Table 20: Pair-wise differentiation between *Venturia* species based on concatenated data set

***Venturia* species**	***V. carpophila***	***V. crataegi***	***V. inaequalis***	***V. pyrina***	***V. populina***
V. carpophila	0.00				
V. crataegi	0.94	0.00			
V. inaequalis	0.88	0.79	0.00		
V. pyrina	0.96	0.93	0.87	0.00	
V. populina	0.95	0.90	0.84	0.95	0.00

All are significant P<0.05

4.5 Population genetic structure of *Venturia* species

The principal component analysis of various *Venturia* species (Fig. 5) based on ITS data set revealed that the first principal coordinate accounted for 68.37 per cent of variation and separated *Venturia* species into three main clusters. Isolates from poplar (*C. humile*) and apple were grouped into two different clusters while the isolates from other hosts were grouped into a single cluster irrespective of the host species from which they were collected. The second principal coordinate accounted for 10.84 per cent of the variation and grouped *Venturia* species into two main clusters. The isolates from crataegus were grouped into a separate cluster while other species were grouped into a single cluster irrespective of the host species from which they were collected, and potentially two off shifters, one from pear and another from cotoneaster, were also observed (Fig. 5). STRUCTURE analysis performed on ITS data set of *Venturia* species revealed six clusters for K = 6; (Fig. 7). The data set partitioned into clusters that does not correspond roughly to host origin. A visual inspection of the individuals clustering (Fig. 6) revealed that the haplotypes of *Venturia* species from apple, pear, poplar (*C. humile*) and peach split from other individuals at K=6, suggesting that these are differentiated genetic units and could be classified into separate clusters, while the isolates of crataegus, poplar (*V. populina*) and almond from different hosts origin consisted of an admixed group sharing genetic ancestry with other populations. Increasing K did not reveal any further subdivision among samples (Fig. 7).

Similarly, the principal component analysis based on β-tubulin data set (Fig. 8) revealed that the first principal coordinate accounted for 24.75 per cent of variation and separated the *Venturia* species into four different clusters, in which the isolates from pear, poplar (*V. populina*) and crataegus were grouped into three different clusters, while the isolates from cotoneaster and apple were grouped into a single cluster irrespective of the host origin from which they were collected. The second principal coordinate accounted for 20.84 per cent of the variation and

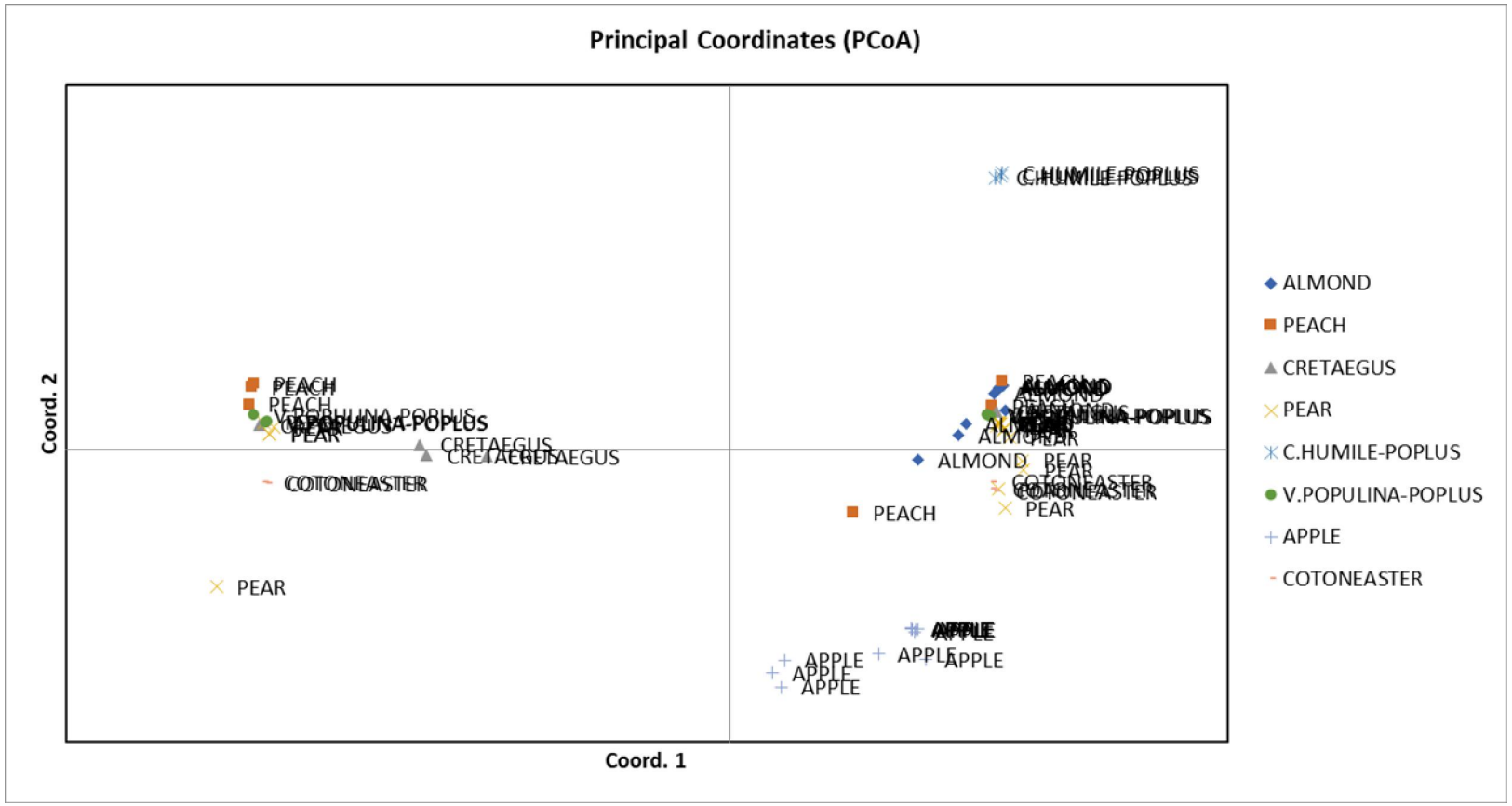


Fig. 5: **Principal coordinate analysis of a matrix of chord distance (Cavalli-Sforza & Edwards 1967) among samples of *Venturia* species based on sequence of ITS data. The first and second principal coordinates account for 68.37 and 10.84 per cent of the variation, respectively**

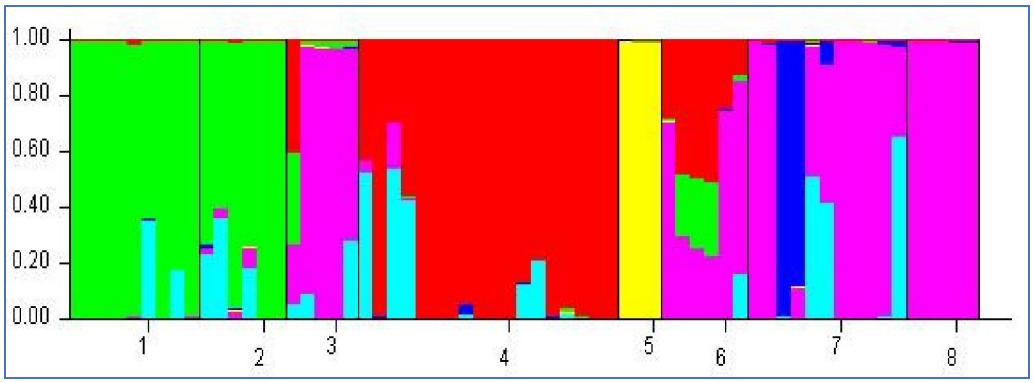


Fig. 6: **Population structure of *Venturia* species inferred using the programme STRUCTURE based on ITS sequence data set**

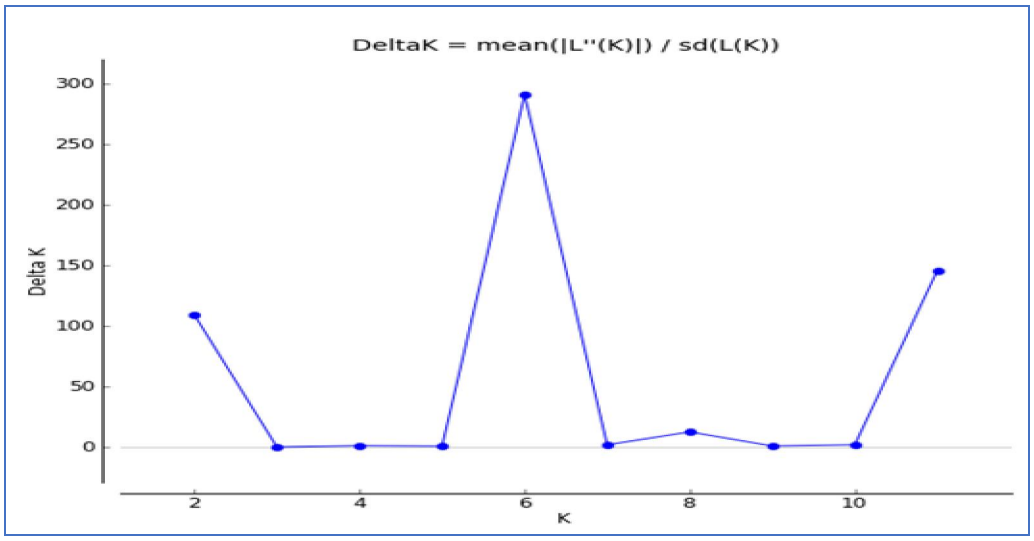


Fig. 7: **Plot of ln likelihood of the data for several value of *K*, the parameters representing the number of populations in Bayesian clustering algorithm in the STRUCTURE program**

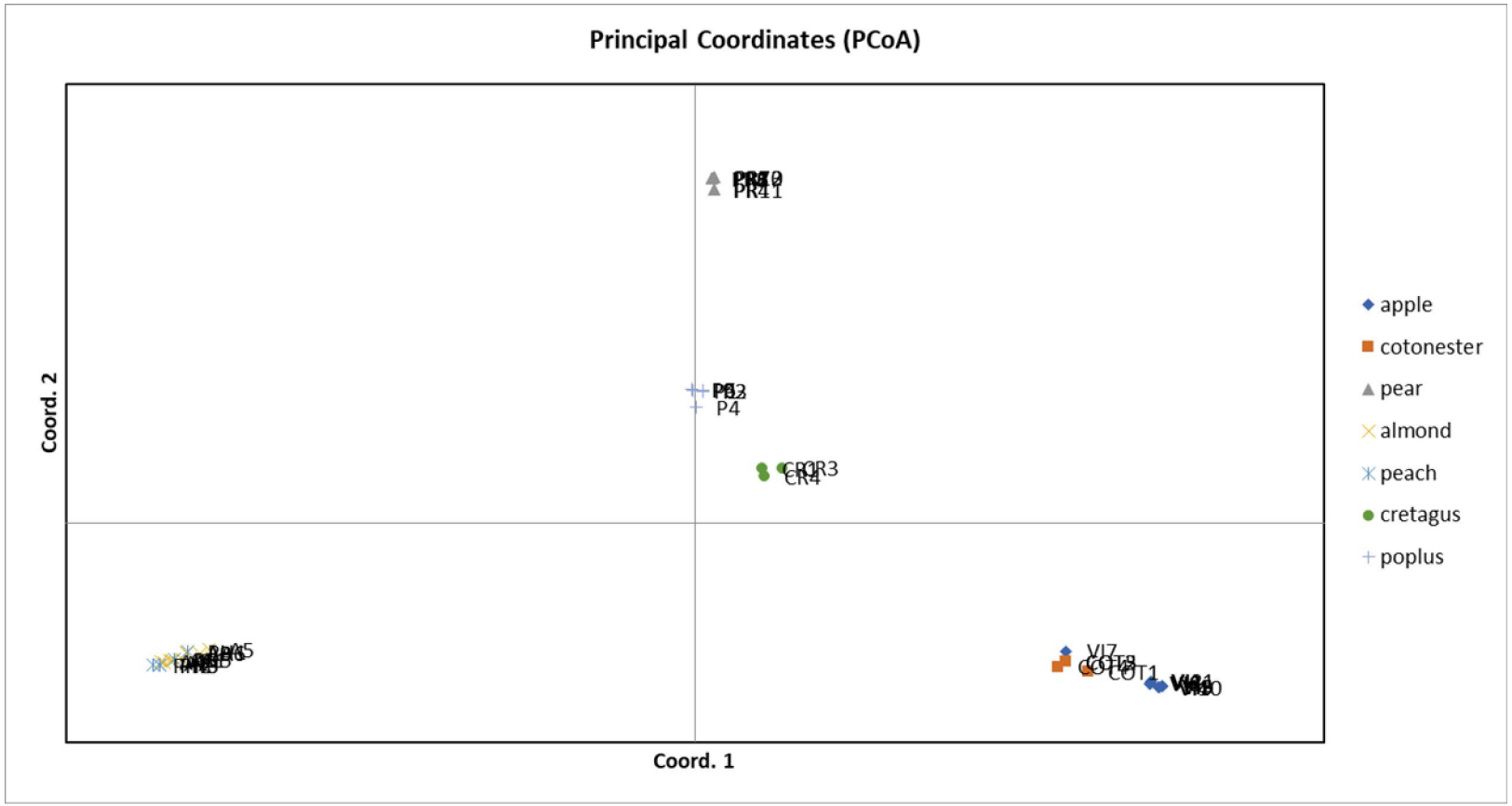


Fig. 8: **Principal coordinate analysis of a matrix of chord distance (Cavalli-Sforza & Edwards 1967) among samples of *Venturia* species based on sequence of β-tubulin. The first and second principal coordinates account for 24.75 and 20.84 per cent of the variation, respectively**

grouped isolates of almond and peach into single main clusters, and potentially no off shifters were observed. The model based on Bayesian clustering algorithms, as implemented in STRUCTURE program, performed on β-tubulin data set grouped Venturia spp. into five clusters (Fig. 10). A visual inspection of the individuals clustering from K value (Fig. 9) revealed that the isolates from apple, pear, almond, crataegus and poplar (*V. populina*) tended to be classified into separate clusters at K=5, thereby suggesting that these are differentiated genetic units. The isolates of cotoneaster and peach having different host origin were assigned into the same clusters of isolates from apple and almond, respectively thereby exhibiting high genetic ancestry and more fractional memberships within these populations. No further subdivision was observed with increasing K among samples (Fig. 10).

The principal component analysis of various *Venturia* species (Fig. 11) based on EF1α data set revealed that the first principal coordinate accounted for 30.56 per cent of variation and separated the *Venturia* species into four clusters, in which the isolates collected from pear and one isolate from almond were grouped two different clusters, while the other isolates collected from almond and peach, crataegus and poplar (*V. populina*) were grouped into two different clusters. The second principal coordinate accounted for 25.04 per cent of variation and grouped all the isolates collected from apple and cotoneaster into a single cluster irrespective of the host origin. STRUCTURE analysis performed on EF1α dataset of various *Venturia* species and the highest-likelihood values of the Δk index was observed for K = 9 (Fig. 13). A visual inspection of the individuals clustering from K value (Fig. 12) revealed that the isolates collected from peach, cotoneaster, crataegus and pear tend to be classified into separate clusters at K=9. Increasing K did not reveal any further subdivision among samples (Fig. 13).

While studying the principal component analysis of various *Venturia* species (Fig. 14) based on concatenated data set, it was evident that the first principal coordinate accounted for 29.93 per cent of variation and separated the

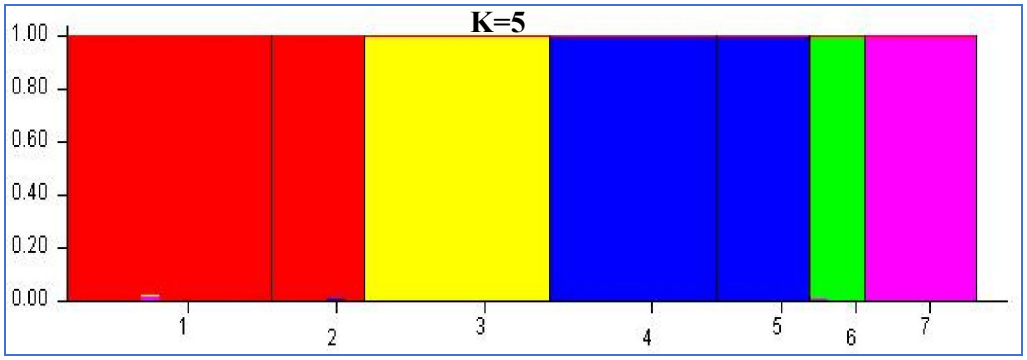


Fig. 9: **Population structure of *Venturia* species inferred using the programme STRUCTURE based on β-tubulin sequence data set**

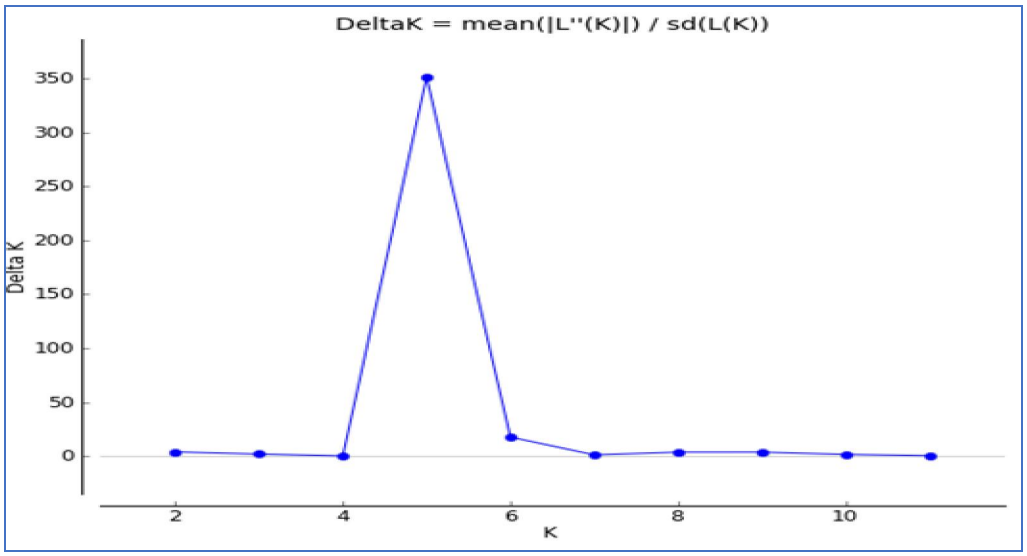


Fig. 10: **Plot of ln likelihood of the data for several value of *K*, the parameters representing the number of populations in Bayesian clustering algorithm in the STRUCTURE program**

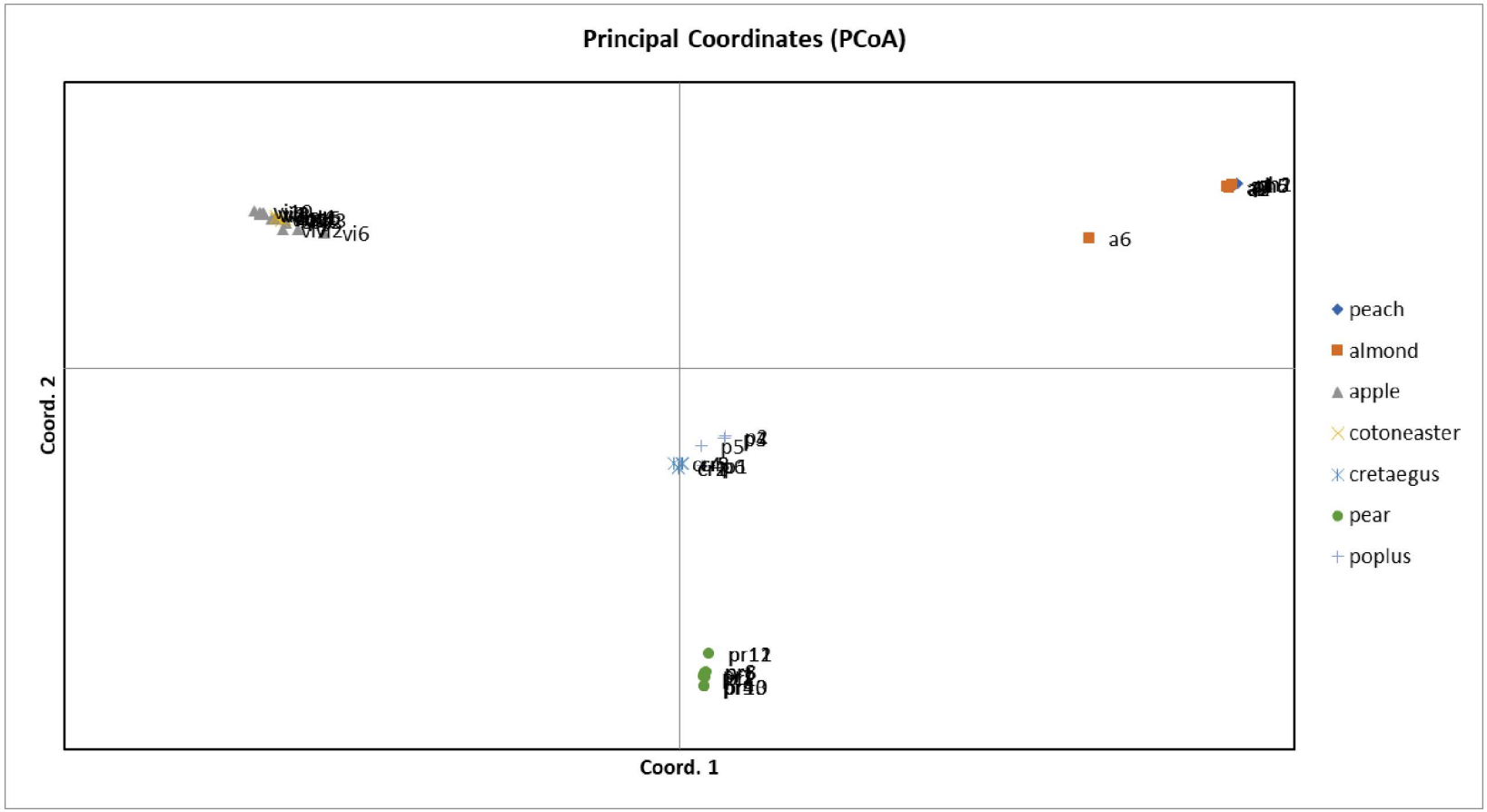


Fig. 11: **Principal coordinate analysis of a matrix of chord distance (Cavalli-Sforza & Edwards 1967) among samples of *Venturia* species based on sequence of EF1α. The first and second principal coordinates account for 30.56 and 25.04 per cent of the variation, respectively**

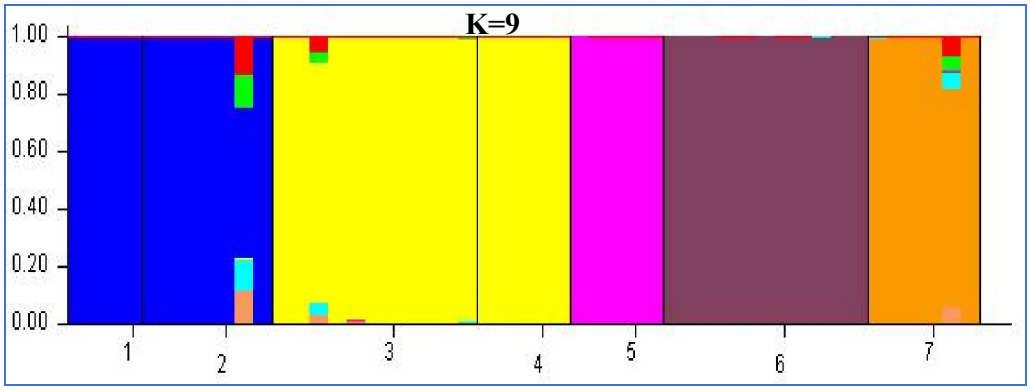


Fig. 12: **Population structure of *Venturia* species inferred using the programme STRUCTURE based on EF1α sequence data set**

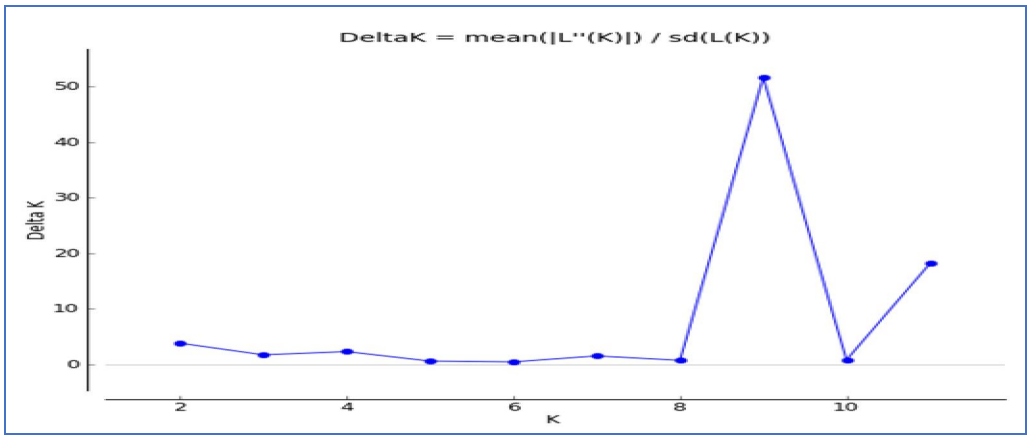


Fig. 13: **Plot of ln likelihood of the data for several value of *K*, the parameters representing the number of populations in Bayesian clustering algorithm in the STRUCTURE program**

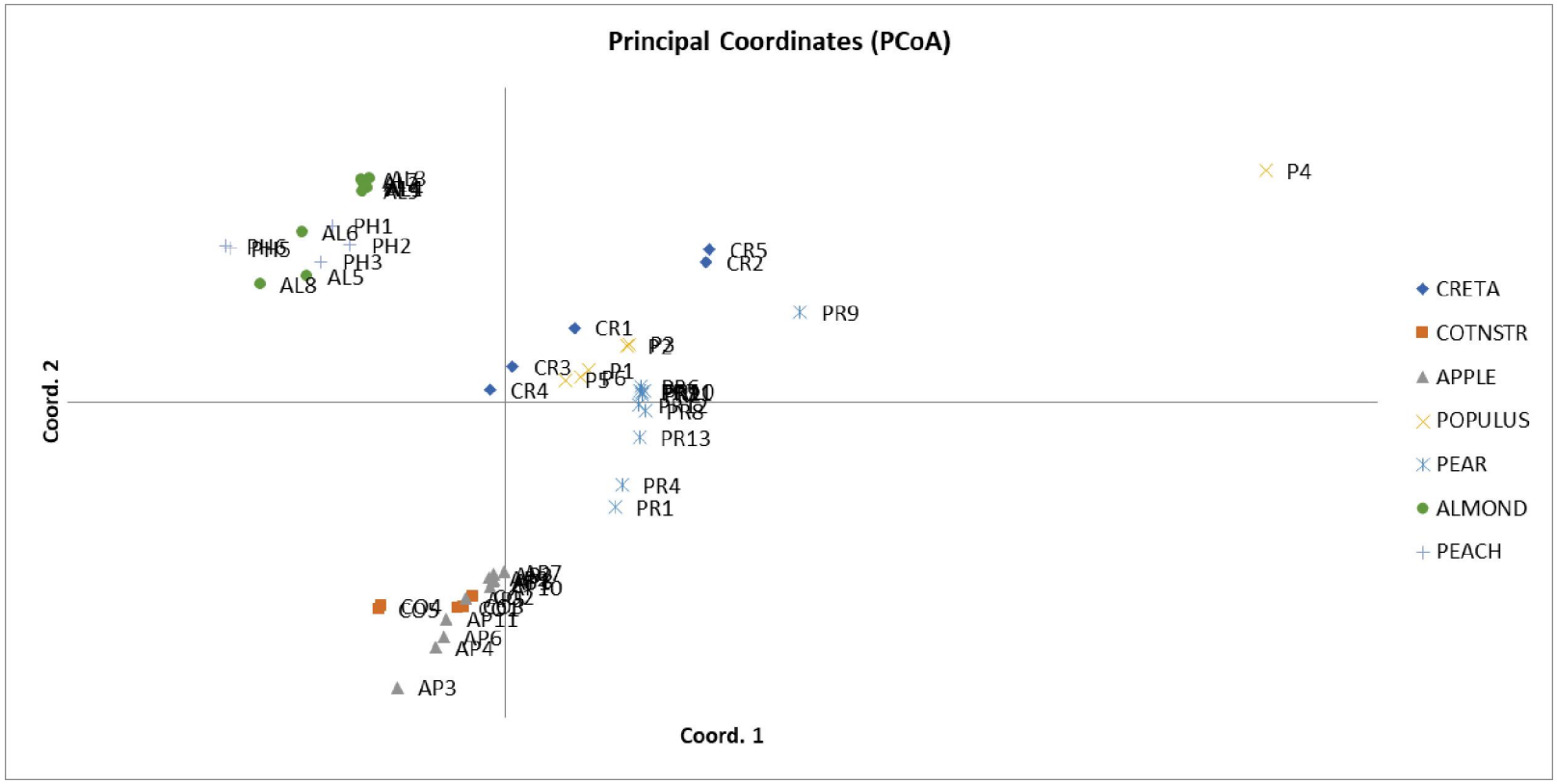


Fig. 14: **Principal coordinate analysis of a matrix of chord distance (Cavalli-Sforza & Edwards 1967) among samples of *Venturia* species based on concatenated data set. The first and second principal coordinates account for 29.93 and 21.37 per cent of the variation, respectively**

three isolates (P4, PR3, CR5) collected from different hosts into separate clusters while, other samples were grouped into a single cluster irrespective of the host species from which they were collected. The second principal coordinate accounted for 21.37 per cent of the variation and clustered all the isolates of *Venturia* species into two main clusters. The isolates collected from almond and peach, apple and cotoneaster were grouped into two separate clusters respectively, irrespective of the host species from which they were collected and potentially off shifters such as one from crataegus and another from apple were observed. STRUCTURE analysis performed on concatenated data set of various *Venturia* species and the highest-likelihood values of the Δk index was observed for K = 5 (Fig. 16). A visual inspection of the individuals clustering on the basis of K value (Fig.15) revealed that the isolates of cotoneaster split from other individuals at K=6, and could be classified into separate clusters. The isolates collected from crataegus, apple, poplar (*V. populina*), pear, almond and peach having different host origin tend to have membership in these distinct clusters, thereby exhibiting fraction of shared genetic ancestry. Further increasing K did not reveal subdivision among samples (Fig. 16).

4.6 Development of species-specific markers and their validation

Primer designing was done based on GC content of three *Venturia* spp. ranging from 38.10 to 47.4 per cent (Table 4). The correlation between GC content and genome size of *Venturia* spp. observed by Pearson's correlation coefficient revealed no significant correlation between them ($r = -0.977$; $p>0.05$). In present investigation, 11, 11 and 12 scaffolds covering 3 to 6.5 Mbp genome of three *Venturia* spp. were used for primer designing. Based on these scaffolds, 37 primers were designed using Primer3 plus software (Table 5). The *in silico* screening of primers showed a single PCR product except a few (Table 5). Out of 12 *V. inaequalis* primers tested on 35 representative isolates of *Venturia* species (Table 21), eight primers (66.6%) showed cross transferability. Among these eight cross-transferrable primers, four primers (Vi9, Vi10, Vi11 and Vi12) amplified *V.*

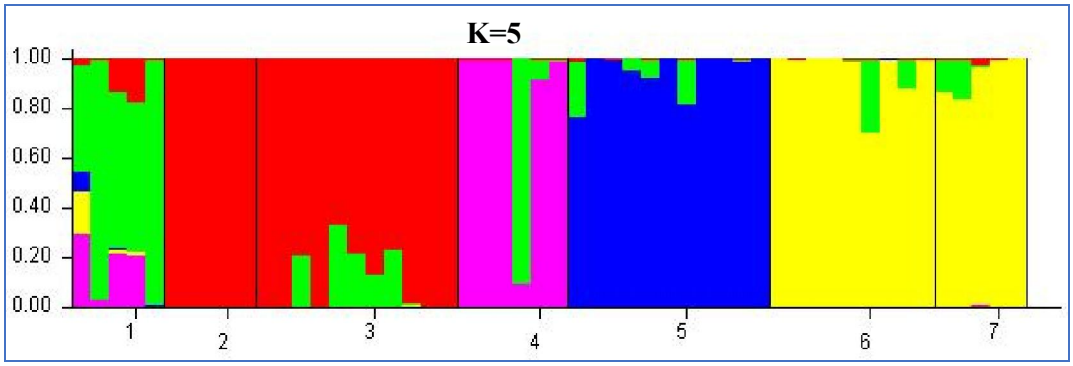


Fig. 15: **Population structure of *Venturia* species inferred using the programme STRUCTURE based on concatenated data set**

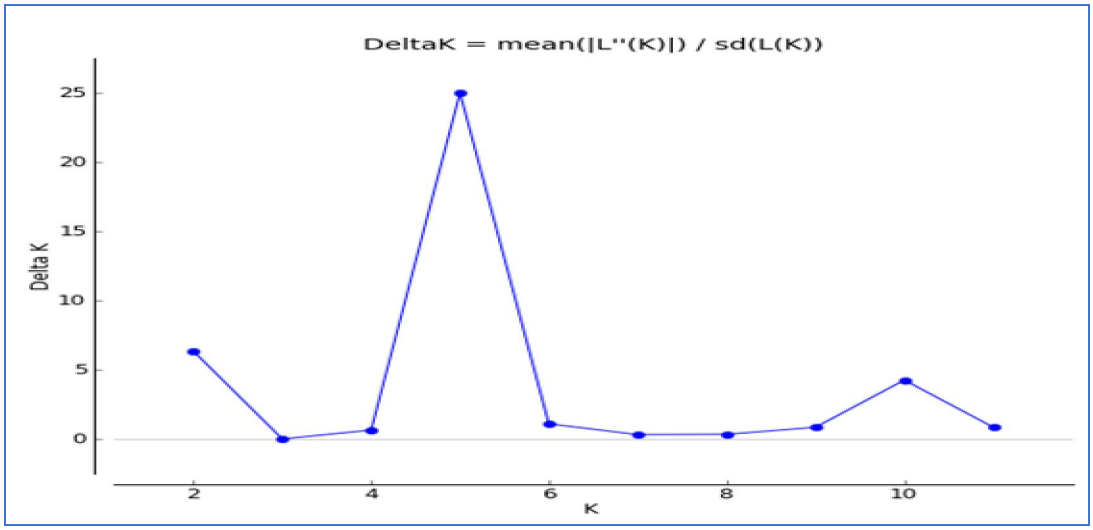


Fig. 16: **Plot of ln likelihood of the data for several value of *K*, the parameters representing the number of populations in Bayesian clustering algorithm in the STRUCTURE program**

inaequalis but also showed cross amplification with *V. pyrina* (Table 21). Similarly, primers Vi6, Vi7, Vi8 amplified *V. carpophila* as well as *V. inaequalis*. One primer Vi3 amplified all the *Venturia* spp. and showed multiple banding patterns (Table 21; Fig. 17d). However, two primers Vi4 and Vi5 could not amplify any *Venturia* spp. (Fig. 18). However, only one primers (Vi1) were found specific to *V. inaequalis*. Primer Vi2 amplified three *V. inaequalis* isolates from apple but failed to amplify *V. inaequalis* isolate from cotoneaster. Therefore only one primer Vi1 could be identified specific to *V. inaequalis* and amplified 250bp product size (Table 21; Fig. 19e-f). This primer successfully led to *in planta* detection of *V. inaequalis* pathogen in the DNA extracted from infected and non-infected leaves (Fig. 20).

Twelve primers specific to *V. pyrina* were designed from 11 scaffolds and among these 83.3 per cent (10) were cross-transferable (Table 21). Primer Pr4 amplified *V. pyrina* at two different regions thus giving two bands of 300 bp and 400 bp. This primer also amplified 5 isolates of *C. humile* with different products (3 isolates with 600 bp and two isolates with 1300 bp) (Table 21; Fig 17a). Primer Pr3 is specific to *V. pyrina* with 200bp product size and did not amplify other *Venturia* species (Fig. 19c-d). However, Pr2 could not amplify any of the species (Fig. 18), whereas, Pr5, Pr6, Pr7, Pr8, Pr9, Pr10, Pr11, Pr12, Pr13 were able to amplify *V. inaequalis* and *V. carpophila* besides *V. pyrina* (Table 21).

Thirteen primers designed from 12 scaffolds of *V. carpophila* were used to investigate their effectiveness and specificity. Ten primers (76.9%) showed cross species amplification (Table 21). Three primers namely C1, C4, C5 were found specific to *V. carpophila* infecting almond. However, primer C1 and C5 amplified only two isolates of *V. carpophila* infecting peach. These results indicated that the C4 primer (300bp product size) was highly specific to *V. carpophila* (Fig. 19a-b). Primers C2 showed undesirable bands in *V. carpophila* and *V. pyrina* in addition to specific bands in *V. carpophila* (Fig. 17b). Similarly, primer C3 produced one specific band 300bp size in *V. pyrina* and 1200bp product size in C. *humile* in

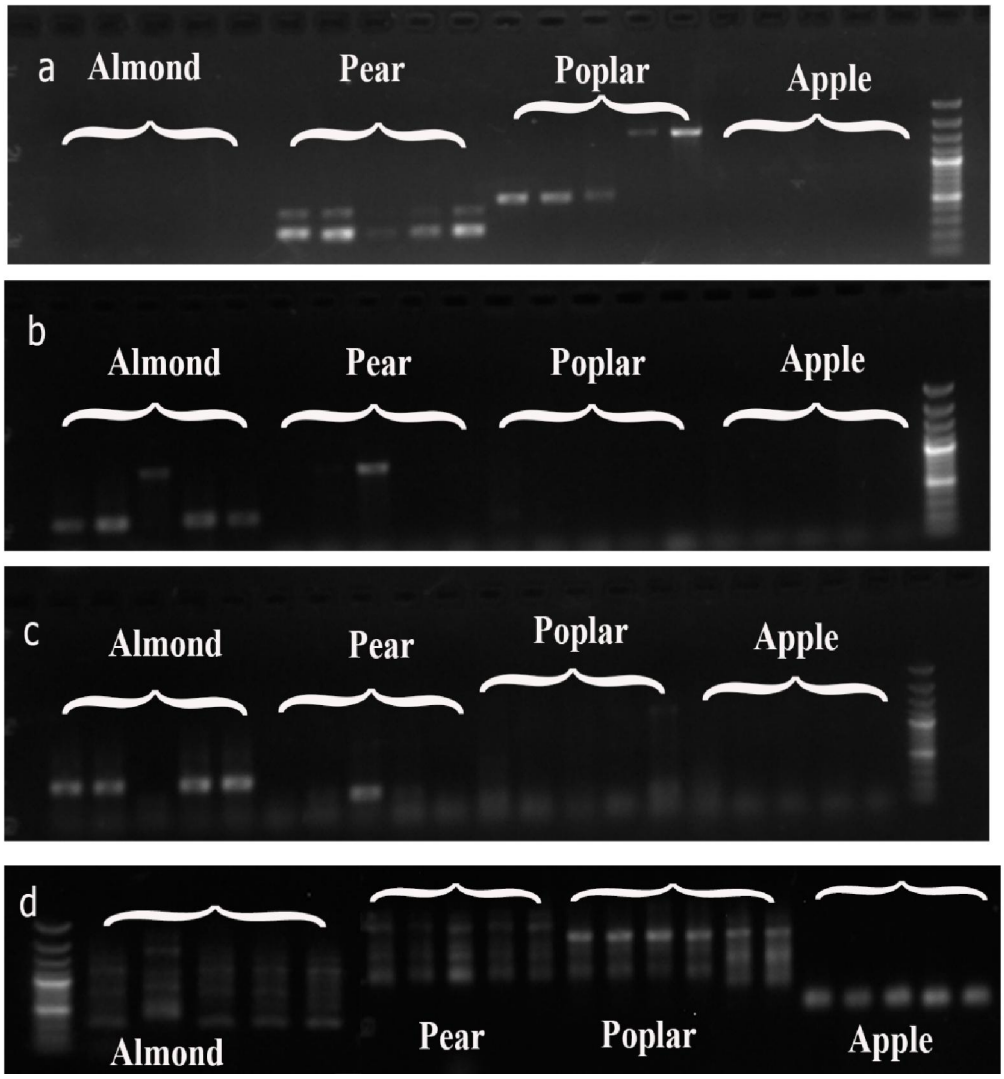


Fig. 17: **Designed primers showing cross amplification between different *Venturia* species; primer [Pr4(a), C2(b), C3(c), Vi3(d)]**

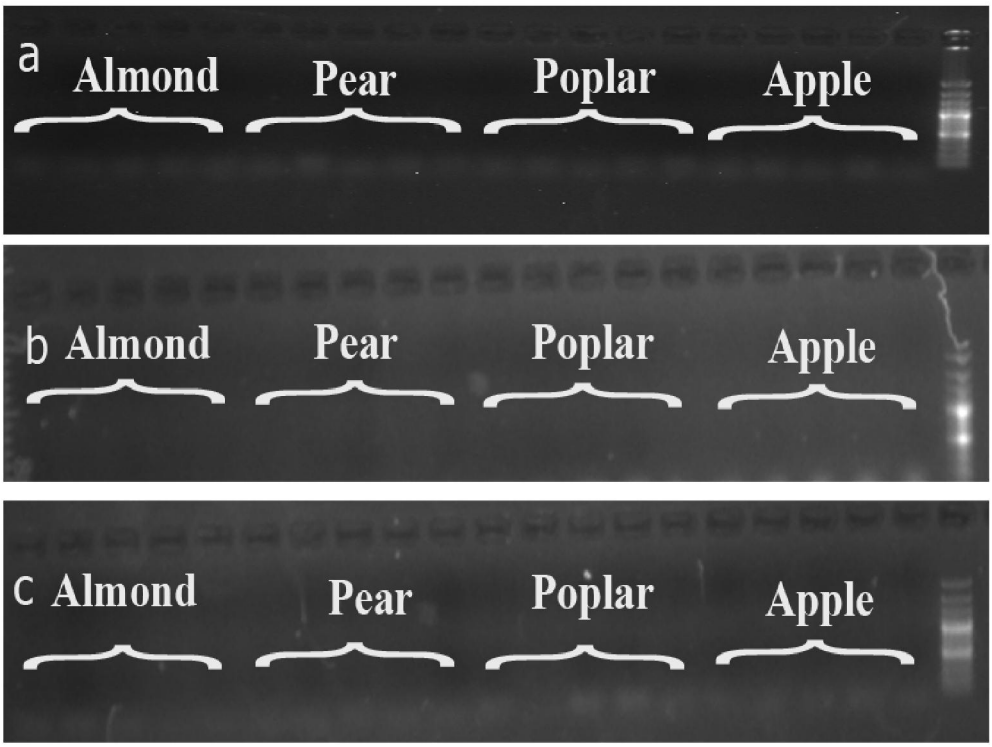


Fig. 18: **Primers that failed to amplify any *Venturia* species; primer [Vi4 (a), Vi5 (b), Pr2 (c)]**

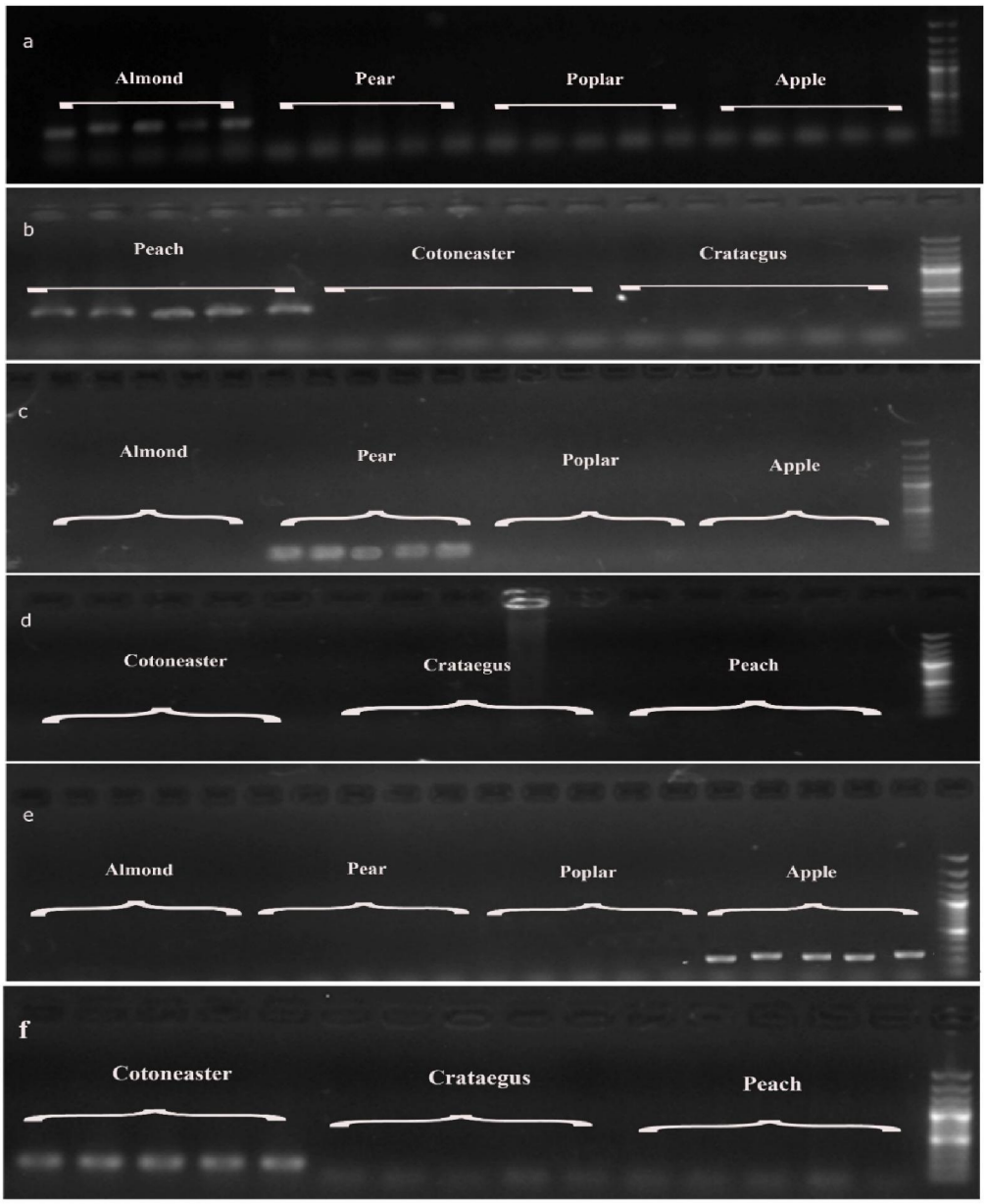


Fig. 19: **PCR based validation of *Venturia* species-specific primer sets [C4 (a &b), Pr3 (c & d) and Vi1 (e & f)]**

Table 21: Cross transferability of *Venturia* species-specific primers

Name of primer	*Venturia* species and host						
	V. inaequalis (apple)	*V. inaequalis* (Cotoneaster)	*V. carpophila* (Almond)	*V. carpophila* (Peach)	*V. pyrina* (Pear)	*C. humile* (Poplar)	*F. crataegi* (Crataegus)
Vi1	Yes	Yes	No	No	No	No	No
Vi2	Yes	No	No	No	No	No	No
Vi3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Vi4	No	No	No	No	No	No	No
Vi5	No	No	No	No	No	No	No
Vi6	Yes	Yes	Yes	Yes	No	No	No
Vi7	Yes	Yes	Yes	Yes	No	No	No
Vi8	Yes	Yes	Yes	Yes	No	No	No
Vi9	Yes	Yes	No	No	Yes	No	No
Vi10	Yes	Yes	No	No	Yes	No	No
Vi11	Yes	Yes	No	No	Yes	No	No
Vi12	Yes	Yes	No	No	Yes	No	No
Pr2	No	No	No	No	No	No	No
Pr3	No	No	No	No	Yes	No	No
Pr4	No	No	No	No	Yes	Yes	No
Pr5	No	No	Yes	Yes	Yes	No	No
Pr6	No	No	No	Yes	Yes	No	No
Pr7	No	No	No	Yes	Yes	No	No
Pr8	No	No	No	Yes	Yes	No	No
Pr9	No	No	No	Yes	Yes	No	No
Pr10	Yes	Yes	No	No	Yes	No	No
Pr11	Yes	Yes	No	No	Yes	No	No
Pr12	Yes	Yes	No	No	Yes	No	No
Pr13	Yes	Yes	No	No	Yes	No	No
C1	No	No	No	No	No	No	No
C2	No	No	Yes	Yes (only few)	Yes	No	No
C3	No	No	Yes	Yes	Yes	No	No
C4	No	No	Yes	Yes	No	No	No
C5	No	No	Yes	Yes	No	No	No
C6	No	No	Yes	Yes	Yes	No	No
C7	No	No	Yes	Yes	Yes	No	No
C8	No	No	Yes	Yes	Yes	No	No
C9	No	No	Yes	Yes	Yes	No	No
C10	Yes	Yes	Yes	Yes	No	No	No
C11	Yes	Yes	Yes	Yes	No	No	No
C12	Yes	Yes	Yes	Yes	No	No	No
C13	Yes	Yes	Yes	Yes	No	No	No

addition to specific bands in *V. carpophila* (Fig. 17c). Primers C6, C7, C8, C9, C10, C11, C12, C13 were transferable to *V. pyrina* and *V. inaequalis* besides *V. carpophila* (Table 21).

4.6.1 Multiplex PCR

Pooled DNA from five different *Venturia* spp. when used for PCR amplification with three species-specific primers singly and in combination successfully produced desired bands in heterogenic DNA of *Venturia* species in multiplex PCR. *V. inaequalis* produced 250bp band, *V. pyrina* produced 200bp band and *V. carpophila* produced 300bp band (Fig. 21). Thus, the primers developed in the present study are robust and detect different *Venturia* spp. individually and in mixed infection.

4.6.2 Realtime PCR (qPCR assay)

A qPCR assay was also performed to know the sensitiveness and detection limit of three *Venturia* species specific primers (Vi1, Pr3, C4). The primer pair for each *Venturia* species showed efficiency above 98 per cent, amplification factor between 1.99 to 2.07 and r^2 value ranging from 0.98 to 0.99 (Table 22). The specificity of each primer pair was confirmed using melt curve analysis. The dissociation curves showed single and sharp peak for all dilutions of DNA except for those dilutions that were not detected. The *V. inaequalis* and *V. pyrina* primer pairs could detect 0.01ng of pathogen DNA. However, *V. carpophila* could detect 0.001ng of DNA concentration (Fig 22). Overall, the primers designed were species specific, highly sensitive to detect *in planta* pathogen infection and can be used for simultaneous detection of three *Venturia* species using multiplex PCR.

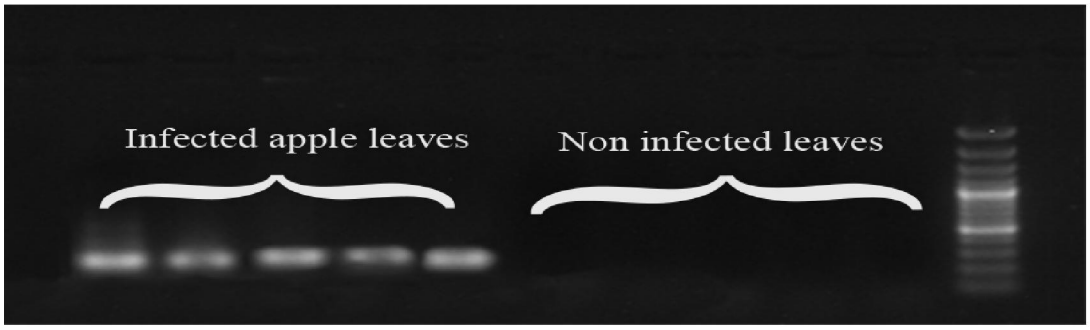


Fig. 20: *Inplanta* detection of apple scab using species-specific primers (Vi1)

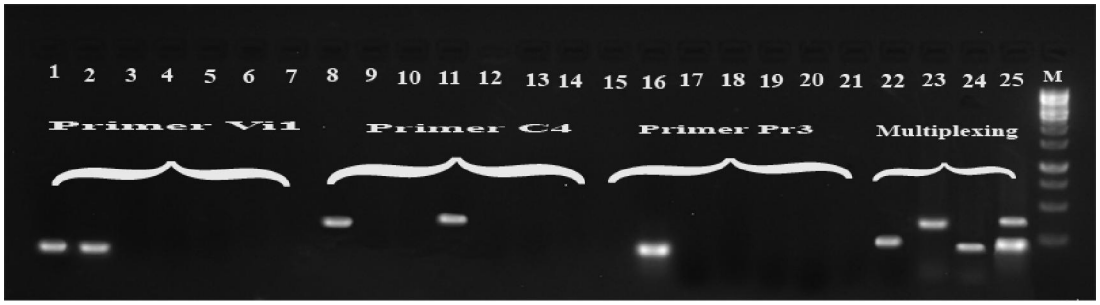


Fig. 21: Simultaneous detection of three *Venturia* spp. with multiplex primer set (Vi1+C4+Pr3 forward and reverse multiplex) and pooled *Venturia* spp.

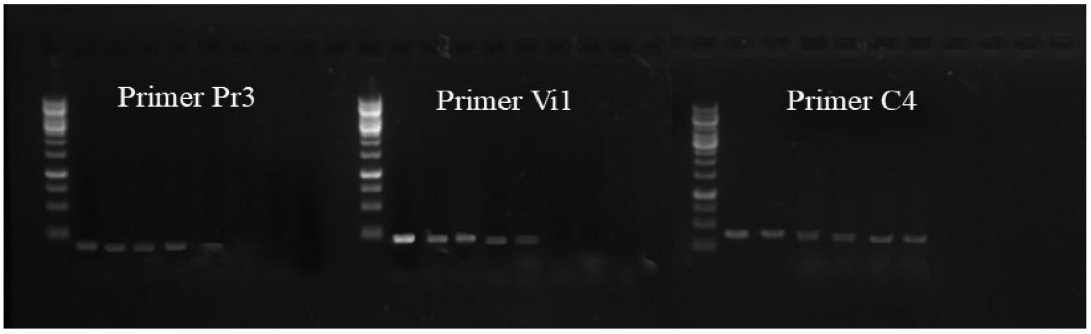


Fig. 22: qPCR product separation of respective *Venturia* species DNA of different concentrations

(Lane: M = 1 Kb DNA ladder; L1 = 100ηg; L2 = 10ηg; L3 = 1ηg; L4 = 0.1ηg; L5 = 0.01ηg; L6 = 0.001ηg; L7=0.0001ηg and L8 = non template control)

Table 22: Efficiency and coefficient of determination (r^2) of three species-specific primers

Species primer	r^2	Amplification efficiency	Amplification factor
V. inaequalis	0.98	104.12%	2.04
V. carpophila	0.99	106.52%	2.07
V. pyrina	0.99	98.96%	1.99

Chapter – 5

DISCUSSION

Venturia species is one of the notorious plant pathogens occurring on a large variety of host plants with haplontic life cycle (Zhang *et al.*, 2015). The various species of *Venturia* are well known for their adverse effects, causing characteristic leaf spots, necrosis, scab as well as leaf and fruit deformations on members of at least 52 angiospermous plant genera (Hashemi *et al.*, 2014). The anamorphic genera namely *Fusicladium*, *Pollaccia* and *Spilocaea* have been reported in the genus *Venturia* (Zhang *et al.*, 2011), however, their separation is not tenable due to their uniform conidiogenous cells, loci and the occurrence of morphologically intermediate species (Braun *et al.*, 2002). DNA barcoding clearly showed that *Venturia* is a monophyletic clade that could not be separated into sub-clades relevant to these anamorphic genera (Beck *et al.*, 2005) requiring thereby their merging in one anamorphic genus. The sexual and asexual morphs of the same genus must have only one name with preference for *Venturia* over *Fusicladium* or *Pollaccia* (Rossman *et al.*, 2015). Little work has been conducted on different *Venturia* species affecting various host species other than apple. This might be due to less investments and specialized management directed towards crops other than apple, and common use of information developed for *V. inaequalis* for managing the scab disease on other crops in Kashmir valley. Thus, an attempt was made to study various *Venturia* species from different hosts prevalent in Kashmir valley for the characterization based on rDNA-ITS, β-tubulin and elongation factor 1α (EF1α) regions for development of DNA barcode(s) and PCR based species-specific markers.

5.1 Isolation, purification, identification and maintenance of *Venturia* species

The isolates of various *Venturia* species *viz*., *V. inaequalis, V. pyrina, V. carpophila, V. populina*, *V. crataegi* and *Cladosporium humile* were isolated and

purified from infected leaves, fruits and twigs of apple, cotoneaster, pear, almond, peach, poplar and crataegus hosts collected from Srinagar, Pulwama and Anantnag districts of Kashmir valley on PDA using single spore technique given by Xu *et al.* (2008). Purification of *Venturia* species by single spore technique has also been reported by other workers (Gladieux *et al.*, 2010a; Zhao *et al.*, 2012; Leroy *et al.*, 2013; Padder *et al.,* 2013; Xu *et al.*, 2012) in India and abroad.

5.1.1 Morpho-cultural charaterisation and identification of the *Venturia* species

On the basis of morphological and cultural characteristic of *Venturia* species such as conidia shape, size, colour, sepetation, and hyphae shape, size, colour, sepetation on culture, and colony colour, texture, margins and shape were studied and compared with authentic description, and the *Venturia* species were identified. Further identity of *Venturia* species were confirmed by sequencing ITS region using ITS1 and ITS2 primer. The ITS sequence was submitted to Genbank of NCBI under Accession number (Table 3).

The morphological characteristics of *Venturia inaequalis* studied on PDA revealed that the fungus produced septate, interwoven compact, light olivaceous to brown mycelium with a width of 4-10 µm. The conidia were solitary, light pale to light olivaceous in colour, obipyriform, straight, without septa but occasionally one septate measuring about 16.23-32.94 x 6.84-11.72 µm with an average of 27.94x 7.84 µm in size. Similar observations were made by Samuels and Sivanesan, (1975), MacHardy, (1996), Schubert *et al.* (2003) and Hashemi *et al*. (2014) who observed that mycelium was septate, while conidia solitary, shape variable, ovoid to obipyriform or obclavate, straight, 12 - 26 x 7 - 9 µm in size, 0-1 septate, narrowly pointed or broadly rounded at the apex, truncate at the base. The fungal colony was first erumpent, spreading, greyish black to dark black with irregular margin. The morphological observation of the pathogen was also corroborated with other workers (MacHardy, 1996; MacHardy *et al*., 2001 and Tarun *et al*., 2018).

The *Venturia pyrina* produced flat to fluffy type of colony on PDA, which was mousy black in colour with irregular margin. The mycelium was septate, compact stromatic, light brown to dark brown coloured with a width of 3-8 µm. Similar morphological descriptions of pathogen were also made by Doliveira, (1937), Puttoo and Chaudhary (1984), Schubert *et al.* (2003) and Hashemi *et al*. (2014), who observed that fungus produces typically sepetate, light coloured mycelium later on turning brown and hyphal width varying from 2.6 to 8.6 µm in size. The conidia were solitary, light yellow to olivaceous brown in colour, fusiform to pyriform, straight to slightly curved, without septa but occasionally one septate, measuring about 15.96-36.92×6.76- 9.40 µm with an average of 28.57×7.66 µm. Present investigation was in completely in accordance with the findings of Esmarch (1930), Saccas (1944), Puttoo and Chaudhary (1984), Schubert *et al.* (2003), Michalska and Polec, (2006) and Hashemi *et al.* (2014) and observed that conidia were mostly 0 – 1 septate, solitary, usually spindle shaped, sometimes ellipsoid to obovoid, straight, 14 - 26 × 6 - 8 µm in size, , olivaceous brownin colour.

The morphological characteristics of *Venturia populina* studied on PDA revealed that the fungus produced septate, swollen, sub-hyaline to light yellowish mycelium with a width of 3-7 µm. The conidia were solitary, sub-hyaline to light olivaceous in colour, fusiform to ellipsoidal, straight, rarely curved having 0 to 2 septa measuring about 15.97- 33.46 × 8.12-12.96 µm with an average of 27.76 × 9.35 µm in size. Similar observations were made by Servazzi, (1940), Dance, (1961), Taris, (1980) and Koul *et al*. (1989) who observed that conidial in culture were ellipsoid to broadly fusoid, olivaceous brown, smooth-walled, slightly constricted at the septa, straight or slightly curved, 25 - 42 x 8 - 14 µm in size. The morphological description of the pathogen was also corroborated with many workers (Schubert *et al*., 2003 and Kasanen *et al*., 2001). The fungal colony on the culture was erumpent, blackish centre surrounded by greyish region with irregular margin. Similar observation was made in colony of the *V. populina*

causing scab on poplar by Servazzi, (1940), Kowalski, (1963) and Kasanen *et al*. (2001) who observed that colonies were greenish black with sporadic outgrowths of hyphal walls or hyphal tips.

The *Venturia crataegi* produced first erumpent, spreading, abundant aerial mycelium with feathery to smooth margins. Upper surface of culture was grey-olivaceous whereas reverse side was dark olivaceous producing septate, branched interwoven, light pale to olivaceous brown mycelium with a hyphal width of 3-5 µm. Earlier worker, Schubert *et al.* (2003) and Michalska and Polec, (2006) have also made similar observations on the morphology of the fungus. The conidia were solitary, light yellow to olivaceous brown in colour, fusiform to obclavate having 0 to 1 septate with slightly constricted at the septum measuring about 11. 47-19.18 × 4.71-6.90 µm in size with an average of 15. 64 × 5.76 µm in size. According to Schubert *et al*. (2003), *Venturia crataegi* forms conidia solitary, fusiform sometimes obclavate, 10-25 x 4-8.5 µm in size, pale olivaceous, 0–1-septate rarely 2-septate.

The morphological characters of *Cladosporium humile* studied on PDA revealed that the fungus produced septate, tubular, light pale to olivaceous colour mycelium with a width of 2-6 µm. The conidia were catenate, sub-hyaline to light olivaceous green in colour, cylindrical to fusiform, straight or slightly curved having scar on the ends, 0 to 1 septate measuring about 15.47-27.46 × 4.43-9.53 µm with an average of 20.12 × 7.71 µm. Similar, observations were made by Plakidas, (1942), Singh *et al*. (1983), Khan *et al*. (1989), Beig, (1995) and Schubert *et al.* (2003) who observed the mycelium were septate, light olive green colour with an average width of 2.36 µm while conida were fusoid to cylinderical, chains, ocassionally one septate and olive green in colour, measuring on average 19.65 x 5.16 µm in size. The fungal colony on the culture was raised, velvety greyish black with un even margins. The morphological description of the pathogen was also corroborated with many workers (Plakidas, 1942; Singh *et al*., 1983 and Beig, 1995) and observed that the fungus formed raised, velvety colony

of grey colour with appressed margins imparting black colour to medium.

The morological characteristics of *Venturia carpophila* studied on PDA revealed that fungus produced septate, interwoven, branched, dark brownish mycelium with a width of 4-6µm. Conidia were mostly simple, sub-hyaline to light brown, cylindrical to fusiform, sometimes ovate, straight, having 0 to 1 septate with slightly constricted at septum measuring 11.67-22.50 × 4.45-7.96 µm with an average of 18.30 × 6.46 µm. Similar observations were made by Schubert *et al.* (2003) and Hashemi *et al*. (2014) who observed that mycelium was septate, olivaceous, hyphae with a width of 3–6 µm, while conida were cylinderical to fusiform, straight, 12.5-22 x 4 x 5 µm in size, aseptate and pale olivaceous in colour, smooth. The morphological description of the pathogen was also corroborated with many workers (Heuchert, 2005 and Khosla *et al*., 2009). The fungal colony on the culture was erumpent, rough blackish with uneven margins. Similar observation was made in colony of the *C. carpohilum* causing scab in peach and cherry by Schweizer, (1958).

5.2 Characterization, sequence analysis and phylogenetic relationships of *Venturia* species based on rDNA-ITS, β-tubulin, EF1α gene sequence data

Venturia species represent one of the most diverse groups of plant pathogens including economically important plant pathogen *V. inaequalis* that has adverse effect worldwide on profitable apple production. Monitoring and predicting effectiveness of management strategies require knowledge of phylogenetic and population genetics/biology of the pathogen, which can be characterized using molecular markers including rDNA-ITS, β-tubulin and EF1α gene sequence data. Most of the sequencing results did not show any ambiguous sequences or double/multiple peaks during chromatogram analysis. In many phylogenetic studies on *Venturia* species, ribosomal and protein-coding genes were used directly for phylogentic reconstruction and species identification (Schnabel *et al.*, 1999; Beck *et al.*, 2005; Sanchez-Torres *et al.*, 2009; Zhao *et al.*, 2012; Padder *et al.,* 2013; Ibrahim *et al.,* 2016) without assessing any intra or

inter-isolate divergence. Under such circumstances, specific divergence might be underestimated in some groups, while species diversity may be overestimated. In the present study, rDNA-ITS, β-tubulin, EF1α gene regions of different *Venturia* isolates were analysed to examine the differences of intra-isolate polymorphism levels between the genes and species to test the implication on molecular identification and phylogenetic analysis in different *Venturia* species. One hundred sixty-two sequences from different geographical origin and host species studied during present study showed maximum similarity with the respective sequences of *Venturia* species *viz*., *V. inaequalis, V. pyrina, V. carpophila, V. populina, V. crataegi* and *C. humile*, available at National Centre of Biotechnology Information (NCBI) using BLASTn programme. Beck *et al.* (2005) also confirmed identity of *Venturia* species by sequencing of ITS region. Thus, sequence analysis of ITS region is a powerful tool for authentic identification of fungal species being conserved within species and supported the monophyly of *Venturia* species. This fact is suppoted by the previous study of Schoch *et al*. (2012) who proved that internal transcribed spacer (ITS) region is the most extensively nuclear ribosomal gene for specific identification and phylogenetic analysis in fungal groups and declared as the DNA barcode for fungi. ITS has been proposed as a universal DNA barcode marker for fungi Schoch *et al*. (2012) as we know, a requirement of molecular markers used in taxonomic and phylogenetic studies is that all copies within the genome are constant and identical, and that intraspecific variation is lower than interspecific variation. Other researchers have also reported the importance of ITS sequence analysis for fungal species delimitation (Schnabel *et al.*, 1999; Beck *et al.*, 2005; Sanchez-Torres *et al.*, 2009; Zhao *et al.*, 2012; Padder *et al.,* 2013; Ibrahim *et al.,* 2016).

The phylogenetic analysis based on ITS sequences led to grouping of *Venturia* species in two main clades. Clade I composed of all isolates of *V. carpophila, V. crataegi, C. humile, V. populina* and *V. pyrina*, while clade II

accommodated *V. inaequalis* isolates. *Venturia* species proved to be the monophyletic group supported by bootstrap values but could not be differentiated on the basis of ITS region. These findings are in agreement with those of Schnabel *et al.* (1999), Beck *et al.* (2005), Zhao *et al.* (2012) and Padder *et al.* (2013) who studied rDNA-ITS data of *Venturia* species in a more comprehensive way and could not differentiate them on the basis of ITS region because of considerable homogeneity between them. In the present study, the sequences of *Venturia* species got grouped together despite having different geographical and host origin (different regions of Kashmir valley and different host species). The clade I was divisible into many sub-clades which indicated partial close coevolution between hosts and *Venturia* species. These results are in agreement with previous studies of different researchers (Schnabel *et al.*, 1999; Beck *et al.*, 2005; Zhao *et al.*, 2012) who also demonstrated partial close coevolution between *Venturia* species and their respective hosts based on ITS phylogenies and that, species boundaries within a host genus are less distinct (Bowen *et al.*, 2011). The switching over of *Venturia* species between the genera was interesting to note, which is evident from clustering of *V. crataegi* on crataegus host with *C. humile* on poplar host indicated their limited ability to colonise the new host plants. These results are also in agreement with Beck *et al.* (2005) who also reported the switching over between *V. saliciperda* on silax host with *Fusicladium* species on poplar host. Four independent lineages were also observed, one from *V. crataegi* and three from *V. populina* in the phylogenetic tree based on the ITS region. Padder *et al.* (2013) also observed similar results in their phylogenetic study of *V. inaequalis* and reported independent lineage of one sequence belonging to *V. inaequalis* (JQ026119). However, the results obtained from ITS region indicated that despite adaptation to diverse hosts during the course of evolution, *Venturia* genus has maintained its ITS region and isolates with different host origin couldn't be differentiated on the basis of this region.

The phylogenetic analysis of *Venturia* spp. based on β-tubulin grouped

them into six clades conspicuously based on species level irrespective of host origin when a consensus tree using bootstrap procedure was drawn. Clade I comprised of *V. inaequalis* isolates, Clade II accommodated *V. carpophila* isolates, Clade III of *V. crataegi* isolates, Clade IV of *V. pyrina* isolates, and Clade V and VI accommodated *V. populina* isolates. Zhao *et al.* (2012) also reported the importance of β-tubulin gene in identification and phylogenetic analysis of *V. pyrina* and *V. nashicola,* but were unable to do so using ITS region (Beck *et al.*, 2005). In the present study, β-tubulin showed high level of inter-specific variation that might be equal or greater than intra-specific variation observed between different *Venturia* species. The isolates of *Venturia* species, in the present study, got grouped on the basis of their species epithet despite having different host origins or geographical locations. Thus, the hypothesis that sequences may form separate clades on the basis of host origin got rejected. This indicated that despite adaptation to diverse hosts during the course of evolution, *Venturia* species maintained its β-tubulin region.

The phylogenetic analysis of *Venturia* species isolates based on EF1α gene grouped them into five clades, obviously based on their species epithet and also lead to little splitting on the basis of their host origin when a consensus tree using bootstrap procedure was drawn. Clade I comprised of isolates of *V. carpophila*, Clade II of *V. crataegi*, Clade III of *V. populina*, Clade IV of *V. pyrina* and Clade V of *V. inaequalis*. Clade I (*V. carpophila*) and Clade V (*V. inaequalis*) indicated partial tendencies of co-evolution between hosts and pathogen. These results are in agreement with those of Hasegawa *et al.* (2010), Zhao *et al.* (2012) and He *et al.* (2016). They also observed the intra isolate heterogeneity level in some fungal species at EF1α locus. Zhao *et al.* (2012) have also reported the importance of EF1α gene in identification and phylogenetic analysis of *V. pyrina* and *V. nashicola*. It could be speculated that the EF1α regions are too polymorphic to use for identification and phylogenetic studies in *Venturia* at the species level, and this holds true for other fungal groups as well. It is also possible because of

differences between mutation rates and DNA repair rates leading to an accumulation of sequence variants in genes with multiple copies (Elder and Turner, 1995). Thus EF1α gene sequences have widespread interspecific variation which might be greater or equal to intra specific variation that leads to grouping of these *Venturia* species into clusters based on their host origin. Therefore, it may be concluded that EF1α is an ideal DNA barcode and could be used in taxonomy and phylogenetic studies in *Venturia* or other fungal groups for the presence of intra-isolate polymorphisms.

Multigene phylogenetic analysis of *Venturia* species using concatenated data set based on rDNA-ITS, β-tubulin, EF1α gene sequences grouped them into nine clades. Clade I comprised of *V. crataegi* isolates, Clade II of *V. populina*, Clade III of *V. pyrina*, Clade IV of *V. inaequalis* and Clade V to Clade IX comprised of *V. carpophila*. In case of clade IV, dichotomy of *V. inaequalis* was observed, which reflects close co-evolution between *V. inaequalis* and their respective hosts, species boundaries within genus also existed. These results are in complete agreement with those of Zhao *et al.* (2012) who also reported co-evolution between *Venturia* species and pears, based on multigene phylogenies. It is also interesting to note that Clade V to clade IX composed of scattered and dispersed isolates of *V. carpophila* from two hosts such as almond and peach, which form independent lineages. It can be suggested that due to infection of *V. carpophila* to a number of hosts *viz.*, almond, peach, apricot and cherry which cause genomic instability and due to early divergence of their hosts that has led to the formation of different evolutionary lineages of *V. carpophila*. Other researchers have also reported the importance of multigene sequences in the identification and phylogenetic analysis of *Venturia* species (Sanchez-Torres *et al.*, 2009; Zhao *et al.*, 2012; Ibrahim *et al.,* 2016). Sanchez-Torres *et al.* (2009) also clearly differentiated *Fusicladium eriobotryae* from *V. inaequalis*, *V. carpophila* and *V. pyrina* on the basis of multigene phylogenetic analysis using not only ITS but also G3PD gene sequences, although multiple gene sequences

evolve with different patterns of substitutions by concatenating all the gene sequences yielding a super gene tree with more accuracy (Gadakar *et al.*, 2005). Our results provide first comprehensive phylogenetic study of *Venturia* species collected from different hosts grown at different locations. In the present study, multigene phylogenetic analysis using not only rDNA-ITS but also β-tubulin and EF1α gene sequence data producing a tree topology with a high reliability and relationship between the *Venturia* species. A species identification criterion based on phylogenetic concordance of multiple unlinked genes was used to indicate lack of genetic exchange among taxa. Multiple gene phylogenetic anaylsis appears more widely applicable and discriminating species recognition criteria in fungi (Giraud *et al.*, 2008). For closely related taxa, a number of analytical approaches such as patterns of genetic variation within and between the taxa are taken in account in order to estimate parameters in a coalescent framework to provide the important insights on the history of divergence (Becquet and Przeworski, 2007). Our results provided the first comprehensive phylogenetic study of *Venturia* species using different isolates from diverse hosts, cultivars and locations. The phylogenetic analysis showed that *V. inaequalis* isolates collected from apple were phylogenetically distinct from those derived from cotoneaster. In addition to this, *V. carpophila* collected from almond and peach form multiple lineages without forming distinct evolutionary clade with respect to host speciation, thus the species boundaries within a host genus are less distinct. In the *Venturia*-host species pathosystem, species boundaries within the genus also existed and co-evolution between *Venturia* species and their hosts were also observed in this study in terms of multi-locus phylogenies. Previous studies on rDNA-ITS phylogenies reflected close co-evolution between *Venturia* species and respective hosts (Schnabel *et al*., 1999; Beck *et al*., 2005). Nevertheless, the species boundaries within a host genus are less distinct (Bowen *et al*., 2011). The co-evolutionary relationship of *V. inaequalis* with apple and cotoneaster, and *V. carpophila* with almond and peach was revealed in three phylogenies and can be postulated that the early divergence of apple and cotoneaster, and almond and

peach as a host led to the different evolutionary lineages of *V. inaequalis* and *V. carpophila*. To protect apple and cotoneaster, and almond and peach from *V. inaequalis* and *V. carpophila*, respectively, resistance breeding is one of the promising ways for disease management. It is thus important to trace the evolutionary pathways of the pathological races of the pathogen. In our study, we also tried to investigate the relationships between genetic variation among and within these species. In *V. inaequalis* isolates belonging to various hosts and locations were used in this study. Results indicated that all phylogenies supported a monophyletic group of *V. inaequalis* isolates and no relationship with geographical origin and host cultivars were observed. On the contrary, in *V. carpophila,* genetic divergence was observed among representative isolates collected from almond and peach. Molecular phylogenies indicated that the genetic divergence in *V. carpophila* was higher than in *V. inaequalis* suggesting that the pathological races of *V. carpophila* might have evolved recently in this species. Higher genetic variation might be attributed to subtle changes in the reproductive mode and epidemiological structure of the fungus because of changing environment or host co-evolution (Gladieux *et al*., 2008). Moreover, it might be possible that *V. carpophila* isolates of peach were derived from other hosts or countries in the past. The present study provided more reliable and qualitative estimation of the interspecific phylogenetic relationships between *V. inaequalis* from apple and cotoneaster, and *V. carpophila* from almond and peach. However, intraspecific relationships were not revealed among isolates. Previous researchers also showed that intraspecific isozyme variation was related to sequence variation of endopolygalacturonase genes and pathogenicity variation in *V. nashicola* (Katoh *et al*., 2008). Further research on pathogenicity related genes and genetic markers needed to be carried out to clarify the evolutionary pathway of pathological races of *V. inaequalis* from apple and cotoneaster, and *V. carpophila* from almond and peach. Application of such approaches is still limited in case of fungi. However, a combination of coalescent analysis was used by Stukenbrock *et al.* (2007) in the pathogen populations of *Mycosphaerella*

graminicola on wheat emerging from uncultivated grasses, due to the lack of current gene flow between populations and rapid speciation associated with a host-shift from wild to domesticated. Consequently, identification and quantification of inter-specific and intra-specific heterogeneity levels is of real importance to ensure reliability as a desirable phylogenetic marker and DNA barcodes for species identification. Our results provided the first comprehensive phylogenetic study of *Venturia* species by using more isolates collected from various host cultivars and locations. Therefore, these results further supported the early taxonomic separation of *Venturia* species.

5.3 Polymorphism, neutral evolution test and recombination survey

The genetic data provided the evidence of divergence in *Venturia* species and indicating its lineages from ancestral populations infecting wild hosts. The polymorphism level observed among *Venturia* species on the basis of rDNA-ITS, β-tubulin and EF1α gene sequence data was very high as indicated by the patterns of variation observed in sequences in terms of base pairs, which ranged from 193 to 556 bp. Such observed pattern of polymorphism depends on speciation events, population size, impact of natural selection, recombination, hybridization and introgression (Stukenbrock *et al.*, 2007). Other researchers have also found ribosomal and protein coding genes suitable for studying polymorphism in fungi (Stukenbrock *et al.*, 2007; Gladieux *et al.*, 2010; He *et al.*, 2017). In the present study, the number of haplotypes were high and a smaller number of shared haplotypes were observed within and among the populations whether based on rDNA-ITS, β-tubulin and EF1α loci, and also on concatenated data set ranging from 2 to 11. These results are in agreement with those of Broughten and Harrison. (2003), Stukenbrock *et al.* (2007), Gladieux *et al.* (2010), Padder *et al.* (2011 and 2013), Chen *et al.* (2017) and Gladieux *et al.* (2018) who also reported high number of haplotypes in the pathogen. The reason being that the sexual recombination in the fungus allows crossing over and gives rise to new combination of alleles, thereby generating new haplotypes without affecting the

linkage disequilibrium (Gladieux *et al.*, 2010). The haplotype diversity in the isolates collected from different hosts based on rDNA-ITS, β-tubulin and EF1α loci, and also on concatenated data set ranged from 0.60 to 1.00 indicating that *Venturia* species has maintained high level of genetic diversity. It is expected to find a high haplotype diversity in isolates collected from uncultivated hosts as the diversifying selections are imposed by different environments and host species (Burdon, 1993). Gladieux *et al.,* (2010) also reported high haplotype diversity in *Venturia* species collected from apple, pyrancantha and loquat. Several other researchers also reported a high haplotype diversity in the fungal pathogen populations (Stukenbrock *et al.*, 2007; Chen *et al.*, 2017; Gladieux *et al.,* 2018). The estimates of intra-nucleotide diversity (π and θ) in the present study ranged from 0.001 to 0.108 and 0.001 to 0.138 respectively, which were generally greater for intra-specific comparison than for inter-specific comparison in the populations collected from different hosts both at individual loci and at concatenated data sets. Over all, the patterns of variations observed in the loci indicated that there were only few variable sites among different isolates and suggested the occurrence of little population subdivision within species. Similar results were reported by Broughten and Harrison (2003), Matute *et al.* (2006), Gladieux *et al.* (2010), Chen *et al.* (2017) and Gladieux *et al.* (2018). It has been observed that during biological speciation, there is conversion of genetic variability within species to between species (Geiser *et al.*, 1998). However, the distribution and origin of genomic mutations are usually false positive, and only few mutations originate from the same donor lineage corresponding to genuine exchange of genetic events resulting in little sub-division in species in the present studies. Tests of neutral evolutions could not be rejected for any loci which showed consistent negative value for isolates collected from apple, almond and poplar, but positive values for peach and cotoneaster, isolates suggesting thereby a possible history of population expansion i.e., the changes in population size are likely the key demographic forces that affected simultaneously all the loci resulting in negative values. The background selection or shrinkage restricts the species to newly shifted hosts

leading to positive values. R_2 statistics is slightly more sensitive test for population growth and the values support the demographic explanation i.e., population expansion and effective population size are the causes for deviation from the neutral expectations of Tajima's D test. Padder *et al.* (2013) has also reported tremendous expansion of apple areas with planting of new cultivars instead of indigenous Ambri cultivar in Kashmir valley. This has led to increase in effective population size of *V. inaequalis* and a recent split, where only a small fraction of ancestral *V. inaequalis* pathogen from apple founded a new population on cotoneaster. However, we can't determine whether increase in population size of the respective pathogen populations occurred in a linear way through time but some historical events such as recombination and segregation of ancestral polymorphism were favorable for the pathogen. Gladieux *et al.* (2008) showed evidence for migration of *V. inaequalis* from out of Central Asia suggesting that the pathogen travelled with its host that resulted in diversification and population expansion of the pathogen. Previous studies have also shown non-neutral evolution of the tested loci for various fugal pathogens (Stukenbrock *et al.*, 2007; Gladieux *et al.,* 2010; Zhong *et al.,* 2017; Gladieux *et al.,* 2018) leading to believe that the observed pattern is most likely due to expansions of populations. The loss of sexual capacity is a powerful factor for isolating the fungi or at least influencing the gene flow among fungal populations (Giraud *et al.*, 2008). Therefore, the level of recombination events and the segregating sites were assessed to determine these factors contributing to differentiation. Our results indicated that there is history of recombination events in *Venturia* species which ranges from 1 to 21 and segregating sites ranging from 1 to 345 in the test loci at individual and combined gene levels. Since recombining population structure of *V. inaequalis* was expected on apple host as the pathogen under goes regular sexual reproduction on fallen leaves in each winter (Gladieux *et al.,* 2008), we found high recombination events in *V. inaequalis* consistent with the expectations. Low recombination events were found in isolates collected from poplar and peach, while high number of segregating sites were found in isolates from poplar

in the present study, thereby suggesting that these are largely clonal and reproduced asexually; the segregating sites showed that there is breakage of ancestral polymorphism, and thus contributes maximum to the evolution of loci. However, Gladieux *et al.* (2010) also found non recombining structure in *V. inaequalis* population, infecting pyracantha and loquat, and accounted it to low sample size and number of loci. Other researches have also found different number of recombination events and segregating sites in various fungal pathogens (Matute *et al.*, 2006; Stukenbrock *et al.*, 2007; Gladieux *et al.,* 2018; Zhong *et al.*, 2018). Thus, recombination events provide a measure to detect the extent to which pathogen is clonal in nature and address the question on mode of reproduction.

5.4 Divergence, differentiation and gene flow

Venturia species collected from various hosts were studied for their origin/emergence from different lineages. Therefore, the present investigations on nucleotide divergence data revealed that weak divergence existed between the isolates of apple and cotoneaster (Dxy = 0.011), and of peach and almond ((Dxy = 0.032). While in other cases the divergence values were high which reflected strong divergence and occurrence of ancient diversification. The divergence values indicated that heterogeneity in genomic and life cycle changes are associated with the emergence and spread of these isolates. Our results are in conformity those of with Gladieux *et al.* (2010) who also studied divergence between isolates of *V. inaequalis* collected from apple, pyracantha and loquat (Dxy=0.0062), which indicated that these isolates are lately diverged. It is the major resistance genes that are determinant to promote the divergence between lineages of pathogen by exerting strong divergent selection on limited number of pathogenicity related genes (Giraud *et al.,* 2010; Liao *et al.*, 2016). Although, our results suggest that divergence has occurred between the isolates of *Venturia* species collected from different hosts, however, we could not determine the initial events of divergence. The group of pathogen isolates from cotoneaster and peach have shared polymorphism with low sequence divergence suggesting that the

common ancestor existed recently and consequently their effective gene flow (Nm=0.06) is very low indicating that these two species have recent origin under the model of gradual speciation in which reproductive barriers arise in early speciation process. The shared polymorphism in the present study ranged from 0 to 12, which is expected when the initial taxa diverge with gene flow and erase the patterns of exclusivity for some loci. These results are in agreement with those of other workers as well (Broughten and Harrison, 2003; Geiser *et al.*, 2006, Gladieux *et al.,* 2010) who also found different level of shared polymorphism. In Kashmir valley, different hosts of *Venturia* spp. are grown together in same orchard, sharing the geographic habitat which results in secondary contact between divergent species that leads to exchange of genetic material, thereby creating shared variation. Balancing selection, demographic expansion and recurrent mutation can also enhance the persistence of shared polymorphism across species (Wakeley and Hey, 1997; Clark, 1999), thus giving impression of species still exchanging genes. It is also clear from the levels of fixed difference in polymorphism among different groups of *Venturia* species that they have resulted in long history of reproductive isolation and thus can be considered different biological species. We estimated four parameters to assess the contribution of genetic differentiation and gene flow in the observed pattern of divergence among *Venturia* species collected from different hosts. The analysis revealed low levels of gene flow among the *Venturia* species. The corresponding F_{ST}, Kst and Snn values have revealed high genetic differentiation, reproductive barriers and non-random matting populations, thereby suggesting a greater role of recent splitting in presence of shared variation among *Venturia* species. It indicates that there is low gene flow because of strong selection against immigrants i.e. host specificity plays a strong and most efficient barrier to gene flow between local and emerging populations. These results are in agreement with those of Gladieux *et al.* (2010), Gladieux *et al.* (2010a), Gladieux *et al.* (2011) and Meng *et al.*, 2018) who also reported low gene flow and high genetic differentiation which accounted for allopatric divergence followed by limited introgression through secondary contact.

Giruad *et al.* (2010) and Rieux *et al.* (2013) also reported a high level of genetic differentiation existing among sub-populations of pathogens due to high level of genetic drift generating changes in allelic frequency during recolonization events. Over all, the results suggest that some other forms of barriers may be active to genetic exchange between population and causing high genetic differentiation such as pre-zygotic and post-zygotic barriers.

5.5 Population genetics

For analysis of population structure, *Venturia* spp. collected from different hosts were clustered on species level and grouped into six populations. The haplotype diversity was high (0.60 to 1.00) indicating a high level of genetic diversity maintained between *Venturia* species. The high amount of genetic diversity observed between *Venturia* species suggests that speciation was not only associated with host adaptation but also accompanied by significant genetic differentiation. Padder *et al.* (2013) also reported a high genetic diversity in *V. inaequalis* from Kashmir valley using ISSR markers. Maintenance of high genetic diversity by fungus in western Himalayas indicated the probable introduction of the fungus from central Asia, the centre of origin of *Venturia* (Gladieux *et al.*, 2008). Nucleotide diversity was less (0.003-0.07) due to conserved regions of rDNA-ITS, β-tubulin and EF1α loci. The variation between nucleotide diversities among different species indicated the occurrence of population subdivisions.

Analysis of molecular variation present in different populations revealed that maximum percentage of variation was distributed among population than within population (90.68 to 77.12%) indicating presence of separate regional populations. The corresponding high pair-wise fixation index (F_{ST}) values (0.71-0.93) also indicated high divergence between the populations. The observed level of genetic variation points towards change in reproductive mode, epidemiological structures of the fungus and probably in combination with significant expansion of the pathogen populations as the host density has increased in new apple growing regions in Kashmir valley. These results are in agreement with the

findings of Gladieux *et al.* (2008) who observed lower level of variation within *V. inaequalis* isolates from non-central Asian populations and suggested that population lost alleles in the process of movement, arrival and establishment outside their native range. Estimation of F_{ST} was very high at species level based on rDNA-ITS, β-tubulin or EF1α loci. It indicated that there is a low gene flow between *Venturia* species because of strong selection against immigrants. Additional intrinsic and extrinsic post zygotic barriers, complete reproductive isolation might be leading to pervasive gene flow. From the present investigation, it is clear that there is high probability of mating between strains of same host than strains residing on different hosts (assortative mating).

The Principal component analysis (PCoA) separated *Venturia* species into distinct clusters with first and second coordinates representing 24.75 to 68.37 and 10.84 to 25.04 per cent of total variation, respectively. These results are in agreement with those of Gladieux *et al.*, (2008), Gladieux *et al.* (2010), Gladieux *et al.* (2011), Leroy *et al.* (2013) and Michalecka *et al.* (2018). Traces of host specialization within the *Venturia* species population were also assessed with the assignment method implemented in the STRUCTURE. The results obtained indicate the genetic distinctness of *Venturia* species due to host selective pressure exerted by different resistance genes. The clustering algorithm also supported distinction of clusters at K = 5-9. At K = 5-9, five genetic populations were obtained and the samples tend to have separate clusters. The distinction between these clusters was also visible in the results of the PCoA. This population split was previously described in commercial orchards between infected *Malus* x *domestica* cultivars with or without *Rvi6* gene (Gurien *et al.*, 2004; Gladieux *et al.*, 2011; Leroy *et al.*, 2013; Michalecka *et al.*, 2018). Since different host species carry many scab resistance genes (Bus *et al.*, 2011) and the presence of resistance gene and other polygenic scab resistance specificities in different host species might have induced an identical split in *Venturia* species populations in Kashmir, despite the very likely presence of other resistance genes in a diverse genetic

background of sampled accessions. However, some individuals at ITS loci and concatenated data showed evidence of admixed ancestry from each cluster. Leroy *et al.* (2013) and Michalecka *et al.* (2018) also observed tendency of admixed ancestry in *V. inaequalis* populations, due to selective favorable mutation occurring in populations and selective sweeps that change the allelic frequency at linked loci, resulting in local reduction of genomic variations. Admixed groups observed in our results are also supported by the fact of presence of potential recent off shifters that lead to gene flow between different species. However, admixture between the subpopulations remained quite low, suggesting the existence of pre-zygotic and/or post-zygotic barriers to gene flow, although different clusters were detected by STRUCTURE in isolates of *Venturia* species and therefore, has evidence of correlations between inferred clusters and host cultivars. The present study cannot however, rule out the existence of other resistance factors shared by the accessions belonging to different host varieties and geographical discontinuities.

5.6 Development of Species-specific marker

With the expansion of plant pathogen diversity, it becomes necessary to conduct identification up to species level. Accurate identification of *Venturia* spp. based on fungal characters requires taxonomic expertise. Alternatively, development of PCR based markers for species delineation is easy, less time consuming and does not require taxonomic expertise. Earlier attempts for development of specific primers for the detection of different *Venturia* spp. (Le-Cam *et al.*, 2001; Stehmann *et al.*, 2001; Koh *et al.*, 2013), failed to develop PCR based accurate detection procedures. Nowadays low-cost sequencing has made surfeit of plant pathogen genomes accessible for development of reliable, robust and informative molecular markers. Due to the easy access to genomes, we can develop species-specific primers. Many of the plant pathogenic genera have different species that are morphologically similar but differ in their pathogenicity. One of such pathogen genus is *Venturia* with 290 species that infect different

hosts. Difference in their pathogenicity will ultimately have discrepancy in genome. Exploiting such differences in the genome we dig out 37 primers from the three *Venturia* spp. genomes. Many of the primers showed cross transferability, some primers could not amplify any region and three primer pairs explicitly showed specificity to *V. inaequalis*, *V. carpophila* and *V. pyrina,* respectively.

In the present study, primers developed from the genome of three *Venturia* species shows cross transferability across species that ranges from 38 to 41 per cent. This much of cross transferability is comprehensible as the genome between the species is similar. As studied in *Alternaria* species, the cross transferability between species were observed to be 45 per cent (Singh *et al.*, 2014); about 42 to 48 per cent cross transferability was observed across *forma speciales* of *Puccinia graminis* (Karaoglu *et al.*, 2013). It can also be accredited to transfer of lineage specific genomic regions in fungi (Thrall and Burdon, 2005; Ma *et al.*, 2010). More closely related the species higher is the rate of transferability because of shared homology and syntenic regions between them, besides the species for which the primers are designed are from the same genus (Kumar *et al.*, 2012). Some of the primers developed showed species specificity that can be due to some sort of disparity between genomes. The comparative genomics has revealed disparity in the genomes of different isolates of *Venturia* spp. on the basis of secreted proteins such as CAZymes, peptidases, lipases, small secreted proteins etc. (Deng *et al.*, 2017). Previous studies have classified *Venturia* spp. on the basis of monophyletic groups keeping *V. inaequalis, V. carpophila and V. pyrina* in separate groups (Le-Cam *et al.*, 2001). The differences in the genomes is anticipated using ITS1-5.8S-ITS2 sequences (Schnabel *et al.*, 1999). These findings revealed that instead of being more syntenous there are some regions that can be attributed to the species specificity. To date many species-specific markers developed previously by many researchers in *Venturia* species are available (Stehmann *et al.*, 2001). However, accuracy and precision are indispensable.

Since the internal transcribed spacer-based PCR markers developed earlier for *V. inaequalis*, *V. pyrina*, *V. carpophila*, *V. asperata* (Stehmann *et al.*, 2001) are based on rDNA sequences that evolve slowly, there is every chance of getting false results; thus these markers can be useful only for studying distantly related organisms (Liyanage *et al.*, 1992). As in case of *V. nashicola,* the internal transcribed spacer-based PCR-RFLP (Le Cam *et al.*, 2001) produced false-positive bands. However, nested PCR improved the accuracy of detection but remains time consuming (Koh *et al.*, 2013). Therefore, designing species-specific primers that are quite reliable, efficient and least time consuming becomes decisive for detection and management of the disease.

We were able to find three primer pairs each specific to *V. inaequalis*, *V. pyrina*, *V. carpophila,* respectively. These primer pairs could amplify the specific species of *Venturia* and showed no cross transferability between the species. Moreover, the GC content of *Venturia* species ranges from 38 to 47.4 per cent depicting stability in the genome that ultimately led to designing of species-specific primers. Further, we studied the detection limit of our primers using real-time PCR.

When compared with the previously developed PCR based methods, our real-time PCR method could detect *V. inaequalis*, *V. pyrina*, *V. carpophila* at 0.01, 0.01, and 0.001ng of DNA concentration, respectively. Real time PCR based primers were previously developed for *V. nashicola* (Yun *et al.*, 2015). However, our species-specific primers for *V. inaequalis*, *V. pyrina*, *V. carpophila* can be easily exploited for the detection of various *Venturia* spp. Besides, these primers can be used for *inplanta* detection of pathogen as the primers could detect very less amount of target DNA, thus can detect the pathogen before symptoms appear.

Chapter – 6

SUMMARY AND CONCLUSION

The present investigations were carried out to ascertain the phylogenetic relationships in *Venturia* species using multigenic approach during the year 2015-2019 in the Division of Plant Pathology, SKUAST-Kashmir, Shalimar, Srinagar. Fifty-five isolates of *Venturia* species collected from different hosts (apple, pear, almond, poplar, peach, crataegus and cotoneaster) grown in Srinagar, Pulwama and Anantnag districts were isolated, purified and maintained on PDA.

Genomic DNA was seperately isolated using CTAB method from 50 days old culture of 55 isolates of *Venturia* species. The PCR amplification of three gene-fragments of rDNA-ITS, β-tubulin and EF1α was carried out successfully in 55 *Venturia* species samples using PCR based gene specific markers. The amplified products were custom-sequenced followed by sequence analysis. One hundred sixty-two sequences were submitted to GenBank using National Centre of Biotechnology Information (NCBI) submission tool Banklt and Accession numbers obtained (MK463637-MK751644). New sequence data of three genes (rDNA-ITS, β-tubulin and EF1α) of *Venturia* species (*V. inaequalis, V. pyrina, V. carpophila, C. humile. V. populina* and *V. crataegi*) was used for phylogenetic analysis along with the known sequences retrieved from GenBank at NCBI. Phylogenetic analysis carried out at individual and combined gene levels using MEGA 6.02 resulted into four cladograms. Phylogenetic analysis based on ITS sequences led to grouping of sixty-four isolates of *Venturia* species in two main clades. Clade I composed of all the isolates of *V. carpophila, V. crataegi, C. humile, V. populina* and *V. pyrina*, and clade II comprised of *V. inaequalis* isolates. *Venturia* species proved to be monophyletic group supported by bootstrap values and could not be differentiated on the basis of ITS region. However, these indicated that despite adaptation to diverse hosts during the course of evolution, *Venturia* genus has maintained its ITS region. Phylogenetic analysis based on β-tubulin grouped them into six different clades *viz*., Clade I composed

of *V. inaequalis* isolates, Clade II accommodated *V. carpophila*, Clade III was comprised of *V. crataegi*, Clade IV contained *V. pyrina* and, Clade V and VI accommodated *V. populina* isolates. The phylogenetic analysis based on EF1α gene sequence grouped them into five different clades based on species level *viz*., Clade I comprised of *V. carpophila* isolates, Clade II of *V. crataegi*, Clade III of *V. populina*, Clade IV of *V. pyrina* and Clade V of *V. inaequalis*, and also lead to little splitting on the basis of host origin. Therefore, it may be concluded that β-tubulin and EF1α are ideal DNA barcodes and could be used in taxonomic and phylogenetic studies in *Venturia* species for the presence of inter-isolate polymorphisms. Multigene phylogenetic analysis of *Venturia* species using concatenated data set of rDNA-ITS, β-tubulin and EF1α gene sequences grouped them into nine clades. Clade I comprised of *V. crataegi* isolates, Clade II of *V. populina*, Clade III of *V. pyrina*, Clade IV of *V. inaequalis* and Clade V to Clade IX comprised of *V. carpophila* isolates. In case of clade IV, dichotomy of *V. inaequalis* was observed, which reflects closely co-evolution between *V. inaequalis* and its respective hosts, and species boundaries within genus also existed. In the multigene phylogenetic analysis using not only rDNA-ITS, but also β-tubulin and EF1α gene sequence data, produced a tree topology with high reliability and the relationships between *Venturia* spp. was also clarified. Therefore, the results further supported early taxonomic separation of *Venturia* species. Consequently, identification and quantification of inter-specific and intra-specific heterogeneity levels are of real importance to ensure reliability as a desirable phylogenetic marker and DNA barcode for species identification.

The polymorphism level observed among the *Venturia* species on the basis of rDNA-ITS, β-tubulin and EF1α gene sequence data was very high. The number of haplotypes for each group of isolates collected from different hosts ranged from 2 to 11 and haplotype diversity ranged from 0.60 to 1.00, indicating that the *Venturia* species has maintained high level of genetic diversity. Estimates of intra-isolate nucleotide diversities in terms of nucleotide differences in sequences (π)

and watterson's (θ) varied from 0.001 to 0.087 and 0.001 to 0.108, respectively, which indicate low nucleotide diversity owing to conserved regions within the three loci *viz*., ITS, β-tubulin, EF1α gene sequences due to only few variable sites among different isolates suggesting the occurrence of little population sub-divisions within the species. Tajima's D test and Ramos-Onsins and Rozas'R_2 test showed evidence of non-neutral evolution suggesting a demographic force affecting all the loci simultaneously, i.e. the population expansion and the effective population size were responsible for deviation from neutral expectations. The history of recombination events in *Venturia* species ranging between 1 and 21 and segregating sites ranged between 1 and 346 in the test loci suggesting the lack of association among alleles and breakage of ancestral polymorphism thus contributing maximum to the evolution of these loci.

The divergence values ranged between 0.011 and 0.165 which revealed that the heterogeneity in genomic and life cycle changes were associated with emergence and spread of these isolates. Fixed difference and shared polymorphism ranged from 0 to 86 and 0 to 12 respectively, indicating the different levels of fixated polymorphism and shared polymorphism among the isolates collected from different hosts. This also showed a long history of reproductive isolation, following secondary contact between *Venturia* species. Genetic differentiation parameters like pair wise fixation index (F_{ST}) and gene flow (Nm) ranged from 0.134 to 0.904 and 0.01 to 1.61, respectively which indicated a low level of gene flow and high genetic differentiation among the *Venturia* species. The corresponding Kst and Snn values also revealed the existence of reproductive barriers and non-random matting populations in different species.

Analysis of molecular variation performed based on ITS, β-tubulin, EF1α and concatenated data set in different populations revealed that maximum percentage of variation was distributed among populations than within population, thereby indicating the presence of separate regional populations. This was also

supported by corresponding high F_{ST} values (0.71-0.93) indicating a high divergence between populations. Estimation of F_{ST} was very high at species level. The high genetic differentiation values reveal a strong genetic differentiation among *Venturia* species. It indicated that there is a low gene flow between the *Venturia* species because of strong selection against immigrants. The Principal component analysis (PCoA) separated isolates belonging to different *Venturia* species into distinct clusters with the first and second coordinate representing 24.75-68.37 and 10.84 -25.04 per cent of total variation. The clustering algorithm in STRUCTURE software also supported the distinction of clusters at K = 5-9, which resulted into five genetic populations and the samples tending to have separate clusters. The distinction between these clusters was also visible in the results of PCoA.

A total of 37 primers were designed for *Venturia* species using Primer3 plus software. *In silico* screening of primers showed a single PCR product in most of the cases, however most of the primers were cross transferable. Only three primer pairs out of 37, Vi1, Pr3 and C4 were found specific to *V. inaequalis*, *V. pyrina*, *V. carpophila,* respectively. Moreover, the GC content of *Venturia* species ranges from 38 to 47.4 per cent depicting stability in the genome that ultimately led to designing of species-specific primers. Pooled DNA from five different *Venturia* species when used for PCR amplification with three species-specific primers singly and in combination (multiplex PCR) successfully produced desired bands in heterogenic DNA of *Venturia* species in multiplex PCR. *Venturia inaequalis* produced 250bp band, *V. pyrina* produced 200bp band and *V. carpophila* produced 300bp band. Further, the lowest detection limits of the developed primers were calculated using real-time PCR. The Vi1 and Pr3 primers, specific to *V. inaequalis* and *V. pyrina*, could detect 0.01ng of pathogen DNA whereas the C4 primer specific to *V. carpophila* could detect 0.001ng of DNA concentration. Besides, these primers could be successfully used for *in planta*

detection of pathogen; as the primers could detect very less amount of target DNA, it can detect the pathogen early even before the symptoms appear.

CONCLUSION

Venturia species is one of the model fungal pathogens that reached the state of ultimate invasion process with well distributed and established geographical populations, and displays high genetic diversity, sexual reproduction and population expansion. Phylogenetic studies based on ITS, β-tubulin and EF1α genes provided the first comprehensive study of *Venturia* species. Phylogeny based on ITS region supported *Venturia* species to be the monophyletic group, and isolates with different host origin couldn't be differentiated. However, β-tubulin and Elf1α gene combination were found to be ideal DNA barcodes which could be used in taxonomic and phylogenetic studies of *Venturia* species for the presence of inter-isolate polymorphisms. Multigene phylogeny based on ITS, β-tubulin and EF1α genes provided a tree topology with high reliability and clarified relationship between *Venturia* species. Consequently, it is evident from the present study that identification and quantification of inter-specific and intra-specific heterogeneity levels is of real importance to ensure the reliability as a desirable phylogenetic marker and DNA barcodes for identification of species in *Venturia*. For molecular based detection of *Venturia* species, three species-specific primers (Vi1, Pr3, C4) were designed for *V. inaequalis*, *V. pyrina* and *V. carpophila*. These species-specific markers were highly sensitive and reliable for early detection of pathogen even before symptoms appearance Different species of *Venturia* could be early detected simultaneously using species specific markers in multiplex PCR.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Dr. Amarjeet Singh, Prof. Plant Pathology, PAU Ludhiana during viva-voce examination held on 17-01-2020 have been incorporated in the manuscript entitled **"DNA barcoding of *Venturia* species infecting various host plants in Kashmir"** submitted by **Mr. Mohammad Saleem Dar (Regd. No. 2015-585-D).**

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