Distinct Species Exist Within the *Cercospora apii* Morphotype

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ABSTRACT

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The genus *Cercospora* is one of the largest genera of hyphomycetes. *Cercospora apii* sensu lato is the oldest name for a large complex of morphologically indistinguishable *Cercospora* spp. occurring on a wide host range. There are currently 659 recognized *Cercospora* spp., and names of another 281 morphologically identical species are included in the synonymy of *C. apii* sensu lato. Two of the species that belong to the *C. apii* complex, *C. apii* and *C. beticola*, cause Cercospora leaf spot on *Apium graveolens* (celery) and *Beta vulgaris* (sugar beet), respectively. In the present study, multilocus sequence data, amplified fragment length polymorphism analysis, and cultural characteristics were used as additional features to characterize morphologically similar *Cercospora* strains occurring on celery and sugar beet. From the data obtained, it is shown that *C. apii* and *C. beticola*, although morphologically similar and able to cross-infect each others' hosts, are distinct functional species that should be retained as separate entities. Furthermore, a third, as yet undescribed species of *Cercospora* was detected in celery fields in Korea and Venezuela, suggesting that additional undescribed species also may be found to cause Cercospora leaf spot on celery. A polymerase chain reaction-based diagnostic protocol distinguishes all three *Cercospora* spp.

In his monograph of the genus *Cercospora* Fresen., Chupp (6) accepted 1,419 species. In total, more than 3,000 species of *Cercospora* have been described, of which 659 presently are recognized (7). Generally, species of *Cercospora* are considered to be host specific (6) at the level of the plant genus or family; this concept has led to the description of a large number of species. Several *Cercospora* spp., which are morphologically indistinguishable from *Cercospora apii* Fresen., were placed in the *C. apii* complex (13). Cross-inoculation studies revealed that isolates in the *C. apii* complex can infect an extremely wide host range, including *Apium graveolens* (celery) and *Beta vulgaris* (sugar beet) (1,2,22,23,38,42). In their revision of the genus *Cercospora*, Crous and Braun (7) referred 281 morphologically indistinguishable species to the *C. apii* sensu lato complex. Recent genetic analyses of *Cercospora* spp. have relied mainly on DNA sequences of the internal transcribed spacers (ITSs) and the 5.8S ribosomal (r)RNA gene. These studies have revealed that most species of *Cercospora*, in particular the members of the *C. apii* complex, are identical or very closely related (18,30,36, 37). Judging from their morphological similarity as well as their proven cross-infectiveness, it is probable that the species in the *C. apii* complex should be considered synonymous.

Species seen as representative of *C. apii* sensu lato lack a known teleomorph. Although the genus *Cercospora* is a well-established anamorph of the genus *Mycosphaerella* (11,18), only a few teleomorphs have been elicited via cultural studies (7,9). Phylogenetic analyses of all *Cercospora* isolates to date have placed them as a well-defined clade in the genus *Mycosphaerella.* Therefore, if a teleomorph were to be found for *C. apii*, it should be a species of *Mycosphaerella* (11,18,30,36).

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C. beticola, causal agent of Cercospora leaf spot on *B. vulgaris*, originally was described by Saccardo (34), and is assumed to have originated in central Europe and the Mediterranean area. *C. apii*, which causes Cercospora leaf spot on *A. graveolens*, was described from the region between The Netherlands and Germany (15), and is assumed to have originated in Western Europe. *C. beticola* is seen as part of the *C. apii* complex (7,13). Several studies so far have suggested that *C. beticola* on sugar beet should be treated as a synonym of *C. apii* (2,13,22,38,42).

Cercospora leaf spot on sugar beet is a serious problem wherever this crop is grown. It is one of the most common and destructive sugar beet diseases, affecting more than a third of all fields worldwide (20,35). A whole sugar beet field can be destroyed by an outbreak of *C. beticola*, resulting in complete loss of the crop (12,32,41).

The similarity in disease symptoms and pathogen morphology seen in celery and sugar beet Cercospora leaf spot diseases led Crous and Braun (7) to conclude that *C. beticola* should be treated as a synonym of *C. apii* sensu lato. Although Cercospora leaf spot is no longer considered the most destructive disease on celery (26), in some parts of the world (e.g., Florida), *C. apii* is still seen as a serious pathogen of this crop (27).

The main objective of the present study was to confirm or reject the synonymy of *C. apii* and *C. beticola*. It was felt that the same study would provide some indication as to the status of a large number of the purported synonyms of *C. apii*. To address these matters, 38 *Cercospora* isolates were collected from sugar beet and celery; representing a total of 13 countries. Isolates were subjected to multigene sequence analysis and amplified fragment length polymorphism (AFLP) analysis, as well as cultural and morphological comparisons. Here, we show that both celery and sugar beet are hosts to two species of *Cercospora*, with one of these species infecting both hosts. Although *C. apii* and *C. beticola* are able to cross-infect each other's hosts and are morphologically similar to one another, they still appear to operate as functional species on their respective primary namesake hosts in nature.

MATERIALS AND METHODS

Fungal isolates. Single-spore isolations were obtained from symptomatic celery and sugar beet leaves, and cultures were established on 2% malt extract agar (MEA) (16) (Table 1). The *Cercospora* isolates were examined morphologically to confirm their identity as *C. apii* sensu stricto as described by Crous and Braun (7). Some reference isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Utrecht, The Netherlands.

Morphological and cultural characterization. *Cercospora* reference strains were selected from celery and sugar beet for morphological and cultural characterization (Table 1). Strains were plated onto 2% MEA and oatmeal agar (OA) (16) and incubated at 24°C in the dark for 8 days. Colony characteristics were determined and colors rated on the different growth media using a color chart (31). Cardinal growth temperatures were determined on MEA (8). These plates were incubated in the dark for 8 days at temperatures beginning at 6°C and progressing to 36°C in 3°C intervals; in addition, growth at 40°C was studied. Several isolates taken from each of the three different groups were used (Table 1). The experiments featured three simultaneous replicates for each isolate; the whole trial was repeated once.

DNA extraction and sequencing. DNA analysis was done on all isolates listed in Table 1. The FastDNA kit (BIO 101, Carlsbad, CA) was used according to the manufacturer's instructions to isolate genomic (g)DNA of 200 to 400 mg of fungal mycelia grown on MEA plates for 8 days at 24°C. A sterile blade was used to scrape the mycelia from the surface of the plate. The primers ITS1 and ITS4 (43) were used to amplify the ITS areas as well as the 5.8S rRNA gene (ITS). Part of the actin (ACT) gene was amplified using the ACT512F and ACT783R primers (4) and part of the translation elongation factor (EF) 1-α gene using the primers EF728F and EF986R (4). The CAL228F and CAL737R primers (4) were used to amplify part of the calmodulin (CAL) gene, and the primers CylH3F and CylH3R (10) to amplify part of the histone H3 (HIST) gene. The polymerase chain reaction (PCR) conditions were the same for all regions, except for the $MgCl_2$ concentration, which was 2 mM for the CAL region and 1.5 mM for the remaining areas. The reaction mixture had a total volume of 12.5 µl and contained 1 µl of diluted gDNA, 1× PCR buffer, 48 µM each of the dNTPs, 2.5 pmol of each primer, and 0.7 units *Taq* polymerase (Bioline GmbH, Luckenwalde, Germany). The amplification reactions were done on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). The initial denaturation step was done at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C (30 s), annealing at 52°C (30 s), and elongation at 72°C (30 s). A final elongation step at 72°C (7 min) was included in the run. The PCR products were separated by electrophoresis at 80 V for 40 min on a 0.8% (wt/vol) agarose gel containing ethidium bromide at 0.1 µg/ml in 1× Tris-acetate-EDTA buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light.

The amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendal, The Netherlands) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Foster City, CA). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNAstar, Madison, WI).

Phylogenetic analysis. The sequences were assembled and added to the outgroups using Sequence Alignment Editor (version 2.0a11; Department of Zoology, University of Oxford, Oxford, UK), and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done in Phylogenetic Analysis Using Parsimony (PAUP; version 4.0b10; Sinauer Associates, Sunderland, MA) and consisted of neighbor-joining analysis with the uncorrected ("p"), Jukes-Cantor, and Kimura 2-parameter substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analysis, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1,000 bootstrap replications (19). Other measures calculated included tree length, consistency index, retention index, and rescaled consistency index (TL, CI, RI, and RC, respectively). The resulting trees were printed with TreeView version 1.6.6 (29). A partition homogeneity test was done in PAUP to test whether the different loci can be used in a combined analysis (14). Sequences were deposited in GenBank (accession numbers listed in Table 1) and the alignments in TreeBASE (accession no. S1285).

AFLP analysis. Restriction enzyme digestion and adaptor ligation were done using 30 ng of gDNA, 1× T4 DNA ligase buffer, 50 mM NaCl, 2 U of *Mse*I, 2 units of *Eco*RI, 40 U of T4 DNA ligase, 10 µg of bovine serum albumin, 50 pmol of *Mse*I adaptor, and 5 of pmol *Eco*RI adaptor made up to a final volume of 11 µl (39). All enzymes were obtained from New England BioLabs (Beverly, MA). This reaction was carried out at 37°C for 12 h. A 1:1 dilution was made with dH_2O and 4 µl was used in the preselective PCR. The preselective PCR was performed in a 20-µl volume containing 25 pmol of primer *Eco*RI-0 (39), 25 pmol of primer *Mse*I-0 (39), 1.5 mM $MgCl_2$, 1× Bioline *Taq* reaction buffer, 0.1 mM each dNTP and, 0.75 units of Bioline *Taq* polymerase. An initial 72°C step was done for 2 min, followed by 20 cycles of denaturation at 94°C (20 s), annealing at 56°C (40 s), and elongation at 72°C (1 min). The preselective amplification was confirmed by electrophoresis on a 0.8% (wt/vol) agarose gel as described above. The preamplified DNA was diluted 1:1 with dH_2O and used as template for selective amplification. Primers used in the selective amplification were *Eco*RI-A [FAM]/*Mse*I-CT, *Eco*RI-AT [JOE]/*Mse*I-C, and *Eco*RI-AG [NED]/*Mse*I-C (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). The reactions contained 1.5 mM MgCl, 0.5 units of Bioline *Taq* polymerase, 1× Bioline *Taq* polymerase buffer, 0.1 mM each dNTP, 0.5 µl of *Eco*RI primer, and 0.5 µl of *Mse*I primer made up to a final volume of 10 µl. Selective PCR products (2 µl), amplified with the different primer combinations for each of the isolates, were mixed together with 0.5 µl of GeneScan 500 (labeled with 6-carboxy-X-rhodamine) (Applied Biosystems) and made up to a final volume of 25 µl with formamide. The products were denatured at 100°C for 5 min, followed by 30-min runs on an ABI 310 genetic analyzer. The AFLP data were analyzed using Bionumerics software (version 2.5; Applied Maths, Kortrijk, Belgium).

Development of a species-specific diagnostic test. The CAL gene was found to be very effective for separating the three species described in the present study; therefore, this area was targeted for the development of a species-specific diagnostic test. Primers CercoCal-F and CercoCal-R (Table 2) were designed from regions of the CAL gene that are conserved for the *Cercospora* spp. in our database. They act as outer primers and their amplification functions as a positive control. Three internal primers (CercoCal-beta, CercoCal-apii, and CercoCal-sp), each specific for one of the three *Cercospora* spp. described in this study, were designed. The species-specific primers were used in separate PCRs together with the outer control primers. Strains of *C. beticola*, *C. apii*, the undescribed *Cercospora* sp., and 13 other species of *Cercospora* (Table 1) were screened with these primers. The sequences and specific nucleotide binding sites of the primers

TABLE 1. *Cercospora* isolates included in the study

Strain, accession no.[a]	Host	Origin	Collector	GenBank number[b]				
				ITS	EF	ACT	CAL	HIST
Cercospora achyranthis								
CPC 10091*	*Achyranthes japonica*	Korea	H. D. Shin	…	…	…	…	…
C. apii								
CBS 119.25; CPC 5086	*Apium graveolens*	…	L. J. Klotz	AY840512	AY840479	AY840443	AY840410	AY840377
CBS 121.31; CPC 5073	*Beta vulgaris*	Austria	…	AY840513	AY840480	AY840444	AY840411	AY840378
CBS 127.31; CPC 5119	*B. vulgaris*	Hungary	…	AY840514	AY840481	AY840445	AY840412	AY840379
CBS 152.52; CPC 5063	*B. vulgaris*	Netherlands	G. van den Ende	AY840515	AY840482	AY840446	AY840413	AY840380
CBS 536.71; CPC 5087	*A. graveolens*	Romania	O. Constantinescu	AY752133	AY752166	AY752194	AY752225	AY752256
CBS 114416; CPC 10925	*Apium* sp.	Austria	…	AY840516	AY840483	AY840447	AY840414	AY840381
CBS 114418; CPC 10924	*A. graveolens*	Italy	Meutri	AY840517	AY840484	AY840448	AY840415	AY840382
CBS 114485; CPC 10923	*A. graveolens*	Italy	Meutri	AY840518	AY840485	AY840449	AY840416	AY840383
CBS 116455; CPC 11556**	*A. graveolens*	Germany	K. Schrameyer	AY840519	AY840486	AY840450	AY840417	AY840384
CBS 116504; CPC 11579	*A. graveolens*	Germany	K. Schrameyer	AY840520	AY840487	AY840451	AY840418	AY840385
CBS 116507; CPC 11582	*A. graveolens*	Germany	K. Schrameyer	AY840521	AY840488	AY840452	AY840419	AY840386
C. beticola								
CBS 116.47; CPC 5074	*B. vulgaris*	Netherlands	G. E. Bunschoten	AY752135	AY752168	AY752196	AY752227	AY752258
CBS 122.31; CPC 5072	*B. vulgaris*	Germany	…	AY752136	AY752169	AY752197	AY752228	AY752259
CBS 123.31; CPC 5071	*B. vulgaris*	Spain	…	AY840522	AY840489	AY840453	AY840420	AY840387
CBS 124.31; CPC 5070	*B. vulgaris*	Romania	…	AY840523	AY840490	AY840454	AY840421	AY840388
CBS 125.31; CPC 5069	*B. vulgaris*	Japan	…	AY840524	AY840491	AY840455	AY840422	AY840389
CBS 126.31; CPC 5064	*B. vulgaris*	Germany	…	AY840525	AY840492	AY840456	AY840423	AY840390
CBS 116454; CPC 11558	*B. vulgaris*	Germany	S. Mittler	AY840526	AY840493	AY840457	AY840424	AY840391
CBS 116456; CPC 11557**	*B. vulgaris*	Italy	V. Rossi	AY840527	AY840494	AY840458	AY840425	AY840392
CBS 116501; CPC 11576	*B. vulgaris*	Iran	A. A. Ravanlou	AY840528	AY840495	AY840459	AY840426	AY840393
CBS 116502; CPC 11577	*B. vulgaris*	Germany	S. Mittler	AY840529	AY840496	AY840460	AY840427	AY840394
CBS 116503; CPC 11578	*B. vulgaris*	Italy	…	AY840530	AY840497	AY840461	AY840428	AY840395
CBS 116505; CPC 11580	*B. vulgaris*	France	S. Garressus	AY840531	AY840498	AY840462	AY840429	AY840396
CBS 116506; CPC 11581	*B. vulgaris*	Netherlands	…	AY840532	AY840499	AY840463	AY840430	AY840397
CPC 5125	*B. vulgaris*	New Zealand	C. F. Hill	AY752137	AY752170	AY752198	AY752229	AY752260
CPC 5128	*B. vulgaris*	New Zealand	C. F. Hill	AY752138	AY752171	AY752199	AY752230	AY752261
CPC 10168	*B. vulgaris*	New Zealand	C. F. Hill	AY840533	AY840500	AY840464	AY840431	AY840398
CPC 10171	*B. vulgaris*	New Zealand	C. F. Hill	AY840534	AY840501	AY840465	AY840432	AY840399
CPC 10197	*B. vulgaris*	New Zealand	C. F. Hill	AY840535	AY840502	AY840466	AY840433	AY840400
C. bizzozeriana								
CBS 258.67; CPC 5061*	*Cardaria draba*	Romania	O. Constantinescu	…	…	…	…	…
C. canescens								
CPC 1138*	*Vigna* sp.	South Africa	S. van Wyk	…	…	…	…	…
C. flagellaris								
CPC 10124*	*Phytolacca americana*	Korea	H. D. Shin	…	…	…	…	…
C. kikuchii								
CBS 135.28; CPC 5067*	*Glycine soja*	Japan	H. W. Wollenweber	…	…	…	…	…
C. malvacearum								
CBS 126.26; CPC 5066*	*Malva* sp.	…	…	…	…	…	…	…
C. penzigii								
CPC 3950*	*Citrus* sp.	South Africa	…	…	…	…	…	…
C. piaropi								
CBS 113127*	*Eichhornia crassipes*	United States	R. Charudattan	…	…	…	…	…
C. polygonacea								
CPC 10117*	*Persicaria* sp.	Korea	H. D. Shin	…	…	…	…	…
C. rautensis								
CBS 555.71; CPC 5082*	*Coronilla varia*	Romania	O. Constantinescu	…	…	…	…	…
C. ricinella								
CPC 10104*	*Ricinus communis*	Korea	H. D. Shin	…	…	…	…	…
C. rodmanii								
CBS 113130*	*Eichhornia crassipes*	United States	R. Charudattan	…	…	…	…	…
Cercospora sp.								
CBS 116457; CPC 10267**	*Apium* sp.	Venezuela	N. Pons	AY840536	AY840503	AY840467	AY840434	AY840401
CBS 116458; CPC 10657	*Apium* sp.	Korea	H. D. Shin	AY840537	AY840504	AY840468	AY840435	AY840402
CPC 10220	*Apium* sp.	Venezuela	N. Pons	AY840538	AY840505	AY840469	AY840436	AY840403
CPC 10248	*Apium* sp.	Venezuela	N. Pons	AY840539	AY840506	AY840470	AY840437	AY840404
CPC 10265	*Apium* sp.	Venezuela	N. Pons	AY840540	AY840507	AY840471	AY840438	AY840405
CPC 10266	*Apium* sp.	Venezuela	N. Pons	AY840541	AY840508	AY840472	AY840439	AY840406
CPC 10279	*Apium* sp.	Venezuela	N. Pons	AY840542	AY840509	AY840473	AY840440	AY840407
CPC 10666	*Apium* sp.	Korea	H. D. Shin	AY840543	AY840510	AY840474	AY840441	AY840408
CPC 10759	*A. graveolens*	Korea	H. D. Shin	AY840544	AY840511	AY840475	AY840442	AY840409
C. violae								
CPC 10725*	*Viola mondshivica*	Korea	H. D. Shin	…	…	…	…	…

[a] Origin of strain numbers: CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands and CPC = Collection of Pedro Crous, The Netherlands; * indicates additional *Cercospora* spp. tested with the species-specific primers; ** indicates *C. apii*, *C. beticola,* and *Cercospora* sp. isolates used for colony characteristics as well as growth rate measurements.

[b] ITS = internal transcribed spacer, EF = elongation factor, ACT = actin, CAL = calmodulin, HIST = histone H3.

are listed in Table 2. The same PCR conditions were used for the detection of all three species. The reaction mixture had a total volume of 12.5 µl and contained 1 µl of diluted gDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 48 µM each of the dNTPs, 1 pmol of CercoCal-F, 3 pmol of each of CercoCal-R and the specific internal primer, and 0.7 units (Bioline) of *Taq* polymerase. The amplification reactions were done on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). The initial denaturation step was done at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C (30 s), annealing at 58°C (30 s), and elongation at 72°C (30 s). A final elongation step at 72°C (7 min) was included to ensure that full-length products were obtained. The PCR products were separated on a 1.5% agarose gel and visualized as described above.

RESULTS

Morphological and cultural characterization. The morphological characteristics of the conidia and conidiophores for all isolates obtained from celery and sugar beet (Table 1) were the same as described for *C. apii* sensu lato by Crous and Brown (7). Isolates from celery obtained from Venezuela and Korea were distinct, however, in that conidiophores were relatively short, 25 to 70 by 4 to 6 µm, and conidia were obclavate-cylindrical, not acicular. They measured (minimum length, 50) 80 to 120 (maximum length, 150) by (minimum width, 3) 4 to 5 µm and were one to six septate.

To facilitate the standardization of further genotypic studies on the *C. apii* complex, we herewith designate new epitype (a specimen selected to serve as an interpretative type in support of other type material, to facilitate the precise application of the published name) materials with cultures for *C. apii* and *C. beticola*. For *C. apii*, the original herbarium material used for the type ("holotype") has been lost, but some of the original material might have been distributed and a lectotype, therefore, can be designated from these duplicates. Isolectotypes are duplicate specimens of the same lectotype. All of the material originally associated with the publication of the name *C. beticola* has been lost; therefore, a specimen has to be designated to serve as if it were the holotype of the species ("neotype"). Isoneotypes are duplicate specimens of the neotype and ex-epitype cultures (to facilitate molecular studies) are derived from the epitype material.

Cercospora apii Fresen., Beitr. Mykol. 3:91. 1863.

Lectotype (proposed here): on *Apium graveolens*, Germany, Oestrich, garden, Fuckel, Fungi rhen. 117, in HAL. Fresenius (15) cited material of *C. apii* obtained from Fuckel. This is an indirect reference to the material distributed by Fuckel as Fungi rhen. 117. Original material in the herbarium of Fresenius could not be traced, and probably is not preserved; therefore, we prefer to select one of the duplicates distributed by Fuckel to serve as lectotype. Isolectotypes: Fuckel, Fungi rhen. 117. Epitype (proposed here): on *Apium graveolens*, Germany, Landwirtschaftsamt Heilbronn, 10.08.2004, K. Schrameyer, culture ex-epitype CBS 116455.

Cercospora beticola Sacc., Nuovo Giorn. Bot. Ital. 8:189. 1876.

Neotype (proposed here): on *Beta vulgaris*, Italy, Vittorio (Treviso), Sept. 1897, Sacc., Fungi ital. 197 (PAD). Isoneotypes: Sacc., Fungi ital. 197. Epitype (proposed here): on *Beta vulgaris*, Italy, Ravenna, 10.7.2003, Rossi V., culture ex-epitype CBS 116456.

Colonies of *C. beticola* and *C. apii* are smooth, erumpent, and regular, with smooth, even margins, and sparse to moderate aerial mycelium. *C. beticola* colonies on MEA are greenish-gray on the surface and dark mouse-gray beneath. On OA, colonies are white to green-olivaceous. *C. apii* colonies on MEA are pale greenish-gray on the surface and dark mouse-gray beneath. The surfaces of the colonies are white to green-olivaceous on OA. Morphologically divergent isolates from Venezuela and Korea are smooth to folded, erumpent with smooth, even to uneven margins, and sparse to moderate aerial mycelium. On MEA, colonies are white to smoke-gray on the surface, and olivaceous-gray to iron-gray beneath. On OA, colonies are white to olivaceous-gray on the surface.

The temperature ranges and colony diameters of three reference isolates (CBS 116455, CBS 116456, and CBS 116457), representing each of the three different species, are given in Figure 1. The Venezuela and Korea isolates can grow at lower temperatures (6°C) than *C. beticola* and *C. apii* (12°C), whereas *C. beticola* and *C. apii* have a higher maximum temperature tolerance (33°C) than the *Cercospora* sp. (30°C). The optimal temperature for growth of the *Cercospora* sp. was observed to be 24°C, whereas the optimal growth temperature for *C. apii* and *C. beticola* is 27°C. The *Cercospora* sp. grows much more slowly than the other two species, growing only 1.72 mm/day at its optimum temperature, whereas *C. beticola* and *C. apii* grew 3.5 and 2.7 mm/day at their respective optimal temperatures. Differences in growth rate between *C. apii* and *C. beticola* were observed for most of the

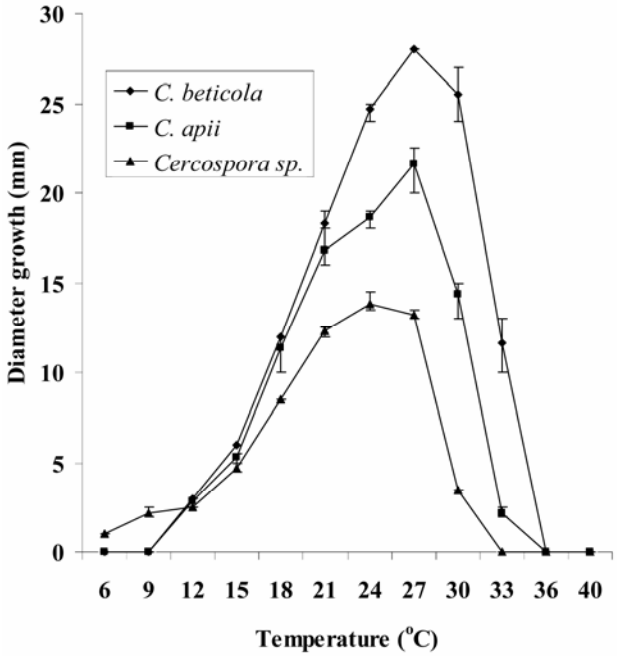

Fig. 1. Colony diameters at different temperatures ranging from 6 to 40°C for 8 days on 2% malt extract agar were calculated for *Cercospora apii* (CBS 116455), *C. beticola* (CBS 116456), and *Cercospora* sp. from Venezuela (CBS 116457).

TABLE 2. Primers designed from calmodulin sequences for the species identification amplifications

Primer	Sequence (5′–3′)	Nucleotide position[a]	Description
CercoCal-F	CGCGAGGCAGAGCTAACGA	61–79	Positive control forward primer
CercoCal-beta	GCCCACCCTCTGCGAATGTA	117–137	*Cercospora beticola*-specific primer
CercoCal-apii	GACCACCCTCTGCAACTGCG	117–137	*C. apii*-specific primer
CercoCal-sp	GCCCACTTTCTGTGACTGCA	117–137	*Cercospora* sp.-specific primer
CercoCal-R	GTGAGGAATTCGGGGAAATC	275–294	Reverse primer

[a] The calmodulin sequence of *C. apii* strain CBS 116455 (GenBank accession no. AY840417) was used to derive the nucleotide positions of the primers.

temperatures tested. *C. beticola* grew faster than *C. apii* (Fig. 1). *C. beticola* was more tolerant of temperatures higher than 30°C (1.46 versus 0.26 mm/day at 33°C).

Phylogenetic analysis. A partition homogeneity test showed that the five data sets were combinable ($P = 0.834$); therefore, the sequence data were analyzed as one combined set. The combined alignment of ITS, ACT, EF, CAL, and HIST contained 41 strains including the three outgroups, and had a total length of 1,611 characters, of which 1,183 were constant, 3 were parsimony uninformative, and 425 were parsimony informative. The topology of the neighbor-joining trees obtained using the different substitution models was the same. A similar topology was found for the most parsimonious trees. Parsimony analysis of the combined data resulted in 12 parsimonious trees, one of which is shown in Figure 2 (TL = 465 steps, CI = 0.989, RI = 0.997, and RC = 0.986). From the phylogenetic analysis (Fig. 2), three distinct and well-supported clades were obtained. The first clade contained isolates of the new *Cercospora* sp. from *Apium* spp. (100% bootstrap support), the second clade contained only *Cercospora* isolates from *B. vulgaris* (91% bootstrap support), and the third clade contained *Cercospora* isolates from both *B. vulgaris* and *Apium* spp. (100% bootstrap support). All the isolates from the third clade were isolated in Europe. The ITS and ACT data sets showed no variation among the isolates from the second and the third clade and no significant variation could be observed between the isolates of these two clades with the EF and HIST data sets. The amount of variation observed within the CAL region of the *C. beticola* and *C. apii* isolates (96% similarity) was significant and placed these species into two distinct phylogenetic clades, each with a high bootstrap support in the combined analysis.

AFLP analysis. Genetic differences between isolates of the different clades also were confirmed using AFLP analysis. Banding patterns obtained with the *Eco*RI-A [FAM]/*Mse*I-CT and *Eco*RI-AT [JOE]/*Mse*I-C primer combinations are shown in Figure 3. The number and sizes of the polymorphic bands obtained for isolates of the *Cercospora* sp., using the *Eco*RI-A [FAM]/*Mse*I-CT primer combination, show major differences with the profiles obtained for the other two species (Fig. 3A). Although isolates from the *C. apii* and *C. beticola* clades are more similar to each other than to the *Cercospora* sp., several bands are specific to each of the species, as seen using the *Eco*RI-A [FAM]/*Mse*I-CT and *Eco*RI-AT [JOE]/*Mse*I-C primer combinations (Fig. 3). The primer combination *Eco*RI-AG [NED]/*Mse*I-C also was tested on isolates from the three *Cercospora* spp. and the banding patterns obtained showed results similar to those obtained with the other two primer combinations (data not shown).

Species identification. Easy and rapid identification of *C. beticola*, *C. apii*, and the new *Cercospora* sp. was possible using three multiplex PCR amplifications, each specific for one of the species. A 234-bp fragment, which serves as the positive control, was present for all three species, whereas a 176-bp fragment was observed only for the *Cercospora* sp. elucidated by the specific internal primer (Fig. 4). Only the 234-bp fragment was present for all other *Cercospora* spp. tested in our database representing 13 *Cercospora* spp. (data not shown). Therefore, primers CercoCal-beta, CercoCal-apii, and CercoCal-sp are specific for *C. beticola*, *C. apii*, and the *Cercospora* sp., respectively, and can be used for their identification and detection.

DISCUSSION

Although morphological characteristics frequently are used to identify newly isolated fungi, it is not possible to distinguish *C. apii* (celery) from *C. beticola* (sugar beet) based solely on morphology. At the onset of this study, these species were considered to be synonymous as part of the *C. apii* sensu lato complex. Our data, however, refute the hypothesis that all morphologically indistinguishable *Cercospora* forms represent one species (7,13). *C. apii* sensu stricto, which typify the *C. apii* sensu lato complex, including *C. beticola*, which is a morphologically similar fungus originally described from sugar beet, are shown to differ genetically and with some cultural characteristics from one another to an extent confirming species-level separation. It is now possible to identify the studied species using these characteristics.

Among the sequence types studied, only CAL strongly supports the split of *C. apii* and *C. beticola* into two distinct phylogenetic groups. This grouping, however, is confirmed in the growth studies as well as in AFLP analysis. This study shows that the choice and number of loci sequenced can be crucial in elucidating phylogenetic relationships of very closely related species and that using the wrong or an insufficient number of sequence loci could result in erroneous synonymies being proposed. It also shows that phenotypic characteristics, such as growth rates and temperature thresholds, can be very important parameters in the identification of species that are morphologically identical.

From the phylogenetic data obtained, it is clear that *C. apii* occurs mainly on celery, whereas *C. beticola* occurs on sugar beet, and that cross-infection of each other's hosts is rare. We did, however, study three isolates, revealed molecularly as *C. apii* sensu stricto, that were obtained from sugar beet in Europe (CBS 121.31 and CBS 127.31, deposited in 1931, and CBS 152.52 in 1952). The origin of *C. apii* is suspected to be Western Europe, and certainly the species was first described from celery collected in Germany. Because all of the *C. apii* isolates available in this study were from European countries, we do not know whether *C. apii* has been introduced on *Apium* spp. in non-European countries. It has been reported that *C. apii* sensu lato isolates can infect hosts other than the ones they were isolated from (7,22, 38,42). Therefore, it is quite possible that *C. apii*, which grows much more slowly than *C. beticola* at high temperatures (Fig. 1), originally was able to infect sugar beet and compete with *C. beticola* in the early 1900s, when Europe was considerably colder than is currently the case (28). Without doubt, *C. beticola* has been introduced from Europe to many other parts of the world, and this species now can be found on almost every continent (7; current study). The absence of *C. apii* on fresh diseased leaf material of *B. vulgaris* obtained for the purposes of this study can be ascribed to the unique growth properties of *C. beticola.* It is very probable that the faster growth rate and its ability to easily grow at higher temperatures allow *C. beticola* to out-compete *C. apii* for infection sites on *B. vulgaris*. It is clear that environmental factors, such as temperature and availability of specific plant species, play an important role in the survival and infection ability of the fungus. Thus, it seems that genotype–environment interactions (24) may play a role in the fitness of species in the *C. apii* complex.

We illustrated an easy PCR-based method which can be used in laboratories that use basic PCR techniques as a diagnostic tool. Although three PCRs are necessary to distinguish between the three *Cercospora* spp. affecting celery and sugar beet, it is possible to limit the number of reactions according to the crop from which the pathogen was isolated. Thus far, the new *Cercospora* sp. has never been isolated from *B. vulgaris*; however, because both *C. beticola* and *C. apii* have been isolated from sugar beet, it is important to test isolates from that source as possible representatives of both these species. None of the *C. beticola* isolates confirmed as such with molecular data have been isolated from *Apium* spp.; therefore, it remains possible that *C. beticola* might not infect celery under field conditions.

Because of the major loss in sugar beet production due to Cercospora leaf spot, naturally derived fungicides and synthetic fungicides with broad chemistries are currently being used to control *Cercospora* spp. infections in this crop (21). Several studies have indicated that *C. beticola* has become resistant to fungicides in the benzimidazole class (17,33,40) and has developed increased tolerance to fungicides in the organotin and triazole classes (3,5,

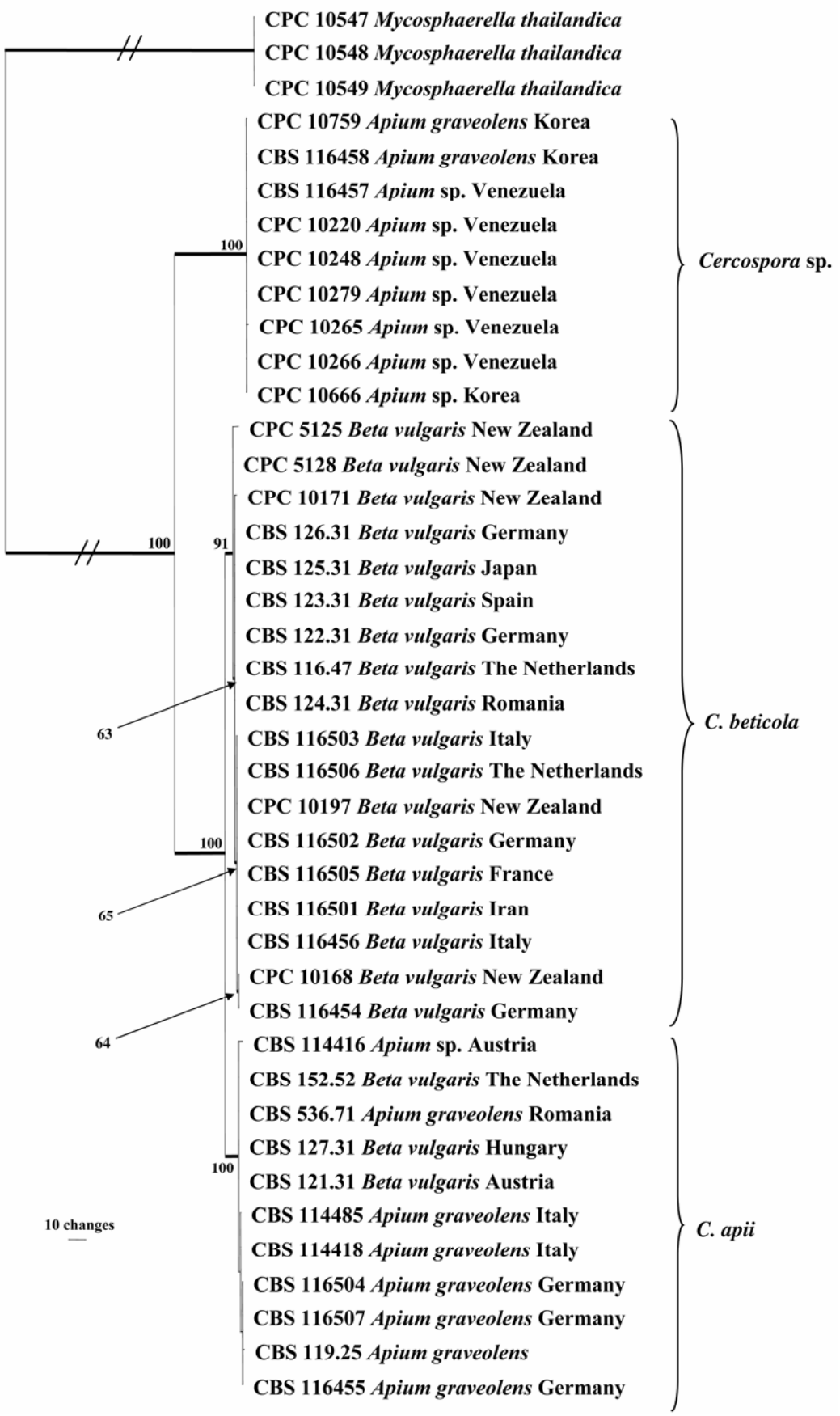

Fig. 2. One of the 12 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined internal transcribed spacer, elongation factor 1-α, actin, calmodulin, and histone H3 sequences alignment. The scale bar shows 10 changes and bootstrap support values from 1,000 replicates are shown in percentages at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted with three *Mycosphaerella thailandica* isolates.

25,41). In order to reduce fungicide tolerance of *Cercospora* spp. and to control the severity of Cercospora leaf spot disease of sugar beet, the frequent rotation of fungicide chemistries as well as the development of crops resistant to *Cercospora* infections have been implemented (21,41). Although *C. beticola* seems to be the main agent of Cercospora leaf spot on sugar beet, this study shows that *C. apii* also can be isolated from Cercospora leaf spot lesions on sugar beet. Fungicide trials must be done on these two species to determine their respective resistance levels against different fungicides. If there is a significant difference in their resistance levels, it might provide an explanation for the buildup of fungicide resistance of Cercospora leaf spot in sugar beet. This also can have major implications for the use of fungicides in other crops to which *Cercospora* spp. are pathogenic.

The relationships of all the other species that have been ascribed to the *C. apii* complex need to be studied in detail. Knowledge of whether species names previously synonymized with *C. apii* are correctly considered superfluous will enable us to better understand the diversity and host specificity of species in this complex, and will enable us to delineate the functional species units that operate in nature. The three species described in this study can be separated from one another not only on the genetic level but also

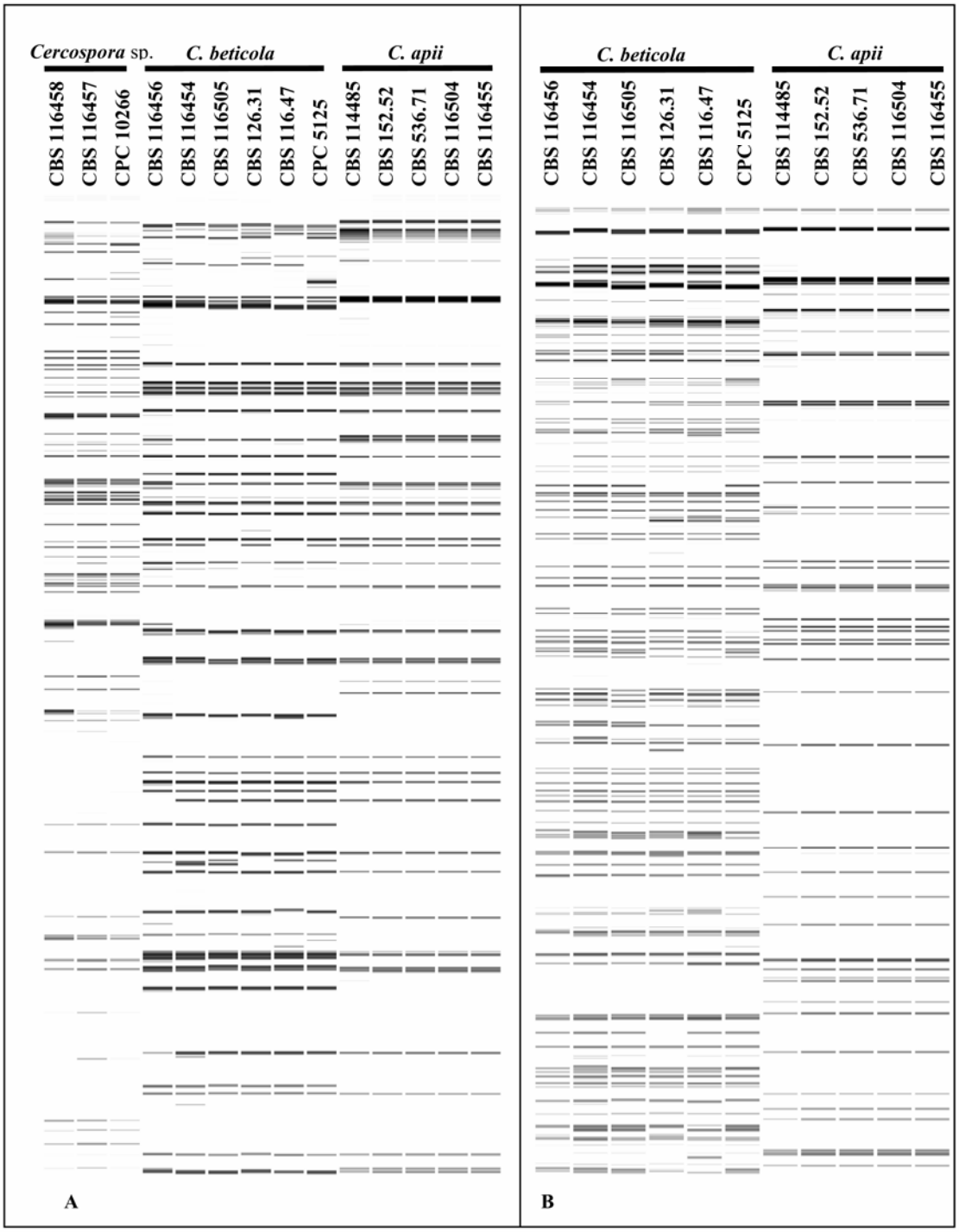

Fig. 3. Visualization of the amplified fragment length polymorphism (AFLP) band patterns were done using Bionumerics software. **A,** AFLP fingerprints of different isolates of the *Cercospora* sp., *Cercospora beticola*, and *C. apii* using primer combination *Eco*RI-A [FAM]/*Mse*I-CT. **B,** AFLP fingerprints of *C. beticola* and *C. apii* isolates using primer combination *Eco*RI-AT [JOE]/*Mse*I-C.

by the ecological niche of each of the species. The genotypic differences observed for the three *Cercospora* spp. can be linked most of the time to the ecological differences between them; for example, cardinal temperature ranges and host identity.

From our data, it is clear that Chupp (6) was not totally incorrect when he proposed that *Cercospora* spp. were restricted to specific host genera or families. If this concept could be used for all the *Cercospora* spp.–host combinations, it would be easy to identify *Cercospora* spp. based on their hosts. Unfortunately, the present study confirms that this concept is not applicable to the genus as whole. For instance, the *Cercospora* sp. present on typical Cercospora leaf spot symptoms of celery in Venezuela and Korea is a distinct species that matches none of the 200 *Cercospora* sequences in our database. This species grows much more slowly than *C. apii*, and is unable to grow at 33°C or above, but can grow at much lower temperatures than *C. apii*; for example, at 6 to 10°C. Based on phylogenetic and AFLP analyses, this species is different from *C. apii* as well as *C. beticola*. A population representing more than 50 celery plants was collected of this species in Venezuela, indicating that it obviously is well established on this host. The fact that this species also occurs on celery in Korea suggests that, rather than representing a pathogen that normally grows on another host but occasionally occurs on celery by chance alone, it is instead an established pathogen of celery. It probably has been overlooked in the past due to its morphological similarity to *C. apii* and similar host symptomatology. This discovery of such a widespread cryptic species on a well-studied host like celery, however, does stimulate one to question whether similar cryptic species could exist within additional "common" pathogens that we currently accept as having wide host ranges. The present study illustrates how important it is to the plant pathology community to lodge reference strains of the pathogens they are working with in long-term storage in publicly accessible collections. Had it not been for the plant pathologists who lodged their *C. apii* strains in the early 1900s, it would not have been possible to prove the presence of different *Cercospora* spp. on celery, or the natural occurrence of *C. apii* on sugar beet. This riddle, in spite of the advanced techniques employed here, remains unresolved to this day.

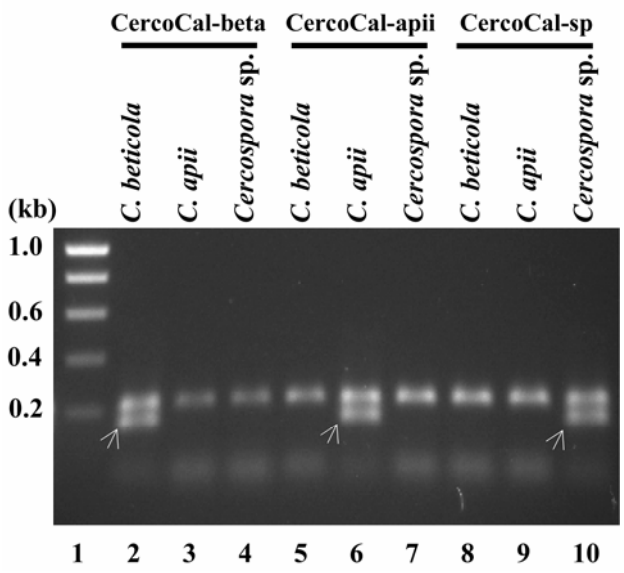

Fig. 4. Identification of *Cercospora beticola*, *C. apii*, and the new *Cercospora* sp. using the different species-specific (CercoCal) primers. Lane 1 contains the DNA marker. The 234-bp fragment, the positive control, is present for all the polymerase chain reaction amplifications done (lanes 2 to 10). The species-specific fragment (176 bp, indicated with an arrow) can be observed only when the amplification reaction contains *C. beticola* (CBS 116456) DNA with primer CercoCal-beta (lane 2), *C. apii* (CBS 116455) with primer CercoCal-apii (lane 6), or *Cercospora* sp. (CBS 116457) with primer CercoCal-sp (lane 10).

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