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Identification of *Colletotrichum* species associated with anthracnose disease of coffee in Vietnam

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Abstract:

Colletotrichum gloeosporioides, *C. acutatum*, *C. capsici* and *C. boninense* associated with anthracnose disease on coffee (*Coffea* spp.) in Vietnam were identified based on morphology and DNA analysis. Phylogenetic analysis of DNA sequences from the internal transcribed spacer region of nuclear rDNA and a portion of mitochondrial

small subunit rRNA were concordant and allowed good separation of the taxa. We found several *Colletotrichum* isolates of unknown species and their taxonomic position remains unresolved. The majority of Vietnamese isolates belonged to *C. gloeosporioides* and they grouped together with the coffee berry disease (CBD) fungus, *C. kahawae.* However, *C. kahawae* could be distinguished from the Vietnamese *C. gloeosporioides* isolates based on ammonium tartrate utilization, growth rate and pathogenictity. *C. gloeosporioides* isolates were more pathogenic on detached green berries than isolates of the other species, *i.e. C. acutatum, C capsici* and *C. boninense*. Some of the *C. gloeosporioides* isolates produced slightly sunken lesion on green berries resembling CBD symptoms but it did not destroy the bean. We did not find any evidence of the presence of *C. kahawae* in Vietnam.

Introduction

The genus *Colletotrichum* consists of several economically important plant pathogenic fungi, occurring predominantly in tropical and subtropical regions on a wide range of crops (Waller. 1992). Single species of *Colletotrichum* can infect multiple hosts. For example, *C. gloeosporioides* Penz, is pathogenic on citrus, avocado, papaya, and other crops. Also, multiple species of *Colletotrichum* can be jointly associated with anthracnose on a single host. For example, several *Colletotrichum* species can cause disease on coffee (Freeman. 2000). *C. kahawae* Waller, causes coffee berry disease (CBD), which can cause yield losses up to 80% (Varzea et al. 2002; Chen et al. 2005) in Africa. *C. gloeosporioides* and *C. acutatum* Simmonds also occur on coffee and may cause minor disease on ripening berries (Masaba and Waller 1992). However, it has also been reported that *C. gloeosporioides* can over grow *C. kahawae* and enhance the CBD infection process when the berries become mature or under conditions of environmental stress (Chen et al. 2003). *C. kahawae* can be distinguished from *C. gloeosporioides* by its inability to utilize citric acid and ammonium tartrate as sole carbon sources, by its high pathogenicity on coffee and by molecular markers as AFLP and VNTR-PCR (Waller et al. 1993; Bridge et al. 2008). Since *C. kahawae* causes a specific disease on coffee, there are practical reasons for considering this as a distinct taxon (Bridge et al. 2008). However, according to Cannon et al. (2008), *C. kahawae* is closely related to the *C. gloeosporioides* complex due to morphological and genetic similarities.

In Vietnam, anthracnose diseases on *Coffea* spp. have occurred during recent years. The highest infection rate on coffee berries usually appears in September during berry development and ripening and up to 50% of the berries on *C. arabica* can be affected (Tran et al. 1998). Symptoms of anthracnose somewhat resemble those of the CBD, *i.e*. slightly sunken or brown blight lesions occur on green and ripening berries that can interfere with pulping during seed processing and also be responsible for shedding of premature berries (Pinkert. 2004; Nguyen et al. 2009). In our previous study (2009) we found that certain Vietnamese isolates of *C. gloeosporioides* were pathogenic on green berries and revealed a high level of genetic variation in the *C. gloeosporioides* populations. *C. acutatum* and *C. capsici* or *C. dematium* have also been found on coffee roots and leaves in Vietnam (unpublished).

Traditionally, identification of *Colletotrichum* species has primarily relied on morphological characters such as colony color, size and shape of the conidia, growth rate, presence or absence of setae, and existence of the teleomorph, *Glomerella* (Smith & Black. 1990). However, the classical identification of *Colletotrichum* species has encountered some limitations due to the plasticity of morphological traits under environmental impact (Sutton. 1992). In addition, differentiation between *Colletotrichum* species based on host range can be unreliable, since taxa such as *C. gloeosporioides, C. dematium, C. acutatum* and *C. graminicola* infect a broad range of host plants (Sutton. 1992).

Molecular approaches have been successfully used in addition to morphological studies in identification of *Colletotrichum* species. Sequencing analysis of the ribosomal DNA (rDNA), a fragment of mitochondrial small subunit rRNA gene (mtSSU) and the ß-tubulin gene have been used for species and subspecies delineation of *Colletotrichum* (Sreenivasaprasad et al. 1993; Vinnere et al. 2002). Species specific primers have been designed from those regions and used for species identification (Sreenivasaprasad et al. 1996).

There have only been a few studies on the causal agents of anthracnose disease on coffee outside Africa. Recently, *C. gloeosporioides* and *C. acutatum* responsible for anthracnose disease on *Coffea arabica* were found in Papua New Guinea (Kenny et al. 2006). To our knowledge, no careful investigation on which *Colletotrichum* species that are associated with anthracnose disease on coffee in Vietnam has ever been carried out. Therefore, the aims of this study were to: i) identify species of *Colletotrichum* that are associated with anthracnose disease on *Coffea* spp. in Vietnam ii) and determine their pathogenic ability

Materials and methods

Fungal isolates

Forty-six *Colletotrichum* isolates (Table 1) were obtained from infected berries, leaves and twigs of *Coffea* spp. from five Vietnamese provinces, Ha Tay, Yen Bai, Son La, Nghe An and Dak Lac (Fig. 1). The majority of collected samples were premature green berries with slightly sunken and dark brown lesions. The diseased samples were surface disinfested with commercial chlorine bleach (Colgate-Palmolive AB, Danderyd, Sweden) containing 1.5% of sodium hypochlorite (pH 12.8) for 7-10 min and rinsed three times in sterile distilled water. Isolations were made by placing small pieces taken from the margin between diseased and healthy tissues on potato dextrose agar (PDA) (Difco, France) amended with tetracycline (100 µg/ml) (Duchefa, the Netherlands), followed by incubation at room temperature (22 ±2 °C). Fungal hyphae growing from the pieces were transferred to fresh PDA.

Isolates of *C. kahawae* (CBD), *C. capsici, C. gloeosporioides, C. acutatum* and *C. boninense* previously identified by reliable sources were used as references (Table 1). The cultures were preserved on 50% PDA slants at 4 °C under paraffin oil, as conidial suspensions in 50% glycerol at -80 °C and as mycelial blocks in sterile distilled water at 4 °C.

Morphology and culture characteristics

Agar plugs from 7-day-old cultures were placed onto fresh PDA medium. Colony and conidial characters were recorded after 6 to 7 days of growth at 25 °C in darkness. To induce sporulation, some cultures were placed in a 12-h photoperiod under near ultra violet (black) light (Phillips TL20W/08) and/or transferred to potato carrot agar (PCA) or oat meal agar (OMA) (Difco, UK). Samples of conidia were taken and mounted in water. The conidial shape was observed under a light microscope (Leica Microsystems) and photographs were taken with a Leica Digital camera DC 300 (Leica Microsystems). For each isolate, length and width of 25 randomly chosen conidia were measured by using the image analysis program Leica Qwin Standard (Leica Microsystems).

To measure growth rates on PDA after 5 days, plates were inoculated with 5 mm-diameter mycelial plugs (5 mm) excised from the margin of a 7-day-old PDA culture. Cultures were incubated in the darkness at 15, 20, 25, 30 and 35 °C with three replicates. The radius was measured at the cardinal points for each plate. The experiment was repeated at 20, 25 and 30 °C with representative isolates, *i.e.* 413(1) and 606b(1)

Substrate utilization tests

Isolates of *C. gloeosporioides* were tested for their ability to utilize ammonium tartrate as a sole carbon source according to the method described by Waller et al.

(1993). *C. kahawae* was included as control since it is known not to be able to metabolize tartrate.

Pathogenicity tests

Twenty-eight Vietnamese isolates of *Colletotrichum* were tested for pathogenicity on detached green berries of the Arabica cultivar 'Catimor'. One isolate of the CBD-fungus (*C. kahawae*) was used as a positive control in the tests (Table 3). A modified method based on the description given by Bock (1956) was used. Green berries were first sterilized as above. Ten non-wounded and ten wounded green berries (the surface was pierced 1-2mm deep with a sterile needle prior to inoculation) were used for each replicate. The berries were placed on moistened sterilized sand in a plastic box maintained at high humidity. A droplet of conidial suspension (2×10^6 conidia/ml) or a 5 mm agar plug of mycelia was placed on the surface of the berries. For the former, the inoculation was repeated after 48 h. Symptoms on coffee berries were observed 11 days after the inoculation. The number of infected berries was counted and the lesion diameter of the infected berries was measured. Three replicates were used for each isolate. The experiment was repeated with some of the pathogenic *C. gloeosporioides* isolates, *i.e.* 119b(1), 702(1),531(7), 606b(4) and SL1a(9) and some representative isolates of the other identified species, *i.e.* BV14, 413(1) and 606b(1).

DNA extraction

Five mycelial disks derived from the edge of 7-day-old actively growing colonies on PDA were transferred into a Petri dish with 20 ml glucose yeast extract medium (GYM). The cultures were incubated for 5 days at 25 °C. Mycelia were washed with sterile distilled water, harvested by vacuum filtration through a Munktell filter paper (grade 3) and grinded under liquid nitrogen. DNA was extracted as previously described (Nguyen et al. 2009). The DNA working concentration was standardized by addition of DNase and RNase free water after assessment of DNA concentration and quality by using spectrophotometry (ND-1000, Nanodrop) and 1.4% (w/v) agarose gel electrophoresis.

Identification by using specific primers

Col1 and Col2 specific primers, designed from 5.8S-ITS region by Martinez-Culebras et al. (2003), were used in identification of *Colletotrichum* genus. DNA amplification of all Vietnamese isolates by using these primers was carried out according to the method previously described by Martinez-Culebras et al. (2003).

A species-specific primer located within the ITS1 region (Sreenivasaprasad et al. 1992; 1996; Ureña-Padilla et al. 2002) was used in combination with the conserved universal primer, ITS4 (White et al. 1990) to confirm morphological identification of *Colletotrichum* isolates. The ITS1 primers were either Cg/fInt1 or CaInt2 that are specific for *C. gloeosporioides* and *C. acutatum*, respectively. Amplification was performed in 20 µl reaction volume containing 50-100ng of DNA, 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP, 1 µM of primer and 1 unit of *Taq*

polymerase (Sigma-Aldrich, St. Louis, MO, USA). The PCR reaction was carried out in a Gen Amp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA), starting with a denaturation step at 95 °C for 5 min, followed by 34 cycles consisting of denaturation at 94 °C for 1min, annealing either at 55 °C (for CaInt2) or 59 °C (for Cg/fInt1) for 1min and 2 min of extension at 72 °C and final extension step at 72 °C for 10 min. The amplified products were separated on 1.5% agarose gels and stained in 0.5xTBE (Tris-Borate-EDTA) buffer with ethidium bromide (0.5µg/ml).

PCR amplification and DNA sequencing

The ITS and mtSSU regions were amplified by using the ITS1/ITS4 primers (White et al. 1990) and NMS1/NMS2 primers (Li et al. 1994) respectively. Both regions were amplified by using PfuUltra II fusion HS DNA polymerase according to the manufacturer's instruction (Stratagene, Delaware, CA, USA). The amplified DNA products were purified using Qiagen QIAquick PCR Purification Kit (Qiagen AG, Basel, Switzerland). The purified DNA was sequenced on both strands using the same primers as used in the PCR reaction by MWG Biotech (Ebersberg, Germany). Sequencing results were evaluated using Phred/Phrap (Ewing & Green. 1998). Stretches of contig sequences having bases showing a continuous quality value equal to or above 30 were accepted and used for further analysis.

Phylogenetic analysis

The resulting sequences were aligned with ClustalW using the BioEdit software 7.0.5 (Tom Hall, North Carolina State University, Raleigh, NC), followed by manual adjustments where necessary. Parsimony analysis was carried out on the aligned sequences of ITS and mtSSU rDNA separately using PAUP (Phylogenetic Analysis Using Parsimony) 4.0b software (Sinauer Associates Inc., Sunderland, MA). Gaps were treated as missing data with the exceptions for several parsimony-informative gaps in the ITS data set that were treated as the 5^{th} character. All characters were of type "unordered" and had equal weight. The reliability of tree topologies was evaluated by bootstrap analysis using 1000 replications in heuristic search combining 10 random replicates by stepwise addition. *Fusarium redolens* with accession number AF008562 and U34507 for the ITS and mtSSU region, respectively, was used as an outgroup.

Statistical analysis

Differences in conidial sizes and growth rates were investigated with Tukey's multiple range test. Differences in pathogenicity between species, *i.e*. frequencies of infected berries, were tested with analysis of variance (GLM) on arcsine transformed frequency values according to Zar (1984):

$$p' = 1/2 \times \arcsin\sqrt{X/(n+1)} + \arcsin\sqrt{(X+1)/(n+1)}$$

Where *p'* is the transformed frequency, *X* is the number of infected berries and *n* is the total number of inoculated berries.

The analyses were carried out with SAS statistical package (SAS Institute Inc., Cary, NC, USA), release 8.2.

Results

Morphological characterization

Mycelia in pure cultures of all Vietnamese isolates were light to dark grey. Some dark grey isolates produced abundant sclerotia. Several isolates, *e.g*. 606b (1), LD35a (T2) and LD11 (L3), formed *Glomerella* teleomorphs in pure culture. All isolates were divided into three groups depending on conidial shape. Thirty-nine isolates had cylindrical conidia rounded at one or both ends, five isolates had fusiform conidia, and finally, conidia of two isolates, BV14 and SL204a, were falcate. The conidia of the Vietnamese *Colletotrichum* varied considerably in length and width (Table 2).

In addition, several isolates with dark grey color had larger cylindrical conidia than any of previously described species of *Colletotrichum*, *e.g*. isolates BMT25 (L3) and LD16 (L2) had conidial sizes ranged 25.4- 40.6 x 8.4-11.7 µm.

Substrate utilization

All Vietnamese *Colletotrichum* isolates, except 606b (1), LD30a (T4), BMT10 (L2), BMT16 (T3) and NA40a (11), were able to use ammonium tartrate as a sole carbon source (Table 1). All three reference CBD isolates (*C. kahawae)* and one of the reference isolates of *C. boninense*, (isolate MAFF 306094) could not metabolize ammonium tartrate.

Fungal growth rate

The mycelial growth rates at different temperatures varied greatly among the studied *Colletotrichum* isolates. All Vietnamese isolates had relatively slow growth at 15 °C. Most isolates that belonged to the group with cylindrical conidia grew rapidly at both 25 and 30 °C, on average 5.4 mm day^{-1} at 25 °C. However, two of the isolates of unknown species (LD30a (T3) and BMT10 (T2)) had slow growth rate, *i.e*. 1.9 to 3.15 mm day^{-1} at 30 °C. The five isolates with fusiform conidia grew slowly compared to the isolates with cylindrical conidia, had an optimal growth rate around 25 °C and did not grow at all at 35 °C. The isolates with falcate conidia grew fast at 30 °C.

The reference CBD isolates grew fast at 20 and 25 °C, slowly at 30 °C and did not grow at all at 35 °C (Fig. 2), whilst the Vietnamese isolates that could not metabolize ammonium tartrate, grew fast at 25 and 30 °C and grew at 35 °C. When all isolates were compared the growth rate at 30 °C differed significantly between the CBD isolates and every Vietnamese isolate identified as *C. gloeosporioides* (Tukey's studentized Range Test, P=0.05) (Fig. 2)

Identification by using specific primers

PCR amplification of DNA from the Vietnamese isolates using Col1/Col2 primers, revealed a fragment of approximate 460bp indicating that all isolates belonged to the *Colletotrichum* genus.

Five Vietnamese isolates of *Colletotrichum* with fusiform conidia and the reference isolate of *C. acutatum* (isolate 397) produced a PCR product of 490bp with CaInt2/ITS4 primers (data not shown).

Twenty-three Vietnamese isolates out of 39 with cylindrical conidia were identified as *C. gloeosporioides* since these isolates and the reference isolate of *C. gloeosporioides* (PR220) produced a 450bp long fragment after PCR amplification with the CgfInt/ITS4 primers. No amplified product was found from the other 16 isolates with cylindrical conidia, five isolates with fusiform and two isolates with falcate conidia.

Phylogenetic analysis

Phylogeny based on the ITS region. The ITS dataset contained 544 characters including alignment gaps, of which 139 characters were found parsimony-informative. After sequencing of the ITS region of all 56 isolates, we discovered that there were several isolates with 100% identical sequences, representing 31 unique sequence types. Therefore, we only included 31 isolates in the phylogenetic study, *i.e*. one representative of each sequence type (Table 1). Six clades were generated from the analysis with high bootstrap support (Fig. 3). Eight Vietnamese representative isolates out of 20 having cylindrical conidia fell in the clade of *C. gloeosporioides* reference isolate (231) and together with the CBD reference isolate (*C. kahawae*, IMI357056) (Clade3) with 100% support. This included 2 Vietnamese isolates that were unable to utilize tartrate. Five isolates with fusiform conidia were placed

together with *C. acutatum* reference isolates (NI90 and 397). Clade 1 containing two isolates with falcate conidia grouped together with *C. capsici* (IMI 56173) with high support. Four isolates with cylindrical conidia, including isolates 606b (1) and BMT10 (L2) that were not able to metabolize ammonium tartrate grouped together with the reference isolates of *C. boninense*. The other isolates with cylindrical broad-sized conidia and dark colony color fell into two separate clades (Clade 3 and clade 6) and were therefore considered as unknown species.

Phylogeny based on the mtSSU region. Similarly, the same groups of isolates as in the case of the ITS data set had identical sequences, thus we used the same subset of taxa as for constructing the ITS trees. The size of the mtSSU region varied substantially among the studied species: 407bp for *C. gloeosporioides*, 481bp for *C. acutatum*, 407bp for *C. capsici*, 412bp for C. *boninense* and 406 to 409bp for the isolates of unknown species. Out of more than 657 total characters, 122 were found parsimony-informative. The topology of the mtSSU tree was concordant with the ITS tree. Four main clades of *C. gloeosporioides*, *C. acutatum*, *C. capsici* and *C. boninense* were delimited with high bootstrap values (Fig. 4). All representative isolates of Vietnamese *C. gloeosporioides* and the reference of CBD (*C. kahawae*) were placed together in the same group as the reference *C. gloeosporioides* isolate 231 with 100% support. Three clades were created from isolates of the unknown species and well supported by high bootstrap, which was higher than in the ITS tree. Clades 3a and 3b (Fig. 4) derived from subclades within clade 3 of the ITS tree.

Isolates that were unable to use ammonium tartrate fell into three different groups of *Colletotrichum* species similar as in the ITS tree.

Pathogenicity tests

The CBD isolate (IMI 357057, *C. kahawae*) was the most pathogenic indicated by the high infection percentage (100%) of both wounded and non-wounded green berries (Table 3). Conversely, most of the Vietnamese *Colletotrichum* isolates caused only moderate damage, mainly on wounded berries, with less severe and less sunken lesions than those caused by the reference CBD isolate (*C. kahawae*). However, several of the Vietnamese isolates also produced clear disease symptoms on non-wounded green berries although at a lower rate. The variability of the results was high as indicated by high SD values. A higher percent infection was found on wounded berries compared to non-wounded berries (P=0.0037 and P=0.0001 for inoculation with agar plug and conidia, respectively). Six of the isolates belonging to *C. gloeosporioides* and one from unknown species were able to induce lesions on non-wounded berries. *C. gloeosporioides* isolate 119b (1) was the most pathogenic among the Vietnamese isolates, and it produced the same size of slightly sunken lesion as the CBD isolate but at lower rate. None of the isolates of *C. acutatum*, *C. capsici* or *C. boninense* produced symptoms on non-wounded berries. The percent infected berries differed significantly between *C. gloeosporioides* and the other species found in Vietnam (P=0.0001 and 0.014 for inoculation with agar plug and conidia,

respectively). The repeated experiment showed the same results as the previous experiment.

Discussion

By combining morphological characterization, species specific primers and sequencing analysis of the portion of mtSSU and ITS region we identified *C. gloeosporioides, C. acutatum, C. capsici*, and *C. boninense* as being associated with anthracnose disease on *Coffea* spp. in Vietnam. In addition, we found ten *Colletotrichum* isolates of unknown species. In total more than 30 isolates of those morphological types were found. Several of the Vietnamese isolates were found to be pathogenic, *i.e*. they produced lesions on detached green berries after inoculation.

Vietnamese *C. acutatum* species derived from coffee could be distinguished from *C. gloeosporioides* by using species-specific primers designated for either *C. acutatum* or *C. gloeosporioides* and by the phylogenetic analysis. *C. acutatum* was reported to be associated with disease on coffee in Papua New Guinea (Kenny et al. 2006). *C. acutatum* is a common and quite an aggressive pathogen on a variety of crops in tropical regions. However, on coffee plants in Africa Masaba and Waller (1992) have regarded this species as mainly saprophytic. We found no symptoms caused by this species on non-wounded berries, thus confirming observations of Massaba and Waller.

The majority of the Vietnamese isolates belonged to *C. gloeosporioides* and was found mainly on berries and not so frequent on twigs and leaves. All isolates of *C. gloeosporioides* and the reference isolate of *C. kahawae* fell in the main group of *C.*

gloeosporioides based on both ITS and mtSSU sequence analyses. This is in agreement with earlier studies and indicates that *C. kahawae* should probably be considered as synonymous to *C. gloeosporioides* (Sreenivasaprasad et al. 1993; Cannon et al. 2008). However, there have been strong debates on taxonomic relevance of these taxa, *e.g*. Waller et al. (1993) stated that *C. kahawae* should be considered as a distinct species due to its high virulence, biochemical features and endemicity to Africa. In a recent study we reported high genetic variation in Vietnamese *C. gloeosporioides* populations causing anthracnose disease of coffee berries, based on RAPD and MpPCR markers (Nguyen et al. 2009). This is typical for *C. gloeosporioides* populations on different hosts (Mills et al. 1992; Abang et al. 2006), while studies of the genetic diversity within populations of the CBD-fungus (*C. kahawae*) revealed low diversity (Derso & Waller. 2003; Bridge et al. 2008). Inability of *C. kahawae* to utilize ammonium tartrate as a sole carbon source can be a distinguishable criterion to differentiate *C. kahawae* from other *Colletotrichum* species associated with coffee berry disease (Waller et al. 1993; Bridge et al. 2008). Furthermore, Bridge et al. (2008) considered *C. kahawae* as a distinct taxon that can be separated from *C. gloeosporioides* by assessing mtDNA RFLP and VNTR. A significant difference in growth rate between *C. gloeosporioides* and *C. kahawae* was found at 25 and 30 $^{\circ}$C in our study. This correlated to previous reports by Waller et al. (1993) and Varzea et al. (2002). Two of the Vietnamese *C. gloeosporioides* isolates could not metabolize tartrate, but grew fast at high temperature. Pathogenicity tests on berries and hypocotyls can separate *C. kahawae* from other species of *Colletotrichum* (Varzea et al. 2002). According to our results, the Vietnamese *C.*

gloeosporioides could be distinguished from *C. kahawae* on basis of growth rate, tartrate utilization and pathogenicity. *C. kahawae* caused deeply sunken lesions on the berry surface, then invaded and destroyed the bean similar to previous studies by Chen et al. (2005) and Masaba and Waller (1992), while Vietnamese *C. gloeosporioides* caused less severe disease symptoms.

Some isolates having somewhat larger cylindrical conidia than *C. gloeosporioides*, were identified as *C. boninense* (Moriwaki et al. 2003). This species has originally been isolated in Japan and has been described as a new species in the *C. gloeosporioides* complex (Moriwaki et al. 2003). Thereafter, it has been reported from South America, China, Australia, Zimbabwe and Brazil (Lu et al. 2004; Lubbe et al. 2004; Farr et al. 2006; Tozze Jr et al. 2009). *C. boninense* has been found on a broad range of hosts, *i.e*. mono/dicotyledonous, herbaceous and woody plants (Farr et al. 2006). We found the teleomorph in pure cultures in almost all Vietnamese *C. boninense* isolates, similar to *C. boninense* isolates derived from other host plants reported by Lu et al. (2004). To our knowledge, presence of teleomorph stage has never been reported in other studies of *C. boninense*. Pathogenicity of this species has not been studied except a short note about virulent ability of the species on *Capsicum annuum* by Tozze Jr et al. (2009). We found only slight symptoms on wounded berries and therefore this species may be regarded as a saprophyte.

Colletotrichum isolates with falcate conidia were identified as *C. capsici*. However, the taxonomic difference between *C. capsici* and *C. dematium* seems to be unclear,

partly due to the lack of holotype material of species with falcate conidia. Lubbe et al. (2004) reported that *C. capsici* grouped together with *C. dematium* according to sequence analysis of either the ITS region or partial β-tubilin gene. *C. capsici* is reported to be a pathogenic fungus on a wide range of host plants in the tropics and subtropics (Sutton. 1980; 1992; Than et al. 2008). In contrast, *C. dematium* has been described as a saprophytic species (Mordue. 1971) and has mainly been found in temperature regions (Sutton. 1980). However, *C. dematium* has been designated as the same taxon as *C. capsici* (von Arx. 1957). Isolates with morphological characters as *C. capsici*, has earlier been found on infected coffee in Vietnam (Tran et al. 1998). The ITS sequences of our isolates showed 99% identity to the *C. capsici* strain (Ccmj7) associated with anthracnose disease on *Capsicum* spp. previously described by Than et al. (2008). They were not pathogenic due to lack of symptoms on non-wounded berries.

Apart from the *Colletotrichum* species described above, we found several *Colletotrichum* isolates having relatively massive, cylindrical conidia, abundant sclerotia and dark color in old cultures. These isolates were grouped into clades distinctly different from the other *Colletotrichum* species and therefore we considered them as unknown species. The cultural and morphological features of those isolates were considerably different from *C. gloeosporioides* and other *Colletotrichum* species described by Sutton (1980; 1992). Interestingly, BLAST search against the GenBank nucleotide database has resulted in close hits (99% similarity and E-values =0) to strains of endophytes of non-identified species of *Colletotrichum* from tropical plants

like *Coffea* spp., *Musa* spp., *Orchis* spp. etc from previous studies (AY438553 and AY442184, Lu et al (2004)) and (AY 26644404, Photita et al .(2005)).

Moreover, two other isolates of unknown species, LD33 (L1) and LD11 (L3), that comprised a separate clade in our phylogenetic trees, were slightly morphologically different from *C. gloeosporioides*. They had dark grey colony color with felt-like surface and almost no aerial mycelium. The ITS sequences of these isolates gave high BLAST scores to sequences deposited by Farr et al. (2006) representing strains of unknown species of *Colletotrichum* originating from orchid genera *Cattleya* and *Dendrobium*. Since one of these isolates caused disease on non-wounded berries whilst other isolates caused minor symptoms on wounded berries further pathogenicity tests should be carried.

The results of the pathogenicity tests on detached green berries indicated that some of the Vietnamese isolates were virulent. Six isolates of *C. gloeosporioides* and one of unknown species produced lesions on non-wounded berries. One *C. gloeosporioides* isolate, 119b (1) also induced symptoms on both hypocotyls and berries in previous study by Nguyen et al. (2009). However, the disease symptoms were less severe than those produced by the CBD-fungus (*C. kahawae*). The Vietnamese isolates from this study seldom infected the coffee beans, although they produced sunken lesions on the skin of the berries. Higher number of *C. gloeosporioides* isolates and other *Colletotrichum* species were able to infect wounded berries than non-wounded berries

indicating that the cuticular wax layer may inhibit the infection process of the pathogens. Wounding makes the host more susceptible as has been demonstrated formerly (Than et al. 2008). Based on our pathogenicity tests we assume that the disease on coffee in Vietnam is mainly caused by *C. gloeosporioides*, but other species may also be involved to enhance infection process especially under nutrient deficiency, physiological stress or during seasons of over-bearing of berries. *C. gloeosporioides* has been found to be responsible for "dieback" disease of branches due to exceeding berries in fruiting period (Chen et al. 2003). One of the isolates of unknown species also infected non-wounded berries and therefore it would be in consideration to describe this putative species and characterize its pathogenic abilities further.

To our knowledge this is the first systematic study of *Colletotrichum* species on coffee in Vietnam. The high biodiversity under tropical climate conditions can be one of reasons for the presence of multiple species of *Colletotrichum* on coffee found in this study. Recently, there have been studies on *Colletotrichum* spp. associated with a single host plant, *e.g*. strawberry (Ureña-Padilla et al. 2002; Martinez-Culebras et al. 2003) and pepper (Than et al. 2008). Taxonomical clarification of the putative unknown species has to be taken in consideration. No evidence of the existence of CBD (*C. kahawae*), the main pathogenic fungus threatening coffee production in Africa, has been found so far in Vietnam. The results of this study can be valuable for

disease management's practices and development of more resistant coffee varieties for sustainable coffee production in Vietnam.

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Legends to figures

Figure1. Locations of coffee growing plantations where samples for *Colletotrichum* isolation were collected in Vietnam.

Figure 2: a. Average growth rate of isolates of *C. gloeosporioides* (Cg, 20 isolates), *C. kahawae* (CBD, 3 isolates) and isolates that could not use tartrate (Cg, tartrate negative, 2 isolates). Bars represent standard deviation of the mean. b. Average growth rate of *C. acutatum* (Cacut), *C. capsici* (Ccapsi), *C boninense* (Cbonin) and the three unknown species. Bar indicate mean standard deviation from ANOVA. Different letter indicate significant differences between group means ($P<0.05$) (Tukey's studentized range test). For vertical comparison only.

Figure 3: Maximum Parsimony (MP) tree based on the ITS sequences of 31 sequence types including 24 Vietnamese *Colletotrichum* isolates and reference *Colletotrichum* species listed in Table 1. The tree was rooted by *F. redolens*; 1000 bootstrap data sets and Kimura-2P model distance matrix were used; bootstrap values above 50% are shown on internal branches.

Figure 4: Maximum Pasimonry (MP) tree based on the mtSSU sequences of 31 sequence types including 24 Vietnamese *Colletotrichum* isolates and reference *Colletotrichum* species listed in Table 1. The tree was rooted by *F. redolens*; 1000 bootstrap data sets and Kimura-2P model distance matrix were used; bootstrap values above 50% are shown on internal branches.

Table 1. Origins of *Colletotrichum* spp isolated from coffee in Vietnam and *Colletotrichum* reference isolates

Name of isolate	Identified as	Location	Tissue[y]/Host		GenBank accession number of representative isolates		Tartrate utilization
				No.[z]	ITS	MtSSU	
SL1a(9)*	*C. gloeosporioides*	N	Coffee berries	1	FJ968590	FJ968627	+
NA100(5)	*C. gloeosporioides*	”	”	1			+
NA302	*C. gloeosporioides*	”	”	1			+
NA200a(3)	*C. gloeosporioides*	”	”	1			+
SL2a(8)	*C. gloeosporioides*	”	”	1			+
SL304a(1)	*C. gloeosporioides*	”	”	1			+
SL207a(6)	*C. gloeosporioides*	”	”	1			+
213(2)	*C. gloeosporioides*	”	”	1			+
NA40a(11)*	*C. gloeosporioides*	”	”	2	FJ968589	FJ968626	−
NA4a(9)	*C. gloeosporioides*	”	”	2			+
LD43(B4)	*C. gloeosporioides*	S	”	2			+
LD11(B3)	*C. gloeosporioides*	”	”	2			+
606b(4)*	*C. gloeosporioides*	”	”	3	FJ968591	FJ968628	+
309b(2)	*C. gloeosporioides*	N	”	3			+
702(1)	*C. gloeosporioides*	S	”	3			+
12c(3)	*C. gloeosporioides*	N	”	3			+
119b(1)*	*C. gloeosporioides*	”	”	4	FJ968596	FJ968631	+
201(1)	*C. gloeosporioides*	”	”	4			+
803b(1)*	*C. gloeosporioides*	”	”	5	FJ968592	FJ968629	+

531(7)	*C. gloeosporioides*	”	”	5			+
478a(3)[*]	*C. gloeosporioides*	S	”	6	FJ968595	FJ968630	+
BMT16(T3)[*]	*C. gloeosporioides*	”	Coffee twigs	7	FJ968593	FJ968632	−
LD46a(L1)[*]	*C. gloeosporioides*	”	Coffee leaves	8	FJ968594	FJ968625	+
SL204a[*]	*C. capsici*	N	Coffee berries	9	FJ968578	FJ968606	+
BV14[*]	*C. capsici*	”	”	10	FJ968579	FJ968607	+
397[*†]	*C. acutatum*	USA	*Fragaria x ananassa*	11	AF411734	AF411749	n.t
BMT26(L1)	*C. acutatum*	S	Coffee leaves	11			+
BMT26(T2)	*C. acutatum*	”	Coffee twigs	11			+
413(1)	*C. acutatum*	S	Coffee berries	11			+
213(1)[*]	*C. acutatum*	N	”	12	FJ968598	FJ968609	+
BMT(HL)19[*]	*C. acutatum*	S	Coffee leaves	13	FJ968601	FJ968610	+
606b(1)[*]	*C. boninense*	”	Coffee berries	14	FJ968580	FJ968611	−
LD35a(T2)[*]	*C. boninense*	”	Coffee twigs	15	FJ968582	FJ968616	+
LD8(L3)[*]	*C. boninense*	”	Coffee leaves	16	FJ968583	FJ968617	+
BMT10(L2)[*]	*C. boninense*	”	”	17	FJ968581	FJ968615	−
LD35b(L1)	*C. boninense*	”	”	17			+
LD43(T4)	*C. boninense*	”	Coffee twigs	17			+
LD35b(B2)[*]	Unknown species[x]	”	Coffee berries	18	FJ968586	FJ968619	+
LD27(T1)	Unknown species[x]	”	Coffee twigs	18			+
LD35b(T1)	Unknown species[x]	”	”	18			+
BMT10(T2)[*]	Unknown species[x]	”	”	19	FJ968588	FJ968618	+
LD30a(T3)	Unknown species[x]	”	”	19			+
LD30a(T4)[*]	Unknown species[x]	”	”	20	FJ968587	FJ968622	−
BMT25(L3)[*]	Unknown species[v]	”	Coffee leaves	21	FJ968585	FJ968620	+
LD16(L2)[*]	Unknown species[v]	”	”	22	FJ968584	FJ968621	+

LD11(L3)*	Unknown species [w]	”	”	23	FJ968600	FJ968624	+
LD33(L1)*	Unknown species [w]	”	”	24	FJ968599	FJ968623	+
231*†	*C. gloeosporioides*	USA	*Fragaria x ananassa*	25	AF411764	AF411733	n.t
NI90*†	*C. acutatum*	UK	*F.* x *ananassa*	26	AF411739	AF411755	n.t
IMI 357056*	*C. kahawae*	Malawi	*C. arabica*	27	FJ968597	FJ968633	−
IMI 357057	*C. kahawae*	”	”	27			−
CBS 396.67	*C. kahawae*	Angola	”	27			−
MAFF 305973*	*C. boninese*	Japan	*Passiflora edulis*	28	FJ968603	FJ968612	+
MAFF 306094*	*C. boninese*	”	*Crinum asiaticum* var. *sincium*	29	FJ968605	FJ968613	−
MAFF 306100*	*C. boninese*	”	*Cymbidium* sp.	30	FJ968604	FJ968614	+
STE-U5304* (IMI 56173)	*C. capsici*	Tanzania (CABI)	*Arachis hypogaea*	31	FJ968602	FJ968608	n.t

N: North; S: South of Vietnam.

+: positive in amonium tatrate utilization; −: negative in amonium tatrate utilization; n.t.: not tested.

[†] reference isolates sequenced by Vinnere et al. (2002).

[v, w, and x] isolates belong to unknown species of *Colletotrichum;* group v, w , and x, respectively.

[y] isolated from *Coffea arabica*, except isolates 606b (1) and 606b (4) isolated from *C. robusta*.

[z] Sequence group No. 1-31. All isolates of each sequence group had identical sequences. Therefore, only one of them was included in the phylogenetic analysis; indicated by * and bold letters.

Table 2: Conidial measurements of *Colletotrichum* species originated from diseased coffee in Vietnam

Species	Number of isolates	Conidial length, width (µm) (conidial shape)	Mean length [a] (µm)	Mean width [a] (µm)
C. gloeosporioides	23	9.6-20.8 x 2.9-8,7 (Cylindrical)	15.0 c	4.2 cde
C. acutatum	5	7.9-19.2 x 2.5-6.2 (Fusiform)	12.8 c	4.0 de
C. capsici	2	15.3-28.0 x 2.5-3.6 (Falcate)	22.5 b	3.1 e
C. boninese	6	11.8-25.2 x 4.4-7.1 (Cylindrical)	14.7 c	5.8 bc
Unknown species [v]	2	25.4-40.6x 8.4-11.7 (Cylindrical)	32.1 a	10.2 a
Unknown species [w]	2	13.6-17.3 x 4.7-6.2 (Cylindrical)	15.3 c	4.8 cd
Unknown species [x]	6	14.8-30.2 x 5.6-8.8 (Cylindrical)	21.2 b	7.11 b

[a] Different letters indicate significant differences. Tukey's multiple range test, $p<0.05$. For vertical comparison only.

Table 3: Pathogenicy tests of *Colletotrichum species* on wounded and non-wounded detached green berries of coffee

Name of isolates	Identified as	Agar plug (A) or Conidia (C)[e]	% infected berries with lesion[f] Means ±SD		Lesion diameter (mm)[g] Mean ±SD			
			Wounded	Non-wounded	Wounded	N	Non-wounded	N
NA4a(9)	*C. gloeosporioides*	C	30±20	3±6	11.1±4.7	9	6.0	1
SL2a(8)	*C. gloeosporioides*	C	20±17	0	5.7±3.2	6	−	0
SL1a(9)	*C. gloeosporioides*	A	7±6	13±12	6.5±0.7	2	8.5±4.4	4
119b(1)	*C. gloeosporioides*	A	47±15	20±10	12.1±3.6	14	11.8±2.7	6
201(1)	*C. gloeosporioides*	A	3±6	0	10	1	−	0
531(7)	*C. gloeosporioides*	A	10±10	0	6.3±3.2	3	−	0
309b(2)	*C. gloeosporioides*	C	0	0	−	0	−	0
702(1)	*C. gloeosporioides*	A	23±32	0	10.9±3.2	7	−	0
606b(4)	*C. gloeosporioides*	C	17±6	3±6	10.2±4.0	5	4.0	1
803b(1)	*C. gloeosporioides*	C	23±15	7±12	10.4±4.9	7	6.0±0.0	2
12c(3)	*C. gloeosporioides*	A	7±12	7±6	10.0±0.0	2	6.0±0.0	2
SL204a	*C. capsici*	A	3±6	0	6.0	1	−	0
BV14	*C. capsici*	C	10±0	0	7.3±2.5	3	−	0
413(1)	*C. acutatum*	C	20±10	0	10.7±4.1	6	−	0
213(1)	*C .acutatum*	C	7±6	0	6.0±0.0	2	−	0
BMT26(L1)	*C. acutatum*	C	13±15	0	5.5±1.0	4	−	0
BMT26(T2)	*C. acutatum*	C	3±6	0	6.0	1	−	0
BMT(HL)19	*C. acutatum*	C	13±12	0	9.5±4.8	4	−	0

LD35a(T2)	*C. boninense*	C	7±6	0	8.5±9.2	2	−	0
LD35b(L1)	*C. boninense*	C	23±6	0	8.6±4.1	7	−	0
BMT10(L2)	*C. boninense*	A	0	0	−	0	−	0
LD16(L2)	Unknown species [v]	A	3±6	0	2.0	1	−	0
LD33(L1)	Unknown species [w]	C	3±6	0	14.0	1	−	0
LD27(T1)	Unknown species [x]	A	0	0	−	0	−	0
LD35b(B2)	Unknown species [x]	A	3±6	0	6.0	1	−	0
LD30a(T4)	Unknown species [x]	A	13±6	7±12	6.5±1.0	4	8.0±2.8	2
LD30a(T3)	Unknown species [x]	A	3±6	0	5.0	1	−	0
BMT25(L3)	Unknown species [v]	A	0	0	−	0	−	0
IMI 357057	*C. kahawae*	C	100±0	100±0	10.5±3.4	30	9.7±3.3	30

−: No lesion

N: Number of infected berries among 30 tested green berries

[e] Inoculation method: agar plugs (A) or conidial suspension (C).

[f] Infected berries were only counted if the lesions were developed from the inoculation point

[g] Diameter of lesions on the same infected berries

Figure 1

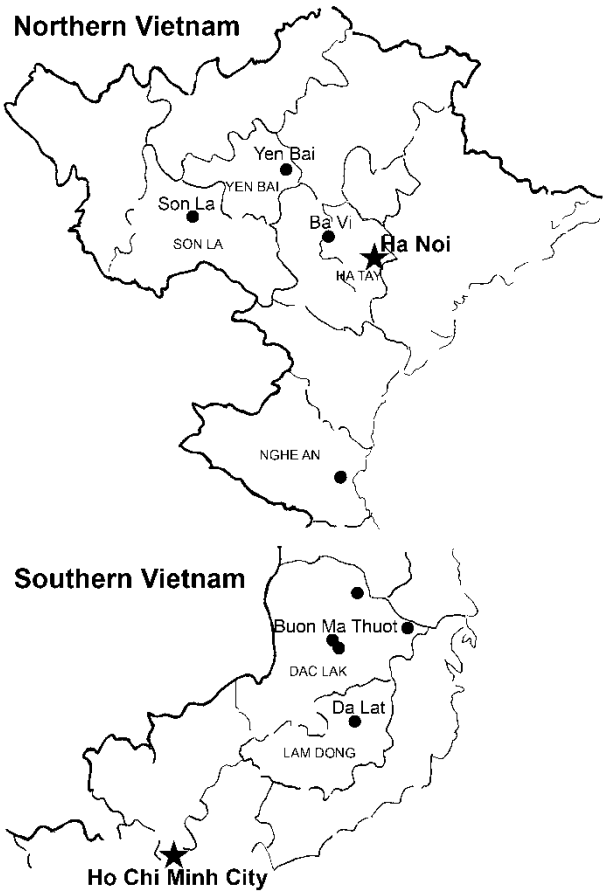

Northern Vietnam
Yen Bai
YEN BAI
Son La
SON LA
Ba Vi
Ha Noi
HA TAY
NGHE AN
Southern Vietnam
Buon Ma Thuot
DAC LAK
Da Lat
LAM DONG
Ho Chi Minh City

Figure 2

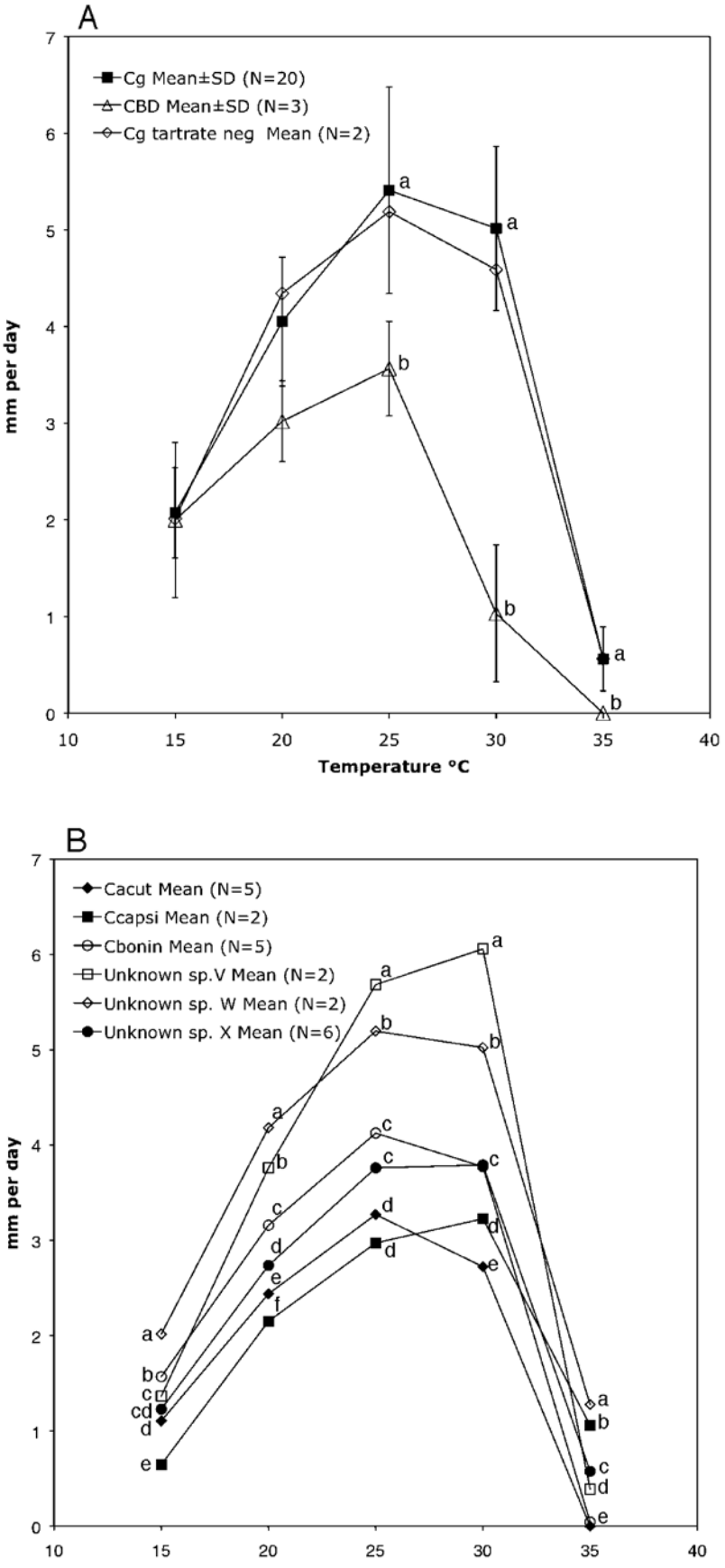

A
Cg Mean±SD (N=20)
CBD Mean±SD (N=3)
Cg tartrate neg Mean (N=2)
mm per day
Temperature °C
B
Cacut Mean (N=5)
Ccapsi Mean (N=2)
Cbonin Mean (N=5)
Unknown sp.V Mean (N=2)
Unknown sp. W Mean (N=2)
Unknown sp. X Mean (N=6)
mm per day

Figure 3

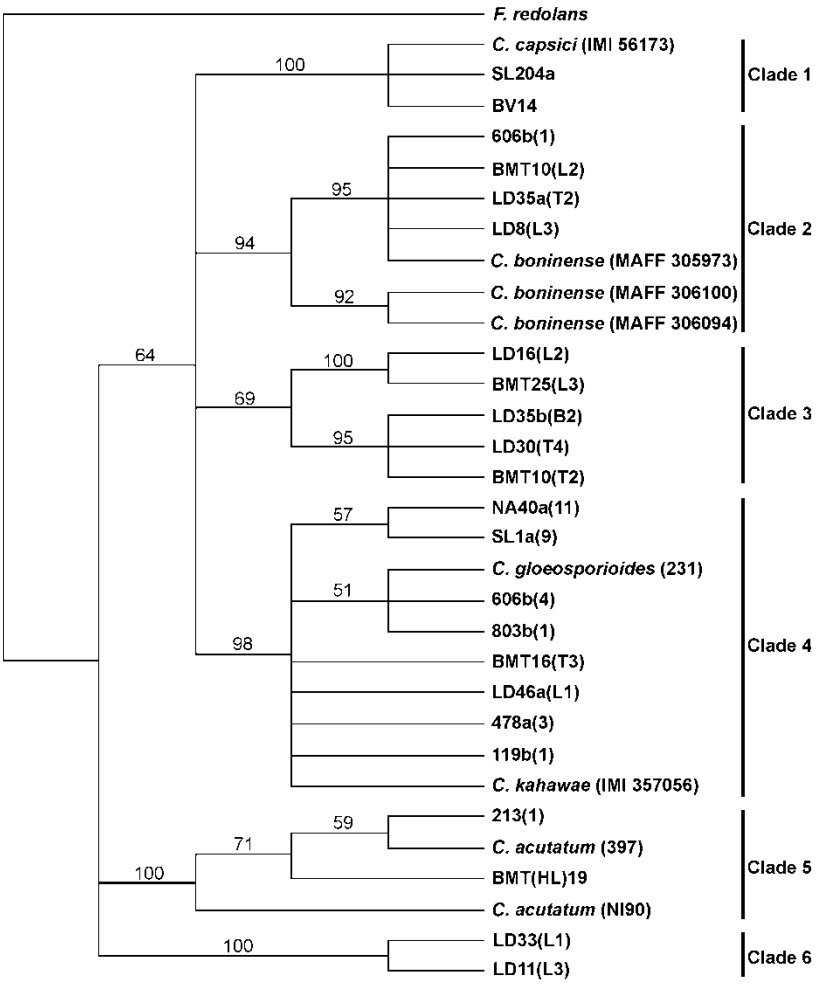
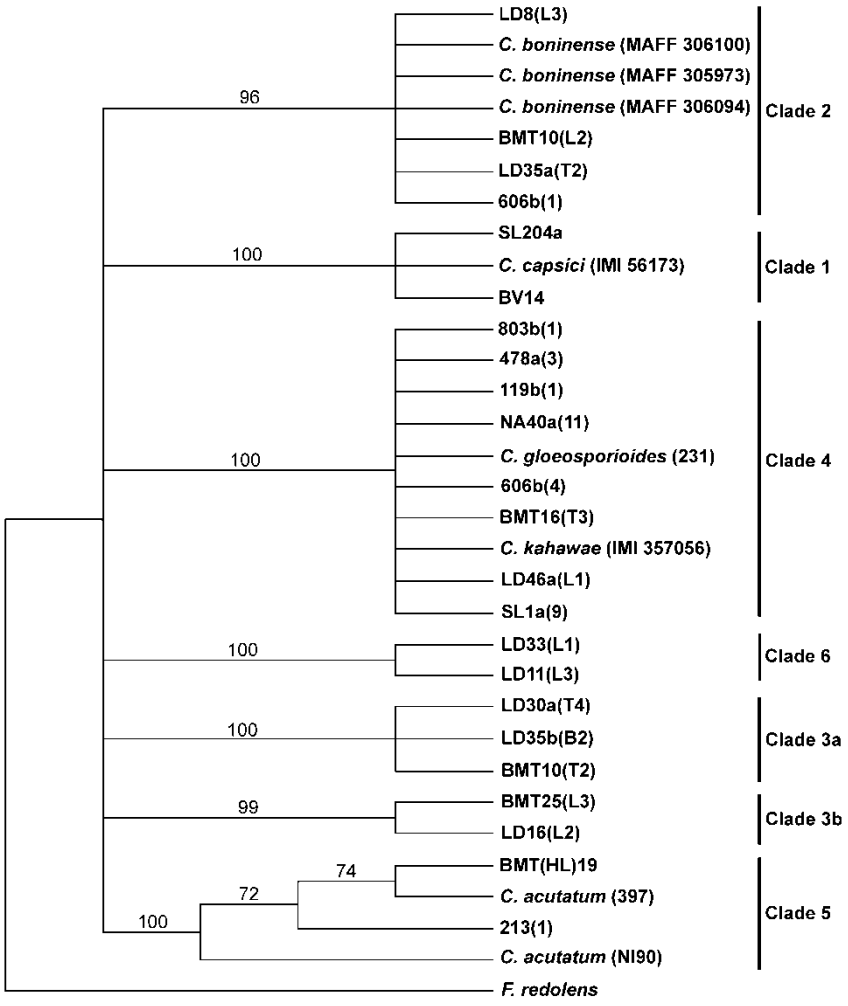

F. redolans
C. capsici (IMI 56173)
SL204a
BV14
606b(1)
BMT10(L2)
LD35a(T2)
LD8(L3)
C. boninense (MAFF 305973)
C. boninense (MAFF 306100)
C. boninense (MAFF 306094)
LD16(L2)
BMT25(L3)
LD35b(B2)
LD30(T4)
BMT10(T2)
NA40a(11)
SL1a(9)
C. gloeosporioides (231)
606b(4)
803b(1)
BMT16(T3)
LD46a(L1)
478a(3)
119b(1)
C. kahawae (IMI 357056)
213(1)
C. acutatum (397)
BMT(HL)19
C. acutatum (NI90)
LD33(L1)
LD11(L3)
Clade 1
Clade 2
Clade 3
Clade 4
Clade 5
Clade 6
100
95
94
92
64
100
69
95
57
51
98
59
71
100
100

Figure 4


LD8(L3)
C. boninense (MAFF 306100)
C. boninense (MAFF 305973)
C. boninense (MAFF 306094)
BMT10(L2)
LD35a(T2)
606b(1)
SL204a
C. capsici (IMI 56173)
BV14
803b(1)
478a(3)
119b(1)
NA40a(11)
C. gloeosporioides (231)
606b(4)
BMT16(T3)
C. kahawae (IMI 357056)
LD46a(L1)
SL1a(9)
LD33(L1)
LD11(L3)
LD30a(T4)
LD35b(B2)
BMT10(T2)
BMT25(L3)
LD16(L2)
BMT(HL)19
C. acutatum (397)
213(1)
C. acutatum (NI90)
F. redolens
96
100
100
100
100
99
74
72
100
Clade 2
Clade 1
Clade 4
Clade 6
Clade 3a
Clade 3b
Clade 5