WITHDRAWN: Leaf Blight Caused by *Colletotrichum Fructicola* of Large Cardamom (*Amomum Subulatam* Roxb.) an Important Cash Crop Grown in Sikkim, India

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Abstract

Large cardamom (*Amommum subulatum* Roxb.) a high valued spice crop grown in Sikkim Himalaya is now facing a devastating leaf blight disease that has brought down the yield drastically. Present study was focused on identification of this major fungal pathogen based on the morphological and molecular characterization. During this study infected leaves of large cardamom with blighted appearance were collected from all the four districts of Sikkim. The pathogen was isolated using Potato Dextrose Agar (PDA) medium, incubated at 25°C. The mycelium was septate, hyaline, and 2-4 µm wide. The conidiospores were cylindrical with both ends rounded, sometimes oblong. Length and breadth were 11-12 µm and 3-4 µm, respectively. On the basis colony morphology, growth and microscopic observations, out of the total 48 samples studied *Colletotrichum* sp. was identified from 14 samples. Based on phylogenetic analysis of the ITS4, ITS5 and ApMAT genes and phenotypic characters (colony morphology, microscopic features) the isolate (No. LC05) isolated from the sample collected from the village Assam Linzey, East Sikkim showed 100% homology with *Colletotrichum fructicola* from NCBI database. The pathogenicity of *C. fructicola* was also confirmed during the study. The fungal culture has been deposited at the NFCCI-ARI, Pune with an accession number NFCCI 4542 and the sequences have been deposited in NCBI GenBank with accession number (ITS) MN710587, (ApMAT) MW348934 respectively. To the best of our knowledge this is the first report of *C. fructicola* causing blight disease of large cardamom. Also the finding is very important to improve the disease control strategies of this high valued cash crop.

Introduction

Large cardamom (*Amommum subulatum* Roxb.), originated and endemic to Sikkim, India, is a shade loving spice crop, grown under the forest trees with wide ranges of altitudes varying from 600 to 2400 m amsl. It is one of the main cash crops cultivated in Sikkim, Darjeeling district of West Bengal, and few other north eastern states in India. The spice crop has played an important role in the rural economy of Sikkim and other north eastern states in the country. More than 80% rural population in the region depends directly or indirectly on large cardamom cultivation for their livings.

But for the past two decades large cardamom cultivation in the region is passing through a critical phase. The mountain regions that once offered good climatic conditions like, favourable temperature, fertile soil, well distributed rain fall, humidity, etc. for this important crop, have turned into a breeding ground for pathogenic diseases. According to studies, various factors are responsible for the current situation including non-availability of quality planting materials, climate change, lack of irrigation facilities during dry season, open cultivation, inadequate nutrient management, unscientific methods of cultivation, diseases and pests, etc. As a result large cardamom plantation is disappearing in the region at an alarming rate[1,2,3,4]. Most importantly large cardamom production has gone down drastically due to various types of fungal diseases[3,4,5,6].

Reports reveal reduction of large cardamom plantation in the region is due to various rot diseases among which blight is highly destructive one in Sikkim and Darjeeling[1,3]. Initially due to this infection lesions are formed on leaves and pseudostem and then gradually affected pseudostems become necrotic resulting in lodging and death of tillers. Rot disease was first noticed in large cardamom in the region about three decades ago in late 1990s and it was described as some mysterious disease[2,7,8]. Then after more than two decades in 2013 a group of scientists reported that *Colletotrichum gloeosporioides* was responsible for the leaf blight[9], but microscopic or molecular characterizations were not found in the report. In 2020 *Curvularia eragrostidis* (family: Pleosporaceae) - a new pathogen has been identified causing leaf blight of large cardamom grown in Sikkim[4]. So far pathogens like *Fusarium* sp., *Rhizoctonia* sp. are also reported for other fungal diseases of large cardamom[2,3,4]. However, till date only few survey and scientific reports are available mainly describing the severity of the problem with no proper identification of causal organism. Hence, keeping the above mentioned facts in mind, the present study was focused to identify the pathogen based on the morphological and molecular characterization.

Results

Disease symptoms assessment in large cardamom

During the study 30-60% severity of leaf blight incidence has been recorded. The survey showed that disease condition is alarming and majority of the plantations in the state are affected with 40-45% blight incidence[10]. Affected leaves from diseased cardamom plantations with blight symptoms were characterized by sunken appearances with the necrotic areas and yellowish-brown irregular spots (Fig. 1A). It was observed that necrotic symptoms spread from the tip and sometimes from the leaf margin (Fig. 1B). Gradually leaves tend to dry out from the tip resulting drying of the whole plant (Fig. 1C), eventually causing death of plants. Fruiting and fruits were abnormal, immature with whitish brown seeds. Initially the disease was noticed with the commencement of pre-monsoon showers in the month of April-May which progressed rapidly during the rainy season. However, in some areas the incidence was started during winter months (January-March) (Unpublished observation by the group). The symptoms shown were water-soaked lesions which appeared either at margins or tips or any other point on the leaves which enlarged rapidly, coalesced and covered major portion or the entire leaf lamina giving a blight appearance. The advancing lesions were blackish brown in colour and margins give a yellow halo. In some cases, the entire lamina became yellowish with blight symptoms. The affected area became necrotic and dried up of plantation. It was also noticed that, among the six varieties grown in the state, *Varlangey, Swaney, Ramla* and *Ramsey* were found with blight symptoms. Further it was also quite prominent that new plantations in open field conditions were worst affected with the disease in comparison to those grown under canopy cover. Among the four districts studied, west district of Sikkim was found to be the severely affected with the disease and with 33-45% disease incidence[4,10].

Growth pattern of fungal pathogen in different medium

Initially isolation and growth of the fungi on PDA at 25 C was found normal. Physico-morphological characteristics of the isolate were observed on five different solid media, namely Potato Dextrose Agar (PDA), Potato Carrot Agar (PCA), Sabouraud Dextrose Agar (SDA), V8 Juice Agar (VJA), and Czapek Dox Agar (CDA) (Hi-media, India). Interestingly maximum mean colony diameter 61.33±0.88 mm was recorded when isolate was grown on PDA. The second best growth was observed when isolate was grown on CDA with mean colony diameter 59.6±0.69 mm. This was followed by SDA and VJA with 53.33±0.88 mm and 52.33±0.88 mm colony diameter respectively. Least colony growth was recorded (47±0.58 mm) on PCA medium (Table 1). The culture produced white colony with an orange shade at the centre (Fig. 1D). From the back side of the culture plates orange pigmentation was observed and this character was common on all the solid media used (Fig. 1E). Mycelium of the isolate was septate, hyaline, and 2-4 µm wide. The conidiospores were cylindrical with both ends rounded and sometimes oblong (Fig. 1F). Length and breadth were 11-12 µm and 3-4 µm, respectively (Table 2). On the basis of colony morphology, microscopic features, and molecular characterization of the isolate (LC05) was identified as *Colletotrichum gloeosporioides* Complex (NFCCI 4542) by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune (Table 2). Further based on phylogenetic analysis of the ITS4, ITS5 and ApMAT genes the isolate was confirmed as *C. fructicola*, causing leaf blight to large cardamom in this region is the first detailed confirmatory report about the pathogen. The pathogenicity of *C. fructicola* was also confirmed during the study by causing similar infection on large cardamom in the experimental field by the isolated pathogen, then through re-isolation, identification, and confirmation of the fungus. On the basis physical characters, i.e., colony morphology, growth and microscopic observations, out of the total 48 samples collected and analysed during this study, *C. fructicola* was identified in 14 samples. Detail molecular analysis was carried with the isolate (LC05) isolated from the large cardamom variety *Varlangey* collected from the village Assam Linzey, East Sikkim. Remaining isolates are stored at 4°C.

Table 1
Effect of different media on the growth of *Colletotrichum fructicola*

PDA	SDA	CDA	VJA	PCA	LSD (*P = 0.05*)
61.33±0.88	53.33±0.88	59.6±0.69	52.33±0.88	47.00±0.58	2.42

Each treatment consisted of three replications. PDA = Potato Dextrose Agar, SDA = Sabouraud Dextrose Agar, CDA = Czapek Dox Agar, VJA = V8 Juice Agar, PCA = Potato Carrot Agar, Mean value (n =3) with ± Standard error (SE), LSD = Least significant difference (*P= 0.05*).

Table 2
Phenotypic and genotypic characters of the fungi isolate

Character	Description
Colony morphology on PDA	Fast growing, white colony with scanty mycelium and orange shade at the centre, reverse white, but turns orange on 5 days incubation at 25°C with maximum mean 61.33±0.88 mm colony diameter.
Microscopic features	Mycelium of the isolate was septet, hyaline, and 2-4 µm wide. The conidiophores were cylindrical with both ends rounded and sometimes oblong. Length and breadth of conidiophores were 11-12 µm and 3-4 µm, respectively.
Physiological characterization (pH and temperature)	Can endure wide range of temperature between 10°C to 40°C (optimum 25°C), and pH 5 to 10 (optimum 7)
Culture accession number: Nucleotide sequence Accession number :	NFCCI 4542 MW348934
Phylogenetic relationship :	
ITS4 & ITS5 (18S rRNA analysis) and top hits upon BLASTn analysis	Maximum (100%) similarity with *Colletotrichum* sp. (MN498096.1, MN498091.1, MN498092.1, *C. gloeosporioides* (MN427973.1) and *C. cereal* (MN486560.1).
Mat1-2-Apn2 intergenic spacer (*ApMAT*) partial gene analysis	*C. fructicola* (MN378631.1, MN378622.1, JQ899290.1, MT396891.1), *C. gloeosporioides* (MN036155.1)

Effect of temperature and pH on the growth

Temperature and pH of medium are very important physical factors influencing growth of fungi. In this study, it was observed that the pathogen could grow in a wide range of temperature between 10-35°C with varying growth rate. Least growth was observed at low temperature (10°C) and at high temperature (40°C), although the overall colony growth was suppressed significantly. No colony growth was observed at 5°C and 45°C temperature or beyond. Maximum colony growth (86.89 mm) was recorded at 25°C temperature. It was significantly high in comparison to that of other temperature tested, and was followed by 20°C with 29.6±0.50 mm colony growth (Fig. 2A).

Effect of different pH on the mycelium growth of the isolate is depicted in Fig. 2B. No mycelium growth was noticed when medium pH was maintained at 3, 11 and 13. Maximum growth of the mycelium (59.6±0.69 mm) was obtained at pH 7, followed by pH 5.5 with 47±0.58 mm colony growth. Minimum growth was recorded (22±0.88mm) at medium pH 10 (Fig. 2B). Previous studies also confirmed that 25°C is suitable for growth of *Colletotrichum* sp. (Table 2).

Pathogenicity and Multigene Phylogenetic Analysis

The pathogenicity of *C. fructicola* was also confirmed by causing infection to the healthy large cardamom plant(s) by the isolated pathogen (Fig. 3A-F), then re-isolation, identification and confirmation of the fungus. During the study first infection was noticed on inoculated plant after 17-18 days (Fig. 3B), showing wilting symptoms. Initially black blight appearance of the leaf margin along with the yellowish dot was noticed (Fig. 3C).

The phylogenetic analysis of ITS4, ITS5 and ApMAT gene sequences data of *Colletotrichum* sp. sequence isolated from leaf blight of large cardamom showed 100% sequence similarity with the top hits of *C. fructicola* sequence available in the public domain, were *C. fructicola* (MN378631.1, MN378622.1, JQ899290.1, MT396891.1), *C. gloeosporioides* (MN036155.1), *Colletotrichum* sp. (MN498096.1, MN498091.1, MN498092.1, *C. gloeosporioides* (MN427973.1) and *C. cereal* (MN486560.1) (Table 2). The phylogenetic tree was generated based on neighbour-joining method with the bootstrap support by taking 1,000 replicates and the resulted five major clad. The phylogenetic tree (Fig. 4) was rooted with sequence of *C. gloeosporioides* (MN036155.1) showed that the sequence share 100% homology with ITS and ApMAT sequences of *C. fructicola* (MT396891.1, JQ899290.1, MN378622.1, MN378631.1), *Colletotrichum* sp. (MN498091.1, MN498096.1, MN498092.1), *C. cereal* (MN486560.1), and *C. gloeosporioides* (MN427973.1, MF036155.1) with 100% bootstraps value. The present isolate *C. fructicola* MW348934.1 performed the close clade with *C. fructicola* (MT396891.1) with similarity coefficient. The ITS and ApMAT gene sequences of the isolate isolated from large cardamom and the combined tree supported by close clade with *C. fructicola* confirmed the identity of the isolate as *C. fructicola* (Fig. 4).

Further to complement the above result another ML phylogenies generated in this study with 29 isolates of *Colletotrichum* spp. comprising the isolate of the present study and 28 reference isolates taken from the GenBank (Table 3) gave identical sequences with 100%, and 99.98% bootstraps value (Fig. 5). Further, isolates were associated with infection of different hosts such as *Oxalis corniculata*, tea, Spider lily and Chinese bean tree etc. and different geographical origin (Fu et al., 2013; Guo et al., 2014; Sun et al., 2019; Ramos et al., 2019). The tree obtained showed that present isolate *C. fructicola* (MW348934.1) performed the close clade with *C. fructicola* (NR144783.1, MK470079.1, KJ954624.1, JQ807845.1). On the contrary, *C. fructicola* (MW348934.1) also showed some distinct clade with other strains available in the database. The strains such as *C. gloeosporioides* (KC913204.1) and *C. gloeosporioides* (MK639180.1) causing leaf diseases in tea and *Oxalis corniculata*, respectively showed a distinct clade with the present strain. The analysis revealed that the homology rate between some different species, such as *C. cereal* (EU859957.1) and *C. lineola* (HQ731491.1) was higher than that along with different host accessions of the same species, suggesting that nucleotide variation among all the *Colletotrichum* spp. was not only related to geographical origin but also host associated (Fig. 5).

Table 3
Isolates of *Colletotrichum* spp. causing leaf disease in different host and geographical origin used in this study for phylogenic analysis

GenBank accession number	Causal organism	Host plant	Disease symptoms	Geographical origin	Source	Gene	Reference
MW348934	*C. fructicola*	Large cardamom	Leaf blight	Sikkim, India	This study	MAT 1-2-1	Present isolate
KX013517.1	*C. fructicola*	Annona leaves	Anthracnose	Brazil	Genbank	ITS	Costa *et al.* 2016[50]
MH721408.1	*C. fructicola*	*Callerya speciosa*	Leaf spot	China	GenBank	ITS	Shu *et al.* 2020[51]
NR144783.1	*C. fructicola*	Cherry	Leaf disease	Thailand	GenBank	ITS	Prihastuti *et al.* 2009[27]
JQ807845.1	*C. fructicola*	Chilli	Anthracnose	India	GenBank	MAT-1-2-1	Sharma and Shenoy 2014[52]
KY007499.1	*C. fructicola*	Papaya	Anthracnose	India	GenBank	GAPDH	Saini *et al.* 2017[53]
MK344200.1	*C. fructicola*	Coffee	Leaf blight disease	Hainan	GenBank	ITS	Cao *et al.* 2019[54]
MN378631.1	*C. fructicola*	Plum	Anthracnose	China	GenBank	MAT-1-2-1, Mat1-2-Apn2	Unpublished
MK114103.1	*C. fructicola*	Apple	Bitter rot	Europe	GenBank	ITS	Nodet *et al.* 201955
MH463893.1	*C. fructicola*	Tea	Leaf disease	China	GenBank	GAPDH	Lu *et al.* 2018[56]
KX859090.1	*C. fructicola*	Apple	Leaf spot	Uruguay	GenBank	ACT	Casanova *et al.* 2017[37]
MK673858.1	*C. gloeosporioides*	*Hymenocallis littoralis* (Spider lily)	Leaf spot	China	GenBank	ITS	Sun et al. 2019 [57]
JN165746.1	*C. gloeosporioides*	Chinese bean tree	Leaf spot	China	GenBank	ACT, CAL, CHS, GAPDH, TUB	Fu et al. 2013[58]
MK639180.1	*C. gloeosporioides*	*Oxalis corniculata*	Leaf with anthracnose	Brazil	GenBank	ITS	Ramos et al. 2019[59]
KC913204.1	*C. gloeosporioides*	Yellow Mountain fuzz tip, a cultivar of tea, (*Camellia sinensis* (L.) Kuntze)	Leaf Brown blight	China	GenBank	ITS	Guo et al. 2014[60]

Source: GenBank[49]

GenBank accession number	Causal organism	Host plant	Disease symptoms	Geographical origin	Source	Gene	Reference
MK251597.1	*C. graminicola*	Maize	Leaf blight	China	GenBank	ITS	Duan et al. 2019[61]
MN520417.1	*C. fioriniae*	*Mahonia aquifolium*	Leaf blight	Italy	GenBank	ITS, ACT, and TUB	Garibaldi et al. 2020[62]
HQ731491.1	*C. lineola*	*Swallow worts* (Cynanchum)	Leaf blight	Russia	GenBank	ITS	Berner et al. 2011[63]
EU859957.1	*C. cereal*	Creeping bent grass	Leaf blight	Mississippi and Alabama	GenBank	ITS	Young et al. 2008[64]
EU000060.1	*C. linicola*	Bindweed	Leaf spot	Turkey	GenBank	ITS	Tunali et al. 2008[65]
MT193091.1	*C. fructicola*	Mandevilla x amabilis	Leaf Spot	China	GenBank	ITS, ACT, and TUB	Sun et al. 2020[66]
MH728827.1	*C. fructicola*	Capsicum annuum	Anthracnose	Asia	GenBank	ITS, ACT, and TUB	de Silva et al. 2019[67]
KJ954624.1	*C. fructicola*	*Camellia sinensis* and other *Camellia* spp	Leaf spot disease	China	GenBank	ACT, ApMat, CAL, GAPDH, GS, ITS, TUB2	Liu et al. 2015[68]
MK470079.1	*C. fructicola*	Citrus	Anthracnose on citrus stems, leaves, and fruit	Australia	GenBank	ITS, gapdh, act, tub2, ApMat, gs, and chs-1	Wang et al. 2021[69]
JQ807838.1	*C. fructicola*	chilli	Anthracnose	India	GenBank	ITS, ACT, and TUB	Sharma and Shenoy 2014[52]
MT921589.1	*C. fructicola*	Pear Fruit in	Anthracnose	Korea	GenBank	ITS, ACT, TUB, ApMAT	Choi and Park 2021[70]
LC469138.1	*C. fructicola*	Shine Muscat (Vitis vinifera L.) is the grape cultivar	Anthracnose	Korea	GenBank	TUB, ApMAT	Lim et al. 2020[20]

Source: GenBank[49]

GenBank accession number	Causal organism	Host plant	Disease symptoms	Geographical origin	Source	Gene	Reference
MT492125.1	*C.* chrysophilum	Apple	Bitter rot	Mid-Atlantic region of the United States	GenBank	ITS	Martin et al. 2021[71]
MT434623.1	*C. siamense*	Areca palm, rubber tree, and coffee	Leaf spot disease	China	GenBank	ITS	**Cao** et al. 2021[72]

Source: GenBank[49]

Hence, based on the colony characteristics like growth, colour, morphology, cylindrical conidia with round ends and application of species-specific PCR using ITS and AM-R primers confirm the pathogen as *C. fructicola* isolated from diseased large cardamom grown in Sikkim. The fungal culture has been deposited at the NFCCI-ARI, Pune with an accession number NFCCI 4542 and the sequences have been deposited in NCBI GenBank with accession number (ITS) MN710587, (ApMAT) MW348934.1. To the best of our knowledge, this is the first report and confirmatory molecular characterization of *C. fructicola* causing blight disease of large cardamom in Sikkim as a major pathogen. The study mirrored the importance of future research on this pathogen to alleviate the risk to the large cardamom cultivation in Sikkim.

Discussion

Large cardamom, one of the most important cash crop grown in Sikkim. It is a spice crop of significant economic importance and contributes substantially to the rural economy of this region and also plays an important role in the environment[11]. In 2017-18 national large cardamom production was 5906 tonnes and Sikkim produced 4862 tonnes which was 82% of total national production. But during the last few decades, gradual decline has been observed in its production in the region[4]. There are numerous factors responsible for the decline in overall productivity, among which the fungal infestation is considered as most important one. Although there is no proper identification of the causes or causal organisms and proper controlling measures so far.

Critical analysis of the situation with time line reveals that in Sikkim drastic change in large cardamom production was recorded in the late 1990s where the crop started to decline rapidly. Reason was unknown at that time or thereafter for about two decades. In 2013 Saju et al.[9] analyzed the situation in detail and reported the *leaf blight* caused by *C. gleosporiodies*. In the plant pathology *blight* caused by *C. gleosporiodies* is considered to be the most devastating disease that results in huge crop loss and even decline in plant population for numerous crops worldwide. The situation in Sikkim is same in case of large cardamom plantation.

C. gloeosporioides is a very important pathogen known for several diseased in a wide range of host plants worldwide[12,13,14]. Basically *C. gloeosporioides* is a complex consists of 22 species and one subspecies which includes *C. asianu*m, *C. cordylinicola*, *C. fructicola*, *C. gloeosporioides*, *C. horii*, *C. kahawae* subsp. *kahawae*, *C. musae*, *C. nupharicola*, *C. psidii*, *C. siamense*, *C. theobromicola*, *C. tropicale*, *C. xanthorrhoeae*, *C. aenigma*, *C. aeschynomenes*, *C. alatae*, *C. alienum*, *C. aotearoa*, *C. clidemiae*, *C. kahawae* subsp. *ciggaro*, *C. salsolae*, and *C. ti*, plus nom. nov. *C. queenslandicum*[15,16]. It is quite difficult to differentiate all these species based on morphological characteristics, i.e., Colony morphology, color, growth rate, shape and size of conidia, etc., as because phenotypes of species varies under different environmental conditions[14,15,17]. While critically examined it was observed that several *Colletotrichum* species were described as *C. gloeosporioides* because of the similarity in physical characters such as round ends and cylindrical conidia, etc.[15,18,19,20,21]. Afterward while studied further, it was observed that several synonymized *C. gloeosporioides* species were recognized as members of the *C. acutatum* and *C. boninense* species complexes[13,15,22].

It is known that fungi exhibit variable morphological characters under the influence of different nutritional composition of the medium[23,24,25,26]. In the present study, colony characters and growth of the pathogen was found to be varied on different media, temperature, pH of the media. When *C. fructicola* was cultured on most conventional PDA medium for fungal isolates, normal growth was obtained. Czapek dox agar medium was found compatible and obtained maximum growth as compared with PDA although statistically non-significant (Table 1). Similar response on growth of *C. fructicola* isolated from diseased Cherry leaf was reported and PDA was found to be the best suited medium[27]. In an earlier study the colony colour of the *C. fructicola* was found to be blackish on water agar medium, white colony was observed when cultured on Richards, oat meal agar and PDA while grayish white on Czapek Dox Agar medium[24,26,28]. The present study colony colour was found to be green and white cottony colour masses in front view and white colour when observed from inverted side of the plate on PDA medium.

The temperature is another important physical environmental factors that affect the growth of fungi in culture. It is important for regulating the growth and reproduction of fungi. The present study revealed that the good growth of *C. fructicola* was observed in a wide range of temperature (20 to 35°C) and maximum growth was recorded at 25°C. The present study is supported by the finding stated that the maximum growth of *Colletotrichum* sp. was found at 25°C[28,29]. Likewise the *C. gloeosporioides* isolated from mango, almond and avocado plants was found to have maximum growth at 25°C[23,24,28,29]. Like temperature, pH also plays an important role in the growth of fungus. In the present study, the maximum growth was recorded at pH 5.5 to 7. Similarly pH also plays an important role in the growth of *Colletotrichum* sp. and in the present investigation the maximum growth of the pathogen was recorded at pH 5.5 to 7. The finding is similar to the results reported earlier, where it was reported that the growth of *C. gloeosporioides* was maximum at pH 7 followed by pH 6[28,30,31].

During the study, the symptoms caused by the pathogen on the plant based bio assay were similar to the symptoms appeared in the field. The symptoms appeared to be blight one, with the necrotic spots along with the die back symptoms, could be observed on the 16th to 18th days of inoculation. Tip burn of leaves was also noticed along with the blighted appearance from the leaf margins. It is known that different species of *Colletotrichum* causes disease in many crops with necrotic tissue and sunken appearance symptoms[31,32,33]. Colony morphology with faster growth, cylindrical conidia with round ends are typical identifying characters for the *C. fructicola*[15,27,34,35] and similar morphology and the microscopic features were observed in the present study.

The important purpose of the study was to confirm that blight of large cardamom in Sikkim by using molecular markers and pathogenecity assay. Colony characteristics like growth, colour, morphology, cylindrical conidia with round ends and application of ITS4, ITS5 genes sequences *C. gloesporioides* was initially described to be associated with leaf blight of large cardamom. Further amplification of ApMAT gene sequences and phylogenic analysis confirmed the pathogen as *C. fructicola* isolated from diseased large cardamom grown in Sikkim. In 2009 the *C. fructicola* – a filamentous Ascomycota of the *C. gloeosporioides* complex was proposed as a pathogen for the first time using polyphasic approach[27]. Now it is identified as a dominant pathogen of apple in Uruguay[36,37], strawberry in Japan and Korea[38,39], sandy pear (*Pyrus pyrifolia*) in southern China[40], etc. Here it should be mentioned that the *C. gloeosporioides*, previously reported to cause different rot, anthracnose disease in numerous plants and crops worldwide, then reclassified and identified as *C. fructicola*, a member of *C. gloeosporioides* complex[27,35]. Moreover, in Korea at first causal agent of strawberry anthracnose was reported as *C. gloeosporioides* now it is identified as *C. fructicola*[35,38]. In the present study initially the causal organism was characterised as *C. gloeosporioides* complex. An additional ApMAT gene analysis confirmed the pathogen as *C. fructicola* (MW348934). To the best of our knowledge, this is the first confirmatory molecular characterization of *C. fructicola* causing blight disease and serious damage to large cardamom as a major pathogen. Hope, this will enable effective targeted measures to protect this important crop – life line for rural hill economy of north east India and Sikkim in particular.

Material And Methods

Collection of sample

A survey was conducted during the month of May - July 2017 in different large cardamom growing areas of Sikkim with the purpose to find the infestation of the fungal diseases. Periodic observations at one month interval for a year were recorded in a cardamom field at the village Assam Linzey, East Sikkim, to understand the dynamics of cyclic epidemics of blights with seasonal variation. The infection index or disease severity was recorded based on the severity scale as described by Sharma and Kolte (1994)[41], with modification[10] (Table 4).

Table 4
Disease descriptive scale

Rating	Description	Infection stage
0	No visible symptom observed on leaf (Disease free healthy plants)	stage –0
2	Visible brown spot with <1% leaf area affected (Infection)	stage –I
4	Brown sunken spots with 1-10% leaf area affected (Infection)	stage –II
6	Brown spots with 11-25% leaf area affected (Moderately infected)	stage –III
8	Spot seen with 26-50% leaf area affected (Highly infected)	stage –IV
10	>51% leaf area affected with circular to irregular spots (Severely infected)	stage –V
Source: Thesis submitted by Gurung K. to the Sikkim University by Gurung K. (2020)[10]		

$$\text{Disease severity or Infection index (\%)} = \frac{\text{Sum of all the disease rating}}{\text{Total number of rating X Maximum disease grade}} \text{ X } 100$$

For sampling, infected leaves of large cardamom showing the blighted appearance was considered as a sample for the study were collected following random sampling method. Samples were collected from 48 villages of all the four districts of Sikkim. Infected leaves were cut from the diseased plants with sterilized scissors, kept in air tight sample bags with proper numbering and brought to the laboratory and stored at 4°C until further study. For this study field survey, sample collection and on field pathogenicity trial were performed in accordance with the Forest (Conservation) Act 1980, Biological Diversity Act 2002, Govt. of Sikkim, Dept. of Forest Environment & Wild Life Management, Gangtok, India.

Isolation of fungus

The fungus was isolated from the lesions of leaves of large cardamom showing the symptoms of blight following the protocol as described by previous researchers[4,42]. Infected large cardamom leaves showing symptoms of blight were washed with running tap water to remove the dirt from leaf surface. Then it was cut into small pieces (10x10 mm^2), treated with mercuric chloride (0.01%) for 2 mins. followed by washing with sterilized distilled water four times to remove the traces of mercuric chloride. The leaf segments of the diseased area were again cut in to small pieces (5x5 mm^2) aseptically and placed on solidified potato dextrose agar (PDA, Hi-media, India) in each Petri plate (Borosil, India 17cm×100cm), with about 30 ml of autoclaved PDA supplemented with streptomycin sulfate (50mg/l) to control bacterial growth and incubated at 25°C. The mycelium growth was observed after two weeks of incubation of infected leaf pieces on PDA. The growing edges of fungal hyphae, developed from the tissues, were then transferred aseptically to the fresh PDA. Germinated spores were picked up with a sterilized needle and transferred to PDA. The process was repeated rapidly six times at 7 days interval to obtain a pure culture. The pure cultures were then transferred to PDA medium slants in 60 ml screw capped culture tubes containing 25ml medium each, incubated at 25°C for one day, and then kept at 4°C for storage for further use.

Characterization and identification of the fungus

Growth characteristics on solid media

The morphological characterization of the isolate (AsLES-2a) was done on the basis of colony features like size, colour, pigmentation, and growth response on different growth medium. In the study five different types of media, i.e., Potato Dextrose Agar (PDA), Potato Carrot Agar (PCA), Sabouraud Dextrose Agar (SDA), V8 Juice Agar (VJA), and Czapek Dox Agar (CDA) (Hi-media, India) were tested for the morphological study of the isolate. Colony diameter was measured everyday at 2 pm for 7 days and growth rate was calculated as mean daily growth (mm per day). After 7 days, colony diameter, colony colour (front and back view) and pigmentation of culture were also recorded.

Influence of abiotic factors on growth of the fungus

The isolate was also grown in different temperatures and pH gradients to understand its physiological tolerance to these abiotic factors. The temperature and pH tolerance was checked by incubating the isolate at different range of temperature (5°C to 45°C) and pH (3 to 13) on PDA plates for five days. For the entire experiment, three replicates for each treatment were taken into consideration for tabulating the data.

Phenotypic and genotypic identification

The fungal morphology was studied macroscopically. Samples were taken from the fungal isolates grown for 72 h on PDA at 25°C, mounted on microscopic glass slides, stained with lacto phenol cotton blue dye. To determine the vegetative and reproductive structures, fungal isolate was grown for 72 h on PDA. A small portion of the mycelium was taken from the freshly grown isolate and mounted on microscopic glass slides, stained with lacto phenol cotton blue and observed under compound microscope (Labophot-2, Nikon, Japan). Microscopic structures i.e., hyphae, conidia, conidiophores and arrangement of spores were characterized, measured and photographed. For identification and confirmation, isolate was sent to the Agharkar Research Institute Fungus Culture Collection (ARIFCC), Pune, India.

Phenotypic and genotypic identification

Five days old fresh culture of the test fungus grown on PDA at 25°C was taken for the pathogenicity trial following the Koch's postulates[45]. The inoculation trial was performed on healthy large cardamom plants. Study was conducted at normal ambient temperature, varying between 25-28°C under poly house condition at 6th mile, Gangtok (latitude: 25.85° N, longitude: 93.77° E and altitude: 1120 m amsl). Inoculum of conidial suspension was prepared by agitating the fresh fungal lawn with the help of sterile glass rod in sterilized distilled water. Then the suspension was sieved with a clean muslin cloth to remove agar, mycelium debris and clean suspension with conidial spores was obtained. The spore count was determined by using hemocytometer. Suspension with 1×10^4 ml^{-1} conidial spores was used for inoculation of large cardamom plants in the nursery. Then healthy large cardamom plants were sprayed with the conidial suspension. All the treatment, for the experiment was performed in triplicates. The control plants were sprayed with distilled water and were kept at an isolation distance to avoid any kind of contamination. After 20 days of inoculation, infected leaves with blighted spots were taken for the further study towards pathogenicity confirmation. Procedures followed for pathogen isolation, analysis, etc. were same as mentioned in the previous section.

Multigene Phylogenetic Analysis

Fungal genomic DNA was extracted by the Hi-PurA^{TM} kit as per the manufacturer protocol (Himedia, India). The Isolate was analyzed based on internal transcribed spacer (ITS) gene and Apn2-Mat1-2 intergenic spacer and partial mating type (Mat1-2) gene (ApMAT) sequences data. Internal Transcribed Spacer genes ITS4 and ITS5 were PCR amplified using two universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Fu et al., 2013; Guo et al., 2014; Duan et al., 2019). The Apn2-Mat1-2 intergenic spacer and partial mating type (Mat1-2) gene (ApMAT) was also

amplified using primers AM-R (5'-CCAGAAATACACCGAACTTGC-3')[44]. PCR was carried out in a total volume of 50 µl using 2.5 µl each dNTPs, 2 µl $MgCl_2$, 2 µl template DNA, 1 µl each primer (ITS4 and ITS5), 1 µl Taq DNA polymerase, and 33 µl nuclease free water (Himedia). Reactions were performed in the Master cycler gradient (Eppendorf, India) with following reaction conditions; 95°C for 5 min. for initial denaturation followed by 30 cycle of 95°C for 30 sec., 55°C for 1 min, 72°C for 1 min and the final extension at 72°C for 10 minutes. The PCR product was purified with the Hi-PurA™ PCR clean up kit (Himedia, India) and sequenced by ABI Applied Biosystems (35000 Genetic Analyzer, SeqGen, Inc. 1725 Del Amo BlvdTorrance, CA 9001, USA) using each of ITS4, ITS5 and AM-R. The sequence was assembled and aligned using Coden-Code Aligner software and compared using NCBI BLASTn tool[45]. The phylogenetic tree was created by using the Maximum Likelihood (ML) method and Kimura 2-parameter model[46] using MEGA X software[47]. Clade stability of the phylogenetic tree was according to bootstrap analysis with 1000 replicates[48]. The sequences of ITS region and *ApMAT* partial gene of the present isolate was analyzed with *Colletotrichum* spp. sequences deposited in the GenBank[49]. The identified sequence was submitted to the NCBI gene bank for the isolate accession number. Further, in order to added clarification, another phylogenic tree was constructed using the nucleotide sequences of the present isolate isolated from the large cardamom causing leaf blight and reference sequences of *Colletotrichum* spp. retrieved from the GenBank reported causing similar types of disease (blight, spot and anthracnose etc.) in various host plants and geographical origin (Table 3).

Statistical Analysis

Least significant differences (LSD) was carried out using the methods of Snedecor and Cochran 1968[73].

Declarations

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Conflict of Interest

The authors declare no conflict of interest.

Author contributions

K.G. performed field survey, experiments and prepared the manuscript. K.D. performed some experiments and provided inputs for manuscript writing. B.S.B. performed some experiments on molecular characterization and provided inputs for manuscript writing. A.P. guided experiments, contributed in editing the manuscript. L.S. guided field survey, contributed in editing the manuscript. N.B. as a supervisor, identified the research problem, conceptualized, designed the study, analyzed results and contributed toward the final shape of the manuscript.

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Figures

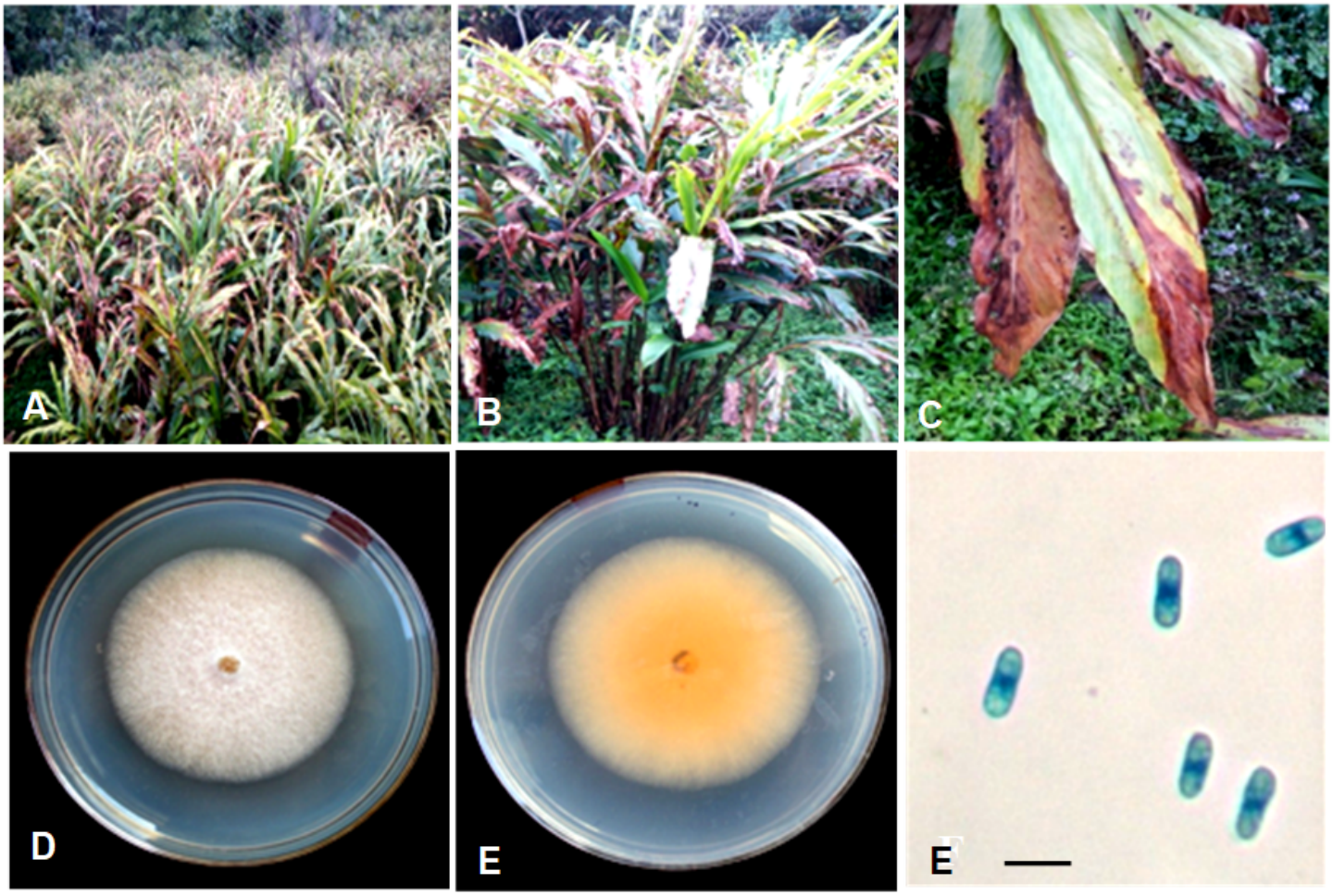

Figure 1

Leaf blight of Large Cardamom (Amomum subalatum Roxb.) var. Varlangey in Sikkim caused by Colletotrichum fructicola. (A) Leaf blight affected large cardamom field, (B) Close view of infected plant, (C-D) Leaf blight symptoms in large cardamom caused by C. fructicola, (E-F) In vitro growth of C. fructicola (E- front view, F- view from reverse side), (G) Mycelium and conidiospores of C. fructicola (Bar = 10 µm).

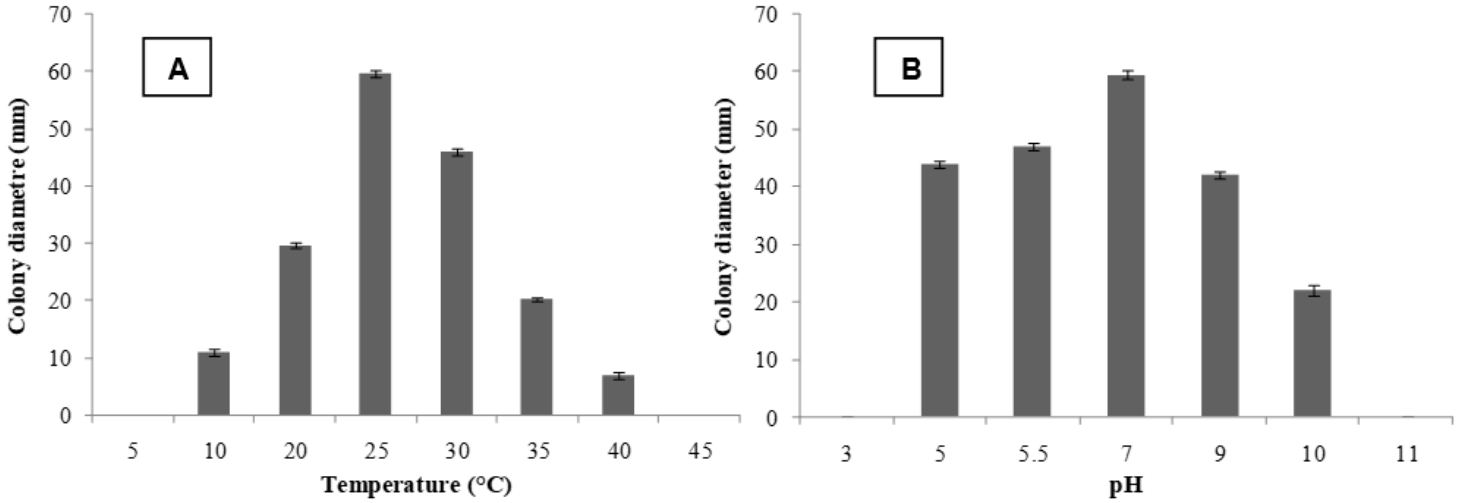

Figure 2

(A) The effect of temperature and (B) pH on the growth of the Colletotrichum fructicola on PDA medium.

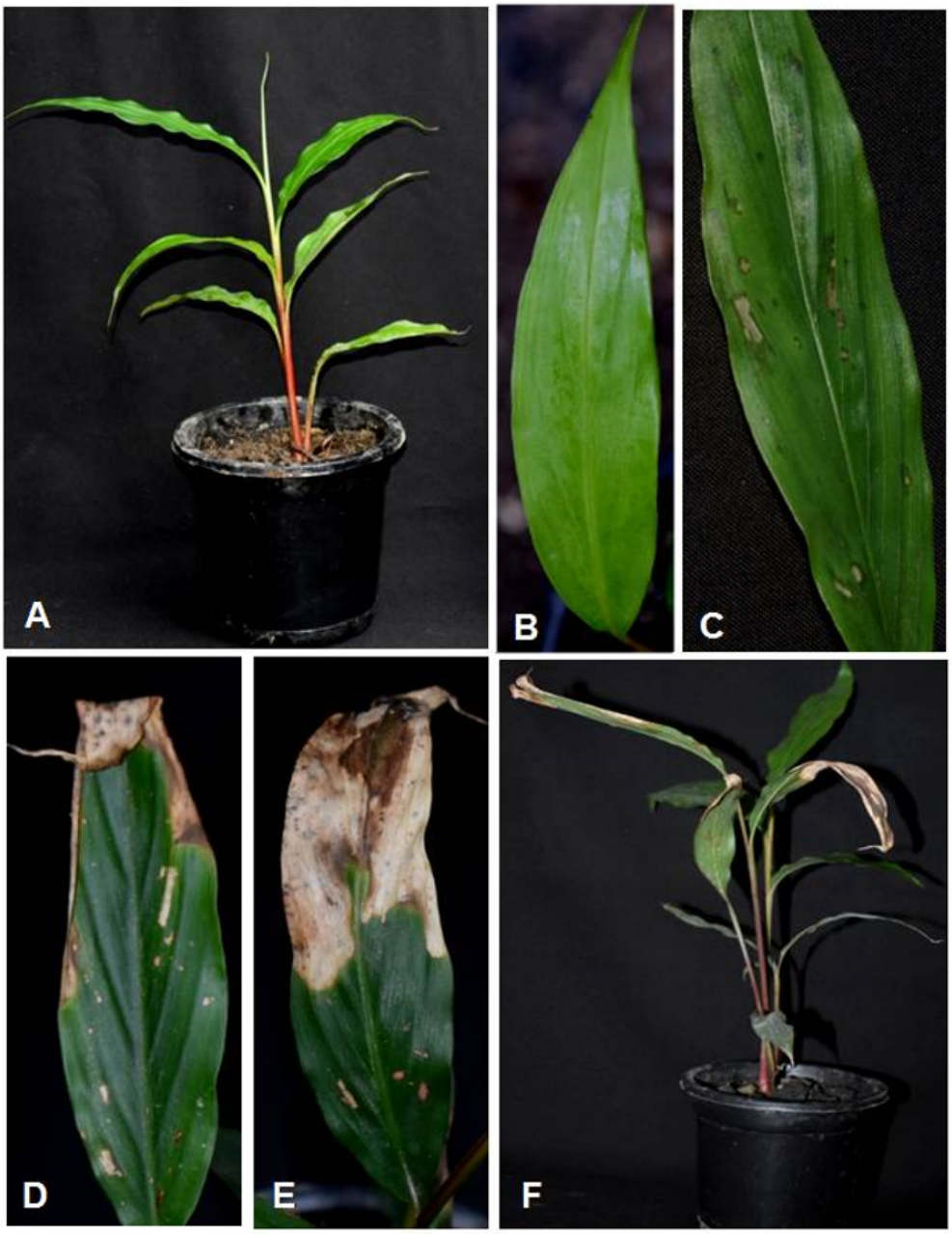

Figure 3

The experiment on confirmation of pathogenicity of Colletotrichum fructicola on healthy large cardamom plant(s) in the nursery: (A) Control plant (Disease free large cardamom plant grown in the nursery , (B) The leaf after inoculation of the inoculum (Spores of Colletotrichum fructicola), (C) Initiation of distinct disease symptoms on the 18th day of inoculation, (D) Tip blight observed on 28th day of the diseases development on the leaf, (E) Enhanced leaf blight covering almost half of the leaf at 33rd day of inoculation, (F) The infected diseased plant.

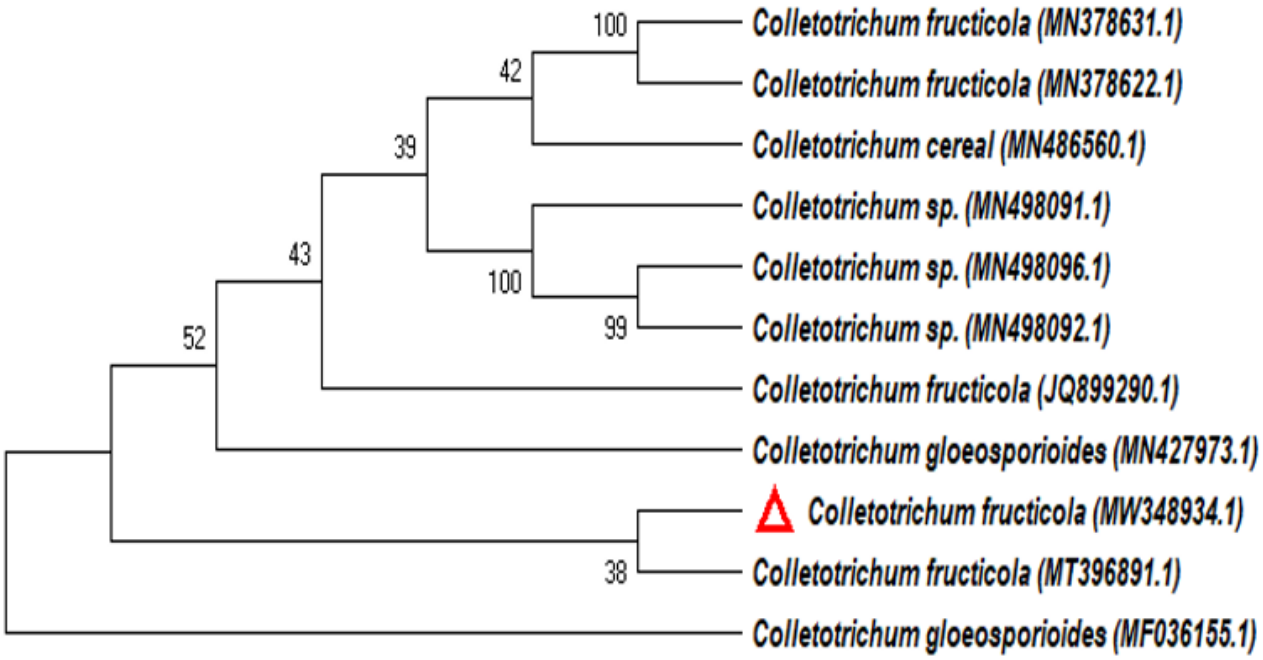

Figure 4

Phylogenetic tree built using neighbor-joining (NJ) methods with ITS, ApMAT nucleotide sequence of Colletotrichum fructicola isolated from large cardamom (Amomum subulatam Roxb.) and other ITS sequences Colletotrichum spp. of plants species using MEGA X. Bootstrap values are indicated for each branch divergence of 1,000 replicates.

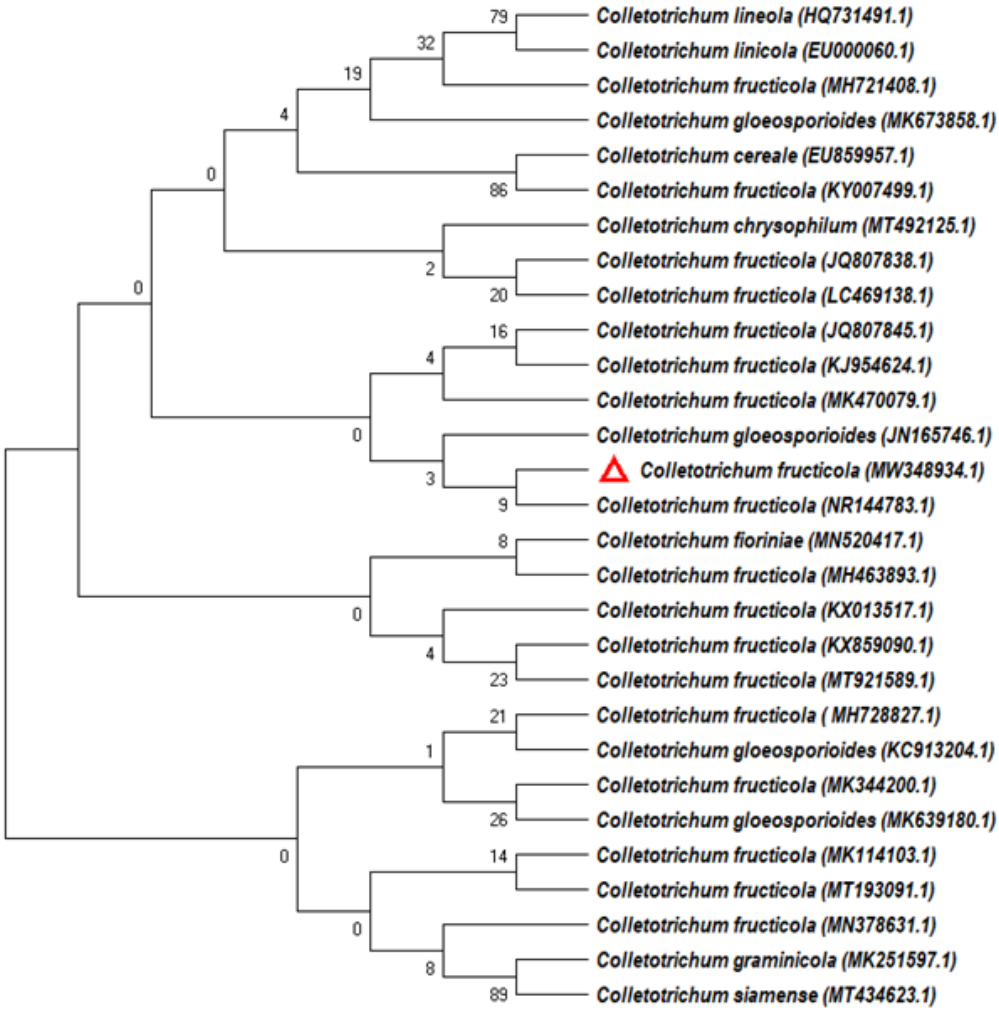

Figure 5

Phylogenetic tree built using neighbor-joining (NJ) methods with ITS, ApMAT nucleotide sequence of Colletotrichum fructicola isolated from large cardamom (Amomum subulatam Roxb.) and ten (28) other reference ITS/ ApMAT sequences of Colletotrichum spp. retrieved from the GenBank reported causing similar types of leaf disease (blight, spot and anthracnose etc.) in various host plants and geographical origin using MEGA X. Bootstrap values are indicated for each branch divergence of 1,000 replicates.