

# Original Research Article

## Morphological and Molecular Identification of *Alternaria* species found in different plants

### Abstract

*Alternaria* belongs to the family Pleosporaceae of the order Pleosporales in the subdivision of Ascomycotina. This fungus is characterized by its ability to produce a number of enzymes able to injure many types of crops. The symptoms of *Alternaria* blight on different agricultural crops such as cabbage, cauliflower, coriander, fenugreek, brinjal, onion, faba bean, dahlia, dracaena, hollyhock, carrot, marigold, tomato and wheat were observed. *Alternaria* produced distinctive "bull's eye" patterned leaf spots in almost all the infected plants. Out of total fourteen infected plant sample, nineteen *Alternaria* isolates were obtained. The observations were recorded from the seven day old culture for colony characteristics on PDA. Based on morphology 10 isolates of *Alternaria alternata*, two isolates *Alternaria tenuissima* and *Alternaria longipes* and one *Alternaria porri*, *Alternaria brassicae*, *Alternaria brassicicola* and *Alternaria* sp. were identified. *Alternaria* species grow rapidly and produced flat, downy to woolly grayish green to black colonies. All the isolates exhibited characteristics dark-coloured multicelled conidia with longitudinal and transverse septa (phaeo-dictyospores) and a beak or tapering apical cells. DNA Isolation of all isolates of *Alternaria* was carried out using CTAB method. Isolated DNA was subjected to amplification with ITS1 and ITS4 primers in a thermal cycler. The amplified products ranged from 580-600bp. These amplified products were sequenced and identify the species of different isolates BLASTn in NCBI online. All the sequences were published in NCBI public domain. The resulting sequences of all isolates were compared to other sequences in the GenBank as 99% identical. Genetic variability was conducted by phylogenetic analysis.

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### Introduction

*Alternaria* belongs to the family Pleosporaceae of the order Pleosporales in the subdivision of Ascomycotina. It is potentially one of the most ubiquitous, worldwide occurrence opportunistic pathogen affecting many cultivated crops in the field and during postharvest storage of fruit and vegetables. *Alternaria* species attacks all the plant parts and causes severe losses to crops in concern to yield and quality. The genus *Alternaria* was originally described by Nees von Esenbeck (1816) with *A. tenuis* as the type. The pathogen produces distinctive "bull's eye" patterned leaf spots. *Alternaria* species grow rapidly and producing flat, downy to woolly colonies, covered with grayish, short, aerial hyphae. The surface is grayish white at the beginning which later darkens and becomes greenish black or olive brown with a light border. *Alternaria* forms conidia that arise as protrusions of the protoplast through pores in the conidiophores cell wall. At the onset of conidial development, apex of the conidiophores thickens and a ring-shaped electron-transparent structure is deposited at the apical dome. The

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classical key taxonomic feature of the genus *Alternaria* is the production of large, multicellular, dark colored (melanized) conidia with longitudinal as well as transverse septa (phaeodictyospores). These conidia are broadest near the base and gradually taper to an elongated beak, providing a club-like appearance. They are produced in single or branched chains on short with erect conidiophores.

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Identification of some *Alternaria* species still offers considerable difficulties owing to their high variability and the polymorphism occurring even in pure culture. The conidial features distinguish *Alternaria* from the closely related genera *Ulocladium*, *Stemphylium*, *Embellisia* and *Nimbya*. The difficulty to distinguish between species or even isolates is demonstrated by the presence of nearly one thousand published species designations of *Alternaria* (Simmons, 1992). Over 300 species occurring worldwide have been described (Refai and Yazid, 2014). However, errors in the taxonomy of *Alternaria* species have arisen due to the variability of its morphological characters, which are not only affected by intrinsic factors but also by environmental conditions. Hypothetically, the difficulties in taxonomic classification of species within the genus *Alternaria* are partly due to the lack of sexual stages (Yu, 1992). The molecular taxonomy of the genus *Alternaria* has been recently reviewed. Molecular methods are faster, more sensitive, more stable and less dependent on external factors than morphological methods (Kim *et al.*, 2001). The characterization of fungal DNA has been employed to detect and differentiate fungi (Gil-Lamaignere *et al.*, 2003). Molecular approaches have been developed for the assessment of microbial diversity in complex communities as well (Gonzalez and Saiz-Jimenez, 2005). Methods based on DNA analysis can reveal fungal diversity in ecosystems, and offer the potential benefits of highly sensitive and rapid detection (Saad *et al.*, 2004). The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal repeat unit has become the primary genetic marker for molecular identification and other species-level pursuits in many groups of fungi (Seifert, 2009). The identification of fungi to species level has been based mostly on the use of variable ribosomal-DNA (rDNA) internally 3transcribed spacer (ITS) regions. The non-coding ITS region consisting of ITS1, 5.8s rDNA and ITS2, produced a highly sensitive assay as the target sequence for amplification, due to its high copy number in the fungal genome as part of tandemly repeated nuclear rDNA. ITS sequences from well-identified fruiting bodies are estimated to be available for <1% of the hypothesized number of fungal species (Nilsson *et al.*, 2005). These ITS regions benefit from a fast rate of evolution, which results in higher variation in sequence between closely related species, in comparison with the more conserved coding regions of the *rRNA* genes. As a consequence, the DNA sequences in the ITS region generally provide greater taxonomic resolution than those from coding regions (Lord *et al.*, 2002; Anderson *et al.*, 2003). Additionally, the DNA sequences in the ITS region are highly variable and might serve as markers for taxonomically more distant groups.

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## Materials and methods

### Sample collection

Fourteen *Alternaria* infected plant samples expressing disease symptoms were collected from adjoining areas of JNKVV campus, Jabalpur.

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cite the plants

### Media preparation: Potato dextrose agar media (PDA)

Peeled potatoes ~200g were sliced into small pieces and boiled in 700ml of distilled water up to softening. Extract was filtered through fine strainer and Dextrose (20g) added into it. Volume of the medium adjusted to 1l and agar (15g) was added and sterilized in autoclave at 121°C for 20 min. for making slants, required quantity of medium distributed in test tubes, sealed with nonabsorbent cotton and tilted till get solid.

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### Isolation of pathogen from host plant

Isolation of *Alternaria* spp. were undertaken from infected plant sample of coriander, cabbage, cauliflower, fenugreek, brinjal, onion, dracaena, fababean, hollyhock, dahlia, marigold, carrot, tomato and wheat. Collected samples were rinsed with sterile water and infected tissues along with adjacent small unaffected tissues were cut into small pieces (2–3 mm length) by using flame-sterilized forceps. Then tissues were further transferred into sterile Petri dishes containing 0.1% mercuric chloride solution for a period of 20 to 30 seconds in laminar air flow and then rinsed three times with sterilized distilled water to ensure freedom from traces of mercuric chloride. Pre-sterilized blotting paper sheet were used to remove excess moisture and then sterilized pieces are aseptically transferred to. Petri dishes containing potato dextrose agar media and kept in BOD incubator at  $25 \pm 2^\circ\text{C}$  temperature with  $85 \pm 5\%$  relative humidity. After growth of fungal mycelium and sporulation, the fungus was under taken for initial identification. Identification of the selected isolates was again confirmed through slide culture method. Seven days old freshly isolated cultures of *Alternaria* spp. on PDA were used for investigations on various aspects.

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### Single spore isolation for pure culture of *Alternaria* spp.

The cultures were purified by single spore technique. A dilute spore suspension was poured on plain agar in Petri dishes and the spores were allowed to settle down on the agar surface. The amount of suspension was so adjusted as to form a very thin layer over the surface of the agar. Spores, which settled quite apart from each other were selected under the microscope, marked and encircled on the back of the bottom of the Petri dish with a glass marking pencil. They were lifted along with agar and transferred to Petri dishes already poured with Potato dextrose agar medium. After the proper growth of the transferred single spore, regular sub-culturing was done to maintain and check further contamination. After purification, the culture was multiplied and maintained at 2% Potato dextrose agar slant in refrigerator at 4°C for further studies. The cultures were multiplied and renewed after every two months.

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All stock cultures were maintained on PDA slants and sub cultured. The slants were incubated at 25°C for 7 days and then stored at 4°C.

### Microscopic observation of the fungal isolates

The observations from the seven day old culture were recorded for colony characters on PDA. The morphological features like colour, branching and septation of the hyphae, conidiophores and conidia of the pathogens were studied under compound microscope. Conidia (100 per isolate) were examined under 10X magnification of microscope in laboratory. A slide culture technique was also used to observe the morphology of the fungi (Booth, 1977).

### Inoculation and incubation of broth culture

In each conical flask 100ml potato dextrose broth was poured, sealed with aluminum foil and autoclaved at 121°C for 20 min. The sterilized PDB were then allowed to cool down upto room temperature. Small pieces of culture was cut from petridish culture by using a sterilized cork

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borer and one piece was kept into each of the conical flasks by sterilized inoculation needle under laminar air flow. The inoculated flasks were cotton plugged and incubated in BOD incubator at  $25 \pm 2^\circ\text{C}$  with  $80 \pm 5\%$  RH for desired growth of the fungus.

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### Harvesting of mycelium

After seven days of incubation, fully grown mycelium mat was harvested. Drain out of medium from conical flask, then mycelium was washed with sterile water 3-4 times to removal of traces of medium and spores from mycelium mat. Invert conical flask on watch glass to drain out excess water from mycelium. Pre-sterilized blotting paper sheet was used to remove moisture from fungal mycelium. Dried mycelium is wrapped into aluminum foil and keeps it into  $-20^\circ\text{C}$  deep freezer for overnight.

### DNA extraction of fungal isolate

Isolation of fungal DNA was carried out by protocol described by Gontia-Mishra *et al.* (2013), with minor modification. Fresh fungal mycelium ~200 mg were carefully grind in liquid nitrogen and crushed with a mortar pestle to a fine powder and 800  $\mu\text{L}$  of extraction buffer (0.1 M Tris-HCl pH 8, 10 mM EDTA pH 8, 2.5 M NaCl, 3.5% CTAB, 150  $\mu\text{L}$  of 20 mg/mL proteinase K) was added. The mixture was vortexed at high speed on a homogenizer (Spinix, Tarsons, India) for 5 min. The samples were placed in a water bath at  $65^\circ\text{C}$  for 30 min. The samples were then centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of phenol-chloroform-isoamylalcohol (25:24:1) was mixed. The samples were again centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was again collected and equal volume of chloroform-iso-amyl-alcohol (24:1) was mixed. Samples were again centrifuged. Supernatant was collected and equal volume of ice-cold isopropanol was added. Samples were incubated at  $-20^\circ\text{C}$  for 1 to 2 h. The samples were centrifuged for 15 min at 13,000 rpm to pellet the DNA. Supernatant was decanted and DNA pellet was washed with 800  $\mu\text{L}$  of 70% ethanol. DNA pellet was air-dried and dissolved in 200  $\mu\text{L}$  TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). 5  $\mu\text{L}$  RNase A (20 mg/ml) was added to DNA samples, mixed and incubated at  $37^\circ\text{C}$  for 1h. DNA was recovered and air-dried. DNA was reconstituted in TE buffer for further use for PCR amplification.

### PCR amplification using ITS primers

The ITS region was amplified by PCR with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCRs were carried out in a 20  $\mu\text{L}$  reaction volume that contained the DNA templates (2  $\mu\text{L}$  of purified DNA), 18  $\mu\text{L}$  Master Mix, contains of 2  $\mu\text{L}$  10xPCR buffer, 2.4  $\mu\text{L}$  of  $\text{MgCl}_2$ , 0.4  $\mu\text{L}$  of dNTPs, 2  $\mu\text{L}$  of primers ITS1 and ITS4, 0.2  $\mu\text{L}$  of *Taq* DNA polymerase and rest of the volume nuclease free water.

Amplification was performed in Agilent Master Cycler Gradient. The optimal annealing temperature for primers ITS1/ITS4 was  $55^\circ\text{C}$ . The PCR amplification conditions included: initial denaturation at  $94^\circ\text{C}$  for 5 min and 35 cycles: Denaturation at  $94^\circ\text{C}$  for 30 sec, annealing at  $55-57^\circ\text{C}$  for 60 sec followed by extension step at  $72^\circ\text{C}$  for 90 sec and a final extension at  $72^\circ\text{C}$  for 7 min. The resulting PCR products were visualized on 1.5% agarose gel containing ethidium bromide at 80 volts. After electrophoresis, the DNA bands were visualized by illumination under ultraviolet (UV) light.

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### DNA sequencing and phylogenetic tree construction

DNA sequencing of PCR products was done by the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977) with outsourcing of Chromous Biotech Pvt. Ltd., Bangalore. The sequence homology search was performed using the BLAST program through the internet server at the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/)). Sequences and accession numbers for compared isolates were retrieved from the GenBank database. Sequence pair distances among related and different fungal isolate were scored with the ClustalW program and phylogenetic tree analysis was performed with the MEGA-7 (Kumar *et al.*, 2016). The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973).

## Results

### Isolation and morphological characterization of *Alternaria* isolates from infected samples

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The present investigations were carried out to study morphological and molecular identification of *Alternaria* species from different plants. Experiments were conducted to study the cultural, morphological characters and molecular characterization of *Alternaria* spp. by using ITS primers. The symptoms of *Alternaria* blight on different crops viz., brinjal, tomato, cabbage, cauliflower, coriander, fenugreek, onion, fababean, dahlia, dracaena, hollyhock, wheat, marigold and carrot were studied during (2017-18). The diseases were appeared on plant parts including leaves, fruits and flower. The characteristic symptoms appeared on different plants were small, circular, brown necrotic spots all over the foliage, lower leaves, the spots gradually enlarged in size and later became irregular in shape, or remained circular with concentric rings or zones (Fig. 1).

The pathogen was successfully isolated from 14 different plant samples and pure culture was maintained on potato dextrose agar. Out of 14 infected plant samples, 19 *Alternaria* spp. isolates were obtained. The developing fungi was sub-cultured and maintained in pure form for further studies. The morphological characters of all isolates were included in Table 1. Similarly colony and spore morphology were presented in Fig. 2 and Fig. 3.

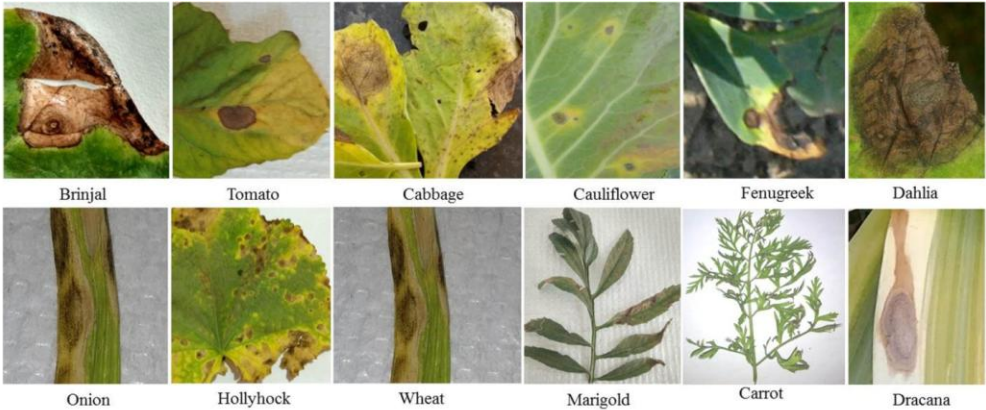
**Table 1. Morphological characteristics of *Alternaria* species**

SN	Host name	Colony characters in PDA	Size of conidia (µm)		Septation	Organisms
			Horizontal	Vertical		
1	Brinjal	Felty to woolly, fluffy mycelial growth and irregular margins.	35.7 × 5.23	3–8	0-2	<i>Alternaria arborescens</i>
2	Brinjal	Grayish green to sage green, fluffy mycelial growth and irregular margins.	45.16 × 12.42	1-3	2-5	<i>Alternaria alternata</i>
3	Tomato	Greenish gray colony, fluffy mycelial growth and irregular margins.	47.16 × 13.49	1-3	13.49	<i>Alternaria alternata</i>
4	Tomato	Greenish gray to dark olivaceous gray, fluffy mycelial growth and	44 × 12	3-8	1-2	<i>Alternaria longipes</i>

		irregular margins.				
5	Cabbage	Smoky grayish, fluffy mycelial growth and irregular margins	67.12 × 12.26	1-5	1- 6	<i>Alternaria brassicicola</i>
6	Cauliflower	Dark Olive gray to grayish green, fluffy mycelia growth and irregular margins.	89 × 29	11-15	6-14	<i>Alternaria alternata</i>
7	Coriander	Dark olive to iron gray, fluffy mycelial growth and irregular margins.	47.56 × 8.95	3-7	1-4	<i>Alternaria sp.</i>
8	Fenugreek	Greenish gray, fluffy mycelial growth and irregular margins.	47.10 × 13.47	2-7	1- 3	<i>Alternaria alternata</i>
9	Onion	Olive gray, fluffy Mycelium with zonation and smooth margins.	87 × 20.13	3-14	0-8	<i>Alternaria porri</i>
10	Fababean	Greenish brown colony, fluffy mycelial growth and irregular margins.	42.12 × 11.45	2- 6	1- 3	<i>Alternaria alternata</i>
11	Dahlia	Brownish green colony, fluffy mycelial growth	43.12 × 11.9	1-3	2-10	<i>Alternaria alternata</i>
12	Dracaena	Pea to sage green colony, fluffy mycelial growth and irregular margins.	46.15 × 16.87	1-3	2-5	<i>Alternaria alternata</i>
13	Hollyhock	Dark Greenish colony, fluffy mycelial growth and irregular margins.	48.19 × 12.22	2- 7	1- 3	<i>Alternaria alternata</i>
14	Wheat	Greenish gray colony, fluffy mycelial growth and irregular margins.	47.14 × 13.46	1-3	2-8	<i>Alternaria alternata</i>
15	Marigold	Sage green to pea green, fluffy mycelial growth and irregular margins.	47.56 × 12.45	1-3	2-10	<i>Alternaria alternata</i>
16	Marigold	Olive buff to dark grayish green colony, fluffy mycelial growth with Zonation and smooth margins.	45.12 × 9.32	2-11	0-10	<i>Alternaria tenuissima</i>
17	Carrot	Greenish gray to olivaceous black, fluffy mycelial growth with zonation and irregular margins.	62.7 × 9.4	3-6	0-2	<i>Alternaria tenuissima</i>
18	Carrot	Dark greenish colony, fluffy mycelial growth and irregular margins.	47.13 × 13.54	1-3	2-10	<i>Alternaria alternata</i>

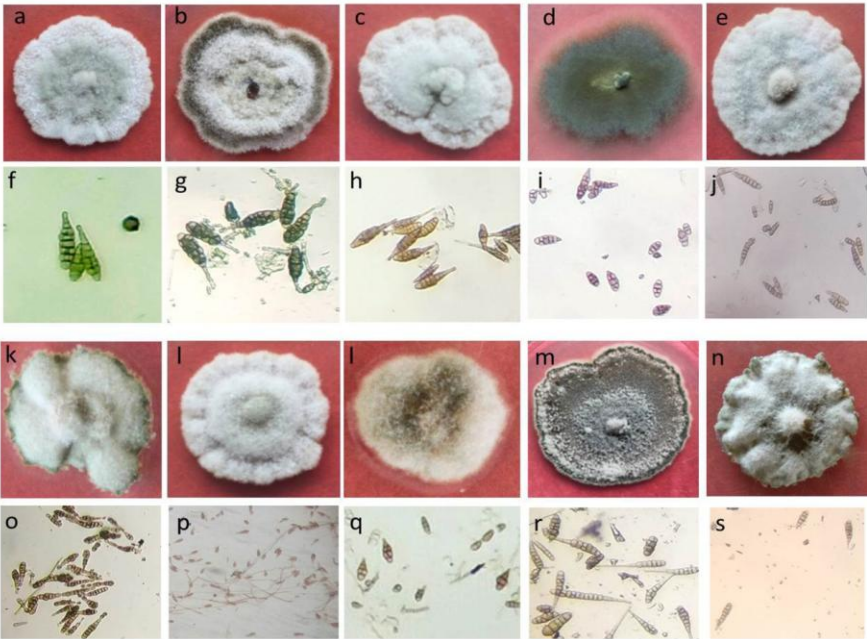


19	Carrot	Pale black to brown, fluffy mycelial growth and irregular margins.	18-20 × 10	3-5	0-2	<i>Alternaria longipes</i>
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**Fig. 1** Symptoms of *Alternaria* Blight on different plants.

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**Fig. 2** Morphology of colony and conidia of isolate no. 1-10



**Fig. 3** Morphology of colony and conidia of isolate no 11-19

#### Molecular identification of different *Alternaria* isolates

ITS regions have been widely used for the identification and differentiation of species as well as in taxonomic, phylogenetic and genetic diversity studies. ITS sequences have been reported as useful for discriminating between different species of fungi. PCR amplified products ranged from (580-600 bp) amplicon using primer ITS1 (F) - ITS4(R). Total 19 PCR products of different isolates were sequenced and resulted in range from 508-528 bp.

#### Similarity based species identification through BLAST tool

Each sequence was subjected to online BLASTn in NCBI ([www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/)) for analysis to identify the species of different *Alternaria* isolates. The searches resolved the different sequences up to genus level. However, due to several hits at the same parameters of max score, total score, query cover, E value and identity %, one of the specie was not identified unambiguously. The closest matches were used to confirm the exact taxonomic group of each isolate. All isolates were determined to have identical sequences in the ITS region. The ITS region and morphological characterization were used to identify *Alternaria* spp. The resulting sequences of all isolates were compared to other sequences in the GenBank and were 99-100 % identical Table 2.

**Table 2.** Identification of *Alternaria* isolates associates with different plants based on ITS sequence

Host	fragment size (bp)	Accession no.	Related species	% Identity
Brinjal	528	MH356755	<i>Alternaria arborescens</i> KU645989	90
Brinjal	522	MH356762	<i>Alternaria alternata</i> MG991256	100

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Tomato	527	MH356763	<i>Alternaria alternata</i> MG025876	90
Tomato	525	MH356770	<i>Alternaria longipes</i> KJ722535	100
Cabbage	522	MH356771	<i>Alternaria brassicae</i> KF542552	100
Cauliflower	522	MH356772	<i>Alternaria brassicicola</i> KU204772	100
Coriander	526	MH356773	<i>Alternaria</i> sp. KT723004	100
Fenugreek	525	MH356764	<i>Alternaria alternata</i> KT192223	100
Onion	508	MH356766	<i>Alternaria porri</i> KY419560	100
Fababean	528	MH356756	<i>Alternaria alternata</i> MF927539	100
Dahlia	527	MH356757	<i>Alternaria alternata</i> KX783404	100
Dracaena	508	MH356758	<i>Alternaria alternata</i> MF422133	100
Hollyhock	525	MH356759	<i>Alternaria alternata</i> MG991256	90
Wheat	522	MH356765	<i>Alternaria alternata</i> MG596645	100
Marigold	522	MH356761	<i>Alternaria alternata</i> MF927539	100
Marigold	522	MH356768	<i>Alternaria tenuissima</i> KX783377	100
Carrot	525	MH356767	<i>Alternaria tenuissima</i> MG132062	100
Carrot	522	MH356760	<i>Alternaria alternata</i> MG025876	90
Carrot	522	MH356769	<i>Alternaria longipes</i> KJ722535	100

### Phylogenetic analysis

A database search for closely related fungi species were aligned using the ClustalW Program. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.03939477 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The phylogenetic tree was divided into major and minor group. Minor group has only *Alternaria* sp. and major group again subdivided into three groups as A, B and C. The subgroup 'A' represents total 12 isolates of *Alternaria alternata* and *A. tenuissima* were closely related. The subgroup 'B' represents total three isolates *A. porri*, *A. brassicicola* and *A. brassicae* whereas *A. brassicicola* and *A. brassicae* were shows 100% similarity. The subgroup 'C' represents total three isolates *A. longipes* and *A. arborescens* (Fig. 4).

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### Pairwise distances amongst sequences

The overall average pairwise distances calculated as 0.006. The pairwise distance amongst sequences ranges 0-0.035. The minimum pairwise distance calculated as 0.00 and maximum value 0.035 found between two pairs of first one *A. longipes* and *Alternaria* sp. and another pair of *A. arborescens* with *Alternaria* sp.

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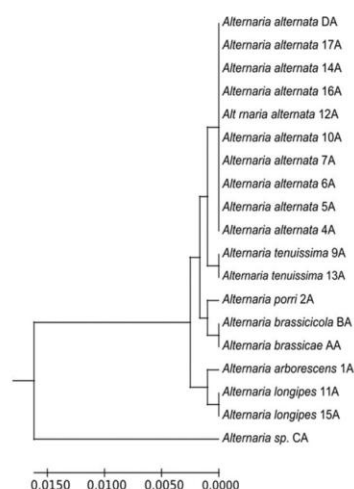
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### Estimation of divergence between sequences

The estimation of evolutionary divergence between all the sequences done to study the evolutionary changes in isolates. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 508 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). The results showed that the range of evolutionary divergence between all the sequences was 0-0.035. Minimum divergence (0) found between *A. brassicicola* and *A. brassicae*. The maximum value (0.035) found between two combinations of *A. longipes* and *A. arborescens* with *Alternaria* sp. most diverse isolates based on molecular study.

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**Fig. 4** Dendrogram representing phylogenetic affiliation of different isolates

## Discussion

All the ten isolates of *Alternaria alternata* isolated produced grayish to green colony. All the isolates produced light brown conidia with varied in shape from obclavate to mostly ellipsoidal, muriform having tapered apex. Conidia and beak length was measured about 42.12-48.19 x 11.9-17.37  $\mu\text{m}$  and 18.7 $\mu\text{m}$  to 23.81  $\mu\text{m}$ . The character agreed with the original descriptions given by Keissler (1912).

Morphological variability like colour of colony, margin of colony, colony growth and sporulation on PDA was studied among ten isolates of *Alternaria alternata* collected from different plant sources. The isolates found to have distinct morphological variability. Ramjegathesh and Ebenezer (2012) also studied the morphology of the ten isolates of *A. alternata* causing leaf blight disease of onion in Tamil Nadu and found conidia were muriform and light brown colour, length of their conidia was varied from 30.99 to 42.47  $\mu\text{m}$ . The width of the conidia was varied from 11.90 to 17.37  $\mu\text{m}$ . Generally, all the isolates produced both beaked and unbeaked conidia. The number of cells in each conidium varied from 2-9. All the isolates took 13-16 days for sporulation.

DNA sequencing has greatly enhanced the ability to accurately and reproducibly identify the fungi. In the present finding *Alternaria brassicicola* and *A. brassicae* were successfully identified on cole crop by using ITS1-ITS4 primer set. Similar experiment was conducted by Mishra et al. (2017) on morphological, biochemical and molecular characterization of *Alternaria brassicicola* and *A. brassicae* using a primer set, ABS28F and ABS28R as an internal diagnostic marker to differentiate and analyze *A. brassicicola* from *A. brassicae* and other fungi associated with leaves, stem and siliques of rapeseed-mustard.

The ITS marker could not distinguish the other *Alternaria* species analyzed and more markers, such as the mitochondrial small subunit (mt SSU) or the glyceraldehyde-3-phosphatedehydrogenase (gpd) gene used by Pryor and Bigelow (2003), are needed. In the present study one of the species was not identified by ITS region unambiguously.

In this study, *Alternaria* sp. isolated from onion was identified as *Alternaria porri* by primer set ITS1-ITS4. Likewise, molecular variability of *A. porri* by using seven RAPD primers and two ITS primers ITS1 and ITS4 in polymerase chain reaction (PCR) were also carried out by Mohsin *et al.* (2015).

Three isolates of *Alternaria* spp. were found from carrot and identified as *Alternaria tenuissima*, *Alternaria alternata* and *Alternaria longipes* using ITS1 and ITS4 primers. However, Tulek and Dolar (2015) reported *A. radicina*, *A. alternata*, *A. tenuissima* and *A. dauci* using ITS1 and ITS4 primer in carrot.

*Alternaria alternata* and *A. longipes* isolated from tomato leaves were identified by using ITS1 and ITS4 primers set. Earlier same experiments were conducted in tomato and identified *Alternaria alternata* using ITS1 and ITS4 primers by Xie *et al.* (2012).

In UPGMA phylogeny analysis, the isolates were grouped into two clusters. Minor group has only one *Alternaria* sp. and major group again subdivided into three sub-groups as A, B and C. The sub-group 'A' represents total 12 isolates of *A. alternata* and *A. tenuissima*. The subgroup 'B' represents total three isolates viz., *A. porri*, *A. brassicicola* and *A. brassicae*. The subgroup 'C' represents two isolates of *A. longipes* and one isolate of *A. arborescens*. Similar clustering patterns have also been reported in *Alternaria* isolates by Pryor and Gilbertson (2000) and Xie *et al.* (2012).

In this study, the ten *Alternaria alternata* isolates MH356764, MH356756, MH35657, MH356758, MH356759, MH356760, MH356761, MH356762 and MH356763 the maximum similarity observed with published ITS sequences of KT192223, MF927539, KX783404, MF422133, MG991256, MF927539. Similarly, Guo *et al.* (2004) also reported the three unknown isolates of *Alternaria* sp. and sequenced, results indicated the best match had high similarity with *A. alternata* with some ITS region of AF455537, AF455441, AF397233, AF397242, and AY160211.

Out of nineteen sequenced isolates two accession MH356767 and MH356768 showing similarity with *A. tenuissima* i.e. MG132062 and KX783377, two accession MH356769 and MH356770 matching with *A. longipes* i.e. KJ722535, KJ722535 and single accession MH356771 showing similarity with *A. brassicae* i.e. KF542552; MH356772 similar with *A. brassicicola* i.e. KU204772; MH356766 similar with *A. porri* KY419560; MH356755 similar with *A. arborescens* i.e. KU645989 and accession MH356773 similar with *Alternaria* sp. i.e. KT723004 were published in public domain NCBI.

## Conclusions

On the basis of morphological characters eight different species were identified viz., *Alternaria alternata*, *A. arborescens*, *A. porri*, *A. longipes*, *A. tenuissima*, *A. brassicicola*, *A. brassicae* and *Alternaria* sp. out of nineteen isolates. Out of nineteen isolates ten were *Alternaria alternata*, although they were different in their colony and conidial characters. For molecular characterization and identification ITS regions was used and the BLASTn analysis revealed sequence similarity of 99-100% with the sequences in database of NCBI GenBank for all the isolates. Phylogenetic analysis revealed minimum divergence between *A. brassicicola* and *A.*

Comment [M30]: italic

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*brassicae* and the maximum divergence between two isolates of *A. longipes* and *A. arborescens* with *Alternaria* sp.

#### COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

**Comment [M34]:** cite the perspectives of this study

#### References

- Anderson, I.C., Campbell, C.D. and Prosser, J.I. 2003. Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environmental Microbiology*. 5(1): 36-47.
- Booth, C. 1977. *Fusarium: laboratory guide to the identification of the major species*. Commonwealth Mycological Institute, Kew, Surrey, England.
- Gil- Laignere, C., Roilides, E., Hacker, J. and Muller, F.M.C. 2003. Molecular typing for fungi-a critical review of the possibilities and limitations of currently and future. *Clinical Microbiology and Infection*. 9: 172-185
- Gontia-Mishra, I., Tripathi, N. and Tiwari, S. 2013. A simple and rapid DNA extraction protocol for filamentous fungi efficient for molecular studies. *Indian Journal of Biotechnology*. 13: 536-539.
- Gonzalez, J.M. and Saiz- Jimenez, C. 2005. A simple fluorimetric method for the estimation of DNA-DNA relatedness between closely related microorganisms by thermal denaturation temperatures. *Extremophiles*. 9: 75-79.
- Keissler, K.V. 1912. *Alternaria alternata*. Beihefte zum botanischen Centralblatt. 29: 395-440.
- Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*. 33: 1870-1874.
- Lord, N.S., Kaplan, C.W., Shank, P., Kitts, C.L. and Elrod, S.L. 2002. Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: comparison of 18S and ITS ribosomal regions. *FEMS Microbiology Ecology*. 42: 327-337.
- Mishra, S., Singh, R., Kumar, S. and Kumar, S. 2017. Morphological, biochemical and molecular characterization of *Alternaria brassicicola* and *Alternaria brassicae*. *American Journal of Biochemistry and Molecular Biology*. 7: 53-64.
- Mohsin, A.M., Islam, M.D.R., Faruq, A.N. and Islam, M.N. 2015. The genetic variability of *Alternaria porri* in Bangladesh. *Journal of Plant Biology*. 60: 47-57.
- Nees von Esenbeck, C.G. 1816. Das system der pilze und schwamme. Wurzburg, In der Stahelschen buchhandlung, Germany 89 p.
- Nilsson, R.H. 2005. Approaching the taxonomic affiliation of unidentified sequences in public databases- an example from the mycorrhizal fungi. *Bioinformatics*. 6:178.
- Pryor, B.M. and Bigelow, D.M. 2003. Molecular characterization of *Embellisia* and *Nimbya* species and their relationship to *Alternaria*, *Ulocladium* and *Stemphylium*. *Mycologia*. 95(6): 1141-1154.

- Pryor, B.M. and Gilbertson, R.L. 2000. Phylogenetic relationships among *Alternaria* and related fungi based upon analysis of nuclear internal transcribed sequences and mitochondrial small subunit ribosomal DNA sequences. *Mycological Research*. 104: 1312-1321.
- Ramjegathesh, R. and Ebenezar, E.G. (2012) Morphological and physiological characters of *Alternaria alternata* causing leaf blight disease of onion. *International Journal of Plant Pathology*. 3(2): 34-44.
- Refai, M. and Yazid, H.A. 2014. Monograph on Dematiaceous fungi <https://www.research.net>.
- Saad, D.S., Kinsey, G.C., Kim, S. and Gaylardec, C.C. 2004. Extraction of genomic DNA from filamentous fungi in biofilms on water-based paint coatings. *International Biodeterioration and Biodegradation*. 54: 99-103.
- Sanger, F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *PNAS USA*. 74: 5463-5467.
- Seifert, K.A. 2009. Progress towards DNA barcoding of fungi. *Molecular Ecology Resources*. 9(1): 83-89.
- Simmons, E.G. 1992. *Alternaria* taxonomy: Current status, viewpoint and challenge. In: Chelkowski, J and Visconti, A. (eds.). *Alternaria: Biology, plant diseases and metabolites*. Amsterdam, Elsevier Science Publishers. pp 1-36.
- Tulek, S. and Dolar, F.S. 2015. Detection and identification of *Alternaria* species causing diseases of carrot in Ankara province, Turkey. *Scientific Papers - Series B, Horticulture*. pp 263-268.
- Xie, G., Tan, S. and Yu, L. 2012 Morphological and molecular identification of pathogenic fungal of post-harvest tomato fruit during storage. *African Journal of Microbiology Research*. 6(22): 4805-4809.
- Yu, S.H. 1992. Occurrence of *Alternaria* species in countries of the Far East and their taxonomy. In: Chelkowski J, Visconti A (eds) *Alternaria: Biology, Plant Diseases and Metabolites*. Elsevier, Amsterdam. pp 37-62.