European Journal of Plant Pathology (2006) 116:107–118
DOI 10.1007/s10658-006-9045-0

Two subpopulations of *Colletotrichum acutatum* are responsible for anthracnose in strawberry and leatherleaf fern in Costa Rica

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Accepted 6 July 2006

Key words: anthracnose, *Fragaria* × *ananassa*, internal transcribed spacer (ITS) region, pathogenicity test, *Rumohra adiantiformis*, Universally Primed polymerase chain reaction (UP-PCR)

Abstract

Strawberry, *Fragaria* × *ananassa*, and leatherleaf fern, *Rumohra adiantiformis*, are two important crops in Costa Rica. One of the most severe diseases affecting these crops is anthracnose, caused by members of the fungal genus, *Colletotrichum* (teleomorph; *Glomerella*). Eighty single-spore isolates from strawberry and leatherleaf fern were identified as *Colletotrichum acutatum* by species-specific PCR, and were further characterised by Universally Primed PCR (UP-PCR) fingerprinting analysis, and sequence analysis of the ribosomal internal transcribed spacer (ITS) region. Morphological differences, genotypic variation revealed by UP-PCR fingerprinting analysis, and a single sequence polymorphism within the ITS2 region were found between the isolates from strawberry and leatherleaf fern, respectively. The UPGMA cluster analysis of the fingerprints clearly separated the isolates derived from strawberry and leatherleaf fern into two different clusters. Pathogenicity assays on detached strawberry fruits confirmed the apparent difference between the two groups of isolates. It is therefore suggested that the pathogens responsible for strawberry anthracnose fruit rot and leatherleaf fern anthracnose in Costa Rica, belong to two distinct subpopulations of *C. acutatum*.

Abbreviations: ITS – internal transcribed spacer; rDNA – ribosomal DNA; UPGMA – unweighted pair-group method using arithmetic average; UP-PCR – Universally primed polymerase chain reaction.

Introduction

Strawberry, *Fragaria* × *ananassa*, and leatherleaf fern, *Rumohra adiantiformis*, are two important crops produced in the Central Valley of Costa Rica. Strawberry is most important for the Costa Rican home market, while leatherleaf fern is exported worldwide and Costa Rica has become the second largest producer of leatherleaf fern. Both strawberry and leatherleaf fern productions in Costa Rica are severely affected by the anthracnose disease. Anthracnoses are caused by members of the genus *Colletotrichum* (teleomorph *Glomerella*), and are some of the economically most important diseases of crop plants in the world. *Colletotrichum* spp. cause a wide range of

diseases in strawberry, where *C. acutatum*, which causes fruit rot or black rot, is considered one of the most problematic species of the genus (Howard and Albregts, 1983, 1984; Ureña-Padilla et al., 2002).

While strawberry anthracnose has been known as long as strawberries have been grown in Costa Rica, the anthracnose on leatherleaf fern has only been known since the late 1970s (E. Vargas, CIPROC, University of Costa Rica, pers. comm.). During the 1980s the disease developed into a serious problem for Costa Rican leatherleaf fern producers with losses up to 50% (Berrocal-Domínguez, 1996). The disease-causing organism infects young, immature fronds or leaves, which results in black necrosis and deformation of leaflets, making all infected fronds unmarketable (Norman and Strandberg, 1997). Strandberg et al. (2002) mention that the causal agent of leatherleaf fern anthracnose in the United States has been identified as *C. acutatum*, and they suggest that the same species may be responsible for anthracnose on leatherleaf fern in Central America.

The species concept of *Colletotrichum* is complicated and a certain degree of confusion still exists (Freeman et al., 1998). However, among mycologists there is a general acceptance of broad species boundaries consisting of several group species or species complexes, including species like *C. gloeosporioides*, *C. acutatum*, *C. dematium* and *C. lindemuthianum*, which are considered cumulative species composed of diverse subpopulations (Sutton, 1992; Freeman et al., 1998; Vinnere et al., 2002). Traditionally, *Colletotrichum* species have been identified by morphological characteristics (Cannon et al., 2000). However, detection and differentiation of *Colletotrichum* spp. based on traditional morphological characteristics may be unreliable and non-predictive, because individual isolates are highly variable and phenotypically plastic (Sutton, 1992; Cannon et al., 2000). To solve the doubts of classical species identification, various molecular techniques are being used currently to characterise and analyse the taxonomic complexity of *Colletotrichum* species (e.g., Sreenivasaprasad et al., 1996a; Lardner et al., 1999; Freeman et al., 2001; Martinez-Culebraz et al., 2002). One of the frequently used techniques is PCR with species-specific primers for amplification of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions. The use of species-specific primers have been shown to be a reliable and fast way to identify and differentiate between morphologically similar *Colletotrichum* spp. (Mills et al., 1992; Brown et al., 1996; Sreenivasaprasad et al., 1996a; Adaskaveg and Hartin, 1997; Ureña-Padilla et al., 2001). A powerful PCR-based fingerprinting technique, not formerly used for analysis of *Colletotrichum* spp., is the Universally Primed PCR (UP-PCR) technique. UP-PCR is similar to the RAPD technique, but has proven to generate more complex banding patterns and provides a high degree of reproducibility (Bulat and Mironenko, 1990; Lübeck et al., 1999; Lübeck and Lübeck, 2005).

To our knowledge, molecular analysis to classify the *Colletotrichum* sp. that is responsible for the devastating anthracnose disease in Costa Rican leatherleaf fern has not been performed. Although there are a few *Colletotrichum* isolates that have been collected from Costa Rican strawberries (Buddie et al., 1999; Martinez-Culebras et al., 2002, 2003), there has been no extensive collection and characterization of isolates from this crop. The aim of this study was to test the hypothesis that anthracnose on strawberry and leatherleaf fern in Costa Rica is caused by the same species of *Colletotrichum* using morphological criteria, species-specific PCR, UP-PCR, ITS sequencing, as well as pathogenicity assays.

Materials and methods

Sampling of Colletotrichum spp. from strawberry and leatherleaf fern

Strawberry fruits and leatherleaf fern leaves with characteristic symptoms of anthracnose were collected at four strawberry and leatherleaf fern production sites each in Costa Rica (Table 1). The samples were maintained 1–3 days in a moist chamber. A total of eighty *Colletotrichum* single-spore isolates, 40 from strawberry and 40 from fern, was collected from lesions (Table 1) and grown on Potato Dextrose Agar (PDA) in the dark at room temperature (~25 °C). Two reference isolates, N1 and GC3, provided by CIPROC, University of Costa Rica, were collected in Costa Rica prior to this study and identified as *C. acutatum* and *C. gloeosporioides*, respectively, based on morphological observations.

Table 1. Colletotrichum acutatum isolates from strawberry and leatherleaf fern in Costa Rica

Isolates[a]	Host plant	Geographical origin
S1.1–S1.10	Strawberry	Vara Blanca, Heredia
S2.1–S2.10	Strawberry	Fraijanes, Alajuela
S3.1–S3.10	Strawberry	Llano Grande, Cartago
S4.1–S4.10	Strawberry	Llano Grande, Cartago
F5.1–F5.10	Leatherleaf fern	Fraijanes, Alajuela
F6.1–F6.10	Leatherleaf fern	Sabana Redonda, Alajuela
F7.1–F7.10	Leatherleaf fern	Oreamuno, Cartago
F8.1–F8.10	Leatherleaf fern	Tablón

[a]Each row of the table includes 10 isolates, which were labelled as follows: One character representing the host origin (S = strawberry and F = leatherleaf fern), and two numbers representing the collection site (i.e., 1–8), and the isolate number (i.e., 1–10).

Species-specific PCR

Fungal DNA was extracted directly from 20 to 80 mg of fresh mycelia using the Wizard® Genomic Purification Kit (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions for isolation of genomic DNA from plant tissue. PCR amplification of the ribosomal ITS region was carried out using the species-specific primer *Ca*Int2 (5′-GGGGAAGCCTCTCGCGG-3′) (Sreenivasaprasad et al., 1996b) for detection of *C. acutatum* in conjunction with the universal primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). All eighty isolates (Table 1) as well as the two reference isolates were tested. The samples were also tested with a *C. gloeosporioides*-specific primer Cg/fInt1 (5′-GACCCTCCCGGCCTCCC GCC-3′) (Ureña-Padilla et al., 2001) and the ITS4 primer.

PCR amplifications were carried out in 20 µl reaction mixtures containing 1–50 ng of genomic DNA and a final concentration of: 1× Perkin–Elmer PCR buffer including 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.0625 µM universal primer ITS4, 0.0625 µM *Ca*Int2 or Cg/fInt1, and 1 U of *Taq* polymerase (Applied Biosystems, Foster City, USA). PCR amplifications were performed using a Gene Cycler (BioRad, Hercules, CA, USA), with initial denaturation at 94 °C for 3 min and 35 cycles consisting of: 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min; and a final extension step at 72 °C for 10 min. Gel electrophoresis was carried out on a 1% agarose gel, followed by ethidium bromide staining and UV visualisation.

Sequence analysis of the ribosomal ITS region

Twenty isolates were selected from the different hosts and localities for sequence analysis of the rDNA ITS1 and ITS2 region including the 5.8S ribosomal subunit (Table 2). The ITS regions were amplified by PCR with the two universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (White et al. 1990).

PCR amplifications were carried out in 50 µl reaction mixtures containing 1–10 ng of template DNA and with final concentrations as described above, except for: 0.5 µM of each primer ITS1 and ITS2, and 1.5 U of Dynazyme (Finnzymes, Espoo, Finland). The PCR programme was as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min. The PCR products were purified using the Qiaquick PCR Purification kit (Quiagen, Valencia, CA, USA), and sequenced in both directions. These sequences were deposited in GenBank (Table 2).

A multiple-sequence alignment was performed by using ClustalW within the BioEdit Sequence Alignment Editor programme (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and a BLAST similarity test also was performed.

Table 2. Isolates used for sequence analysis of the ribosomal ITS1, 5.8S and ITS2 regions and pathogenicity tests

Isolate	Host	GenBank acc. number
S1.2	Strawberry	DQ018737
S1.7	Strawberry	DQ018736
S2.2	Strawberry	AY770553
S2.8	Strawberry	DQ018738
S2.9[a]	Strawberry	DQ018739
S3.2	Strawberry	DQ018740
S3.7	Strawberry	DQ018741
S3.9[a]	Strawberry	DQ018742
S4.3	Strawberry	DQ018743
S4.10	Strawberry	AY770554
F5.2	Leatherleaf fern	DQ018744
F5.9	Leatherleaf fern	DQ018745
F6.4	Leatherleaf fern	DQ018746
F6.6	Leatherleaf fern	DQ018747
F7.4	Leatherleaf fern	DQ018748
F7.8	Leatherleaf fern	AY770555
F8.4	Leatherleaf fern	DQ018749
F8.10	Leatherleaf fern	AY770556
N1[a]	Orange	AY770557
GC3[a,b]	*Annona muricata*	AY770558

[a]These isolates were not included in the pathogenicity test.
[b]*C. gloeosporioides*.

UP-PCR amplification and data analysis

To test for polymorphism between isolates, a UP-PCR fingerprint analysis was performed on the *C. acutatum* isolates. A few of the isolates were not included in the analysis as the DNA quality was too poor and the cultures were lost upon storage. Three different primers were applied: 3-2 (5′-TAAGGGCGGTGCCAGT-3′); AA2M2 (5′-CTGCGACCCAGAGCGG-3′); and L15/AS19 (5′-GAGGGTGGCGGCTAG-3′) (Lübeck and Poulsen, 2001). UP-PCR amplifications were carried out as described by Lübeck and Poulsen (2001). Markers were identified for construction of a dendrogram by recording the presence (1) or absence (0) of bands into a binary matrix. Binary data were analysed for band similarity using Dice's coefficient that measures the proportion of common discrete bands among isolates. A dendrogram of genotypic similarity of the *C. acutatum* isolates was constructed from the distance matrix by the Unweighted Pair-Group Method using Arithmetic Average (UPGMA), followed by bootstrapping 1000 times. For this purpose the programme FREE TREE (Hampl et al., 2001) was applied. The output data was transferred to the TREE-VIEW programme for displaying the dendrogram (Page, 1996).

Pathogenicity tests and statistical analysis

The isolates from strawberry and leatherleaf fern mentioned in Table 2, except isolates S2.9, S3.9, N1 and GC3, were used for this assay. Inoculum was prepared one day before inoculation by flooding 2- to 4-week-old PDA cultures with 10 ml of sterile distilled water (SDW), and rubbing carefully with a sterile glass rod to release conidia. The resulting conidial suspensions were adjusted to a concentration of 1×10^6 conidia ml^{-1}.

The pathogenicity assays on detached strawberries were conducted using the susceptible cultivar 'Oso Grande'. Reddish-green fruits of physiological maturity were harvested at a production site, on the day or one day prior to inoculation. Due to local circumstances, it was not possible to request certified disease-free strawberries for these experiments. Crowns were removed to avoid latent infection from the sepals, and fruits were washed twice in tap water. Unblemished fruits were selected and surface-sterilised by immersing in 70% ethanol for 1 min, followed by 1% sodium hypochlorite solution for 30 s. Finally, the fruits were rinsed in SDW and air-dried. Fruits then were placed on paper towels in plastic boxes (size: $30 \times 21.5 \times 6.5$ cm). Two boxes, each containing eight fruits, were used per treatment. Spray inoculation of fruits with spore suspensions of each isolate was performed until run-off by using a hand-held atomizer (Sagola Badger 250 Aerografo Hobby) connected to an air compressor. Two control treatments were included, consisting of inoculation with SDW and one non-inoculated control. The plastic boxes were left at room temperature. Disease response was recorded 4 days post-inoculation (dpi) based on a 0–5 disease severity scale, as follows: 0 = no symptom, 1 = a few necrotic patches, 2 = necrotic patches up to 25% of the fruit covered, 3 = 26–50% covered, 4 = 51–90% covered and 5 = 91–100%. The assay was repeated three times ('a', 'b' and 'c'). A two-sample *t*-test was applied for the statistical analysis, using the SAS programme version 8.2 to test the null hypothesis regarding the difference between the strawberry and leatherleaf fern group of *C. acutatum* isolates.

To complete Koch's postulates, isolates were recovered from inoculated strawberries. The recovered isolates from the strawberry assays were subjected to DNA extraction, species-specific PCR, and UP-PCR profiling to compare with original isolates, as described above. Two distinct UP primers, L15/AS19 and AA2M2, were used to ensure an unambiguous result.

Results

Colony growth characteristics

The colony characteristics of the *Colletotrichum* spp. single-spore isolates collected from strawberry were distinct from those collected from leatherleaf fern. Isolates from strawberry were white- to creamy-coloured, with a cottony to cushion-like aerial growth and a centre filled with salmon- to peach-coloured acervuli (Figure 1). Most isolates produced black stroma or compacted mycelia. This tendency was more pronounced in older cultures.

The leatherleaf fern isolates showed a flat and creeping growth with dark, pigmented hyphae penetrating the agar. Young isolates were white to

beige or grey with white borders and a beige to grey reverse side (Figure 1), whereas older cultures turned darker with a grey to olivaceous-black reverse side. The fern isolates also produced dark stroma or compacted mycelia. Young cultures of some isolates produced large amounts of salmon-coloured acervuli.

Molecular identification with species-specific PCR

All eighty *Colletotrichum* spp. isolates, plus the *C. acutatum* N1 reference isolate, tested positive in the PCR amplification of the ribosomal ITS region using the *C. acutatum*-specific primer set *Ca*Int2/ITS4. None of the *Colletotrichum* spp. isolates or the N1 isolate tested positive with the *C. gloeosporioides*-specific primer set *Cg*/fInt1/ITS4, except the *C. gloeosporioides* reference isolate GC3 (result not shown).

Sequence analysis of the ribosomal ITS1 and ITS2 region

The rDNA ITS1 and ITS2 including the 5.8S region of the isolates mentioned in Table 2, were successfully amplified, and sequenced. A single sequence polymorphism in ITS2 was present between isolates collected from strawberry and leatherleaf fern (Figure 2). The BLAST similarity search confirmed the results obtained by the species-specific PCR analysis, as the ITS sequences obtained from the *C. acutatum* isolates shared sequence identity with published ITS sequences of *C. acutatum*. ITS sequences obtained from the

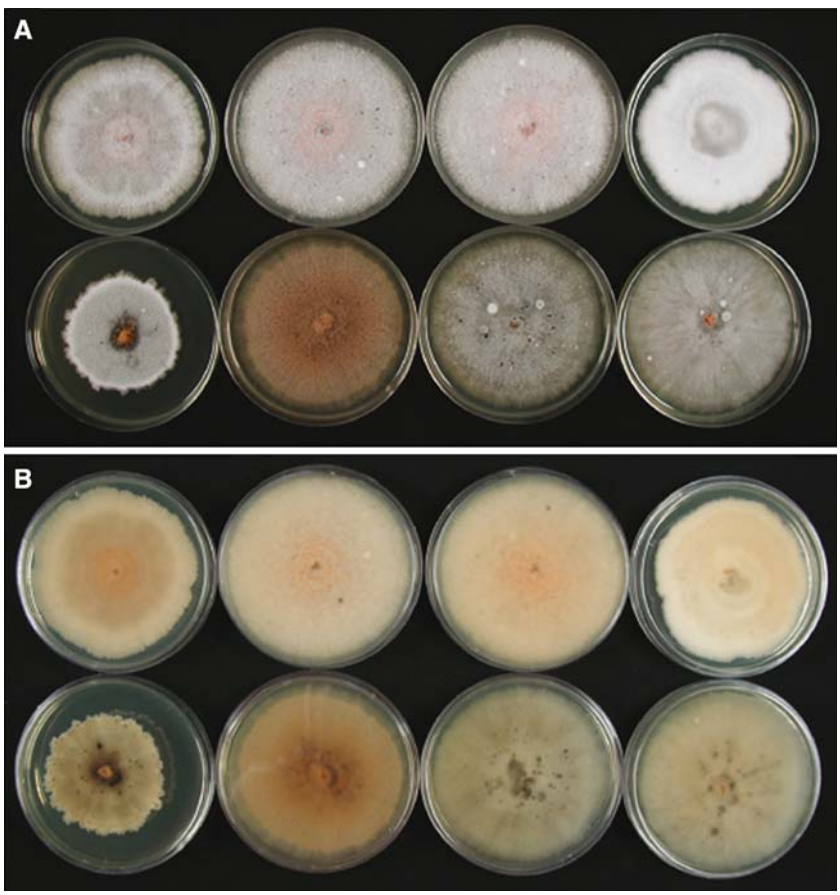

Figure 1. Colonies of *Colletotrichum acutatum* derived from single conidia grown on PDA in the dark for 14 days at 26 °C. (A) upper colony surface and (B) lower colony surface. Upper row from left to right shows strawberry isolates S1.2, S2.2, S3.2, and S4.3. Lower row from left to right shows fern isolates F5.2, F6.6, F7.8, and F8.4. In both (A) and (B) isolates are presented in the same order.

C.gloeosporioides reference isolate, GC3, shared sequence identity with other published *C. gloeosporioides* isolates (data not shown).

UP-PCR-generated DNA profiles

UP-PCR amplification with all three primers, 3-2, AA2M2 and L15/AS19, yielded complex banding patterns for all isolates tested. From the UP-PCR fingerprinted isolates, sixty-eight recordable markers were found representing both polymorphic and conserved bands. Generally, the fingerprints revealed more conserved bands among isolates from the same host (Figure 3). The banding profile of the *C. acutatum* reference isolate N1 from orange was dissimilar to the isolates from strawberry and fern. In total, 13 genotypes were identified based on identical fingerprints when combining the information from the three primers of the isolates (Table 3). All the strawberry isolates except one had identical banding profiles, while the fern isolates could be divided into 10 genotypes that differed in a minority of the bands (Figure 3). In order to ensure the reproducibility of the banding profiles, UP-PCR was repeated at least twice for each isolate.

The dendrogram of genotypic similarity between isolates derived from the UPGMA analysis shows that the 13 genotypes are separated into three clusters comprising strawberry isolates, leatherleaf fern isolates, and the references isolate, respectively (Figure 4). The clusters are supported by bootstrap analysis showing a 100% probability of the divergence between the strawberry and leatherleaf fern isolates.

Pathogenicity test

The pathogenicity test was performed to assess whether *C. acutatum* isolates from strawberry and leatherleaf fern were equally aggressive towards strawberry fruits. *C. acutatum* isolates from strawberry and leatherleaf fern revealed a significant difference ($P < 0.0001$) in their ability to cause disease on strawberry fruits (Table 4, Figure 5). *C. acutatum* isolates collected from strawberry were highly pathogenic on strawberry. The fruits became soft, due to the extensive invasion, which covered most of the fruit, 4 days post-inoculation. This result was consistent throughout the three repetitions, with a mean score of 3.91, including a total of 48 fruits per strawberry isolate. *C. acutatum* isolates originally collected from fern, yielded a mean score of 1.38 four days post-inoculation. Many fruits inoculated with fern isolates did not show any visible symptoms. However, when observed using a stereo microscope, it was possible to see hyphae of germinated conidia on the surface of the fruits (data not shown). Many fruits also produced necrotic spots, which possibly were caused by germinating conidia that in most cases were unable to continue any further growth.

Two out of three water controls resulted in a significantly lower disease score ($P < 0.0001$ to $P = 0.0448$) compared with fruits treated with isolates corresponding to either the strawberry or leatherleaf fern group. All control treatments without water yielded a significantly lower ($P < 0.0001$, $P = 0.0076$, $P = 0.0334$) disease score than the strawberry group, whereas only two

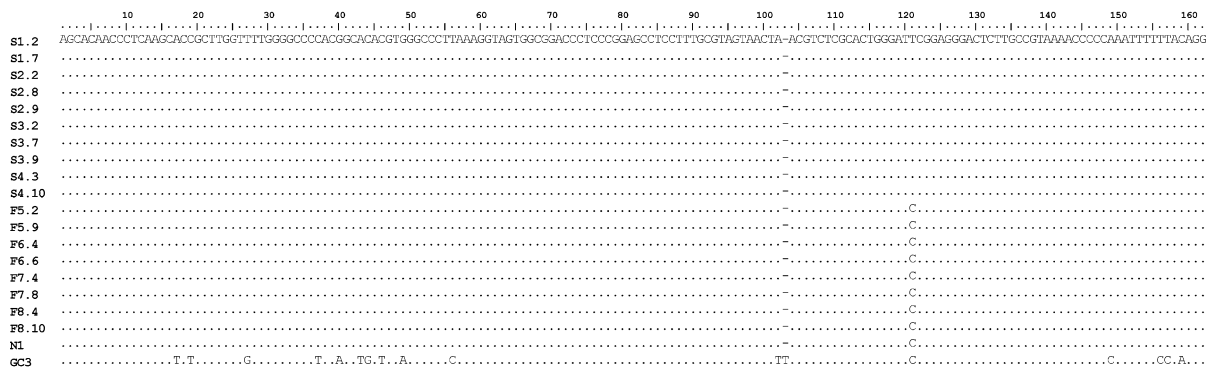

Figure 2. Sequence alignment of the rDNA ITS2 -region showing the polymorphism between *Colletotrichum acutatum* isolates originating either from strawberry (beginning with S) or fern (beginning with F). Sequences from the *C. acutatum* reference isolate N1 and *C. gloeosporioides* reference isolate GC3 also are included.

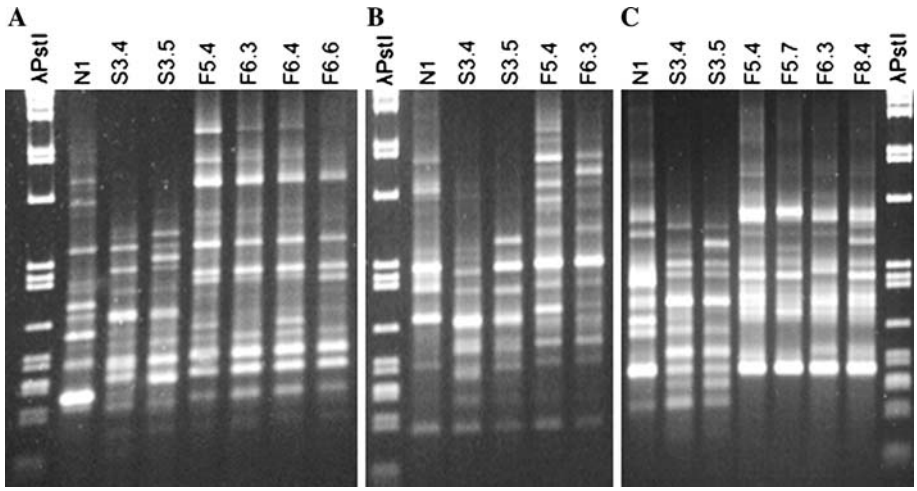

Figure 3. Examples of UP-PCR banding profiles of *Colletotrichum acutatum* isolates using three different primers: A: AA2M2, B: 3–2 and C: L15/AS19. Isolate numbers are indicated above each lane. λPst1 is the molecular size marker.

out of three control treatments without water were significantly different ($P < 0.0001$, $P = 0.0104$) from the leatherleaf fern group.

To complete Koch's postulates, *C. acutatum* isolates were recovered from the three pathogenicity assays (Table 4). All recovered isolates tested positive with the *C. acutatum* specific primer set *Ca*Int2/ITS4. Furthermore, by using two UP-primers, L15/AS19 and AA2M2, all isolates recovered from fruits inoculated with *C. acutatum* isolates originating from strawberry had the same DNA profile as the strawberry genotype reference. However, 6 out of 21 isolates recovered from fruits inoculated with isolates originating from leatherleaf fern, turned out to share the DNA profiles with the strawberry genotype references. The remaining 15 isolates had the same DNA profile as the leatherleaf fern reference.

Table 3. Distribution of the *Colletotrichum acutatum* isolates from strawberry and leatherleaf fern into genotypes on the basis of identical profiles with three UP primers

Genotype	Isolate
I	N1
II	S1.1–S1.10, S2.1–S2.10, S3.1–S3.4, S3.6–S3.10, S4.1–S4.10
III	S3.5
IV	F5.1–F5.6, F5.8, F5.9, F7.1, F7.8, F7.9, F8.3, F8.6
V	F5.7
VI	F6.1, F7.2, F7.3, F7.4, F7.5, F7.6, F7.10
VII	F6.2, F6.5
VIII	F6.3, F6.8
IX	F6.4, F6.10
X	F6.6, F6.7
XI	F6.9
XII	F7.7
XIII	F8.1, F8.4, F8.7, F8.10

Discussion

The overlapping morphology between the closely related species *C. acutatum* and *C. gloeosporioides* makes reliable identification on the basis of morphological characteristics difficult. In this study, a combination of morphological characterisation, pathogenicity tests and molecular tools, allowed a differentiation between the pathogens that are responsible for anthracnose in strawberry and leatherleaf fern in Costa Rica.

Fungal isolates originating from strawberry and leatherleaf fern turned out to have a distinct polymorphism regarding colony colour and growth pattern as well as a sequence polymorphism in ITS2 and different UP-PCR profiles. In spite of these differences, all eighty isolates were identified as *C. acutatum* by means of morphology and species-specific PCR. Concerning strawberry anthracnose, this result is consistent with the findings of Ureña-Padilla et al. (2002), who only recovered isolates belonging to *C. acutatum* from anthracnose fruit lesions under field conditions. It is also possible to recover *C. gloeosporioides* on crowns and stolons, especially as *C. gloeosporioides*

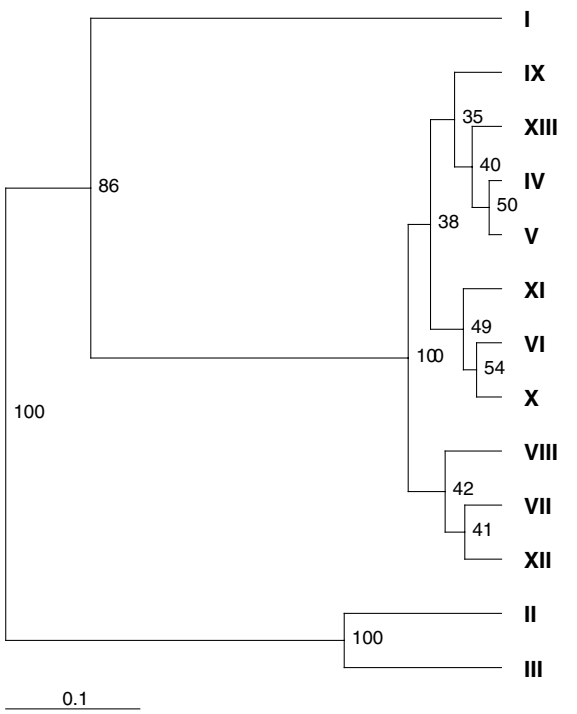

Figure 4. Dendrogram showing the two clusters of *Colletotrichum acutatum* isolates. The dendrogram was generated using the Unweighted Pair-Group Method with arithmetic mean and the Dice coefficient. Genotypes are indicated with Roman numerals. Genotype II and III represent *C. acutatum* isolates collected from strawberry and genotype III–XIII represent *C. acutatum* isolates collected from leatherleaf fern. Genotype I is the reference isolate N1 collected from orange. Bootstrap values from 1.000 bootstrap replications are shown.

is found to give rise to anthracnose epidemics on strawberry crowns, whereas *C. acutatum* seems to be the main contributor to epidemics of anthracnose fruit rot (Ureña-Padilla et al., 2001, 2002). As shown by Leandro et al. (2001, 2003), it also may be possible to find *C. acutatum* on symptomless strawberry leaves, which could provide inoculum for flower and fruit infections and thereby contribute substantially to disease development.

While Norman and Strandberg (1997) refer to *C. acutatum* as the causal pathogen of leatherleaf fern anthracnose in Florida, the identity of the causal pathogen in Costa Rican ferns has not been confirmed. Strandberg et al. (2002) suggested that the causal pathogen of Central American leatherleaf fern anthracnose is *C. acutatum*, which is now confirmed for Costa Rica by the results of this study. As both strawberry and leatherleaf fern are produced all year round in Costa Rica, it must be expected that *Colletotrichum* spp. prevail throughout the year, and give rise to a continuous disease development, if proper control measures are not applied. The species complex of *C. acutatum*, which has been widely studied for its inherent genetic variation, is known to be highly polyphagous, with a worldwide distribution on 34 host genera within 21 families (Walker et al., 1991). Johnston and Jones (1997) and Lardner et al. (1999) studied *C. acutatum* using both morphological and molecular methods. They recognised *C. acutatum* as a group species and introduced the terms '*C. acutatum sensu lato*' and '*C. acutatum sensu* Simmonds'. Freeman et al. (2001) used different molecular techniques to study isolates belonging to *C. acutatum sensu* Simmonds which showed genetic variation, but no correlation was found between host and subgroup. Correll et al. (2000) studied *C. acutatum* isolates from various hosts. They found a considerable diversity in RFLP profiles but no accordance with host plants. However, examples of host specificity have been found within *C. acutatum*. *C. acutatum* f.sp. *pineum* is the causal agent of Crook disease of pine seedlings (Sutton, 1992) and *C. acutatum* f.sp. *hakeae* appears to be specific to *Hakea* spp. (Lubbe et al., 2004).

The profiling of genomic DNA from a number of isolates with three highly sensitive primers resulted in a banding pattern with unique markers for *C. acutatum* strawberry and leatherleaf fern isolates, respectively. The separation into different clusters, as illustrated with the UPGMA-generated dendrogram, indicates the presence of two different subpopulations. The strawberry isolates, except a single isolate, had identical profiles using the three primers, indicating clonality. In contrast, more variation was found in the leatherleaf fern isolates where 10 genotypes could be found.

Denoyes-Rothan et al. (2003) identified a *C. acutatum* clonal group, only containing isolates from strawberry, and a *C. acutatum* variable group, isolated from various hosts. By obtaining and inspecting the sequences from their study, we observed that the clonal group and the variable group have a sequence polymorphism in ITS2 corresponding to the polymorphism found here between strawberry and fern isolates. At this position, the strawberry clonal group have a 'T', whereas fern isolates and the variable group have a 'C'. The two genotypic and morphologically

distinct subgroups of *C. acutatum*, recognised in this study, might also have developed certain host specificity, at least the one originating from leatherleaf fern. This possibility was addressed by pathogenicity tests, which supported the apparent separation of the two groups of isolates. *C. acutatum* isolates originally collected from strawberry were highly aggressive towards strawberry fruits, whereas *C. acutatum* isolates collected from fern were not aggressive towards strawberry, although they were able to infect some of the fruits by developing minor lesions within the strawberry epidermis. The symptom development was mostly too slow to become readily established within the 4 days when data were recorded. Because it was not possible to request certified disease-free fruits for the pathogenicity tests, the disease development after inoculation may have been masked by inherent field contamination. The level of inherent field contamination was assessed by the control treatments, and turned out in the majority of cases to be significantly lower compared to fruits inoculated with the *C. acutatum* isolates. The UP-PCR analysis revealed that 6 out of 21 isolates recovered from strawberries inoculated with *C. acutatum* isolates originating from leatherleaf fern shared the DNA profile with the strawberry genotype reference. This indicates that the fern isolates in some cases were not able to establish on the fruits due to competition from the field inoculum. These new strawberry isolates most probably belong to the same genotypes or the same clonal population as the reference isolates that were recovered a year and a half previously, since they share similar UP-PCR profiles.

Interestingly, the necrotic spots on strawberry, which apparently were caused by isolates from the leatherleaf fern group, could indicate a host

Table 4. Mean disease scores of detached strawberry fruits inoculated with *Colletotrichum acutatum* isolates from strawberry and leatherleaf fern

C. acutatum isolates from strawberry											
Repetition		S1.2	S1.7	S2.2	S2.8	S3.2	S3.7	S4.3	S4.10	Mean/Rep[e]	SD/Rep[f]
A	Mean[a]	4.13[b]	4.00	3.88	3.44	4.13	3.75	4.13	3.88	3.91	0.24
B		4.00	3.94	3.94	4.00	3.81	4.00	3.50	3.50	3.84	0.22
C		4.06	4.00	3.75	4.19	4.00	4.00	3.94	4.00	3.99	0.12
	Mean/Isolate[c]	4.06	3.98	3.85	3.88	3.98	3.92	3.85	3.79	3.91	0.09
	SD/Isolate[d]	0.24	0.25	0.65	0.49	0.33	0.35	0.46	0.46		
C. acutatum isolates from leatherleaf fern											
Repetition		F5.2	F5.9	F6.4	F6.6	F7.4	F8	F8.4	F8.10	Mean/Rep	SD/Rep
A	Mean	0.63	1.38	1.38	1.67	1.38	2.13	2.00	1.94	1.56	0.48
B		0.81	0.81	1.56	1.69	1.81	1.75	0.88	1.00	1.29	0.45
C		0.81	0.81	1.56	1.69	1.81	1.75	0.88	1.00	1.29	0.45
	Mean/Isolate	0.75	1.00	1.50	1.68	1.67	1.88	1.25	1.31	1.38	0.38
	SD/Isolate	1.23	1.16	1.47	1.33	1.31	1.40	1.35	1.38		
Repetition		Control with water[g]	Control without water[g]								
A	Mean	0.50	0.44								
B		0.94	0.50								
C		0.44	1.06								
	Mean/Control	0.63	0.67								
	SD/Control	1.16	1.17								

[a]The table displays the mean disease score for 16 fruits for each isolate and repetition 'a', 'b' and 'c'.
[b]The disease response was recorded four dpi based on a 0–5 disease severity scale.
[c]Mean score and [d]Standard Deviation per isolate for all repetitions.
[e]Mean score and [f]Standard Deviation per repetition produced by the group of strawberry and leatherleaf fern isolates, respectively.
[g]The control treatments with and without water resemble the level of the inherent field contamination.

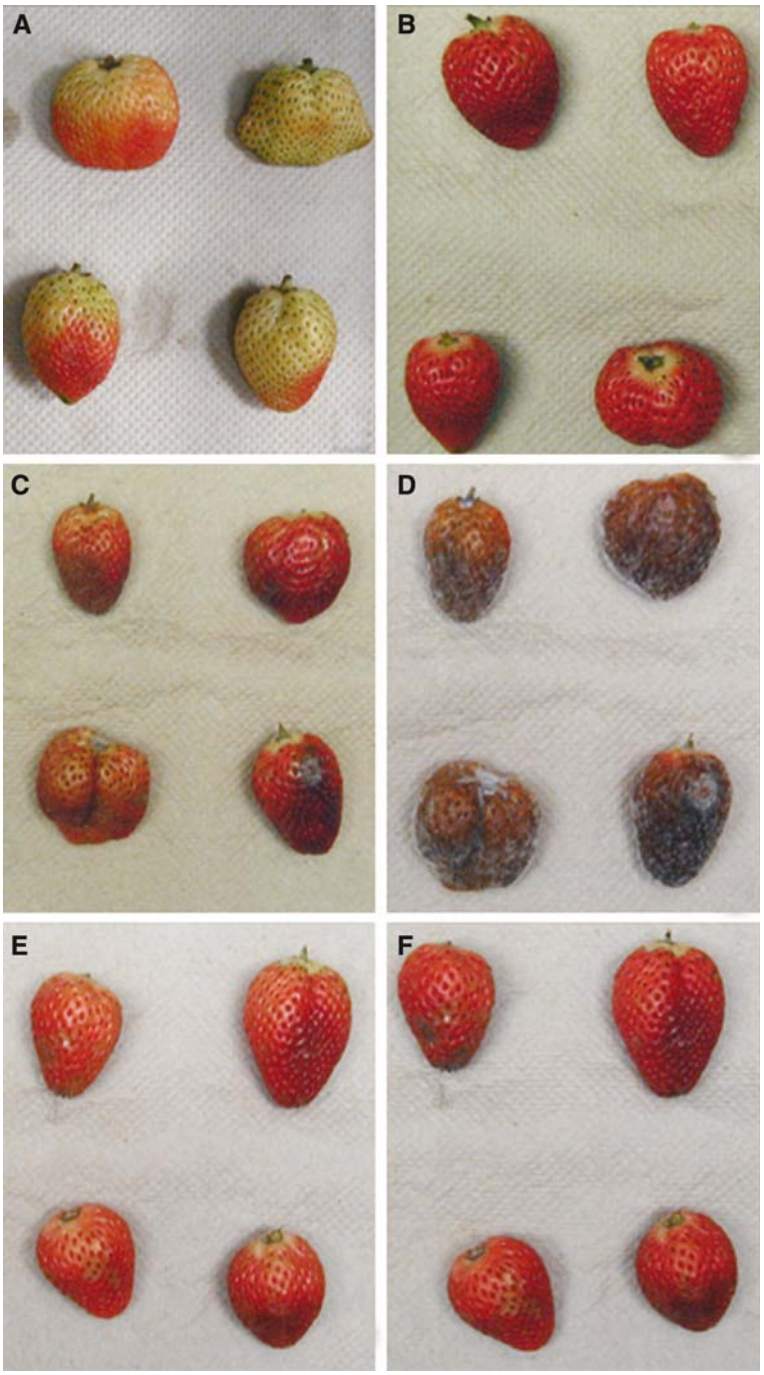

Figure 5. Symptom development on detached strawberry. A: Before inoculation. B: Water-inoculated control 4 days post-inoculation (dpi). C: Three dpi with isolate S3.7 from strawberry. D: Four dpi with isolate S3.7. E: Three dpi with isolate F7.4 from leatherleaf fern. D: Four dpi with isolate F7.4.

defence response that actively prevented these isolates from further development. This could be further assessed by microscopy and by applying various biochemical assays for defence reactions.

A preliminary pathogenicity test on leatherleaf fern showed that isolates derived from strawberry seemed to be able to infect fern, although to a lesser extent than isolates derived from fern

(unpublished results). These results indicate a host specialisation of the two sub-populations. This seems not to be the general conclusion from *C. acutatum* research as several genetic sub-groups have been found with little indication of host specificity although some isolates show specializations on certain hosts (Peres et al., 2005). Future studies should address the question of possible host specialisation in more detail by testing for pathogenicity on an array of other potential hosts known to be infected by *C. acutatum*.

The clear advantages of UP-PCR analysis are that it is a cheap, fast and sensitive method for detecting genotypic variation within species. This sensitive method for identifying specific genotypes, could be used to reveal population structures within different species of *Colletotrichum*. Genotypic markers may serve as pathogenicity markers, if pathogenicity patterns can be correlated with specific genotypic markers, as seems to be the case for *C. acutatum* on strawberry and leatherleaf fern. UP-PCR-derived pathogenicity markers may hence provide a fast method to predict the degree of pathogenicity conferred by certain isolates, and the markers could be used for development of 'pathotype-specific' PCR primers.

On the basis of eighty single-spore isolates, it was shown that the causal agent of leatherleaf fern and strawberry fruit anthracnose is *C. acutatum*, but that there exists an intra-species variation between the isolates attacking the two hosts. The fact that two distinct sub-populations apparently prevail in the Central Valley of Costa Rica with one being pathogenic to strawberry and the other to leatherleaf fern, is shown in this study by morphological observations, molecular evidence and by means of their differential pathogenicity towards strawberry.

Acknowledgements

We would like to acknowledge the support received by Amy Wang and Felipe Aráuz (CIPROC). We are grateful to Alexis Serrano, M.Sc., Milagro Granados, Dr. Edgar Vargas, and M.Sc., Juan Diego Román (CIPROC) for invaluable technical assistance. Thanks to Dr. Eigil de Neergaard and Dr. Hans Lyngs Jørgensen, Department of Plant Biology, for fruitful discussions on *Colletotrichum* and statistical analysis. M.S. is very grateful for the financial support received from Hedvig og Johannes Sørensens Fond; Valborg Hartings Fond; Arnborg Hedegaards Mindelegat and Gårdejer Lauritz Rasmussens Legat til Minde om Slægten på Nysøgaard i Hvalsø, Oticon Fonden, Agronom Fonden, Lauritz Smidts Fond, Nordsjællands Landbrugskoles Fond and Gårdejer Peter Hansen og Hustru Ellen Hansens Mindelegat.

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