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Morphological and Molecular Characterization of *Colletotrichum* Species Causing Anthracnose of Grape in India

Pallem Chowdappa* • **Gaddam Subhash Reddy** • **Ashok Kumar** • **B. Madhusudhan Rao** • **Ram D. Rawal**

Division of Plant Pathology, Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore -560 089, India

Corresponding author: * **pallem@iihr.ernet.in**

ABSTRACT

Anthracnose, caused by *Colletotrichum* sp., is a major disease causing severe yield losses. The objective of the present study was to understand diversity and distribution of *Colletotrichum* species prevalent in major grape-growing areas in India. A total of 30 isolates of *Colletotrichum*, collected from major grape growing regions of Andhra Pradesh, Karnataka and Maharashtra between 2003 and 2005, were characterized using morphological criteria, species-specific PCR and ITS sequencing. Three morphological types were distinguished based on colony growth rate and conidial morphology. Morphological type I (*C. gloeosporioides*) produced cylindrical conidia with both ends rounded and colony growth rates ranged from 11.21 to 12.08 mm/day. Morphological type II (*C. acutatum*) produced fusiform conidia with tapered/rounded ends and growth rate was 6.29 mm/day. Morphological type III (*C. acutatum*) had fusiform and elongated conidia with mostly tapered ends and colony growth rate was 4.66 mm/day. The universal primer pair ITS1 and ITS4 amplified, a 560-bp fragment from genomic DNA of isolates belonging to all three morphological types. Morphological type I isolates were identified as *C. gloeosporioides* on the basis of species-specific diagnostic PCR and rDNA-ITS sequences while morphological types II (isolate CG 5) and III (CG33) were identified as *C. acutatum*. All three morpho groups could also be distinguished based on ITS-RFLP profiles as generated by restriction enzyme, *Mbo*I. The present study, thus, clearly established *C. gloeosporioides* as the dominant pathogen (93.33%) with very limited occurrence of *C. acutatum* (6.66%). This is the first report of occurrence of *C. acutatum* and existence of diverse molecular groups among *C. gloeosporioides* and *C. acutatum* populations associated with grape anthracnose in India.

Keywords: diagnostic PCR, detection, internal transcribed spacer (ITS) region, phylogentic analysis, rDNA, species-specific primer, sequencing

Abbreviations: ITS, internal transcribed spacer; **rDNA**, ribosomal DNA

INTRODUCTION

Grape, a highly remunerative horticultural crop, grown in 57,800 ha with an annual production of 14,72,800 tones in India, nearly 94% of the area falls in Maharashtra, Karnataka, Tamil Nadu and Andhra Pradesh (Chadha 2006). Anthracnose is one of the major threats to the grape industry, not only causing damage to fruit but also affecting leaves and canes (Sawant and Sawant 2006). Symptoms include depressed black lesions on fruit and canes with pink spore masses in lesions and round to irregular necrotic lesions with shot holes on leaves. *Colletotrichum gloeosporioides* (Penz). Penz & Sacc) in Penz and *C. acutatum* J. H. Simmonds have been reported to cause anthracnose on grape in Australia and Portugal (Bernstein *et al.* 1995; Talhinhas *et al*. 2005; Steel and Greer 2006).

The genus *Colletotrichum* (telemorph *Glomerella*) is an important and wide spread group with more than 900 species reported from wide range of host, some of which are serious plant pathogens (Jeffries *et al.* 1990). But based on conidial morphology, the number of species has been reduced to 40 accepted taxa (Sutton 1992). Although the genus has been fairly well defined, the species concept is still complicated and a certain degree of confusion still exists (Freeman *et al*. 1998), especially between C. *gloeosporioides* and *acutatum* groups, which are considered cumulative species composed of diverse subpopulations (Sutton1992; Freeman *et al*. 1998, 2001; Vinnere *et al*. 2002; Talhinhas *et al*. 2005; Schiller *et al*. 2006). Traditionally, identification of species in the genus *Colletotrichum* has been based on the shape and size of conidia, appressorial, production of telemorphs, presence or absence of setae and colony morphology (Gunnell and Gubler 1992; Smith and Black 1990; Sutton 1992; Urena-Padilla *et al*. 2002), cross inoculation studies (Agostini *et al.* 1992; Hayden *et al*. 1994; Freeman *et al.* 1998; Kelemu *et al.* 1996) and physiology (Agostini *et al.* 1992). These criteria are highly variable and differentiation based on morphology, physiology and cross inoculation may be unreliable (Chakraborty *et al*. 1999; Sutton 1992). This situation had led to misidentification of species in numerous cases (Agostini *et al*. 1992; Sutton 1992; Timmer *et al*. 1998). For instance, a group of isolates from apple and peach originally identified as *G. cingulata* which were later classified as *C. acutatum* in California (Gunnell and Gubler 1992) and *C. gloeosporioides* isolates from citrus were also reclassified as *C. acutatum* in Florida (Agostini *et al.* 1992; Brown *et al*. 1996). The importance of differentiation of species is critical for control purposes. For instance, in mixed population of *C. acutatum* and *C. gloeosporioides*, sensitivity of one species to benomyl as against to other may cause a shift in population structure (Liyanage *et al*. 1993; Bernstein *et al*. 1995; Adaskaveg and Hartin 1997).

To resolve the doubts of classical species identification, various molecular techniques such as protein profiles (Dale *et al*. 1998), isozyme analysis (Bonde *et al*. 1991) and dsRNA patterns (Dale *et al*. 1988), RFLPs (Sreenivasaprasad *et al*. 1993; Bernstein *et al*. 1995; Munaut *et al*. 1998), RAPD (Alahakoon *et al.* 1994; Hayden *et al*. 1994; Munaut *et al*. 2002) and analysis of internal transcribed spacer (ITS) regions of rDNA (Sreenivasaprasad *et al*. 1992; Munaut *et al*. 1998) have been successfully employed. The use of PCR with species-specific primers, designed from the ITS region of rDNA, has been found to be most efficient and reliable

Received: 30 October, 2008. Accepted: 12 July, 2009.

Original Research Paper

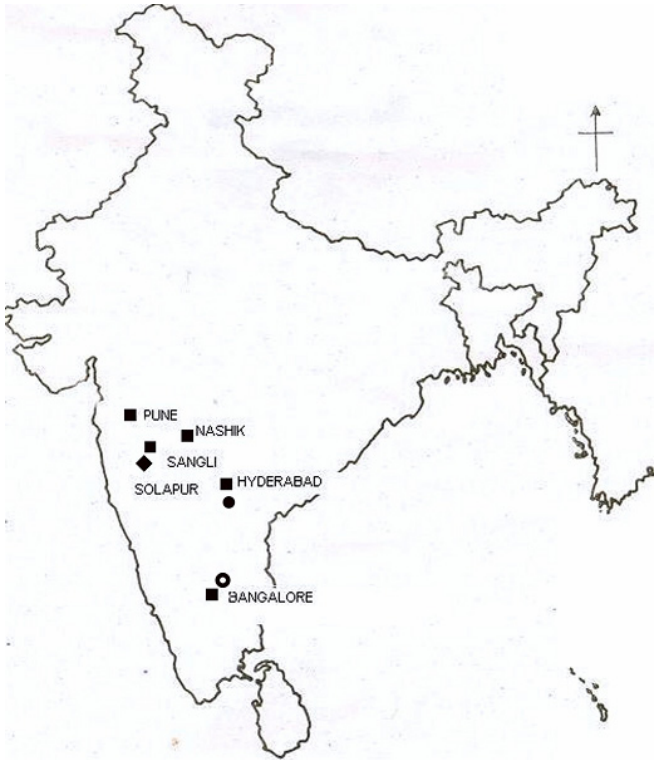

Fig. 1 Map of India depicting the origin of *Colletotrichum* spp. isolates. *C. acutatum* group A1 (♦), *C. acutatum* group A2 (O), *C. gloeosporioides* group G1 (■), *C. gloeosporioides* group G1 (●). Isolate details are given in **Table 1**.

tool for detection and differentiation of morphologically similar *Colletotrichum* species (Mills *et al*. 1992; Brown *et al*. 1996; Sreenivasaprasad *et al*. 1996; Freeman *et al*. 2000). To date, molecular characterization to classify *Colletotrichum* species that are responsible for anthracnose disease on grapes in India has not been done. The objective of the present study was to identify the *Colletotrichum* spp. associated with grape in India through morphology, validate the identification and to identify the sub groups within species, if any, using species-specific primers and ITS sequencing.

MATERIALS AND METHODS

Sampling of *Collectotrichum* spp. from grape

Grape samples such as leaves, canes and fruits showing typical symptoms of anthracnose were collected from major grape-growing regions of Andhra Pradesh, Karnataka and Maharashtra between 2003 and 2005 (**Fig. 1**). Diseased plant tissues were surface sterilized in 0.1% mercuric chloride for 1 min and plated onto potato dextrose agar (PDA) amended with streptomycin sulphate (20 mg/l) and incubated in dark at 24 ± 1°C for 3-7 days. Single conidia isolates were produced for each isolate. Cultures were maintained in PDA slants at 10°C by sub culturing at 4-week intervals. Three agar plugs (3 mm diam.) from actively growing cultures on PDA were suspended in 3 ml of 20% glycerol: 17% skimmed milk (1: 1) solution and stored at -80°C. The geographic origin of isolates is given in **Table 1**.

Colony and conidial morphology

The growth, the appearance of the colonies, and reproductive structures were recorded on PDA after 5 days of incubation at 25 ± 1°C in light. The size of 100 conidia was recorded at X400 magnification under a Zeiss bright field microscope using Axio Imager A1 software.

DNA extraction

Total fungal DNA was extracted from mycelia obtained from 100 ml of potato dextrose broth for 4-6 days at 25 ± 1°C. Mycelium was harvested from liquid cultures by filtration through Whatman No. 3 filter paper, blot dried and subsequently ground into a fine powder in liquid nitrogen. DNA was extracted from the frozen mycelial powder employing a slightly modified method of Raedor and Broda (1985) by incubating at 37°C for 10 min after the phenol: chloroform: isoamyl alcohol (25: 24: 1) precipitation. This was followed by precipitation with 0.54 volumes of isopropyl alcohol and centrifugation at 10,000 × *g* for 2 min. The DNA pellet was washed with 70% cold ethanol, dried at room temperature overnight (16 h) and then pellet was re-suspended in 30 µl 10 mM TE buffer (pH 8.0). DNA was stored at -20°C.

Table 1 Details of *Colletotrichum* isolates used in the study.

Isolate	Year of isolation	Geographic origin	Morpho group	Genetic group
CG 1	2004	Hessaraghatta, Karnataka	CG	CG I
CG 3	2004	Yelahanka, Karnataka	CG	CG I
CG 4	2004	Nagadasanahalli, Karnataka	CG	CG I
CG 5	2004	Rajanakunta, Karnataka	CA	CA I
CG 8	2004	Himmaguda, Andhra Pradesh	CG	CG II
CG 9	2004	Lemor, Andhra Pradesh	CG	CG II
CG 10	2004	Kandukur, Andhra Pradesh	CG	CG II
CG 13	2004	Akanapalle, Andhra Pradesh	CG	CG II
CG 15	2004	Maheswaram, Andhra Pradesh	CG	CG II
CG 17	2004	Dubbacherla, Andhra Pradesh	CG	CG II
CG 18	2004	Hyderabad, Andhra Pradesh	CG	CG I
CG 19	2004	Muddanahalli, Andhra Pradesh	CG	CG II
CG 20	2005	Vijayapura, Karnataka	CG	CG I
CG 24	2005	Pune, Maharastra	CG	CG I
CG 25	2005	Pune, Maharastra	CG	CG I
CG 26	2005	Narayangoan, Maharastra	CG	CG I
CG 27	2005	Theur, Maharastra	CG	CG I
CG 28	2005	Theur, Maharastra	CG	CG I
CG 29	2005	Narayangoan, Maharastra	CG	CG I
CG 30	2005	Nasik, Maharastra	CG	CG I
CG 31	2005	Hoveli, Maharastra	CG	CG I
CG 32	2005	Baramati, Maharastra	CG	CG I
CG 33	2005	Sangli, Maharastra	CA	CA II
CG 36	2005	Medichalla, Andhra Pradesh	CG	CG I
CG 37	2005	Medichalla, Andhra Pradesh	CG	CG I
CG 38	2005	Shamaripet, Andhra Pradesh	CG	CG I
CG 41	2005	Srinagar, Andhra Pradesh	CG	CG I
CG 42	2005	Mehaboobnagar, Andhra Pradesh	CG	CG I
CG 43	2005	Turkapalle, Andhra Pradesh	CG	CG I
CG 45	2005	Padmaja nagar, Andhra Pradesh	CG	CG I

CA: *C. acutatum*, CG: *C. gloeosporioides*

Diagnostic PCR with species-specific primers

The ITS region of rDNA of fungal isolates were amplified by PCR with universal primer pairs (White *et al*. 1990): ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), primer pair specific to *C. acutatum* (CaInt1, 5′-CCGGCCCCCACCACGGGGAC-3′ and CaInt2, 5′-GGGGAAGCCTCTCGCGG-3′) together with ITS 4 (expected fragment size of amplified product, 490 bp) and with a primer specific to *C. gloeosporioides* (CgInt, 5′-GGCCTCCCGCCTCCGGGCGG-3′) together with ITS4, expected to yield a 450-bp PCR product. PCR reactions (50 µl) were performed with primer pair ITS1/ITS4 and species-specific primer/ITS4 in an Eppendorf master cycler by 34 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 1.5 min with an initial denaturation of 5 min at 94°C before cycling and final extension of 5 min at 72°C after cycling. Amplified PCR products were separated in 2% agarose gels in Tris-Borate-EDTA (TBE) buffer and visualized under UV after staining with ethidium bromide (0.5 µg/ml). Primers, all the reagents and restriction enzymes used in this study were procured from Fermentas Life Sciences, Bangalore, India.

Table 2 Cultural and conidial morphology of *C. gloeosporioides* and *C. acutatum*.

Morphological group	№ of isolates	Colony colour	Growth rate/day (mm)	Conidial shape	Mean length (l) (µm)	Mean width (w) (µm)	l/w ratio
Morphological group I (*C. gloeosporioides*)	30	Cream/gray	11.21 ± 3.44	Cylindrical	12.60 ± 1.01	4.40 ± 0.47	2.86
Morphological group II (*C. acutatum*)	01	Pink/orange	6.29 ± 1.94	Fusiform	11.37 ± 1.32	3.40 ± 0.40	3.44
Morphological group III (*C. acutatum*)	01	Pink/orange	4.66 ± 1.33	Fusiform and elongated	23.81 ± 2.50	7.84 ± 1.00	3.03

Conidial measurements were made on 120 conidia
Growth rate was determined using six replications and each replication consisted of 6 plates

Restriction enzyme digestion of amplified rDNA

PCR-amplified rDNA products from representative fungal isolates, using primer pair ITS1 and ITS4, was digested with restriction enzyme, *Mbo*I. The reactions were performed in 10 µl enzyme reaction mixture containing 5 µl PCR product and 3 units of enzyme at 37°C. The resultant restriction products were analysed in 2.5% agarose gels in TBE buffer.

Sequencing of the ribosomal ITS region

Amplified ITS region of rDNA products (560 bp) with primer pair (ITS 1 and ITS 4) were purified using PCR product purification kit KT 72 (Genei, Bangalore). The purified product (10-12 ng) was used for PCR cycle sequencing using Big Dye Terminator ready reaction Mixture kit (Applied Bio systems, USA) and analyzed with ABI 3100 analyzer capillary machines. The nucleotide sequences generated from *C. gloeosporioides* (CG4, CG15, CG18, CG29, CG37, CG41), *C. acutatum* (CG5) and *C. acutatum* (CG33) were deposited in the NCBI data base (http://www.ncbi.nlm.nih.gov/) with the following accession numbers: EF025933, EFO25931, EFO25034, EFO25947, EFO25935, EFO25932, EFO25968 and EFO25973, respectively.

Phylogenetic analysis

A multiple-sequence alignment was performed with similar reference sequences of other *Colletotrichum* isolates available in the NCBI database using CLUSTAL X (Thompson *et al.* 1994) and a BLAST similarity test was also performed. The regions of sequence ambiguity and positions that were not available for all of the sequences compared were omitted before undertaking the phylogenetic analysis. Phylogenetic trees were constructed from the aligned sequences with the original data set and 100 bootstrap data sets generated by Clustal X. The trees generated for the ITS region of rDNA sequences using *C. graminicola* as the out group sequences allowed the trees to be rooted. The final trees were displayed using TREEVIEW (Page 1996) and NJ PLOT program (Periere and Gouy 1996).

RESULTS

Cultural and conidial morphology

Three morphological types were distinguished based on colony growth rate and conidial morphology (**Table 2**). Morphological type I (*C. gloeosporioides*) produced cylindrical conidia with both ends rounded (**Fig. 2**) and colony growth rates ranged from 11.21 to 12.08 mm/day. Morphological type II (*C. acutatum*) produced fusiform conidia with tapered ends and growth rate was 6.29 mm/day. Morphological type III (*C. acutatum*) had fusiform and elongated conidia with tapered ends and growth rate was 4.6 mm/day. None of the isolates produced setae. The cultures of morphological type I was either cream or white when viewed from above and gray from the underside. Morphological type II and III produced cultures with pink on the upper surface and orange on the lower surface. The conidial length (12.40 µm) and breadth (4.30 µm) of type I was almost similar to type II (12.10 and 3.40, respectively). The length and breadth of conidia of type III were 24.24 and 7.84 µm, respectively, which are larger than those of types I and II. Perithecia were not observed with any isolate on PDA or in any of the collections of tissues from the field.

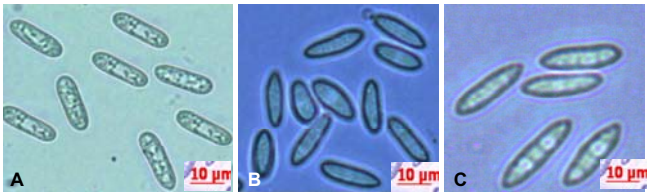

Fig. 2 Conidial morphology of *Colletotrichum* isolates. (**A**) *C. gloeosporioides* (CG25); (**B**) *C. acutatum* (CG5); (**C**) *C. acutatum* (CG33).

Molecular identification with species-specific PCR

The *C. acutatum* species-specific primers, CaInt and CaInt 1, in conjunction with ITS 4 amplified a 490-bp fragment from DNA of isolates of *C. acutatum* (CG5) and *C. acutatum* (CG33) but not from DNA of *C. gloeosporioides* (**Fig. 3**). In contrast, a 450-bp fragment was amplified from the DNA of *C. gloeosporioides* isolates with *C. gloeosporioides* species-specific primer *Cg*Int and ITS primer (**Fig. 4**). *Cg*Int did not amplify a product from the DNA of other isolates.

Restriction digestion patterns of ITS region of rDNA

The restriction enzyme *Mbo*I distinguished the isolates belonging to *C. gloeosporioides* and *C. acutatum* by recognizing unique restriction sites (**Fig. 4**).

Sequence analysis of the ribosomal ITS region

The ITS 1 and ITS2 region of rDNA of *Colletotrichum* isolates were successfully amplified and sequenced. The BLAST similarity search confirmed the results obtained by the species-specific PCR analysis as the ITS sequences obtained from *C. gloeosporioides* and *C. acutatum* shared sequence identity with sequences of *C. gloeosporioides* (AJ

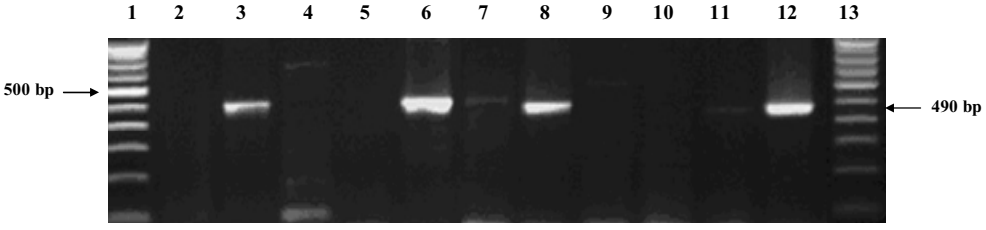

Fig. 3 PCR amplification of ITS region of ribosomal DNA of isolates with primer pairs CaInt and ITS 4. Lanes: 1 and 13, marker (100 bp ladder); 3, 6, 8 and 12, *C. acutatum*. 2, 4, 5, 7, 9, 10 and 11 (*Colletotrichum gloeosporioides* CG41, CG15, CG10, CG34, CG29, CG27, CG24, respectively).

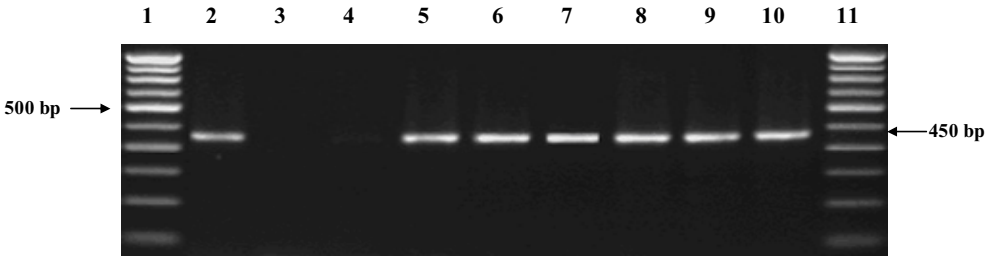

Fig. 4 PCR amplification of ITS region of ribosomal DNA of grape isolates with primer pair Cglnt and ITS 4. Lanes: 1 and 11, marker (100 bp ladder); 2, 5, 6, 7, 8, 9 and 10 (*C. gloeosporioides* CG41, CG15, CG10, CG34, CG29, CG27, CG24, respectively); 3 and 4 (*C. acutatum* CG5, CG33, respectively).

311884) and *C. acutatum* (AJ749678 and AY266405) isolates available in GenBank. The nucleotide sequences of *C. gloeosporioides* and *C. acutatum* (CG5 and CG33) isolates are shown in **Fig. 5**.

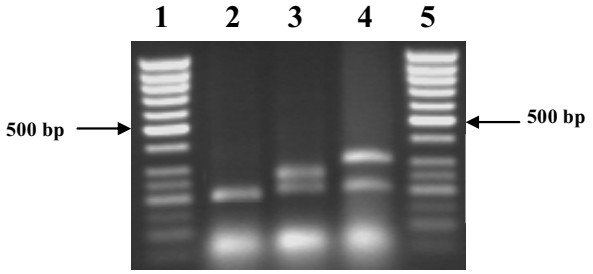

Fig. 5 Restriction digestion patterns of *Colletotrichum* with *Mbo*I restriction enzyme based on ITS region of ribosomal DNA. Lanes: 1 and 5, marker (100 bp ladder); lane 2, CG42 (*C. gloeosporioides*); 3 and 4 (*C. acutatum* CG5, CG33, respectively).

Phylogenetic analysis

A dendrogram of genetic similarity between isolates derived from sequences of ITS region of rDNA indicated that *C. gloeosporioides* and *C. acutatum* isolates were clusterd as separate groups (**Fig. 6**). Two molecular subgroups were evident within each species of *C. gloeosporioides* and *C. acutatum*.

DISCUSSION

A range of morphological and colony characters such as cylindrical conidia with rounded ends and fast growing gray colonies for identification of *C. gloeosporioides* and fusiform conidia with acutate ends and slow growing pink colonies for *C. acutatum* have been used for separation of these species (Talhinhas *et al*. 2002; Afandor-kafuri *et al*. 2005; Talhinhas *et al*. 2005; Schiller *et al*. 2006). Further, the acute-ended nature of conidia was one of the major criterion for the identification of *C. acutatum*, but it has been reported that only certain isolates of *C. acutatum* group ex-

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CG5_C.acut    -GAGTATCCTTTCGTCGGTTCCGGA-GGACTCTCTCCCTTTGT-GAACATACCTA--ACCGTTGCTTCGG
CG33_C.acut   -GAGTGATCTTTACTCGAGATTGCC-GCTCTATCACCCTTTGTCGAACATACCTA--ACCGTTGCTTCGG
CG18_C.glo    GGAGTGATCTTTACTCGAGATTGTACGCTCTATAACCCTTTGCTGAACATACCTATAACTGTTGCTTCGG

CG5_C.acut    CGGGCAGGGGAAGCCTCTCGCGGGCCTCCCCTCCCGGCGCCGGCCCCCACCAGGGGGACGGGGCGCCCGC
CG33 C.acut   CGGGCAGGGGAAGCCTCTCGCGGGCCTCCCCTCCCGGCGCCGGCCCC-ACCACGGGGACGGGGCGCCCGC
CG18_C.glo    CGGGTAGGG-----TCCCCGTGACCCTCCGGGCCT----CCCGCCCC--CCGGGCGGGTCGG-CGCCCGC

CG5_C.acut    CGGAGGA-AACCAAACTCTATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTTTA
CG33_C.acut   CGGAGGA-AACCAAACTCTATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTTTA
CG18_C.glo    CGGAGGATAACCAAACTCTGATTTAACGACGTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTA

CG5_C.acut    ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
CG33_C.acut   ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
CG18_C.glo    ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA

CG5_C.acut    ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGA
CG33_C.acut   ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGA
CG18_C.glo    ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGCATGCCTGTTCGA

CG5_C.acut    GCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCCCTTAAAGGTAGT
CG33_C.acut   GCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCCCTTAAAGGTAGT
CG18_C.glo    GCGTCATTTCAACCCTCAAGGTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCCCTCAAAGGTAGT

CG5_C.acut    GGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTA-ACGTCTCGCACTGGGATCCGGAGGGACTCTTG
CG33_C.acut   GGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTA-ACGTCTCGCACTGGGATTCGGAGGGACTCTTG
CG18_C.glo    GGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTTACGTCTCGCACTGGGATCCGGAGGGACTCTTG

CG5_C.acut    CCGTAAAACGCCCCAATTTTTTACAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATA
CG33_C.acut   CCGTAAAAG-CCCCAATTTTTTACAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATA
CG18_C.glo    CCGTAAAAC-CCCCAATTTTCCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATA

CG5_C.acut    TCATTAGCGGGAGGGAA
CG33_C.acut   T-ATTAAGCCGAGAGA-
CG18_C.glo    TCAGTAAAGAGAAGGAA
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Fig. 6 Clustal sequence alignments of the 560 bp fragment produced during PCR with rimer pair ITS1 and ITS4 of isolates of *C. gloeosporioides* and *C. acutatum*. Sequence differences are unshaded.

Table 3 Comparison of *C. acutatum* and *C. gloeosporioides* isolates used in this study with previous studies.

Colletotrichum species	Host	Country	Growth rate at 25°C (mm/day)	Colony colour on PDA	Spore shape On PDA	Spore size (µm)	Species-specific PCR*	Grouping based on ITS nucleotide sequences	β-tubulin 2 nucleotide sequences	Reference
C. acutaum	Woody herbaceous crops, ornamentals, fruits and conifers, almond, citrus, lupin, olive and strawberry	World-wide	3.60- 9.00	White to beige, yellow, light pink to carmine/flesh-colored, purple to red to grey	Fusiform with tapered-ends	12.4-15.0 × 3.2 to 4.5	+	A8	A8	1, 2, 3
	Grape	India	4.60-6.30	Cream/grey	Fusiform with tapered ends	11.37-23.80 × 3.40-7.84	+	02	---	Present study
C. gloeosporioides	Wide host range	World-wide	10.40-12.40	Pink/orange, gray	Round-ended conidia	13. 60-16.71 × 4.72-4.90	+	01	01	2, 3, 4
	Grape	India	11.22	Pink/orange	Cylindrical with rounded ends	12.60-4.40	+	01	--	Present study

*Species-specific diagnostic primer PCR using primers, CaInt2 for *C. acutatum* and CgInt for *C. gloeosporioides*
References: 1, Sreenivasaprasad and Talhinhas (2005); 2, Talhinhas *et al.* (2002); 3, Talhinhas *et al.* (2005); 4, Manaut *et al.* (2002)

hibit such identity (Talhinhas *et al*. 2002). In this study, a combination of molecular and morphological characterization of 30 *Colletotrichum* isolates associated with grape anthracnose led to the identification of 28 *C. gloeosporioides* and two *C. acutatum* isolates. Using colony growth rate and conidial morphology, *Colletotrichum* isolates from the grapes in the present study were grouped into two morpho species: *C. acutatum* isolates exhibited pink colonies with orange colour on the reverse side, fusiform conidia with acute ends and slow growing while *C. gloeosporioides* formed gray phenotype colonies, cylindrical conidia with rounded ends, and rapidly growing. These descriptions correspond to *C. acutatum* and *C. gloeosporioides* reported on various hosts (**Table 3**; Smith and Black 1990; Bonde *et al*. 1991; Sutton 1991; Gunnell and Gubler 1992; Liyanage *et al*. 1993; Bernstein *et al*. 1995; Adaskaveg and Hartin 1997; Freeman *et al*. 2001; Talhinhas *et al*. 2002, 2005; Schiller *et al*. 2006).

Application of species-specific PCR using rRNA gene-ITS primers developed for *C. acutatum* and *C. gloeosporioides* (Mills *et al*. 1992; Sreenivasaprasad *et al.* 1996) in the current study, further provided confirmatory evidence for reliable diagnosis of isolates belonging to *C. acutatum* and *C. gloeosporioides* from grape whereas rDNA restriction fragment length polymorphism (RFLP) and ITS sequences sharply divided *C. acutatum* isolates into two molecular subgroups, corresponding to the morphological groups. Previous studies identified four sub groups within *C. acutatum* causing strawberry fruit rots, originated in New Zealand and Australia, from *Lupinus* spp. in New Zealand, Canada, France and the U.K., and from *Pinus radiata* in New Zealand and Australia, using RAPDs and morphological criteria (Lardner *et al*. 1999) and ITS- sequencing analysis (Freeman *et al*. 2001) and three rDNA-RFLP subgroups on strawberry in the UK (Buddie *et al*. 1999). Studies by Talhinhas *et al*. (2005) identified six molecular groups within *C. acutatum* populations associated with olive anthracnose and other hosts in Portugal which could be related to various *C. acutatum sensu lato* groups described based on morphology and randomly amplified polymorphic DNA analyses (Lardner *et al*. 1999) and mitochondrial DNA-RFLP and sequence variations in introns of two different genes (Guerber *et al*. 2003). Talhinhas *et al*. (2005) identified the *C. acutatum* isolates occurring on grapes in Portugal as molecular group A3 with characteristic pink colonies similar to the isolates of grape used in the present study.

C. acutatum was first recognised on strawberry as a pathogen (Simmonds 1965, 1968) and now it has been identified to cause anthracnose diseases on 34 host genera in 22 families (Suton 1992; Buddie *et al.* 1999). Johnston and Jones (1997) and Lardner *et al*. (1999), who recognized *C. acutatum* as group species, coined the terms C. *acutatum sensu lato* and *C. acutatum sensu* Simmonds based on morphological and molecular criteria. *C. acutatum* was unknown on grapes until it was reported from Australia (Melkham *et al*. 2002; Stele and Greer 2006) and Portugal (Talhinhas *et al*. 2005). To our knowledge, *C. acutatum* was reported only on rubber from India (Saha *et al.* 2002). Rubber is being grown mainly in high rainfall and tropical humid areas in Kerala and Karnataka and grape is grown in low rain fall and semi arid tropical zones of Maharashtra and Karnataka. The movement of rubber material between these zones is rare. But, the grape planting material was frequently brought from Australia to Maharashtra. It is surmised that *C. acutatum* might have been moved to Maharashtra through grape cuttings, which have been brought from Australia. Further cross inoculation studies on rubber and grapes will reveal from where the pathogen has come.

Based on a dendrogram constructed using rDNA sequences (**Fig. 7**), isolates of *C. gloeosporioides* fell into two genetically distinct sub groups in this study. The largest population (Group I) consisted of 21 isolates and the smallest population (Group II) contained only seven isolates. Group I is widespread and present in all grapegrowing regions of India while Group II is restricted only to Andhra Pradesh. The diversity in the population of *C. gloeosporioides* population in Andhra Pradesh may be due to either partly adverse environmental factors compared to other grape growing regions or heavy fungicide loads and cultural practices adopted by the farmers or combination of both factors. Morphologically both these groups were indistinguishable. Earlier, various researchers identified two distinct genetic groups within *C. gloeosporioides* isolates from different fruit crops in temperate, sub-tropical and tropical regions (Alahakoon *et al.* 1994; Freeman and Shab 1996; Johnston and Jones 1997; Xiao *et al.* 2004). Co-occurrence of *C. gloeosporioides* and *C. acutatum* was previously reported on many tropical fruit crops and symptoms caused by them are also highly indistinguishable (Talhinhas *et al*. 2005). Thus, the role and relative importance of *C. acutatum* in grape anthracnose and co-occurrence with *C. gloeosporioides* needs to be addressed. The grape anthracnose pathogen epidemiology needs to be studied as *C. acutatum* has been reported to exhibit epiphytic, endophytic and non-pathogenic life styles on other crops (Freeman *et al.* 2001).

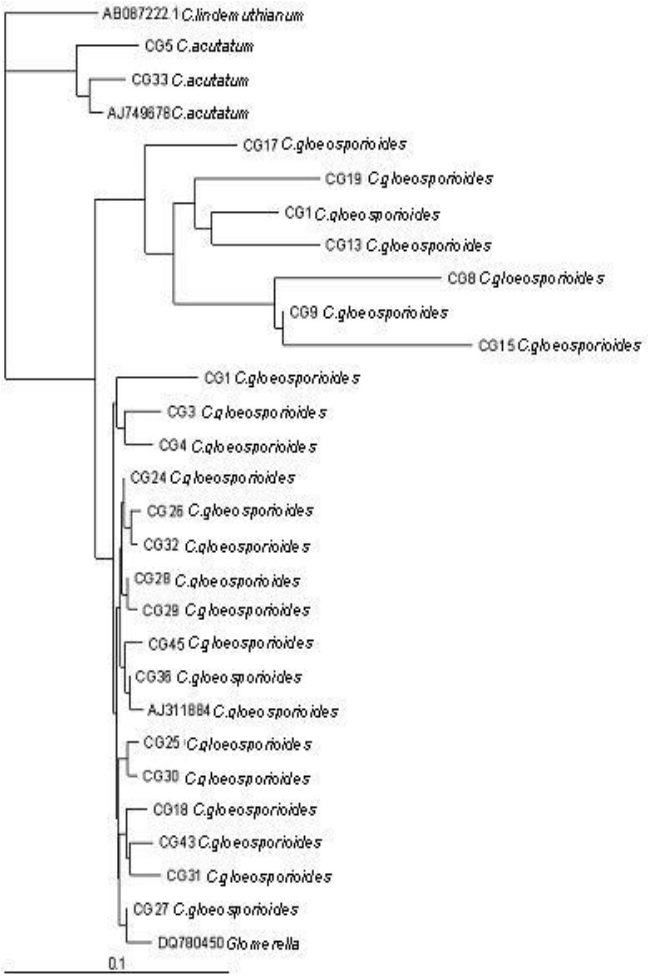

Fig. 7 Internal Transcribed Spacer based phylogenetic tree of *Colletotrichum* spp. isolates and published sequences.

There is need to study the pathogenicity of these two sub groups on different grape varieties and their sensitivity to fungicides. So it is critical to accurately diagnose the pathogen and understand its epidemiology to develop effective disease management. Moreover, differential sensitivity of these mixed *Colletotrichum* populations to fungicides such as benomyl can pose problems in disease control, as well as lead to shifts in pathogen populations (Freeman *et al*. 2000).

The present study, thus, clearly established *C. gloeosporioides* as the dominant pathogen (93.33%) with very limited occurrence of *C. acutatum* (6.66%). This is the first report of occurrence of *C. acutatum* and existence of diverse molecular groups among *C. gloeosporioides* and *C. acutatum* populations associated with grape anthracnose in India. Considering the importance of out break of new disease epidemics, *C. acutatum*, at present a minor pathogen, may become major pathogen and cause severe disease epidemics in the future.

ACKNOWLEDGEMENTS

We thank Department of Biotechnology, Government of India for financial support in the form of project No.BT/PR/4223/Agr./16/352/2003 and Dr. S. Sreenivasaprasad and Dr. Pedro Talhinahas, HRI, University of Warwick, Wellesbourne, United Kingdom for valuable comments on the manuscript.

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