



Identification of *Colletotrichum* spp. isolated from strawberry in Zhejiang Province and Shanghai City, China*

Liu XIE, Jing-ze ZHANG^{†‡}, Yao WAN, Dong-wei HU

(Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture, Institute of Biotechnology, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, China)

[†]E-mail: jzzhang@zju.edu.cn

Received June 22, 2009; Revision accepted Nov. 5, 2009; Crosschecked Dec. 15, 2009

Abstract: Strawberry anthracnose, caused by *Colletotrichum* spp., is a major disease of cultivated strawberry. This study identifies 31 isolates of *Colletotrichum* spp. which cause strawberry anthracnose in Zhejiang Province and Shanghai City, China. Eleven isolates were identified as *C. acutatum*, 10 as *C. gloeosporioides* and 10 as *C. fragariae* based on morphological characteristics, phylogenetic and sequence analyses. Species-specific polymerase chain reaction (PCR) and enzyme digestion further confirmed the identification of the *Colletotrichum* spp., demonstrating that these three species are currently the causal agents of strawberry anthracnose in the studied regions. Based on analysis of rDNA internal transcribed spacers (ITS) sequences, sequences of all *C. acutatum* were identical, and little genetic variability was observed between *C. fragariae* and *C. gloeosporioides*. However, the conservative nature of the *Mvnl* specific site from isolates of *C. gloeosporioides* was confirmed, and this site could be used to differentiate *C. gloeosporioides* from *C. fragariae*.

Key words: *Fragaria x ananassa*, Taxonomic identification, *Colletotrichum acutatum*, *C. gloeosporioides*, *C. fragariae*
doi:10.1631/jzus.B0900174 **Document code:** A **CLC number:** Q945.8

1 Introduction

Colletotrichum species cause economically significant diseases including wilts, rots and anthracnose in production areas of strawberry (*Fragaria x ananassa* Duch.) worldwide (Buddie *et al.*, 1999; Debode *et al.*, 2009). Three species have been reported as causal agents of strawberry anthracnose: *C. acutatum* Simmonds (teleomorph *Glomerella acutata* J.C. Guerber & J.C. Correll), *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. [teleomorph *Glomerella cingulata* (Stoneman) Spauld. & Schrenk], and *C. fragariae* Brooks (Gunnell and Gubler, 1992; Denoyes-Rothan *et al.*, 2003). *C. gloeosporioides* and *C. acutatum* are distributed

worldwide on a number of hosts, while *C. fragariae* has a very narrow host range (MacKenzie *et al.*, 2008). The disease causes up to 80% of plant deaths in nurseries and over 50% of yield losses in strawberry production fields (Sreenivasaprasad and Talhinhas, 2005). Likewise, in the Zhejiang region, nearly 50% of seedling deaths in nurseries and over 40% of yield losses in strawberry production fields are caused by this disease according to our investigations.

Strawberry is cultivated extensively in some districts of China and severe levels of strawberry anthracnose have been prevalent for many years. Shao (1992) first observed strawberry anthracnose disease in Shanghai on cv. Lego introduced from Japan and the pathogen was identified as *C. acutatum* (Dai *et al.*, 2006). Subsequently, Ye *et al.* (1997) reported that the pathogens responsible for strawberry anthracnose in Shanghai belonged to *C. fragariae* and *C. gloeosporioides*, based on pathogen morphological characteristics. However, based on a combination of

[‡] Corresponding author

* Project supported by the National Natural Science Foundation of China (No. 30571208) and the Key Scientific and Technological Project of Hangzhou City (No. 200432239), China

© Zhejiang University and Springer-Verlag Berlin Heidelberg 2010

morphology and rDNA internal transcribed spacers (ITS) sequence analysis, *C. acutatum* and *C. gloeosporioides* were identified as the causal agents (Ren et al., 2008). Zhejiang Province, adjacent to Shanghai, is an important strawberry-producing area with about 6000 ha under cultivation. Although the disease occurs every year, isolation and molecular identification to clarify which *Colletotrichum* spp. are responsible for anthracnose disease in the area have not been performed. The main purpose of this study was to establish which species are currently causing anthracnose disease in strawberry in Zhejiang Province and to compare them with isolates from strawberry plants from Shanghai.

2 Materials and methods

2.1 Fungal isolates

Colletotrichum spp. were isolated from diseased strawberry plants (fruit, vegetative parts, and root crowns) from different cultivars and sites from the summer of 2006 to the spring of 2007. Infected plant organs were surface-sterilized [0.5% (w/v) sodium hypochlorite] for 2–5 min before they were placed onto potato dextrose agar (PDA) containing streptomycin. Isolates selected were transferred to PDA, and single conidial isolates were prepared from each isolate. Cultures were grown on PDA at 25 °C. Plugs (5 mm) were cut from the margin of fresh colonies and stored in 20% (w/v) glycerol in a freezer at –70 °C.

2.2 Morphological examination

Isolates representative of different sites were selected for morphological species identification. These isolates were cultured on PDA in darkness at 25 °C for 5 d. Mycelial discs (5 mm) were taken from the growing edge of colonies and transferred onto plates of strawberry leaf agar (SLA) (Gunnell and Gubler, 1992). The SLA plates were placed in an incubator under continuous fluorescent light at 25 °C. Conidia were harvested after 14 d, and setae after 22–30 d, from acervuli produced on the leaf pieces. Conidia were mounted in water, and examined for size and shape, using a Zeiss Axiophot 2 microscope with an AxioCam CCD camera and AxioVision digital imaging software (AxioVision Software Release

3.1, version 3-2002; Carl Zeiss Vision Imaging Systems). Colony color was determined after 7 d on PDA at 25 °C under conditions of alternating cycles of 12 h light and 12 h darkness.

2.3 DNA extraction

Fungal isolates were cultured in darkness at 25 °C for 5 d. Mycelial disks were excised from the margins of colonies and inoculated into 100 ml of liquid growth media potato dextrose broth (PDB) in 250 ml flasks, shaken at 200 r/min at 25 °C for 5 d. Mycelia were harvested by filtration, freeze-dried, ground to a fine powder in liquid nitrogen, and then stored at –70 °C. About 50 mg of mycelial powder was removed into a sterile 1.5-ml tube, rehydrated in 600 µl of 2× cetyltrimethylammonium bromide (CTAB) buffer [100 mmol/L Tris (pH 8.0), 1.4 mol/L NaCl, 30 mmol/L ethylenediaminetetraacetic acid (EDTA), 2% (w/v) hexadecyltrimethylammonium bromide], and incubated in a water bath at 65 °C for 30–60 min. Following a phenol/chloroform extraction, the genomic DNA was precipitated by isopropanol in the presence of sodium acetate. Genomic DNA was visualized in 1% (w/v) agarose gels after ethidium bromide staining. DNA concentration was determined by ultraviolet (UV) spectroscopy.

2.4 Amplification of rDNA-ITS regions

The ITS regions of rDNA were amplified using the primers ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for direct sequencing (Cooke and Duncan, 1997). Polymerase chain reaction (PCR) amplifications were performed in a total volume of 50 µl containing 40 mmol/L of Tris-HCl (pH 8.4), 100 mmol/L of KCl, 3 mmol/L of MgCl₂, 200 µmol/L of each dNTP, 1 µmol/L of each primer, and 0.5 U of *Taq* (Sangon Co., Shanghai, China). PCR amplifications were carried out using a PTC-150 MiniCycler (MJ Research Corp., America). Following an initial denaturation at 95 °C for 4 min, the DNA templates were amplified for 35 cycles. Each cycle consisted of a denaturation step at 95 °C for 1 min, an annealing step at 52 °C for 1 min, and an extension step at 72 °C for 1 min. A final extension step (72 °C for 10 min) was included at the end. Aliquots of 4 µl of the amplification products were electrophoresed on 1% (w/v) agarose gels, and the PCR products were

subsequently stained with ethidium bromide. Successful PCR amplification products resulted in a single DNA band (corresponding to ~560 bp). The PCR products were purified using a Biolight PCR Purification Kit (Shanghai Biolight Technology Co. Ltd., China) according to the manufacturer's instructions.

2.5 Sequencing and analysis of rDNA-ITS regions

The purified PCR products were submitted to the Hangzhou Genomics Institute for sequencing in both directions. Sequence files were assembled and edited, and consensus sequences were constructed using DNAMAN 4.0 (Lynnon bioSoft). The ITS sequences were submitted to the GenBank database. Sequences were deposited in GenBank (accession numbers given in Table 1) and compared against those sequences already found in the databases using the BLAST search. The ITS sequences for reference isolates were downloaded from the GenBank database (Table 1), along with that of *Neurospora crassa* (M13906) as an outgroup. Sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic analyses were performed using test version 4.0b10 (PPC) of PAUP (Swofford, 2002). Phylogenetic trees were inferred from the ITS sequence data set using parsimony analysis and all characters were weighted equally and gaps were treated as missing data. Clade stability was assessed using a bootstrap analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa.

2.6 PCR with *C. acutatum*-specific primers

PCR primers for detection of the pathogen included the ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') coupled with the specific primer for *C. acutatum* (*CaInt2*) (5'-GGGGAAGCCTCTCGCGG-3') (Sreenivasaprasad *et al.*, 1996; White *et al.*, 1990). The genomic DNA of *Trichoderma virens* was used as a negative control. *C. acutatum*-specific PCR reactions were performed in a total volume of 50 μ l, containing 50 ng of genomic DNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl, 100 μ mol/L of each dNTP, 1.5 mmol/L MgCl₂, 1 U of *Taq* DNA polymerase (TaKaRa Corp., Japan), and 0.5 μ mol/L of each primer. The reaction mixtures were incubated in a PTC-150 MiniCycler (MJ RESEARCH Corp., America) for 35 cycles consisting of 1 min at 95 °C, 30 s at 54 °C, and

1 min at 72 °C. Amplification products were separated in 1% (w/v) agarose gels, stained with the nucleic acid stain GoldView™ (Shanghai SBC Genetech Co. Ltd., China), and viewed under UV light for detection of amplification products.

2.7 PCR reaction and DNA digestion

The 5.8S-ITS regions of representative isolates of *C. gloeosporioides* and *C. fragariae*, identified by morphological characteristics, were amplified by PCR using universal primers ITS1 (5'-GCCGTAGG TGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTT ATTGATATGC-3') (Martinez-Culebras *et al.*, 2000). The genomic DNA of *Trichoderma virens* was used as a negative control. PCR reactions were performed in a total volume of 50 μ l, containing 150–200 ng of DNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl, 200 μ mol/L of each dNTP, 1 μ mol/L of each primer, 1.5 mmol/L MgCl₂, and 2.5 U of *Taq* DNA polymerase (Sangon Co., Shanghai, China). The reaction mixtures were incubated in a PTC-150 MiniCycler (MJ RESEARCH Corp., America). Following an initial denaturation at 95 °C for 4 min, the DNA templates were amplified for 35 cycles. Each cycle consisted of a denaturation step at 95 °C for 1 min, an annealing step at 56 °C for 1 min, and an extension step at 72 °C for 1 min. A final extension step (72 °C for 10 min) was included at the end. Amplification products were separated on 1% (w/v) agarose gels in Tris-acetate-EDTA (TAE) buffer.

To specifically identify strawberry isolates of *C. gloeosporioides*, PCR products from the ITS1/ITS4 primer pair were digested with the restriction enzyme *MvnI* (supplied under the name Bsh1236I by Fermentas, MBI, Republic of Lithuania) at 37 °C for 16 h (Martinez-Culebras *et al.*, 2003). PCR products for species-specific identification and restriction fragments from the PCR fragment primed with ITS1 and ITS4 were separated on 1% and 3% (w/v) agarose gels, respectively, with 0.5 \times Tris-borate-EDTA (TBE) buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 μ g/ml), and the DNA bands were visualized with UV light. DNA sizes were estimated by comparison with a DNA length standard (100–3000 bp molecular marker, Bio Basic Inc., Canada), as described by Martinez-Culebras *et al.* (2003).

3 Results

3.1 Morphological comparisons

Isolates of *Colletotrichum* spp. obtained from fruit, vegetative parts, and root crowns from different cultivars showing typical anthracnose symptoms, were selected for morphological identification. Based on taxonomic criteria of morphological characteristics (colony characters, conidial and setal morphology

and production of a teleomorph) (Gunnell and Gubler, 1992), 10 *C. gloeosporioides*, 10 *C. fragariae* and 11 *C. acutatum* isolates were recognized among 31 isolates (Table 1). On SLA, most of the conidia of *C. fragariae* isolates were narrowly obovate, straight or occasionally slightly curved, [(13.0–) 14.5–16.5 (–19.0)] $\mu\text{m} \times [(3.5–) 4.2–4.8 (–5.5)] \mu\text{m}$ (mean=16.0 $\mu\text{m} \times 4.5 \mu\text{m}$) (Fig. 1a). Conidia of *C. acutatum* isolates were elliptic to fusiform, [(11.0–) 11.2–15.5

Table 1 Isolates of *Colletotrichum* spp. from Zhejiang Province and Shanghai City, China used in this study and the reference isolates used for ITS ribosomal DNA sequence comparisons

Isolate/ strain	Species	Host plant	Geographical origin	GenBank accession No.	Reference
Isolates used					
WY01	<i>C. gloeosporioides</i>	Toyonoka/leaf*	Jiande City, Zhejiang Province	FJ608625	In this study
WY02	<i>C. gloeosporioides</i>	Toyonoka/petiole	Jiande City, Zhejiang Province	FJ608626	In this study
WY03	<i>C. gloeosporioides</i>	Toyonoka/leaf	Jiande City, Zhejiang Province	FJ608627	In this study
WY04	<i>C. gloeosporioides</i>	Toyonoka/leaf	Jiande City, Zhejiang Province	FJ608628	In this study
WY05	<i>C. gloeosporioides</i>	Toyonoka/leaf	Zhaotun, Shanghai City	FJ608629	In this study
WY06	<i>C. gloeosporioides</i>	Toyonoka/crown	Zhaotun, Shanghai City	FJ608630	In this study
WY07	<i>C. gloeosporioides</i>	Hongjia/leaf	Hangzhou City, Zhejiang Province	FJ608631	In this study
WY08	<i>C. gloeosporioides</i>	Toyonoka/leaf	Hangzhou City, Zhejiang Province	FJ608632	In this study
WY09	<i>C. gloeosporioides</i>	Toyonoka/crown	Jiande City, Zhejiang Province	FJ608633	In this study
WY10	<i>C. gloeosporioides</i>	Toyonoka/petiole	Hangzhou City, Zhejiang Province	FJ608634	In this study
WY11	<i>C. fragariae</i>	Toyonoka/crown	Jiande City, Zhejiang Province	FJ608635	In this study
WY12	<i>C. fragariae</i>	Toyonoka/leaf	Jiande City, Zhejiang Province	FJ608636	In this study
WY13	<i>C. fragariae</i>	Toyonoka/crown	Zhaotun, Shanghai City	FJ608637	In this study
WY14	<i>C. fragariae</i>	Toyonoka/leaf	Zhaotun, Shanghai City	FJ608638	In this study
WY15	<i>C. fragariae</i>	Toyonoka/crown	Zhaotun, Shanghai City	FJ608639	In this study
WY16	<i>C. fragariae</i>	Tochiotome/crown	Hangzhou City, Zhejiang Province	FJ608640	In this study
WY17	<i>C. fragariae</i>	Toyonoka/leaf	Hangzhou City, Zhejiang Province	FJ608641	In this study
WY18	<i>C. fragariae</i>	Akihime/petiole	Hangzhou City, Zhejiang Province	FJ608642	In this study
WY19	<i>C. fragariae</i>	Toyonoka/leaf	Hangzhou City, Zhejiang Province	FJ608643	In this study
WY20	<i>C. fragariae</i>	Toyonoka/crown	Jiande City, Zhejiang Province	FJ608644	In this study
WY21	<i>C. acutatum</i>	Toyonoka/leaf	Zhaotun, Shanghai City	FJ608645	In this study
WY22	<i>C. acutatum</i>	Toyonoka/fruit	Zhaotun, Shanghai City	FJ608646	In this study
WY23	<i>C. acutatum</i>	Toyonoka/leaf	Zhaotun, Shanghai City	FJ608647	In this study
WY24	<i>C. acutatum</i>	Toyonoka/fruit	Zhaotun, Shanghai City	FJ608648	In this study
WY25	<i>C. acutatum</i>	Toyonoka/fruit	Jiande City, Zhejiang Province	FJ608649	In this study
WY26	<i>C. acutatum</i>	Toyonoka/leaf	Jiande City, Zhejiang Province	FJ608650	In this study
WY27	<i>C. acutatum</i>	Toyonoka/leaf	Jiande City, Zhejiang Province	FJ608651	In this study
WY28	<i>C. acutatum</i>	Toyonoka/fruit	Jiande City, Zhejiang Province	FJ608652	In this study
WY29	<i>C. acutatum</i>	Toyonoka/leaf	Jiande City, Zhejiang Province	FJ608653	In this study
WY30	<i>C. acutatum</i>	Toyonoka/leaf	Jiande City, Zhejiang Province	FJ608654	In this study
WY31	<i>C. acutatum</i>	Toyonoka/petiole	Jiande City, Zhejiang Province	FJ608655	In this study

(To be continued)

Isolate/ Strain	Species	Host plant	Geographical origin	GenBank accession No.	Reference
Reference isolates					
IMI 345047	<i>C. fragariae</i>	Strawberry	USA	AJ536223	Martinez-Culebras et al., 2003
IMI 348167	<i>C. fragariae</i>	Strawberry	USA	AJ536222	Martinez-Culebras et al., 2003
CG 231	<i>C. gloeosporioides</i>	Strawberry	USA	AF272780	Freeman et al., 2001
IMI 324983	<i>Glomerella cingulata</i>	Strawberry	USA	AJ536226	Martinez-Culebras et al., 2003
IMI 347765	<i>G. tucumanensis</i>	<i>Saccharum</i> sp.	Bangladesh	AJ536231	Martinez-Culebras et al., 2003
IMI 345051	<i>G. cingulata</i>	Strawberry	Canada	AJ536224	Martinez-Culebras et al., 2003
IMI 345027	<i>G. acutata</i>	Strawberry	France	AJ536199	Martinez-Culebras et al., 2003
IMI 348160	<i>G. acutata</i>	Strawberry	USA	AJ536200	Martinez-Culebras et al., 2003
IMI 360086	<i>G. acutata</i>	Strawberry	Japan	AJ536205	Martinez-Culebras et al., 2003
IMI 345575	<i>G. acutata</i>	Strawberry	New Zealand	AJ536215	Martinez-Culebras et al., 2003
MAFF 511343	<i>G. graminicola</i>	unknown	Japan	AB057436	Moriwaki et al., 2002
CBS 151.28	<i>G. lindemuthiana</i>	<i>Phaseolus vulgaris</i>	unknown	AJ301946	Nirenberg et al., 2002
BBA 70709	<i>C. trifolii</i>	<i>Trifolium</i> sp.	USA	AJ301941	Nirenberg et al., 2002
UQ 343	<i>C. destructivum</i>	Unknown	Australia	AF451908	Unpublished
STE-U 5304	<i>C. capsici</i>	<i>Arachis hypogaea</i>	Tanzania	AY376526	Lubbe et al., 2004
STE-U 5301	<i>C. coccodes</i>	<i>Lycopersicon esculentum</i>	Zimbabwe	AY376528	Lubbe et al., 2004
STE-U 5299	<i>C. dematium</i>	<i>Peperomia</i> sp.	Unknown	AY376531	Lubbe et al., 2004
BBA 70535	<i>C. fuscum</i>	<i>Digitalis lanata</i>	unknown	AJ301938	Nirenberg et al., 2002
STE-U 5296	<i>C. orbiculare</i>	<i>Xanthium spinosum</i>	Argentina	AY376541	Lubbe et al., 2004
STE-U 5294	<i>G. truncata</i>	<i>Arachis hypogaea</i>	Gambia	AY376543	Lubbe et al., 2004

* Cultivar/Organ from which fungus isolated

(-16.0)] $\mu\text{m} \times [(3.8-)$ 4.1-4.7 (-5.0)] μm (mean=13.5 $\mu\text{m} \times 4.4 \mu\text{m}$) (Fig. 1b). The conidia of *C. gloeosporioides* isolates were oblong with obtuse ends, straight (Fig. 1c), and were shorter and broader than those of *C. fragariae* and *C. acutatum*, [(11.8-) 12.5-14.8 (-16)] $\mu\text{m} \times [(4.6-)$ 5.0-6.0 (-6.8)] μm (mean=13.5 $\mu\text{m} \times 5.5 \mu\text{m}$). The shapes of the conidia of *C. fragariae*, *C. gloeosporioides* and *C. acutatum* agreed with previous descriptions (Gunnell and Gubler, 1992), but the average conidial size of each of the three species was slightly different from the range described by Gunnell and Gubler (1992), namely 18 $\mu\text{m} \times 4 \mu\text{m}$, 15.5 $\mu\text{m} \times 3.7 \mu\text{m}$, and 15 $\mu\text{m} \times 4.3 \mu\text{m}$ for *C. fragariae*, *C. acutatum*, and *C. gloeosporioides*, respectively.

Mature setae of *C. fragariae* were dark brown, uniform in width except for the apical cells which functioned as phialides, and produced conidia in individual isolates (Figs. 1e and 1f). Setae of *C. gloeosporioides* were dark brown, gradually tapered along their entire length to their apices, straight, produced singly, and did not produce conidia (Fig. 1g). Setae of *C. acutatum* were brown to dark brown, tapered,

generally aseptate (Fig. 1h), did not produce conidia, and were shorter than those of *C. fragariae* and *C. gloeosporioides*. The isolates of *C. gloeosporioides* readily produced perithecia containing asci and ascospores (Fig. 1d), but none of the isolates of *C. fragariae* or *C. acutatum* produced perithecia.

On PDA, the colony color of *C. fragariae* isolates ranged from beige to dark grey. Colonies of *C. gloeosporioides* isolates showed a dense, white mycelial growth turning to a dark olive-grey color. The colony color of *C. acutatum* isolates was white for 4-5 d but later became grey-brown.

3.2 Sequence analysis of the ribosomal DNA spacer sequence

Sequence alignment showed that among 11 isolates (WY21-WY31), all sequences were identical to that of a strawberry isolate of *C. acutatum* (IMI 360086) from Japan. Among the other isolates (WY01-WY20), all sequences were very similar.

Reference sequences were selected cautiously from GenBank, with only those from voucher

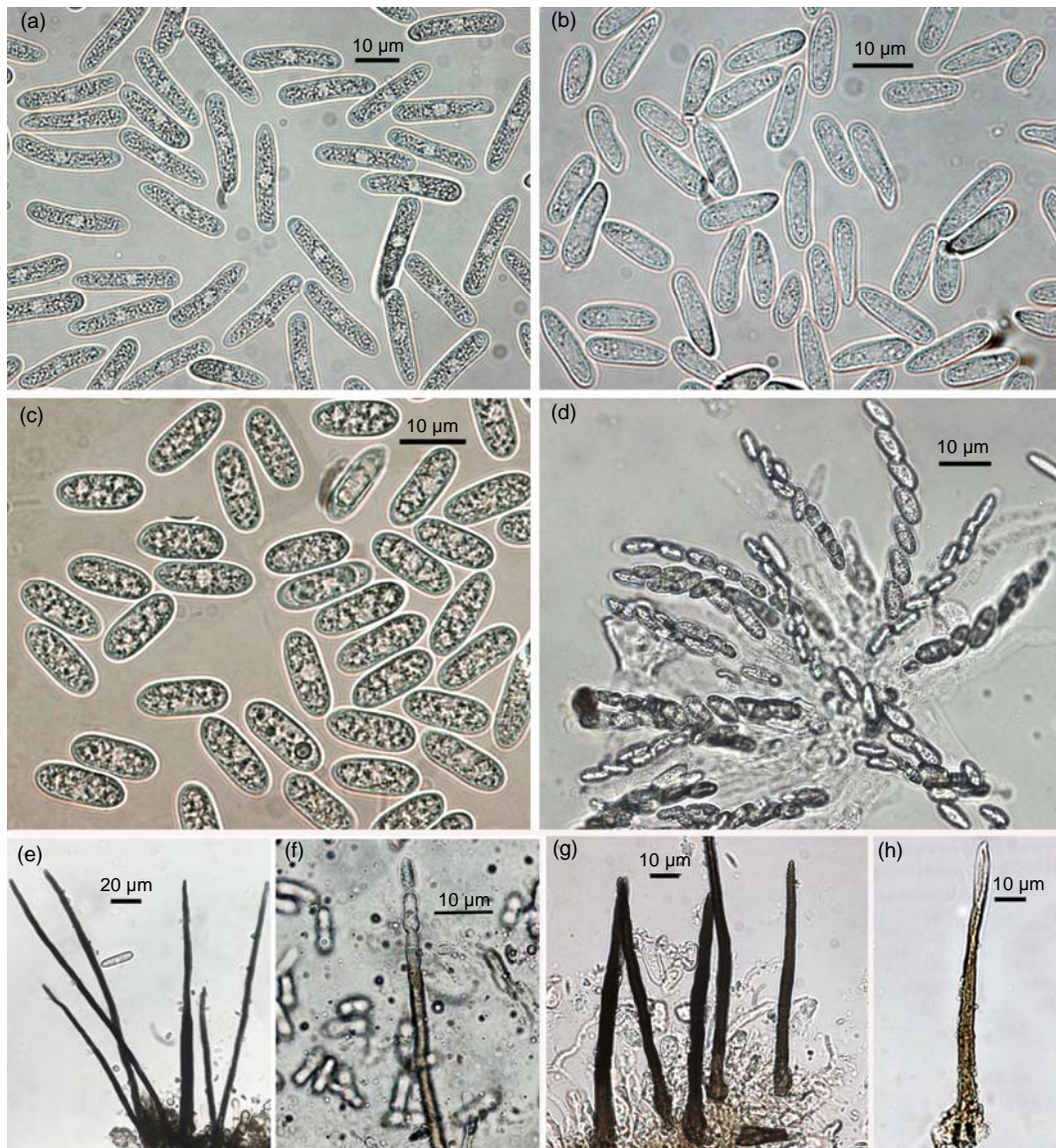
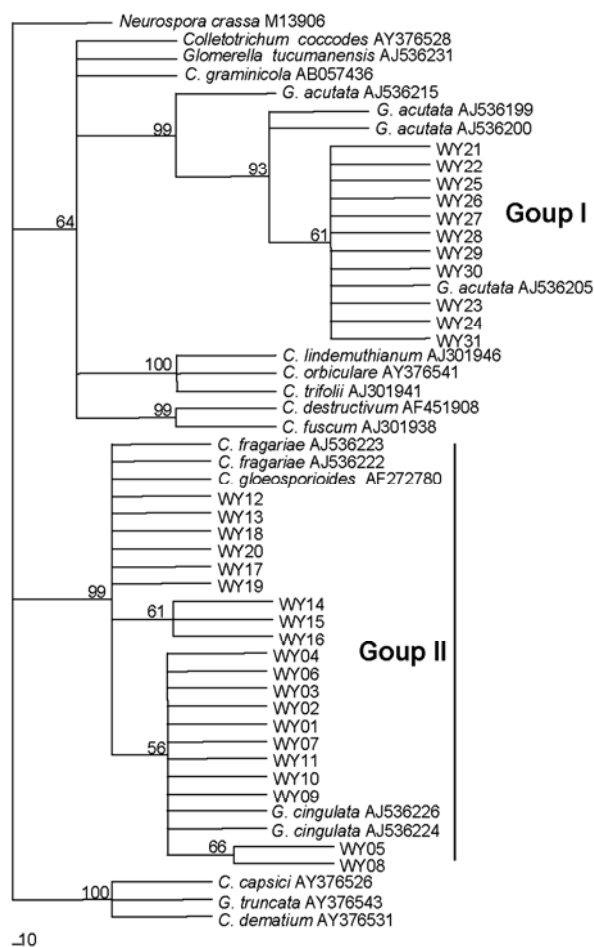


Fig. 1 Conidial morphology of *Colletotrichum* species isolates from strawberry. (a) Obovate conidia of *C. fragariae*; (b) Elliptic to fusiform conidia of *C. acutatum*; (c) Cylindrical conidia of *C. gloeosporioides*; (d) Asci and ascospores of *C. gloeosporioides*; (e) & (f) Setae of *C. fragariae*; (f) A seta producing a conidium from its apical cells; (g) Setae of *C. gloeosporioides*; (h) Seta of *C. acutatum*

specimens being selected. The final ITS/5.8S dataset included 52 sequences with 530 characters after alignment. Phylogenetic analysis showed that the 31 isolates in this study were clustered into two groups, group I and group II (Fig. 2). In group I, 11 isolates, identified as *C. acutatum* based on morphological characteristics, were clustered together with 4 reference isolates of *C. acutatum* (teleomorph *G. acutata*) from strawberry. The isolate IMI 345575 (New Zealand) formed a single subgroup and did not group with any others. While 11 isolates produced a clade

with the reference sequence of *C. acutatum* IMI 360086 from Japan, they were clustered with the isolates IMI 348160 (USA) and IMI 345027 (France) to form another subgroup with 93% bootstrap support, as described in Table 1 of Sreenivasaprasad and Talhinhas (2005). In group II, 20 isolates (WY01–WY20), classified as *C. fragariae* and *C. gloeosporioides* based on morphology, were in a poorly resolved group that included the reference isolates of *C. fragariae* and *C. gloeosporioides* (teleomorph *G. cingulata*) from strawberry (Fig. 2).



-10

Fig. 2 Phylogram generated from parsimony analysis based on complete sequence of ITS1-5.8S-ITS2 ribosomal RNA genes of 52 sequences with *Neurospora crassa* as outgroup

Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Bootstrap values $\geq 50\%$ are shown above or below branches. The numbers near each branch represent percentages out of 100 bootstrap replications

However, by analyzing 5.8S-ITS sequences, an *MvnI* restriction site was found to be present within the ITS1 region in only 10 isolates (WY01–WY10) and no *MvnI* restriction sites were found in the other isolates. Previous studies have shown an *MvnI* restriction site present within the ITS1 region of the 5.8S-ITS sequences of only *C. gloeosporioides* isolates but not in other isolates of *Colletotrichum* spp. from strawberry plants (Martinez-Culebras *et al.*, 2000; 2003). So in group II, 10 isolates (WY01–WY10) were related to *C. gloeosporioides* and the other isolates were attributed to *C. fragariae*, consistent with morphological identification (Table 1).

The ITS1-5.8S-ITS2 rDNA sequences of the 10 *C. gloeosporioides* isolates fell into two groups: the first group comprised sequences from eight isolates (WY01, WY02, WY03, WY04, WY07, WY09, WY10 and WY06) and the second group comprised sequences from the remaining two (WY05 and WY08). Sequences within each group were identical but the two groups differed by three base pairs. Sequences of the *C. fragariae* isolates fell into three groups comprising four (WY18, WY20, WY12 and WY13), three (WY14, WY15 and WY16), and three (WY19, WY17 and WY11) isolates, respectively. Again, sequences within each group were identical but the three groups differed by three base pairs. Analysis of the rDNA ITS sequences revealed that the *MvnI* restriction site in *C. gloeosporioides* isolates was conservative and a useful tool for distinguishing *C. gloeosporioides* from *C. fragariae* from strawberry plants. It also showed the genetic diversity of population structures in *C. gloeosporioides* and *C. fragariae*.

Based on 5.8S-ITS sequences and phylogenetic analysis, the 31 isolates could be classified clearly into three species. Eleven isolates (WY21–WY31) were identified as *C. acutatum* (all identical), 10 (WY01–WY10) as *C. gloeosporioides* (8 clustering in a group that is 3-bp different from the remaining 2) and 10 (WY11–WY20) as *C. fragariae* (4 clustering in a group that is 3-bp different from the remaining 6 that are in two groups of 3), consistent with the results of morphological identification (Table 1). Based on host origin, 7 isolates of *C. acutatum*, 8 of *C. gloeosporioides* and 7 of *C. fragariae* were isolated from the Zhejiang area, and 4, 2 and 3, respectively, from the Shanghai region. This confirms that the species of *Colletotrichum* causing anthracnose disease of strawberry in Zhejiang and Shanghai are the same.

3.3 Species-specific PCR and enzyme digestion

Representative isolates, WY01, WY06 and WY08 for *C. gloeosporioides*, WY12, WY19 and WY14 for *C. fragariae*, and WY27, WY29 and WY31 for *C. acutatum*, were selected for species-specific PCR and enzyme digestion.

The nine representative isolates were detected by using the *C. acutatum*-specific primer *CaInt2* in conjunction with the ITS4 primer. About 490-bp fragments were amplified from the genomic DNA of

the *C. acutatum* isolates but no amplification products were produced from the isolates representative of *C. gloeosporioides* or *C. fragariae*, along with the negative control (*Trichoderma virens*) (Fig. 3). This species-specific PCR result further confirmed the species identity of *C. acutatum*, consistent with phylogenetic and morphological identification.

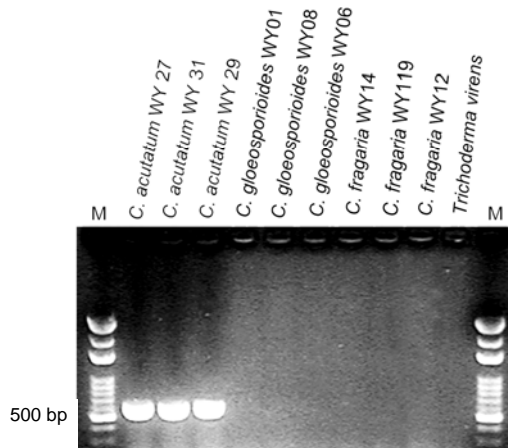


Fig. 3 PCR amplification of a specific fragment from *Colletotrichum* species using the *C. acutatum* specific primer *CaInt2* in conjunction with the primer *ITS4*

Lanes 1–3 correspond to representative *C. acutatum* isolates; Lanes 4–6 are the representative *C. gloeosporioides* isolates; Lanes 7–9 correspond to representative *C. fragariae* isolates; Lane 10 is a negative control (*Trichoderma virens* genomic DNA); Lane M corresponds to the 100–3000 bp molecular weight marker (Bio Basic Inc., Canada)

The isolates representative of *C. fragariae* and *C. gloeosporioides* were analyzed using the endonuclease *MvnI*. The restriction endonuclease *MvnI* cut all *C. gloeosporioides* PCR products amplified by PCR using the universal primers *ITS1* and *ITS4* to produce two fragments of about 480 and 100 bp. The species-specific restriction pattern could be obtained from the *C. gloeosporioides* isolates but this endonuclease did not cut any of the *C. fragariae* isolates (Fig. 4). This result confirms the established method for species identification using *MvnI* digestion data (Martinez-Culebras *et al.*, 2000; 2003).

4 Discussion

Most strawberry cultivars grown in Zhejiang Province and Shanghai City were introduced from

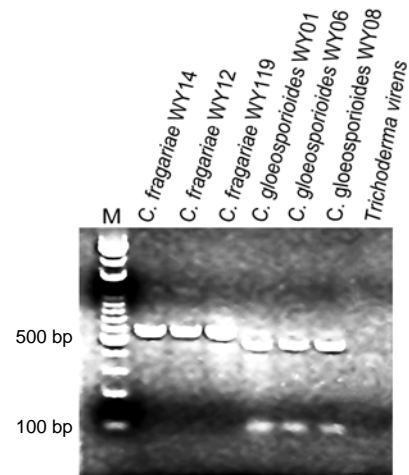


Fig. 4 Ribosomal DNA restriction patterns shown by *Colletotrichum* species after amplification with universal primers *ITS1* and *ITS4* followed by digestion with the restriction endonuclease *MvnI*

Lanes 1–3 correspond to representative *C. fragariae* isolates; Lanes 4–6 correspond to representative *C. gloeosporioides* isolates; Lane 7 is a positive control (*Trichoderma virens* genomic DNA); Lane M corresponds to the 100–3000 bp molecular weight marker (Bio Basic Inc., Canada)

Japan and the cv. Toyonoka is presently the principal cultivar. Almost all of the isolates of *Colletotrichum* spp. responsible for the anthracnose disease originated from this cultivar. In this study, we identified the *Colletotrichum* spp. causing strawberry anthracnose, and aimed to determine whether the same or different species occur in strawberry nurseries and production fields in Zhejiang and Shanghai.

Traditionally, three species from strawberry were considered to be differentiated by morphological characters (Gunnell and Gubler, 1992). However, morphological criteria can be unreliable and non-predictive, because of highly variable morphological characteristics (Sreenivasaprasad *et al.*, 1996; Sutton, 1992; Johnston and Jones, 1997). So Ye *et al.* (1997) possibly failed to distinguish *C. gloeosporioides* from *C. fragariae* from strawberry plants from Shanghai.

Molecular genetic studies have provided useful data for clarifying the systematics of the genus *Colletotrichum* (Martinez-Culebras *et al.*, 2003). However, our studies showed that the divergence of 5.8S-ITS region sequences among isolates of *C. fragariae* and *C. gloeosporioides* was too low to separate them confidently as different species, confirming the observations of Sherriff *et al.* (1995),

Sreenivasaprasad *et al.* (1996) and Martinez-Culebras *et al.* (2003). Similarly, Ren *et al.* (2008) found that the ITS sequences of 17 isolates from strawberry were 99% identical, but they all were mistaken for *C. gloeosporioides*. Although the similarity between the nucleotide sequences of *C. gloeosporioides* and *C. fragariae* prevented the development of specific primers for these species based on the 5.8S-ITS region, Martinez-Culebras *et al.* (2000) found that there was an *MvnI* specific site among ITS1 region of isolates of only *C. gloeosporioides* and not *C. fragariae*. This trait was confirmed subsequently by different original sequence data and could be used for differentiating *C. gloeosporioides* from *C. fragariae* (Martinez-Culebras *et al.*, 2003). Martinez-Culebras *et al.* (2003) pointed out that this conclusion still needed to be confirmed by studying a higher number of isolates of *C. fragariae* and *C. gloeosporioides* from strawberry and also *C. gloeosporioides* from different hosts (Martinez-Culebras *et al.*, 2003). Our results provide new evidence that the *MvnI* site within the ITS1 region from isolates of *C. gloeosporioides* from strawberry is conservative.

In *C. acutatum*, phylogenetic analysis based on 5.8S-ITS region sequences indicated that different original isolates of pathogens of strawberry form a polyphyletic taxon, consistent with other observations (Martinez-Culebras *et al.*, 2003; Freeman *et al.*, 2000; 2001). The sequences of our 11 isolates of *C. acutatum* from Zhejiang and Shanghai, however, were identical to that of a strawberry isolate of *C. acutatum* (IMI 360086) from Japan. Although currently there are over 600 rDNA-ITS sequences from *C. acutatum* isolates available in public databases, a BLAST search showed that our sequences were identical to those of AL-9 (GenBank accession No. AB219025), AL-5 (GenBank accession No. AB219024) and IMI 360086 found only on strawberry in Japan. This suggests that this species has been introduced into Zhejiang and Shanghai from Japan through infected propagation material and has rapidly become established locally. However, analysis of sequences showed that there were two sequence types in *C. gloeosporioides* and three in *C. fragariae*. These may reflect the genetic diversity of population structures within them. Within *C. gloeosporioides*, two sequence types were distributed in Zhejiang and Shanghai but based on the frequency of isolation, and

the sequence types of the isolates WY01, WY02, WY03, WY05, WY06, WY07, WY09 and WY10 might be the predominant forms. Within *C. fragariae*, there were three sequence types of which two were distributed in Zhejiang and Shanghai and one, from isolates WY19, WY17 and WY11, was found only in Zhejiang (Table 1).

Based on isolation data (Table 1), *C. acutatum* can be isolated from fruits, leaves and petioles, *C. gloeosporioides* from leaves, petioles and root crowns, and *C. fragariae* from root crowns, leaves and petioles. Differences in pathogenicity among them, however, still need to be evaluated by pathogenicity tests. Although pathogenicity tests had been performed and typical symptoms could be seen, control transplant seedlings also could produce similar symptoms. Apparently, *Colletotrichum* spp. may be present in the form of latent infections in strawberry. The presence of latent infections of *Colletotrichum* spp. in strawberry was detected by a freeze treatment method and *Colletotrichum* spp. could be found on up to 50% of leaves and 10% of petioles in transplant seedlings (Mertely and Legard, 2004). For this reason, currently we still have not obtained reliable results from pathogenicity tests. However, this may be resolved in future studies by using pathogen-free strawberry plants obtained by propagating plantlets from meristem culture.

Based on morphological characters, phylogenetic analysis, species-specific PCR and enzyme digestion, three species, *C. acutatum*, *C. gloeosporioides* and *C. fragariae*, are considered the cause of anthracnose in strawberry-growing areas of Zhejiang and Shanghai. Although molecular studies are helpful for resolving relationships within the genus *Colletotrichum*, studies to date can be regarded as only preliminary (Photita *et al.*, 2005). Many questions remain regarding the interspecies relationships within the genus, such as between *C. gloeosporioides* and *C. fragariae*. Sequence analysis from ITS regions has enabled some progress to be made towards a better understanding of the taxonomy of *Colletotrichum*, but the analysis of a larger collection of *C. gloeosporioides* and *C. fragariae* isolates, preferably based on a multilocus comparison (e.g., of ITS, actin, beta-tubulin, calmodulin, glutamine synthetase), is required before the two taxa can be differentiated with confidence.

References

- Buddie, A., Martínez-Culebras, P., Bridge, P., García, M., Querol, A., Cannon, P., Monte, E., 1999. Molecular characterization of *Colletotrichum* strains derived from strawberry. *Mycological Research*, **103**(4):385-394. [doi:10.1017/S0953756298007254]
- Cooke, D.E.L., Duncan, J.M., 1997. Phylogenetic analysis of *Phytophthora* species based on the ITS1 and ITS2 sequences of ribosomal DNA. *Mycological Research*, **101**(6):667-677. [doi:10.1017/S0953756296003218]
- Dai, F.M., Ren, X.J., Lu, J.P., 2006. First report of anthracnose fruit rot of strawberry caused by *Colletotrichum acutatum* in China. *Plant Disease*, **90**(11):1460. [doi:10.1094/PD-90-1460A]
- Debode, J., Hemelrijck, W., Baeyen, S., Creemers, P., Heungens, K., Maes, M., 2009. Quantitative detection and monitoring of *Colletotrichum acutatum* in strawberry leaves using real-time PCR. *Plant Pathology*, **58**(3):504-514. [doi:10.1111/j.1365-3059.2008.01987.x]
- Denoyes-Rothan, B., Guérin, G., Délye, C., Smith, B., Minz, D., Maymon, M., 2003. Genetic diversity and pathogenic variability among isolates of *Colletotrichum* species from strawberry. *Phytopathology*, **93**(2):219-228. [doi:10.1094/PHYTO.2003.93.2.219]
- Freeman, S., Minz, D., Jurkevitch, E., Maymon, M., Shabi, E., 2000. Molecular analysis of *Colletotrichum* species from almond and other fruits. *Phytopathology*, **90**(6):608-614. [doi:10.1094/PHYTO.2000.90.6.608]
- Freeman, S., Minz, D., Maymon, M., Zveibil, A., 2001. Genetic diversity within *Colletotrichum acutatum* sensu Simmonds. *Phytopathology*, **91**(6):586-592. [doi:10.1094/PHYTO.2001.91.6.586]
- Gunnell, P.S., Gubler, W.D., 1992. Taxonomy and morphology of *Colletotrichum* species pathogenic to strawberry. *Mycologia*, **84**(2):157-165. [doi:10.2307/3760246]
- Johnston, R.P., Jones, D., 1997. Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. *Mycologia*, **89**(3):420-430. [doi:10.2307/3761036]
- Lubbe, C.M., Denman, S., Cannon, P.F., Groenewald, J.Z., Lamprecht, S.C., Crous, P.W., 2004. Characterization of *Colletotrichum* species associated with diseases of Proteaceae. *Mycologia*, **96**(6):1268-1279. [doi:10.2307/3762144]
- MacKenzie, S.J., Mertely, J.C., Seijo, T.E., Peres, N.A., 2008. *Colletotrichum fragariae* is a pathogen on hosts other than strawberry. *Plant Disease*, **92**(10):1432-1438. [doi:10.1094/PDIS-92-10-1432]
- Martinez-Culebras, P.V., Barrio, E., García, M.D., Querol, A., 2000. Identification of *Colletotrichum* species responsible for anthracnose of strawberry based on the internal transcribed spacers of the ribosomal region. *FEMS Microbiology Letter*, **189**(1):97-101. [doi:10.1016/S0378-1097(00)00260-3]
- Martinez-Culebras, P.V., Querol, A., Suarez Fernandez, M.B., Garcia Lopez, M.D., Barrio, E., 2003. Phylogenetic relationships among *Colletotrichum* pathogens of strawberry and design of PCR primers for their identification. *Journal of Phytopathology*, **151**(3):135-143. [doi:10.1046/j.1439-0434.2003.00694.x]
- Mertely, J.C., Legard, D.E., 2004. Detection, isolation, and pathogenicity of *Colletotrichum* spp. from strawberry petioles. *Plant Disease*, **88**(4):407-412. [doi:10.1094/PDIS.2004.88.4.407]
- Moriwaki, J., Tsukiboshi, T., Sato, T., 2002. Grouping of *Colletotrichum* species in Japan based on rDNA sequences. *Journal of General Plant Pathology*, **68**(4):307-320. [doi:10.1007/PL00013096]
- Nirenberg, H.I., Feiler, U., Hagendorn, G., 2002. Description of *Colletotrichum lupini* comb. nov. in modern terms. *Mycologia*, **94**(2):307-320. [doi:10.2307/3761809]
- Photita, W., Taylor, P.W.J., Ford, R., Lumyong, P., McKenzie, H.C., Hyde, K.D., 2005. Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Diversity*, **18**:117-133.
- Ren, X.J., Liang, Y., Lu, J.P., Yang, B.R., Xu, J.Y., Dai, F.M., 2008. Identification of *Colletotrichum* species from strawberry in Shanghai. *Acta Phytopathologica Sinica*, **38**(3):325-328.
- Shao, X.H., 1992. New disease of strawberry: strawberry anthracnose. *Acta Agriculturae Shanghai*, **8**(2):86-87.
- Sherriff, C., Whelan, M.J., Arnold, G.M., Bailey, J.A., 1995. rDNA sequence analysis confirms the distinction between *Colletotrichum graminicola* and *C. sublineolum*. *Mycological Research*, **99**(4):475-478. [doi:10.1016/S0953-7562(09)80649-7]
- Sreenivasaprasad, S., Talhinhas, P., 2005. Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. *Molecular Plant Pathology*, **6**(4):361-378. [doi:10.1111/j.1364-3703.2005.00291.x]
- Sreenivasaprasad, S., Mills, P.R., Meehan, B.M., Brown, A.E., 1996. Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. *Genome*, **39**(3):499-512. [doi:10.1139/g96-064]
- Sutton, B.C., 1992. The Genus *Glomerella* and Its Anamorph *Colletotrichum*. In: Bailey, J.A., Jeger, M.J. (Eds.), *Colletotrichum*, Biology, Pathology and Control. CAB International, Oxford, UK, p.1-26.
- Swofford, D.L., 2002. PAUP: Phylogenetic Analysis Using Parsimony (and Other Methods). Version 4b10, Sinauer Associates, Sunderland, USA.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**(24):4876-4882. [doi:10.1093/nar/25.24.4876]
- White, T.J., Bruns, S.L., Taylor, J.W., 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols. A Guide to Methods and Applications. Academic Press Inc., San Diego, p.315-322.
- Ye, Z.W., Zheng, H.Q., Tong, Y.M., 1997. First report on species of strawberry anthracnose pathogen in Shanghai suburb and resistance of some strawberry cultivars to anthracnose. *Acta Agriculturae Shanghai*, **13**(1):75-80.