Molecular Analyses of *Colletotrichum* Species from Almond and Other Fruits

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ABSTRACT

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Isolates of *Colletotrichum* spp. from almond, avocado, and strawberry from Israel and isolates of the pink subpopulation from almond from the United States were characterized by various molecular methods and compared with morphological identification. Taxon-specific primer analysis grouped the avocado isolates within the species *C. gloeosporioides* and the U.S. almond and Israeli strawberry isolates within the species *C. acutatum*. However, the Israeli almond isolates, previously identified morphologically as *C. gloeosporioides*, reacted with *C. acutatum*-specific primers. Arbitrarily primed polymerase chain reaction and A+T-rich DNA analyses determined that each population from almond and strawberry was distinct and clonal. Sequence analysis of the complete internal transcribed spacer (ITS) region (ITS 1–5.8S–ITS 2) revealed a similarity of between 97.03 and 98.72% among almond isolates from Israel, *C. acutatum* almond isolates from the United States, and *C. acutatum* strawberry isolates from Israel. Similarity of the above populations to that of *C. gloeosporioides* of avocado was between 92.42 and 92.86%. DNA sequence analysis of the entire ITS region supported the phylogeny inferred from the ITS 1 tree of 14 different *Colletotrichum* species. Although morphological criteria indicated that the Israeli isolates from almond are unique, this population was grouped within the *C. acutatum* species according to molecular analyses.

Additional keywords: *Glomerella*, rDNA (ITS 1 to ITS 2) sequence, species-specific primers.

The filamentous fungal plant pathogens *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk (anamorph *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz.) and *C. acutatum* Simmonds cause anthracnose diseases on various crops worldwide (2, 5,15). Almond, avocado, and strawberry anthracnose are major diseases that occur in the United States, Israel, and elsewhere (3,8,19, 22) and can be inflicted by single or multiple species of *Colletotrichum* (1,4,7,10,14,17).

Although almond and avocado crops are cultivated in close proximity in Israel, where anthracnose of both crops caused by *Colletotrichum* spp. occur, no cross-infection has been evident (9). Likewise, although strawberry infected by *C. acutatum* is cultivated in the same geographic regions and under the same climatic conditions as those of avocado and almond crops in Israel, cross-infection among these crops has not been established (8). Therefore, host specificity within a species of *Colletotrichum* seems to exist in certain cases. In the past, the causal agent of almond anthracnose in Israel was identified as *C. gloeosporioides* based on morphological characteristics alone, such as size of conidia and colony color (22). Recently, Förster and Adaskaveg (7) have reported that two distinct clonal subpopulations of *C. acutatum* infecting almond exist in California. In general, isolates of the species *C. gloeosporioides* are sensitive to benzamidazole fungicides, such as benomyl, and have optimal growth temperatures ranging from 25 to 28°C (4). However, the almond pathogen in Israel is insensitive to benomyl and has a low optimal growth temperature (20 to 22°C). These features are in contrast to the two *C. acutatum* subpopulations (gray and pink) from almond in the United States, which are also insensitive to benomyl but have a higher optimal growth temperature of 25°C (7). Previously, it was reported that the pink U.S. population belongs to a single vegetative compatibility group, distinct from that of the Israeli population from almond (1,9,16). Deviation of the Israeli almond isolates from the *C. gloeosporioides* standards raised a doubt regarding its species identity (10). The implication of species identification has direct impact on control measures, as demonstrated by differential sensitivity of *C. gloeosporioides* and *C. acutatum* to benomyl.

Morphotaxonomic criteria, as illustrated by the anthracnose pathogens from almond, are not accurate enough to discriminate between *Colletotrichum* spp.; therefore, much work has been done recently to determine the genetic complexity of isolates infecting subtropical and tropical fruits. A variety of molecular approaches have been used. The GcpR1 repetitive nuclear DNA element from *C. lindemuthianum* (20) and A+T-rich DNA were used for grouping various isolates of *C. gloeosporioides* from strawberry (11) and for determining that the almond pathogen in Israel belongs to a unique clonal lineage (9). Likewise, by using arbitrarily primed polymerase chain reaction (ap-PCR) analysis, it was demonstrated that DNA from eight U.S. isolates of *C. acutatum* (of the pink subpopulation) causing almond anthracnose were distinct from Israeli isolates (9), also corroborated by Förster and Adaskaveg (7). Random amplified polymorphic DNAs (RAPDs) were also used to demonstrate that the U.S. gray subpopulation of *C. acutatum* from almond is very similar to the Israeli almond population (7). Polymorphisms in ribosomal DNA (rDNA) and sequence data of the internal transcribed spacer (ITS) 1 and 2 regions have been used for differentiation of *Colletotrichum* spp. (6,18,23,24). Similarly, sequencing of the variable D2 domain from the 5′ end of the large rDNA subunit has been also used for species delimitation in the genus *Colletotrichum* (15).

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A primary objective of this study was to determine whether various molecular methods would give results consistent with morphotaxonomic descriptors for delimiting species of the genus *Colletotrichum* from various hosts in general, and almond from the United States and Israel in particular. For this purpose, we used representative isolates of *Colletotrichum* from almond, avocado, and strawberry in Israel, and from the pink subpopulation of almond in the United States. The isolates were compared by five independent molecular methods: (i) PCR amplification using taxon-specific primers, (ii) ap-PCR amplification of genomic DNA, (iii) A+T-rich DNA patterns associated with the mitochondrial genome, (iv) restriction endonuclease digest analysis of PCR-amplified rDNA, and (v) sequencing of the complete rDNA ITS region (ITS 1–5.8S–ITS 2). Molecular data were used to determine the relatedness among isolates and the accuracy and reliability of these methods for species designation. Phylogenetic analysis was performed to determine whether the ITS 1 and complete ITS 1 to ITS 2 sequences support previous published data (24). These results were further compared with morphotaxonomic characteristics previously published and those recently reidentified.

MATERIALS AND METHODS

Fungal cultures and growth conditions. The monoconidial *Colletotrichum* cultures used in this study included 63 Israeli isolates of *C. gloeosporioides* from avocado fruit, 57 Israeli isolates of *Colletotrichum* from almond fruit, 19 U.S. isolates of *C. acutatum*, all belonging to the pink subpopulation from almond fruit, and 230 Israeli isolates of *C. acutatum* from strawberry (Table 1). The Israeli *C. gloeosporioides* isolates from avocado originated from infected fruit collected from packinghouses, supermarkets, and orchards in Israel during the years 1993 to 1997. The *C. acutatum* isolates responsible for almond anthracnose in the United States were provided by B. Teviotdale (University of California, Davis). The Israeli almond cultures were isolated during the years 1993 to 1998 from infected fruit from groves situated in northern and southern regions of the country. *C. acutatum* isolates from strawberry originated from infected plants collected during the years 1995 to 1997 (8).

All fungi were cultured in the dark on modified Mathur's medium (MS; 0.1% yeast extract, 0.1% bactopeptone, 1% sucrose, 0.25% $MgSO_4 \cdot 7H_2O$, 0.27% KH_2PO_4, and 2% agar supplemented with 25 mg of ampicillin in 1 liter of sterile distilled water) (26). For DNA extraction, liquid cultures comprising 100 ml of MS devoid of agar in 250-ml Erlenmeyer flasks were inoculated with five mycelial disks derived from colony margins. The cultures were agitated for 5 to 6 days on a rotary shaker at 150 rpm and maintained at 25°C. Twelve hours before harvesting mycelia, the cultures were fragmented by blending for 10 s at 24,000 rpm with a tissue homogenizer (Ultra-Turrax T25; Janke & Kunkel, IKA Labortechnik, Staufen, Germany) under sterile conditions and returned to the shaker. To avoid contamination, the homoginizer was washed in sterile water before blending each culture.

Fungal morphological identification. *Colletotrichum* isolates from representative almond and avocado isolates were sent to P. F. Cannon at CABI Bioscience (Egham, United Kingdom) for independent identification based on morphological characteristics. The U.S. almond isolate (ALM-US-1B) of the pink subpopulation was identified as *C. acutatum*, Israeli avocado isolates (AVO-38-7C and AVO-39-1C) were identified as *C. gloeosporioides*, and seven Israeli almond isolates (ALM-IKS-7Q, ALM-KSH-10, ALM-BZR-9A, ALM-KN-17Q, ALM-NRB-30K, ALM-NRB-98-UEF, and ALM-NRB-98-NPU) were identified as *C. gloeosporioides*, although some of these exhibited conidial forms that were intermediate between the two *Colletotrichum* spp. *C. gloeosporioides* and *C. acutatum* (P. F. Cannon, *personal communication*). Conidia of Israeli *Colletotrichum* isolates from almond varied in form but were often narrow and tapered toward the base, with a small proportion possessing acute ends, and measured 13 to 22 µm in length. Conidia of *C. acutatum* from almond (United States) and strawberry (Israel) were elliptic-fusiform, narrow, tapered and acute at both ends, and measured 13 to 20 µm in length. Conidia of Israeli *C. gloeosporioides* isolates from avocado were broader than those of the *C. acutatum* and Israeli almond isolates, cylindrical with obtuse ends, and measured 13 to 24 µm in length. All isolates were grown on MS medium for identification.

Isolation and purification of fungal DNA. Mycelia from 100-ml MS liquid cultures were collected by vacuum filtration and lyophilized until dry. DNA was extracted and purified as previously described (11). The DNA was dissolved in 0.5 ml of Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) to an approximate concentration of 200 to 500 µg/ml and diluted to a concentration of 10 to 100 ng/µl for PCR reactions.

PCR amplification. For ap-PCR, primers were derived from minisatellite or repeat sequences as follows: CAGCAGCAGCAGCAG (20), TGTCTGTCTGTCTGTC (11), GACACGACACGACAC (13), and GACAGACAGACAGACA (27). In the text, these primers have been designated $(CAG)_5$, $(TGTC)_4$, $(GACAC)_3$, and $(GACA)_4$, respectively. Universal PCR primers were used (ITS 1, TCCGTAGGTGAACCTGCGG and ITS 4, TCCTCCGCTTATTGATATGC) for amplification of the ITS 1 and ITS 2 regions between the small and large nuclear rDNA, including the 5.8S rDNA, as described (28). PCR primers for taxon-specific amplification included the ITS 4 primer coupled with specific primers for *C. acutatum* (*Ca*Int2) (GGGGAAGCCTCTCGCGG) and *C. gloeosporioides* (*Cg*Int) (GGCCTCCCGCCTCCGGGCGG) (5). PCR reactions were performed in a total volume of 20 µl, containing 10 to 100 ng of genomic DNA; 50 mM KCl; 10 mM Tris-HCl; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 1.5 mM $MgCl_2$; 1 unit *Taq* DNA polymerase (Promega Corp., Madison, WI); and 1 µM primer. The reactions were incubated in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA) starting with 5 min of denaturation at 95°C. For ap-PCR, this was followed by 30 cycles consisting of 30 s at 95°C, 30 s at either 60°C (for $(CAG)_5$) or 48°C (for $(GACA)_4$, $(GACAC)_3$, and $(TGTC)_4$), and 1.5 min at 72°C. For rDNA amplification, denaturation was followed by 40 cycles consisting of 30 s at 95°C, 30 s at 50°C, and 1.5 min at 72°C. Taxon-

TABLE 1. Isolates of *Colletotrichum* species used in this study

Species	Isolate	Origin	Host plant
C. gloeosporioides	AVO-37-4B[a]	Israel	Avocado
C. gloeosporioides	AVO-AS-2	Israel	Avocado
C. gloeosporioides	AVO-47-1	Israel	Avocado
Colletotrichum	ALM-BZR-9A[b]	Southern Israel	Almond
Colletotrichum	ALM-GVA-6A	Northern Israel	Almond
Colletotrichum	ALM-IKS-7Q	Northern Israel	Almond
Colletotrichum	ALM-KN-17Q	Northern Israel	Almond
Colletotrichum	ALM-NRB-30K	Southern Israel	Almond
Colletotrichum	ALM-KSH-10	Northern Israel	Almond
C. acutatum	ALM-US-1A[c]	California	Almond
C. acutatum	ALM-US-1B	California	Almond
C. acutatum	ALM-US-2A	California	Almond
C. acutatum	ALM-US-3A	California	Almond
C. acutatum	ALM-US-4	California	Almond
C. acutatum	ALM-US-6B	California	Almond
C. acutatum	ALM-US-7A	California	Almond
C. acutatum	TUT-5954[d]	Israel	Strawberry
C. acutatum	TUT-136A	Israel	Strawberry
C. acutatum	TUT-137A	Israel	Strawberry

[a] Other representative Israeli avocado isolates were AVO-38-7C, AVO-39-1C, and an additional 54 isolates as described (9).

[b] Other representative Israeli almond isolates were ALM-GOZ-93, ALM-NRB-98-UEF, ALM-NRB-98-NPU, and an additional 52 clonal isolates as described (9).

[c] Other representative U.S. almond isolates of the pink subpopulation were ALM-US-2B, ALM-US-3B, ALM-US-5A, ALM-US-5B, ALM-US-6A, ALM-US-7B, ALM-US-8A, ALM-US-8B, ALM-US-10A, ALM-US-10B, ALM-US-11A, and ALM-US-12A.

[d] Additional 138 clonal strawberry isolates from Israel as described (8).

specific PCR reactions were performed under reaction conditions for primer $(CAG)_5$, with 0.5 µM ITS 4 primer coupled with either 0.5 µM primer *Ca*Int2 for *C. acutatum*-specific detection or 0.5 µM primer *Cg*Int for *C. gloeosporioides*-specific detection. Amplification products were separated in agarose gels (1.5%, wt/vol; 15 × 10 cm, width×length) in Tris-acetate-EDTA buffer (21) electrophoresed at 80 V for 2 h.

A+T-rich DNA analyses. A+T-rich DNA was analyzed by *Hae*III digestion of total genomic DNA, which cleaves DNA at GGCC sites. *Hae*III digests nuclear DNA to fragments mainly less than 2 kb in size, whereas A+T-rich sequences are cleaved less frequently (11,12). A+T-rich DNA is partially associated with the mitochondrial genome, although contaminating nuclear A+T-rich DNA may also be present. Procedures used for *Hae*III restriction enzyme digestion and agarose gel electrophoresis were similar to those previously described (21).

Restriction enzyme digestion of amplified rDNA and sequencing procedure. PCR-amplified rDNA products from representative isolates of *Colletotrichum* spp., using the primer pair ITS 1 and ITS 4 (28), resulted in a product of approximately 560 bp, which was extracted from agarose gels using the Jetsorb kit (Genomed GmbH, Bad Oeynhausen, Germany). Recovered DNA was digested with the restriction enzymes *Bam*HI, *Hae*III, *Hha*I, *Hin*dIII, *Msp*I, *Pst*I, *Rsa*I, *Stu*I, and *Taq*I and separated on agarose gels.

The Big Dye Terminator DNA sequencing kit (Perkin-Elmer Inc., Branchburg, NJ) was used for determining sequence of the ITS 1 to ITS 2 regions (28). The sequence was determined using an ABI prism 377 DNA sequencer (Applied Biosystem Inc., Foster City, CA) and was performed at the sequencing unit of the Hebrew University of Jerusalem, Israel.

TABLE 2. Internal transcribed spacer 1 sequences of *Colletotrichum* isolates used in this study

Species	Isolate	Host	EMBL accession
C. lindemuthianum	CLD2[a]	*Phaseolus vulgaris*	Z 32987
C. orbiculare	172.59[a]	*Cucumis sativus*	Z 33379
C. fructigenum	4885[a]	*Acmena smithii*	Z 32907
C. acutatum	TUT-5954[b]	*Fragaria* × *ananassa*	AF 207794
C. acutatum	397[a]	*Fragaiae* × *ananassa*	Z 32915
C. acutatum	ALM-US-4[b]	*Prunus dulcis*	AF 207793
C. acutatum	179[c]	*Fragaria* × *ananassa*	Z 32913
C. acutatum	602/91.326[a]	*Phormium* spp.	Z 32924
Colletotrichum	ALM-KSH-10[b]	*Prunus dulcis*	AF 207791
C. acutatum	534/90.368[a]	*Ceanothus* spp.	Z 32918
C. coccodes	527.77[a]	*Lycopersicon esculentum*	Z 32930
C. dematium	343673[a]	*Piper nigrum*	Z 32906
C. musae	489[a]	*Musa nana*	Z 32991
C. kahawae	319406[a]	*Coffea arabica*	Z 32983
C. gloeosporioides	SAS 1[a]	*Citrus paradisi*	Z 32945
C. gloeosporioides	498[a]	*Zizyphus jujuba*	Z 32955
C. gloeosporioides	AVO-37-4B[b]	*Persea americana*	AF 207792
C. gloeosporioides	503[a]	*Malus domestica*	Z 32959
C. gloeosporioides	SAS 8[a]	*Dioscorea* spp.	Z 32963
C. gloeosporioides	501[a]	*Mangifera indica*	Z 32957
C. gloeosporioides	203[a]	*Aeschynomene virginica*	Z 32947
C. gloeosporioides	561[a]	*Fragaria* × *ananassa*	Z 32961
C. gloeosporioides	AV3/1[a]	*Persea americana*	Z 32965
C. gloeosporioides	G201[a]	*Persea americana*	Z 32967
C. fuscum	120[a]	*Digitalis lanata*	Z 32900
C. gloeosporioides	502[a]	*Hevea brasiliensis*	Z 32958
C. fragaria	63-1[a]	*Fragaria* × *ananassa*	Z 32943
C. gloeosporioides	HM335[d]	*Stylosanthes viscosa*	Y 16198
C. graminicola	DR1[e]	*Poa annua*	AF 059676
C. trichellum	84989[a]	*Hedera helix*	Z 33003

[a] Isolates sequenced by Sreenivasaprasad et al. (24).
[b] Isolates sequenced in this study.
[c] Isolate sequenced by Sreenivasaprasad et al. (24) and submitted directly to GenBank.
[d] Isolates sequenced by Munaut et al. (18).
[e] EMBL accession no. AF 059676.

Phylogenetic analysis. Analyses of ITS sequences were carried out using the program package ARB (25). Alignment of sequences was performed with the implemented ARB automated alignment tool, and alignments were refined manually. Phylogenetic analyses were performed by applying ARB parsimony, distance matrix, and maximum likelihood methods. To determine the robustness of phylogenetic trees, analyses were performed both on the original data set and on a data set from which highly variable positions were removed by use of a 50% conservation filter for members of the genus *Colletotrichum*, to reduce potential tree artifacts that may result from multiple base changes. Analysis was conducted on the ITS 1 sequences of the U.S. *C. acutatum* isolates (ALM-US-1B, ALM-US-4, and ALM-US-6B) from almond, the Israeli isolates (ALM-GVA-6A, ALM-KSH-10, and ALM-NRB-30K) from almond, the Israeli *C. acutatum* isolate (TUT-5954) from strawberry, the Israeli *C. gloeosporioides* isolate (AVO-37-4B) from avocado, and a number of *Colletotrichum* isolate sequences retrieved from GenBank (Table 2). In addition, sequences of the entire ITS region consisting of the ITS 1, 5.8S rRNA, and ITS 2 sequences complemented this analysis. Complete ITS 1 to ITS 2 sequences of the U.S. *C. acutatum* isolate ALM-US-4 from almond, the Israeli *Colletotrichum* isolate ALM-KSH-10 from almond, the Israeli *C. acutatum* isolate TUT-5954 from strawberry, and the Israeli *C. gloeosporioides* isolate AVO-37-4B from avocado were submitted to GenBank (Table 2).

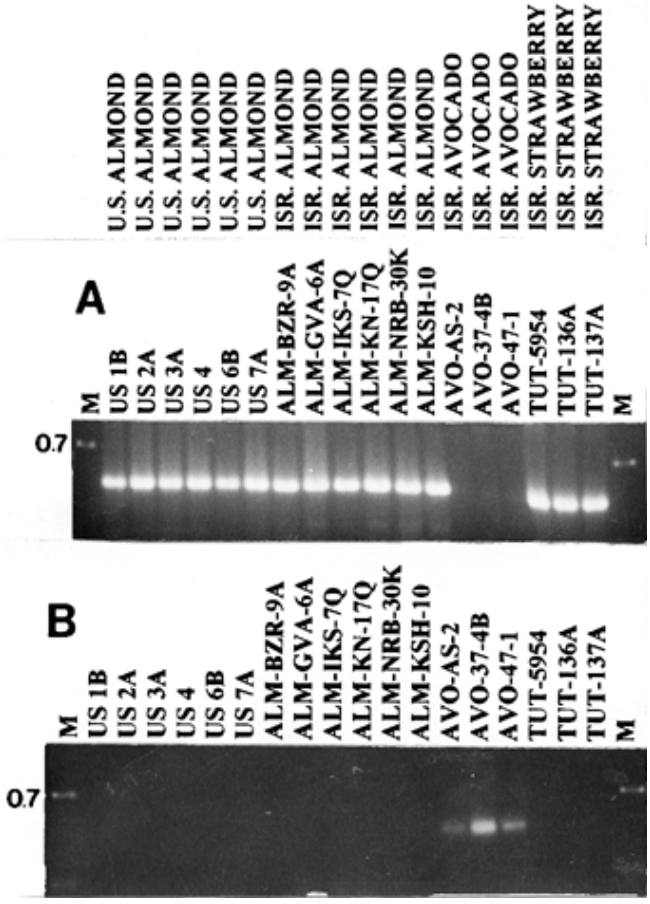

Fig. 1. Taxon-specific **A,** *Colletotrichum acutatum* and **B,** *C. gloeosporioides* amplification products of genomic DNA from U.S. *C. acutatum* isolates from almond (ALM-US-1B, ALM-US-2A, ALM-US-3A, ALM-US-4, ALM-US-6B, and ALM-US-7A), Israeli *Colletotrichum* isolates from almond (ALM-BZR-9A, ALM-GVA-6A, ALM-IKS-7Q, ALM-KN-17Q, ALM-NRB-30K, and ALM-KSH-10), Israeli *C. gloeosporioides* isolates from avocado (AVO-AS-2, AVO-37-4B, and AVO-47-1), and Israeli *C. acutatum* isolates from strawberry (TUT-5954, TUT-136A, and TUT-137A). Lane M: DNA markers with sizes in kilobases.

RESULTS

Taxon-specific primer analysis. DNA from 63 Israeli avocado isolates of *C. gloeosporioides*, 57 Israeli almond isolates, 19 U.S. pink isolates of *C. acutatum* from almond fruit, and 230 Israeli isolates of *C. acutatum* from strawberry were PCR amplified with the taxon-specific primers. Isolates within each population reacted identically and amplified the same size product. Representative isolates were then chosen for presentation of data. DNA of representative Israeli *C. acutatum* isolates from strawberry (TUT-5954, TUT-136A, and TUT-137A), U.S. *C. acutatum* almond isolates (ALM-US-1B, ALM-US-2A, ALM-US-4, ALM-US-6B, and ALM-US-7A), and Israeli *Colletotrichum* almond isolates (ALM-BZR-9A, ALM-GVA-6A, ALM-IKS-7Q, ALM-KN-17Q, ALM-KSH-10, and ALM-NRB-30K) were PCR amplified with the taxon-specific *C. acutatum* primers (Fig. 1A). *C. gloeosporioides* isolates from avocado (AVO-AS-2, AVO-37-4B, and AVO-47-1) were PCR amplified with the taxon-specific *C. gloeosporioides* primers (Fig. 1B).

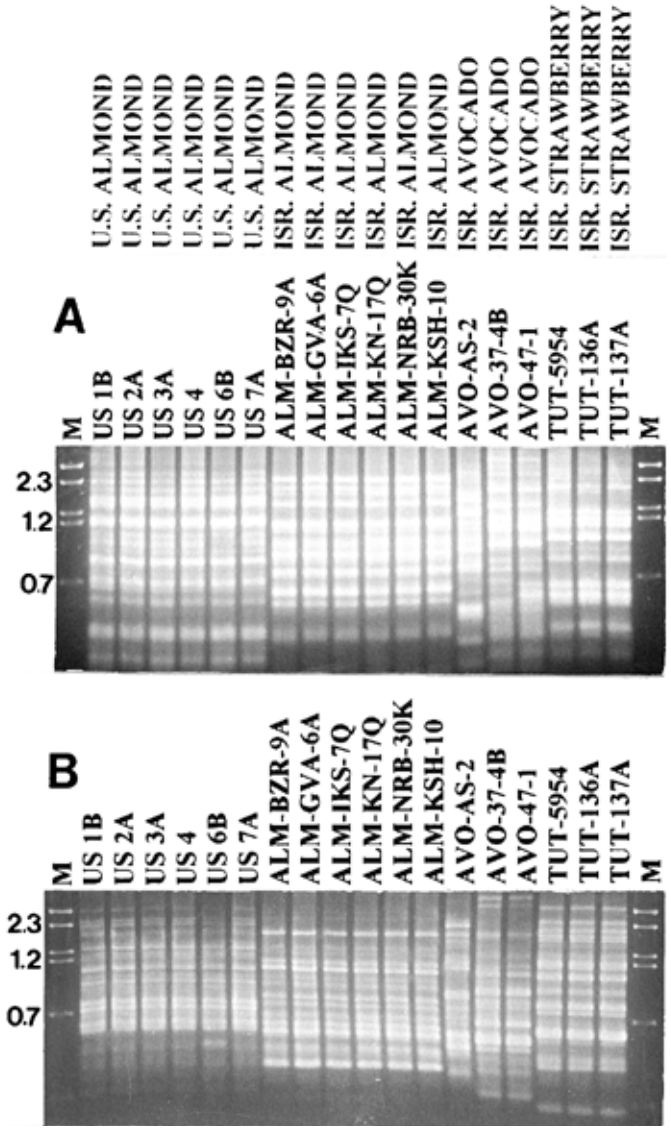

Fig. 2. Band patterns of arbitrarily primed polymerase chain reaction-amplified genomic DNA from U.S. *Colletotrichum acutatum* isolates from almond (ALM-US-1B, ALM-US-2A, ALM-US-3A ALM-US-4, ALM-US-6B, and ALM-US-7A), Israeli *Colletotrichum* isolates from almond (ALM-BZR-9A, ALM-GVA-6A, ALM-IKS-7Q, ALM-KN-17Q, ALM-NRB-30K, and ALM-KSH-10), Israeli *C. gloeosporioides* isolates from avocado (AVO-AS-2, AVO-37-4B, and AVO-47-1), and Israeli *C. acutatum* isolates from strawberry (TUT-5954, TUT-136A, and TUT-137A) using primers **A,** $(CAG)_5$ and **B,** $(GACAC)_3$. Lane M: DNA markers with sizes in kilobases.

The *Ca*Int2-specific primer for *C. acutatum* in conjunction with the ITS 4 primer amplified a 490-bp fragment from genomic DNA of the U.S. and Israeli almond isolates, as well as the *C. acutatum* strawberry isolates, but not from DNA of the *C. gloeosporioides* avocado isolates (Fig. 1A). In contrast, DNA of the U.S. *C. acutatum* and Israeli almond isolates, as well as the *C. acutatum* strawberry isolates, were not amplified by the *C. gloeosporioides*-specific *Cg*Int primer in conjunction with the ITS 4 primer, whereas a 450-bp fragment was amplified from DNA of the *C. gloeosporioides* avocado isolates alone (Fig. 1B). Due to uncertainty regarding apparent morphological and molecular identities of the Israeli almond population, we have decided for convenience to refer to this population as Israeli *Colletotrichum* isolates from almond, without reference to species.

ap-PCR analysis. Genomic DNA from all isolates included in the taxon-specific analysis were amplified by ap-PCR using primers $(CAG)_5$, $(GACAC)_3$, $(TGTC)_4$, and $(GACA)_4$ (Fig. 2). Amplified products from U.S. almond, Israeli almond, and Israeli strawberry isolates were identical among isolates within each population, indicating clonality, whereas heterogeneity was observed within the avocado population according to diversity of the amplified products. The same representative isolates that were used in the taxon-specific analysis were also used for ap-PCR. Identical banding patterns were produced with DNA of isolates within each population of the U.S. *C. acutatum* isolates from almond, Israeli *Colletotrichum* isolates from almond, and Israeli *C. acutatum* isolates from strawberry when amplified with primers $(CAG)_5$ and $(GACAC)_3$ (Fig. 2A and B, respectively) and with primers $(TGTC)_4$ and

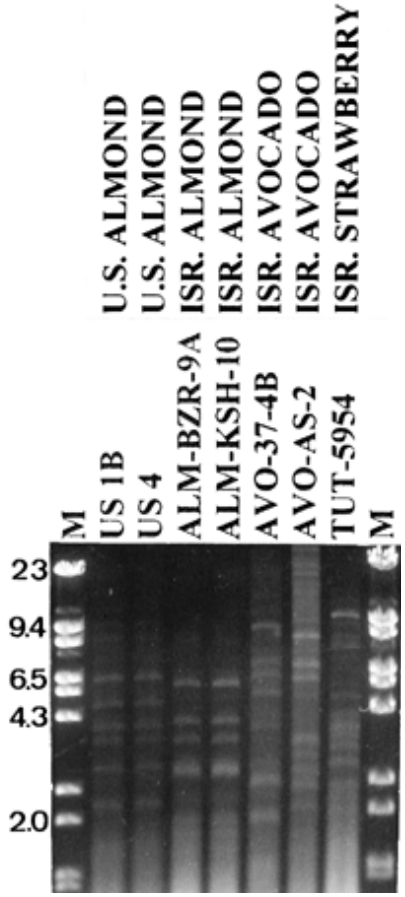

Fig. 3. Band patterns of genomic DNA from U.S. *Colletotrichum acutatum* isolates from almond (ALM-US-1B and ALM-US-4), Israeli *Colletotrichum* isolates from almond (ALM-BZR-9A and ALM-KSH-10), an Israeli *C. gloeosporioides* isolate from avocado (AVO-37-4B), and an Israeli *C. acutatum* isolate from strawberry (TUT-5954), digested with *Hae*III for A+T-rich DNA analysis. DNA was electrophoresed until the major G+C-rich fragments were eluted from the gel. Lane M: DNA markers with sizes in kilobases.

$(GACA)_4$ (data not shown). No identity was observed between the different populations, namely, Israeli Colletotrichum almond isolates, U.S. *C. acutatum* almond isolates, *C. acutatum* strawberry isolates, and *C. gloeosporioides* avocado isolates (Fig. 2).

A+T-rich DNA analysis. A+T-rich DNA was analyzed by observing *Hae*III digestions of genomic DNA from representative U.S. *C. acutatum* isolates (ALM-US-1B and ALM-US-4) and Israeli isolates (ALM-BZR-9A and ALM-KSH-10) from almond, *C. gloeosporioides* isolates from avocado (AVO-AS-2 and AVO-37-4B), and *C. acutatum* isolate TUT-5954 from strawberry (Fig. 3). Band patterns of two isolates from avocado (AVO-37-4B and AVO-AS-2) were distinct from those of the almond isolates and that of strawberry. Ten additional avocado isolates had a different restriction fragment length polymorphism pattern compared with the above six avocado, almond, and strawberry isolates (data not shown). The populations of almond isolates from the United States and Israel each showed a unique, uniform band pattern indicating two distinct genotypes. The strawberry isolate was unique in its banding pattern. An additional 10 isolates from each population were analyzed by this method, with each being identical to the representative isolates of its population (data not shown).

Polymorphism of rDNA ITS 1 to ITS 2 restriction digests. Restriction endonuclease digest patterns of PCR-amplified rDNA ITS 1 to ITS 2 regions were compared for the representative U.S. *C. acutatum* isolates (ALM-US-1B, ALM-US-4, and ALM-US-6B) and Israeli *Colletotrichum* isolates (ALM-GVA-6A, ALM-KSH-10, and ALM-BZR-9A) from almond, *C. gloeosporioides* isolate AVO-37-4B from avocado, and *C. acutatum* isolate TUT-5954 from strawberry. The enzyme *Rsa*I distinguished between the representative isolates of *Colletotrichum* spp. (Fig. 4) by recognizing unique restriction sites, in contrast to the enzymes *Bam*HI, *Hae*III, *Hha*I, *Hin*dIII, *Msp*I, *Pst*I, *Stu*I, and *Taq*I that did not possess unique sites among the different species. *Rsa*I cleaved the amplified fragment of the U.S. almond isolates at a single site, whereas the fragment of the Israeli almond and strawberry isolates remained uncut (Fig. 4). The *C. gloeosporioides* isolate AVO-37-4B from avocado had a unique digestion pattern. An additional 10 isolates from each population were analyzed by this method, with each producing an identical pattern to that of the representative isolates (data not shown).

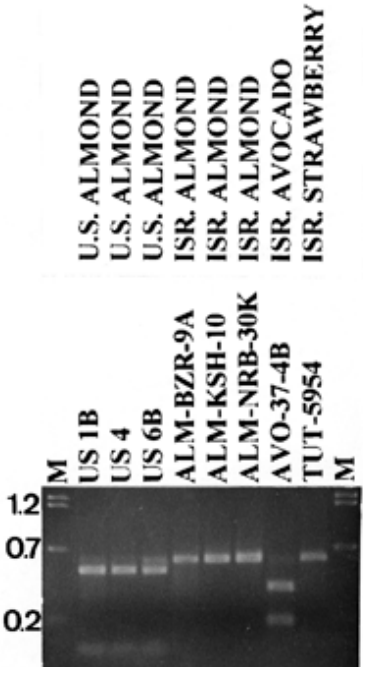

Fig. 4. *Rsa*I restriction enzyme digests of polymerase chain reaction-amplified ribosomal DNA of the entire internal transcribed spacer (ITS) region (ITS 1–5.8S–ITS 2) from U.S. *Colletotrichum acutatum* isolates from almond (ALM-US-1B, ALM-US-4, and ALM-US-6B), Israeli *Colletotrichum* isolates from almond (ALM-BZR-9A, ALM-KSH-10, and ALM-NRB-30K), an Israeli *C. gloeosporioides* isolate from avocado (AVO-37-4B), and an Israeli *C. acutatum* isolate from strawberry (TUT-5954). Lane M: DNA markers with sizes in kilobases.

Sequence and phylogenetic analysis. The U.S. *C. acutatum* almond isolates were uniform in sequence; therefore, further analysis was conducted using representative isolate ALM-US-4. Similarly, the Israeli almond isolates were uniform; therefore, isolate ALM-KSH-10 was used for further analysis.

Phylogenetic analysis was performed using both the ITS 1 sequence (Fig. 5) and that of the ITS 1 to ITS 2 fragment (Table 3). Comparative analysis of ITS 1 sequences produced a phylogenetic tree (Fig. 5) that supported the previous analysis published by Sreenivasaprasad et al. (24). This analysis confirmed grouping of the Israeli strawberry isolate TUT-5954 in a clade with U.S. *C. acutatum* strawberry isolates 179 and 397 and the U.S. *C. acutatum* almond isolate ALM-US-4. Isolate ALM-US-4 was found to be closely related to isolate 179. Isolate ALM-KSH-10, previously identified as *C. gloeosporioides* (22; P. F. Cannon, *personal communication*), grouped together with *C. acutatum* isolate 534 from a *Ceanothus* sp. and isolate 602 from a *Phormium* sp. (24). Israeli *C. gloeosporioides* isolate AVO-37-4B from avocado grouped with *C. gloeosporioides* isolate 498 from *Ziziphus jujuba* and isolate 503 from *Malus domestica*.

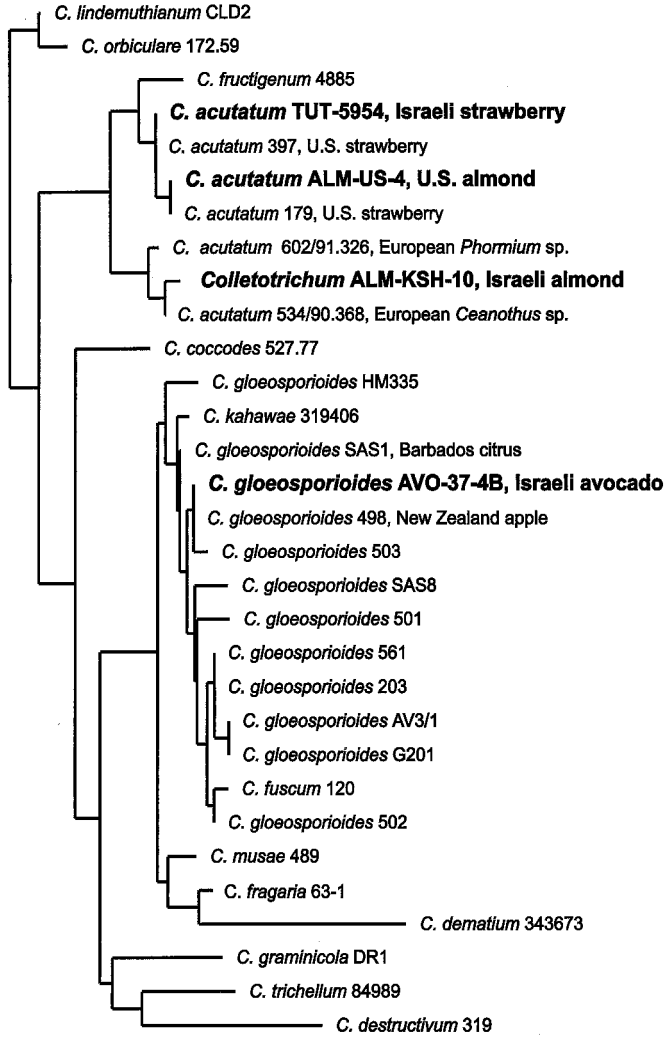

Fig. 5. Internal transcribed spacer 1-based phylogenetic tree of *Colletotrichum* isolates and published sequences. The tree was produced using the neighbor-joining algorithm. The orders of branching was similar in all tree construction approaches used. Scale bar indicates estimated 10% sequence divergence

Analysis of sequences of the ITS 1 to ITS 2 fragment supported the phylogeny inferred from the ITS 1 tree. The analysis was performed on the sequences of five isolates from which this sequence was available (*C. acutatum* isolate TUT-5954 from strawberry in Israel, *C. acutatum* isolate ALM-US-4 from the pink subpopulation of almond in the United States; *Colletotrichum* isolate ALM-KSH-10 from almond in Israel; *C. gloeosporioides* isolate AVO-37-4B from avocado in Israel, as described in this study; and *C. graminicola* isolate DR1 [EMBL accession no. AF059676]). Sequence analysis of the ITS 1 to ITS 2 regions revealed a similarity of between 97.03 to 98.72% among the *C. acutatum* isolates from almond and strawberry and the Israeli isolates from almond (Table 3). Similarity of the above populations to that of *C. gloeosporioides* of avocado was between 92.42 to 92.86%. The *C. graminicola* isolate DR1 was more closely related to the *C. gloeosporioides* cluster (95.11% similar) than that of the *C. acutatum* populations (92.4 to 92.64% similar) (Table 3).

DISCUSSION

The main objective of this study was to determine whether the various molecular methods used in this study were consistent with morphotaxonomic criteria for delimiting species of the genus *Colletotrichum*. Of particular interest were the almond populations of *Colletotrichum, C. acutatum* from the United States and that from Israel. Using ap-PCR and A+T-rich, we were able to differentiate between the different populations' DNA at the subspecies level. The taxon-specific primers and ITS sequence analysis were useful for grouping the isolates according to species. However, the molecular analyses of species identification were not in strict accordance to morphotaxonomic characteristics.

Previous nonmolecular studies have distinguished between the almond pathogens, *C. acutatum* pink subpopulation from the United States, and the population from Israel (10). It was shown that the optimal growth temperature, average growth rate, and morphology in culture varied significantly for each population. In addition, each population had a single unique vegetative compatibility group. Moreover, each of the almond populations was clearly distinguished from the *C. gloeosporioides* avocado pathogen according to the above and additional criteria, such as presence of the teleomorph, fungicide sensitivity, and infection process. Recently, an additional *C. acutatum* gray subpopulation was reported from California, and this subpopulation is apparently similar to the Israeli almond pathogen according to molecular analyses (7). However, the morphological differences between these two populations still need to be addressed.

Each molecular method was evaluated for the ability to assess isolates within or between species. Assessment of intraspecies variation relied on ap-PCR and A+T-rich DNA analyses, each of which gave a single pattern for all Israeli *Colletotrichum* almond isolates (Figs. 2 and 3). This may be indicative of a clonal population of the almond pathogen that has probably spread throughout Israel. Similarly, by these methods, the U.S. pink subpopulation of *C. acutatum* almond isolates appeared uniform and unique, but different from the Israeli *C. acutatum* strawberry isolates and the Israeli population of almond isolates. Likewise, the *C. acutatum* strawberry population was homogeneous and apparently clonal (Fig. 2). In contrast, ap-PCR and A+T-rich DNA analyses indicated multiple genotypes within the *C. gloeosporioides* avocado population, as previously described (9).

Due to its high rate of genetic variation, the ITS 1 region, ranging from 170 to 181 bp within the rDNA gene cluster, is considered suitable for species delimitation (24). In this study, the complete 560-bp rDNA region, encompassing the ITS 1 to ITS 2 regions and the 5.8S rDNA gene, was used for DNA sequence analysis for a more comprehensive analysis. Sequence analysis of this ITS 1 to ITS 2 fragment supported the phylogeny inferred from the ITS 1 tree (Fig. 5). The analysis was performed on five isolates from which this sequence was available (*C. acutatum* strawberry isolate TUT-5954 from Israel, *C. acutatum* almond isolate ALM-US-4 from the United States belonging to the pink subpopulation, *Colletotrichum* almond isolate ALM-KSH-10 from Israel, *C. gloeosporioides* avocado isolate AVO-37-4B from Israel described here, and *C. graminicola* isolate DR1 [EMBL accession no. AF059676]).

Phylogenetic analysis of the ITS 1 region confirmed that the Israeli strawberry isolate TUT-5954 clusters within the *C. acutatum* species, within a clade consisting of isolates 179, 397, 534, and 602, the U.S. *C. acutatum* almond isolate ALM-US-4, and the Israeli *Colletotrichum* almond isolate ALM-KSH-10 (Fig. 5). Analysis of the sequence data for the *Rsa*I restriction site further indicated that variation exists within the *C. acutatum* species cluster. The *C. gloeosporioides* isolate of avocado from Israel clustered with *C. gloeosporioides* isolates 503 and 498, and possessed a unique *Rsa*I cleavage site that differed from isolates of the *C. acutatum* species (Fig. 4).

The phylogenetic grouping based on sequence data did not appear to be congruent with conidial morphology. For example, it has previously been shown that straight cylindrical conidial species, such as *C. fragaria* and *C. gloeosporioides*, clustered in the same group as those of *C. capsici*, which possess falcate conidia (24). Therefore, although conidial morphology is one of the important criteria for species delimitation, its reliability as the sole basis for this purpose is questionable, due to the possibility of intermediate shapes and sizes of conidia being present within a given, even clonal, population. This appears to be the case for the almond population from Israel that was identified previously as *C. gloeosporioides* (22), although some isolates exhibited conidial morphology and culture characteristics that were intermediate between *C. gloeosporioides* and *C. acutatum* (P. F. Cannon, *personal communication*). However, based on ITS sequence data, this population grouped within the *C. acutatum* species cluster. It is possible, therefore, that the morphological characteristics of the Israeli almond population are as yet ill defined and in a state of flux, despite the fact that it is considered a clonal population according to molecular analyses. This is in contrast to the U.S. pink subpopulation from almond, which conforms with the morphotaxonomic criteria of the species *C. acutatum*.

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TABLE 3. Percent similarity, according to nucleotide sequence distance among representative *Colletotrichum* isolates, was based on the entire internal transcribed spacer (ITS) region (ITS 1–5.8S–ITS 2)[a]

Species (isolate)	AVO-37-4B	TUT-5954	ALM-US-4	ALM-KSH-10	DR1
C. gloeosporioides (AVO-37-4B)	100.00	92.84	92.86	92.42	95.11
C. acutatum (TUT-5954)		100.00	98.72	97.03	92.63
C. acutatum (ALM-US-4)			100.00	97.6	92.63
Colletotrichum (ALM-KSH-10)				100.00	92.4
C. graminicola (DR1)					100.00

[a] Percent similarity, based on pairwise analysis of isolates, was calculated using the ARB software (25).

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