

Cercosporoid diseases of *Citrus*

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Abstract: *Citrus* leaves and fruits exhibiting disease symptoms ranging from greasy spot, yellow spot, small or large brown spot, black dot, and brown dot were sampled from Fujian, Guangdong, Guizhou, Hunan, Jiangxi, Yunnan, Zhejiang provinces and the Guangxi Zhuang Autonomous Region in China. In total 82 isolates representing various cercosporoid genera were isolated from these disease symptoms, which were supplemented with eight *Citrus* cercosporoid isolates collected from other countries. Based on a morphological and phylogenetic study using sequence data from the nuclear ribosomal DNA's ITS1-5.8S-ITS2 regions (ITS), and partial actin (*act*), β -tubulin (*tub2*), 28S nuclear ribosomal RNA (28S rDNA) and translation elongation factor 1- α (*tef1*) genes, these strains were placed in the following genera: *Cercospora*, *Pallidocercospora*, *Passalora*, *Pseudocercospora*, *Verrucisporota* and *Zasmidium*. All isolates tended to be sterile, except the *Zasmidium* isolates associated with citrus greasy spot-like symptoms, which subsequently were compared with phylogenetically similar isolates occurring on *Citrus* and other hosts elsewhere. From these results four *Zasmidium* species were recognized on *Citrus*, namely *Z. indonesianum* on *Citrus* in Indonesia,

Z. fruticola and *Z. fructigenum* on *Citrus* in China and *Z. citri-griseum*, which appears to have a wide host range including *Acacia*, *Citrus*, *Eucalyptus* and *Musa*, as well as a global distribution.

Key words: citrus greasy spot, multigene phylogenetic analyses, *Mycosphaerella*, *Pseudocercospora*, *Zasmidium*

INTRODUCTION

Several species of cercosporoid fungi have been associated with leaf and fruit spot diseases of *Citrus* spp. (Pretorius et al. 2003), of which two are regarded as particularly serious. Greasy leaf spot, caused by *Zasmidium citri-griseum* (sexual morph *Mycosphaerella citri*) (Sivanesan 1984, Timmer and Gottwald 2000, Braun et al. 2014), and Phaeoramularia fruit and leaf spot, caused by *Pseudocercospora angolensis* (Seif 2000), which is also of quarantine concern in Europe (Quaedvlieg et al. 2012, Crous et al. 2013).

In former years these genera were treated as asexual morphs of the genus *Mycosphaerella* (Corlett 1991, Crous et al. 2004b), although recent phylogenetic studies have shown that this complex consists of more than 40 genera and that these asexual morphs represent genera in their own right (Crous et al. 2007, 2009b). Many of these represent important phytopathogenic genera, including *Cercospora* (Crous et al. 2006, Groenewald et al. 2013), *Passalora*, *Pseudocercospora* (Crous et al. 2013), *Ramularia* (Braun 1995, 1998; Videira et al. 2015), *Septoria* (Quaedvlieg et al. 2013, Verkley et al. 2013), *Zasmidium* (Braun et al. 2013, 2014) and *Zymoseptoria* (Quaedvlieg et al. 2011, Stukenbrock et al. 2012), to name but a few.

Several leaf spot diseases of citrus have been associated with cercosporoid fungi in the past. Greasy leaf spot of citrus originally was associated with *Cercosporaci-tri-grisea* in Florida, although the fungus was isolated from brown leaf spot symptoms (Fisher 1961), in contrast to the greasy leaf spots linked to *Mycosphaerellacitri* (Whiteside 1972). Presently this disease is still a major problem in Florida and has also been reported from many other areas including Mexico, Central America, the Caribbean islands and Egypt (Mondal et al. 2004, Haggag 2012). At the same time similar diseases of citrus have been observed in Argentina, Australia and Japan (Tanaka and Yamada 1952, Marcó and Whiteside 1986, Timmer and Gottwald 2000). A different disease, called citrus pseudo greasy spot was reported from Japan (Koizumi 1986, Furuya et al. 2012). Although Yamada (1956) linked *Mycosphaerella horii* to citrus leaf spot in this country, the illustration suggests

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it could be a species of *Didymella*. Fisher (1961) reported *Cercospora gigantea* from grapefruit leaves in Florida, but an examination of the type specimen suggests this is probably a synonym of *Corynespora citricola* (Pretorius et al. 2003). Crous and Braun (2003) reported *Passalora citricola* from leaves of *Citrus* (origin unknown) and *P. citrigena* from leaf spots of *Citrus* sp. occurring in Mexico. Several species of *Cercospora*, identified as *C. penzigii*, have been associated with leaf spots on a range of *Citrus* spp. from Algeria, Argentina, Azerbaijan, Bhutan, Caucasus, China, Cuba, Dominican Republic, India, Italy, Japan, Mexico, Papua New Guinea, Senegal, South Africa, Swaziland, USA (FL, MS, TX) and Venezuela (Pretorius et al. 2003). Phylogenetically, however, these isolates appear to represent unresolved species in the *C. canescens*, *C. fagellaris* and *Cercospora* sp. P species complexes (Groenewald et al. 2013).

Citrus represents a significant and rapidly growing global industry. Among all citrus-producing countries, China is regarded as one of the most important centers of origin of *Citrus*, having a citrus-growing history of more than 4000 y (Deng et al. 2008, Zhou and Ye 2010). Presently citrus represents a significant industry in more than nine provinces, autonomous regions and municipalities in China (Deng et al. 2008). The major cultivars grown here include grapefruit, kumquat, lemon, mandarin, orange, pomelo and tangerine (Deng et al. 2008). All these cultivars suffer from citrus greasy spot, which has been recorded under three different names based on their different occurring symptoms, including greasy spot (Huang 1943, Zhang and Yin 1987), yellow spot (Yan 1984, Jiang et al. 2012) and small brown spot (Zhang and Yin 1987).

In the present study we collected citrus materials with a range of leaf spot symptoms throughout China and supplemented this collection with *Citrus* isolates collected in other countries. We aimed to characterize and compare the cercosporoid species associated with citrus greasy spot in China and elsewhere with the consolidated species concept introduced by Quaedvlieg et al. (2014). Under this species concept, species are recognized based on the combination of morphological, ecological and phylogenetic species concepts as a whole and not just based on phylogenetic data. Finally we wanted to examine the correlation between different genera and species and different citrus leaf spot symptoms.

MATERIALS AND METHODS

Isolates.—Symptomatic leaves were collected from major citrus growing areas in China including Fujian, Guangdong, Guizhou, Hunan, Jiangxi, Yunnan, Zhejiang provinces and the Guangxi Zhuang Autonomous Region (Fig. 1). The main sampled *Citrus* varieties were grapefruit, mandarin,

orange, pomelo and tangerine (TABLE I). Collected symptomatic leaves, fruits and other citrus tissues were washed with running water to remove the dust and were dried with absorbent paper. To obtain the fungal strains, the dried tissues were surface-disinfected by scrubbing thoroughly (5 times) with a cotton ball dipped in 75% ethanol. After drying under a laminar flow hood, a small piece (3 × 3 mm) of tissue was removed from the junction of infected and healthy tissue and plated on 2% potato dextrose agar (PDA, according to Crous et al. 2009b) plates amended with 100 µg/mL ampicillin and streptomycin to inhibit bacterial growth and incubated at 25 C. Emerging colonies were transferred to fresh PDA plates, and fertile colonies single-spored and stored at 4 C (see Crous et al. 2009b) (TABLE II). Additional strains were obtained from the working collection of Pedro Crous (CPC) housed at CBS, as well as the CBS culture collection (Utrecht, the Netherlands).

DNA extraction, PCR amplification and sequencing.—Isolates were cultured 7 d on PDA at 25 C, the mycelia harvested with a sterile blade and placed in centrifuge tubes. DNA was extracted with the Biospin Fungus Genomic DNA Extraction Kit (Bio-Flux, Bior Technology Co., China) and used for PCR amplification. Six primer pairs were used to amplify partial sequences of nuclear ribosomal RNAs ITS1-5.8S-ITS2 regions (ITS), and partial actin (*act*), β -tubulin (*tub2*), 28S ribosomal RNA gene (28S rDNA) and translation elongation factor 1- α (*tef1*) genes. Sequences of primers followed the studies, ITS4/ITS5 (White et al. 1990), ACT-512F/ACT783-R (Carbone and Kohn 1999), Bt2a (Glass and Donaldson 1995) or T1 (O'Donnell and Cigelnik 1997), /Bt2b (Glass and Donaldson 1995) or β -Sandy-R (Stukenbrock et al. 2012), LSU1Fd (Crous et al. 2009a), /LR5 (Vilgalys and Hester 1990) and EF1-728F (Carbone and Kohn 1999), /EF-2 (O'Donnell et al. 1998). The amplified products were sequenced in both directions and a consensus sequence determined (Sunny Biotechnology Co. Ltd, Shanghai, China) for the phylogenetic analyses. All sequences produced in this study were submitted to GenBank (TABLE II).

Phylogenetic analyses.—Resulting sequences were used to identify additional related sequences deposited in the NCBI GenBank nucleotide databases using megablast queries and subjected to multiple sequences alignments with the online interface of MAFFT 6 (Kato and Toh 2010) and manually corrected where necessary. Two datasets were constructed; the first using the 28S rDNA data only to determine the generic relationships among the obtained isolates (Fig. 2) and the second from a combined ITS, *tef1*, 28S rDNA and *tub2* alignment representing species of *Zasmidium* (Fig. 3). The *act* phylogeny was not congruent with the other phylogenies in that three isolates of *Zasmidium fructicola* (ZJUM50, ZJUM77, ZJUM89) and a single isolate of *Z. fructigenum* (ZJUM99) formed separate lineages intermediate between the *Z. fructigenum* and *Z. fructicola* clades, with *Zasmidium xenoparkii* (CBS 111185) clustering basal within the *Z. fructigenum* clade. Therefore this locus was excluded from the combined analysis. Congruency of the sequence datasets for the separate loci were determined with tree topologies of 70% reciprocal neighbor-joining bootstrap trees with maximum

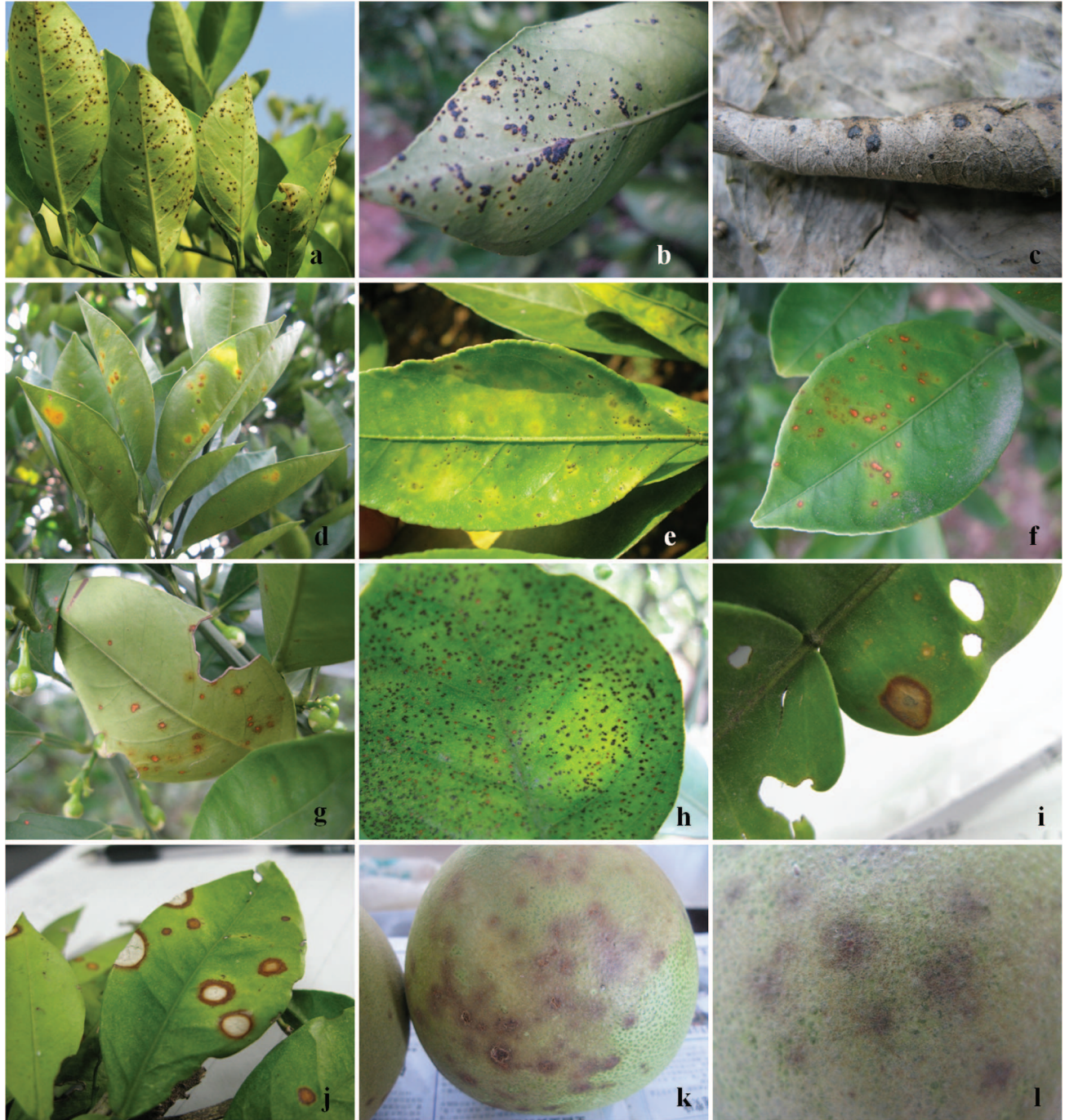


FIG. 1. Symptoms of citrus greasy spot in China. a, b. Greasy spot. c. Greasy spot on decayed leaf of grapefruit; d, e. Yellow spot. f, g. Upper and lower side of brown small round spot on leaves of grapefruit, respectively. h. Black or brown dot on leaf of grapefruit. i, j. Big round spot on leaves of pomelo and grapefruit, respectively. k, l. Similar greasy spot on fruits of pomelo.

likelihood distances that were compared visually to identify conflicts between partitions (Mason-Gamer and Kellogg 1996, Gueidan et al. 2007). A maximum parsimony analysis was performed on the alignments with PAUP (Phylogenetic analysis using parsimony) 4.0b10 (Swofford 2003) using the heuristic search option with 100 random sequence additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed,

and the first 1000 multiple, equally most parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis and Bull 1993). All characters were weighted equally, and alignment gaps were treated as new state data. Tree length, consistency indexes (CI), retention indexes (RI), rescaled consistency indexes (RC) and homoplasy indexes (HI) were calculated for the resulting trees. The resulting phylogenetic trees

TABLE I. The sampling information of Chinese citrus greasy spot-associated material

Province (8 total)	County (17 total)	Host	Tissue	No. of isolates (82 total)
Fujian	Nanjing	<i>Citrus grandis</i> (pomelo, Guanximiyou)	Fruit	2
	Pinghe	<i>Citrus grandis</i> (pomelo, Guanximiyou)	Fruit	6
Guangdong	Dabu	<i>Citrus grandis</i> (pomelo, Guanximiyou)	Fruit	1
	Pingyuan	<i>Citrus sinensis</i> (orange)	Fruit	1
Guangxi	Yangsuo	<i>Citrus</i> sp.	Leaf	1
		<i>Citrus sinensis</i> (orange)	Leaf	1
		<i>Fortunella margarita</i> (kumquat)	Leaf	1
		<i>Citrus grandis</i> (pomelo, Shatianyou)	Fruit	4
Guizhou	Congjiang	<i>Citrus reticulata</i> (tangerine, Ponkan)	Leaf	2
			Twig	2
Hunan	Jishou	<i>Citrus reticulata</i> (tangerine, Ponkan)	Fruit	3
Jiangxi	Nanfeng	<i>Citrus reticulata</i> (tangerine, Nanfengmiju)	Fruit	2
Yunnan	Mengdian	<i>Citrus limon</i> (lemon)	Leaf	2
		<i>Citrus aurantifolia</i> (key lime)	Leaf	1
Zhejiang	Cangnan	<i>Citrus grandis</i> (pomelo, Sijiyou)	Leaf	3
			Fruit	2
	Changshan	<i>Citrus paradisi</i> × <i>C. sp.</i> (grapefruit, Changshanhuoyou)	Leaf	12
			Fruit	8
			Leaf	1
			Fruit	2
	Huangyan	<i>Citrus reticulata</i> (tangerine, Ponkan)	Leaf	1
			Fruit	2
	Jiangshan	<i>Citrus unshiu</i> (mandarin)	Fruit	2
			Fruit	2
	Jiangshan	<i>Citrus paradisi</i> × <i>C. sp.</i> (grapefruit, Changshanhuoyou)	Leaf	1
			Leaf	1
	Linhai	<i>Citrus unshiu</i> (mandarin)	Fruit	1
			Fruit	1
			Fruit	1
	Longyou	<i>Citrus paradisi</i> × <i>C. sp.</i> (grapefruit, Changshanhuoyou)	Leaf	6
Leaf			6	
Quzhou	<i>Citrus paradisi</i> × <i>C. sp.</i> (grapefruit, Changshanhuoyou)	Leaf	1	
		Fruit	2	
		Fruit	1	
		Leaf	1	
Yuhuan	<i>Citrus grandis</i> (pomelo, Wendanyou)	Leaf	1	
		Fruit	2	

(FIGS. 2, 3) were printed with Geneious 5.5.4 (Drummond et al. 2011) and the layout of the tree for publication was done in Adobe Illustrator CS5.1. Sequences derived in this study were lodged at GenBank, the alignments and trees in TreeBASE (www.treebase.org/treebase/index.html).

Morphology.—Colonies were studied on PDA, oatmeal agar (OA) (Crous et al. 2009c) and pine needle agar (PNA) (Smith et al. 1996), and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation. Observations were made under a Zeiss V20 Discovery stereomicroscope and with a Zeiss Axio Imager 2 light microscope using differential interference contrast (DIC) illumination and an Axio-Cam MRc5 camera and ZEN software. Measurements and photographs were made from structures mounted in clear lactic acid. The 95% confidence intervals were derived from 30 observations (1000× magnification), with the

extremes in parentheses. Ranges of the dimensions of other characters are provided. Colony characters and pigment production were noted after 2 wk growth on media incubated at 25 °C in the dark. Colony colors (surface and reverse) were rated according to the color charts of Rayner (1970). Morphological descriptions were based on cultures sporulating on PNA. Reference strains were deposited in CBS, descriptions and nomenclatural data in MycoBank (Crous et al. 2004a).

RESULTS

Isolates.—In total 82 isolates were collected from China, supplemented with 15 isolates from other countries (TABLE II). Of the Chinese isolates, nine were recorded without any specific foliar disease symptoms, while 73 isolates were associated with citrus greasy spot

TABLE II. Collection details and GenBank accession numbers of isolates included in this study

Species	Culture No. ^a	Host	Location	GenBank accession No. ^b				
				28S rDNA	<i>act</i>	ITS	<i>tef1</i>	<i>tub2</i>
<i>Cercospora</i> cf. <i>flagellaris</i>	ZJUM 121	<i>Citrus grandis</i> , leaf	China: Zhejiang, Longyou	KP895883	KP895965	KP896013	KP896062	KP896109
<i>Pallidocercospora crystallina</i>	ZJUM 2	<i>Citrus grandis</i> , fruit	China: Zhejiang, Cangnan	KP895884	KP895966	KP896014	KP896063	KP896110
	ZJUM 16	<i>Citrus grandis</i> , leaf	China: Zhejiang, Cangnan	KP895885	KP895967	KP896015	KP896064	KP896111
	ZJUM 92	<i>Citrus grandis</i> , fruit	China: Fujian, Nanjing	KP895886	KP895968	KP896016	KP896065	KP896112
	ZJUM 128	<i>Citrus reticulata</i> , leaf	China: Guizhou, Congjiang	KP895887	KP895969	KP896017	KP896066	KP896113
	ZJUM 129	<i>Citrus reticulata</i> , leaf	China: Guizhou, Congjiang	KP895888	KP895970	KP896018	KP896067	KP896114
	ZJUM 138	<i>Citrus grandis</i> , fruit	China: Fujian, Pinghe	KP895889	KP895971	KP896019	—	KP896115
<i>Pallidocercospora heimioides</i>	ZJUM 71	<i>Citrus sinensis</i> , leaf	China: Guangxi, Yangsuo	KP895890	KP895972	KP896020	KP896068	KP896116
	ZJUM 85	<i>Citrus unshiu</i> , fruit	China: Zhejiang, Huangyan	KP895891	KP895973	KP896021	KP896069	KP896117
<i>Passalora loranthi</i>	ZJUM 66	<i>Citrus grandis</i> , fruit	China: Guangdong, Dabu	KP895892	—	KP896022	KP896070	KP896118
<i>Pseudocercospora</i> sp.	ZJUM 11	<i>Citrus grandis</i> , leaf	China: Zhejiang, Cangnan	KP895893	KP895974	KP896023	—	KP896119
	ZJUM 35	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Changshan	KP895894	KP895975	KP896024	KP896071	KP896120
	ZJUM 73	<i>Citrus grandis</i> , fruit	China: Guangxi, Yangsuo	KP895895	KP895976	KP896025	KP896072	KP896121
	ZJUM 75	<i>Citrus grandis</i> , fruit	China: Guangxi, Yangsuo	KP895896	KP895977	KP896026	KP896073	KP896122
	ZJUM 130	<i>Citrus reticulata</i> , twig	China: Guizhou, Congjiang	KP895897	KP895978	KP896027	KP896074	KP896123
	ZJUM 131	<i>Citrus reticulata</i> , twig	China: Guizhou, Congjiang	KP895898	KP895979	KP896028	KP896075	KP896124

TABLE II. Continued

Species	Culture No. ^a	Host	Location	GenBank accession No. ^b				
				28S rDNA	<i>act</i>	ITS	<i>tef1</i>	<i>tub2</i>
<i>Verrucisporota</i> sp.	ZJUM 135	<i>Citrus grandis</i> , fruit	China: Fujian, Pinghe	KP895999	KP895980	KP896029	KP896076	KP896125
	ZJUM 136	<i>Citrus grandis</i> , fruit	China: Fujian, Pinghe	KP895900	KP895981	KP896030	KP896077	KP896126
	ZJUM 137	<i>Citrus grandis</i> , fruit	China: Fujian, Pinghe	KP895901	KP895982	KP896031	KP896078	KP896127
<i>Zasmidium citri-griseum</i>	ZJUM 72	<i>Fortunella margarita</i> , leaf	China: Guangxi, Yangshuo	KP895902	KP895983	KP896032	KP896079	KP896128
	CBS 116366 = CPC 10522 = CMW 11730	<i>Acacia mangium</i>	Thailand	KF902138	AY752207	KF901780	KF903386	KF903080
<i>Zasmidium citri-griseum</i>	CBS 116426 = X115	<i>Musa</i> sp.	USA: Florida	KF901987	AY752208	KF901648	KF903387	KF903081
	CBS 122455 = X126	<i>Citrus</i> sp.	USA: Florida	KF902156	AY752206	KF901797	KF903388	KF903082
	CPC 13467	<i>Eucalyptus</i> sp.	Thailand	KF902137	KF903650	KF901779	KF903379	KF903075
	CPC 15285	<i>Citrus</i> sp.	USA: Florida	KF902150	—	KF901791	KF903380	KF903076
	CPC 15289	<i>Citrus</i> sp.	USA: Florida	KF902151	—	KF901792	KF903381	—
	CPC 15291	<i>Citrus</i> sp.	USA: Florida	KF902152	KF903676	KF901793	KF903382	KF903077
	CPC 15293	<i>Citrus</i> sp.	USA: Florida	KF902153	—	KF901794	KF903383	—
	CPC 15294	<i>Citrus</i> sp.	USA: Florida	KF902154	—	KF901795	KF903384	KF903078
	CPC 15296, ex-epitype	<i>Citrus</i> sp.	USA: Florida	KF902155	—	KF901796	KF903385	KF903079
	ZJUM 5 = CPC 24464	<i>Citrus grandis</i> , leaf	China: Zhejiang, Cangnan	KP895903	KP895984	KP896033	KP896080	KP896129
	ZJUM 25 = CPC 24468	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895904	KP895985	KP896034	KP896081	—
	ZJUM 27 = CPC 24469	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895905	KP895986	KP896035	KP896082	KP896130
	ZJUM 54 = CPC 24474	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895906	KP895987	KP896036	KP896083	KP896131
ZJUM 81 = CPC 24488	<i>Citrus reticulata</i> , leaf	China: Zhejiang, Huangyan	KP895907	KP895988	KP896037	KP896084	KP896132	
ZJUM 97 = CPC 24497	<i>Citrus grandis</i> , leaf	China: Zhejiang, Yuhuan	KP895908	KP895989	KP896038	KP896085	KP896133	

TABLE II. Continued

Species	Culture No. ^a	Host	Location	GenBank accession No. ^b				
				28S rDNA	<i>act</i>	ITS	<i>tef1</i>	<i>tub2</i>
<i>Zasmidium fructicola</i>	ZJUM 103 = CPC 24500	<i>Citrus limon</i> , leaf	China: Yunnan, Mengdian	KP895909	KP895990	KP896039	KP896086	KP896134
	ZJUM 104 = CPC 24501	<i>Citrus limon</i> , leaf	China: Yunnan, Mengdian	KP895910	KP895991	KP896040	KP896087	KP896135
	ZJUM 105 = CPC 24502	<i>Citrus aurantifolia</i> , leaf	China: Yunnan, Mengdian	KP895911	KP895992	KP896041	KP896088	KP896136
	ZJUM 127 = CPC 24504	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Jiaogshan	KP895912	KP895993	KP896042	KP896089	KP896137
	ZJUM 9 = CPC 24465	<i>Citrus grandis</i> , fruit	China: Zhejiang, Cangnan	KP895913	KP895994	KP896043	KP896090	KP896138
	ZJUM 48 = CPC 24472	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Changshan	KP895914	KP895995	KP896044	KP896091	—
	ZJUM 50 = CPC 24473	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Changshan	KP895915	KP895996	KP896045	KP896092	KP896139
	ZJUM 55 = CPC 24475	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Changshan	KP895916	KP895997	KP896046	KP896093	KP896140
	ZJUM 58 = CPC 24477	<i>Citrus grandis</i> , fruit	China: Fujian, Pinghe	KP895917	KP895998	KP896047	KP896094	KP896141
	ZJUM 68 = CPC 24479	<i>Citrus sinensis</i> , fruit	China: Guangdong, Pingyuan	KP895918	KP895999	KP896048	KP896095	KP896142
	ZJUM 77 = CPC 24484	<i>Citrus reticulata</i> , fruit	China: Hunan, Jishou	KP895919	KP896000	KP896049	KP896096	KP896143
	ZJUM 78 = CPC 24485	<i>Citrus reticulata</i> , fruit	China: Hunan, Jishou	KP895920	KP896001	KP896050	KP896097	KP896144
ZJUM 79 = CPC 24486	<i>Citrus reticulata</i> , fruit	China: Hunan, Jishou	KP895921	KP896002	KP896051	KP896098	KP896145	
ZJUM 80 = CPC 24487, ex-type	<i>Citrus reticulata</i> , fruit	China: Zhejiang, Huangyan	KP895922	KP896003	KP896052	KP896099	—	
ZJUM 84 = CPC 24489	<i>Citrus unshiu</i> , fruit	China: Zhejiang, Huangyan	KP895923	KP896004	KP896053	KP896100	KP896146	

TABLE II. Continued

Species	Culture No. ^a	Host	Location	GenBank accession No. ^b				
				28S rDNA	act	ITS	tef1	tub2
<i>Zasmidium fructigenum</i>	ZJUM 89 = CPC 24494	<i>Citrus sinensis</i> , fruit	China: Zhejiang, Linhai	KP895924	KP896005	KP896054	KP896101	KP896147
	ZJUM 90 = CPC 24495	<i>Citrus grandis</i> , fruit	China: Fujian, Nanjing	KP895925	KP896006	KP896055	KP896102	KP896148
	ZJUM 36 = CPC 24471, ex-type	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Changshan	KP895926	KP896007	KP896056	KP896103	KP896149
	ZJUM 86 = CPC 24491	<i>Citrus reticulata</i> , fruit	China: Jiangxi, Nanchang	KP895927	KP896008	KP896057	KP896104	KP896150
	ZJUM 87 = CPC 24492	<i>Citrus reticulata</i> , fruit	China: Jiangxi, Nanchang	KP895928	KP896009	KP896058	KP896105	KP896151
	ZJUM 88 = CPC 24493	<i>Citrus unshiu</i> , fruit	China: Zhejiang, Linhai	KP895929	KP896010	KP896059	KP896106	KP896152
	ZJUM 99 = CPC 24498	<i>Citrus grandis</i> , fruit	China: Zhejiang, Yuhuan	KP895930	KP896011	KP896060	KP896107	KP896153
	ZJUM 100 = CPC 24499	<i>Citrus grandis</i> , fruit	China: Zhejiang, Yuhuan	KP895931	KP896012	KP896061	KP896108	KP896154
	CPC 15300	<i>Citrus</i> sp.	Indonesia	KF902086	—	KF901739	KF903377	—
	CBS 110988 = CPC 1090	<i>Eucalyptus grandis</i> , leaf	Colombia	KF901975	KF903418	DQ303021	KF903271	KF902975
CBS 110999 = CPC 1087, ex-type	<i>Eucalyptus grandis</i> , leaf	Colombia	KF901977	KF903419	DQ303023	KF903273	KF902977	
CBS 111049 = CPC 1089	<i>Eucalyptus grandis</i> , leaf	Colombia	KF901976	KF903426	DQ303025	KF903272	KF902976	
<i>Zasmidium</i> sp.	ZJUM 28	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895932	—	—	—	—
	ZJUM 34	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895933	—	—	—	—
	ZJUM 38	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Changshan	KP895934	—	—	—	—

TABLE II. Continued

Species	Culture No. ^a	Host	Location	GenBank accession No. ^b				
				28S rDNA	act	ITS	tef1	tub2
<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	ZJUM 40	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895935	—	—	—	—
<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	ZJUM 41	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895936	—	—	—	—
<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	ZJUM 42	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895937	—	—	—	—
<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	ZJUM 43	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895938	—	—	—	—
<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	ZJUM 44	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895939	—	—	—	—
<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	ZJUM 46	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895940	—	—	—	—
<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	ZJUM 47	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Changshan	KP895941	—	—	—	—
<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	ZJUM 49	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Changshan	KP895942	—	—	—	—
<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	ZJUM 51	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Changshan	KP895943	—	—	—	—
<i>Citrus grandis</i> , fruit	ZJUM 69	<i>Citrus</i> sp., leaf	China: Guangxi, Yangsuo	KP895944	—	—	—	—
<i>Citrus grandis</i> , fruit	ZJUM 74	<i>Citrus grandis</i> , fruit	China: Guangxi, Yangsuo	KP895945	—	—	—	—
<i>Citrus grandis</i> , fruit	ZJUM 76	<i>Citrus grandis</i> , fruit	China: Guangxi, Yangsuo	KP895946	—	—	—	—
<i>Citrus reticulata</i> , fruit	ZJUM 82	<i>Citrus reticulata</i> , fruit	China: Zhejiang, Huangyan	KP895947	—	—	—	—

TABLE II. Continued

Species	Culture No. ^a	Host	Location	GenBank accession No. ^b				
				28S rDNA	<i>act</i>	ITS	<i>lef1</i>	<i>tub2</i>
	ZJUM 93	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Quzhou	KP895948	—	—	—	—
	ZJUM 94	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Quzhou	KP895949	—	—	—	—
	ZJUM 95	<i>Citrus paradisi</i> × <i>Citrus</i> sp., fruit	China: Zhejiang, Quzhou	KP895950	—	—	—	—
	ZJUM 96	<i>Citrus reticulata</i> , fruit	China: Zhejiang, Quzhou	KP895951	—	—	—	—
	ZJUM 106	<i>Citrus unshiu</i> , leaf	China: Zhejiang, Quzhou	KP895952	—	—	—	—
	ZJUM 111	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Longyou	KP895953	—	—	—	—
	ZJUM 112	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Longyou	KP895954	—	—	—	—
	ZJUM 113	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Longyou	KP895955	—	—	—	—
	ZJUM 114	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Longyou	KP895956	—	—	—	—
	ZJUM 116	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Longyou	KP895957	—	—	—	—
	ZJUM 117	<i>Citrus paradisi</i> × <i>Citrus</i> sp., leaf	China: Zhejiang, Longyou	KP895958	—	—	—	—
	ZJUM 119	<i>Citrus grandis</i> , leaf	China: Zhejiang, Longyou	KP895959	—	—	—	—
	ZJUM 122	<i>Citrus grandis</i> , leaf	China: Zhejiang, Longyou	KP895960	—	—	—	—

TABLE II. Continued

Species	Culture No. ^a	Host	Location	GenBank accession No. ^b				
				28S rDNA	act	ITS	tef1	tub2
	ZJUM 123	<i>Citrus grandis</i> , leaf	China: Zhejiang, Longyou	KPS95961	—	—	—	—
	ZJUM 124	<i>Citrus grandis</i> , leaf	China: Zhejiang, Longyou	KPS95962	—	—	—	—
	ZJUM 126	<i>Citrus grandis</i> , leaf	China: Zhejiang, Longyou	KPS95963	—	—	—	—
	ZJUM 139	<i>Citrus grandis</i> , fruit	China: Fujian, Pinghe	KPS95964	—	—	—	—
<i>Zasmidium xenoparkii</i>	CBS 111185 = CPC 1300, ex-type	<i>Eucalyptus grandis</i> , leaf	Indonesia	KF902002	KF903438	KF901663	KF903274	KF902978

^a CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; CMW: Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CPC: Culture collection of Pedro Crous, housed at CBS; X: personal culture collection of Mahdi Arzanlou, Iran; ZJUM: culture collection at Zhejiang University, China.

^b 28SrDNA: 28S nuclear ribosomal RNA gene; act: partial actin gene; ITS: nuclear ribosomal RNA's ITS1-5.8S-ITS2 regions; tef1: partial translation elongation factor 1-alpha gene; tub2: partial beta-tubulin gene.

and a range of other symptoms. Of these 17 isolates were collected from greasy spot symptoms (FIG. 1a–c), 16 from yellow spot symptoms (FIG. 1d, e), 14 from small, brown spots (FIG. 1f, g), nine from black dot symptoms, resembling melanose (FIG. 1h), six from big round spots (FIG. 1i, j), five from greasy spot slacking black protuberances (FIG. 1k, l), four from citrus black spots (Wang et al. 2012) and one isolate from brown dot (FIG. 1h) and healthy fruit, respectively.

Phylogenetic analyses.—In this study we aimed to classify cercosporoid isolates from *Citrus* by employing a consolidated species concept incorporating morphological and phylogenetic data. However, many isolates proved to be sterile. An analysis of the 28S rDNA sequences was performed to determine the generic relationships of the obtained isolates; this analysis was based on 108 isolates, including the outgroup sequence, and the resulting dataset of 754 characters, including alignment gaps which are treated as fifth base, consisted of 595 constant characters, 44 variable parsimony-uninformative characters and 115 parsimony-informative characters. Twenty equally most parsimonious trees were retained, the first of which is presented (FIG. 2). Based on the 28S rDNA phylogeny (FIG. 2), isolates from China clustered in clades corresponding to the genera *Cercospora* (one isolate), *Pallidocercospora* (eight isolates), *Passalora* (one isolate), *Pseudocercospora* (three isolates, with a further six isolates not included in the phylogeny), *Verrucisporota* (one isolate) and *Zasmidium* (23 isolates, with a further 39 isolates not included in the phylogeny). Most of the isolates were found to be members of the genus *Zasmidium*, and these could be induced to sporulate on PNA.

The *Zasmidium* isolates were selected for a multigene (ITS, *tef1*, 28S rDNA, *tub2*) phylogenetic analysis (FIG. 3), which could resolve six species, four of which occurred on *Citrus*. This analysis was based on 46 isolates, including the two outgroup sequences, and the resulting dataset of 1822 characters (488, 328, 747 and 259 characters for ITS, *tef1*, 28S rDNA and *tub2*, respectively), including alignment gaps which were treated as fifth base, consisted of 1276 constant characters, 185 variable parsimony-uninformative characters and 361 parsimony-informative characters. The maximum of 1000 equally most parsimonious trees were retained, the first of which is presented (FIG. 3).

TAXONOMY

In the present study isolates were found to cluster in clades corresponding to the genera *Cercospora*, *Pallidocercospora*, *Passalora*, *Pseudocercospora*, *Verrucisporota* and *Zasmidium*. Of these, however, only those in the genus

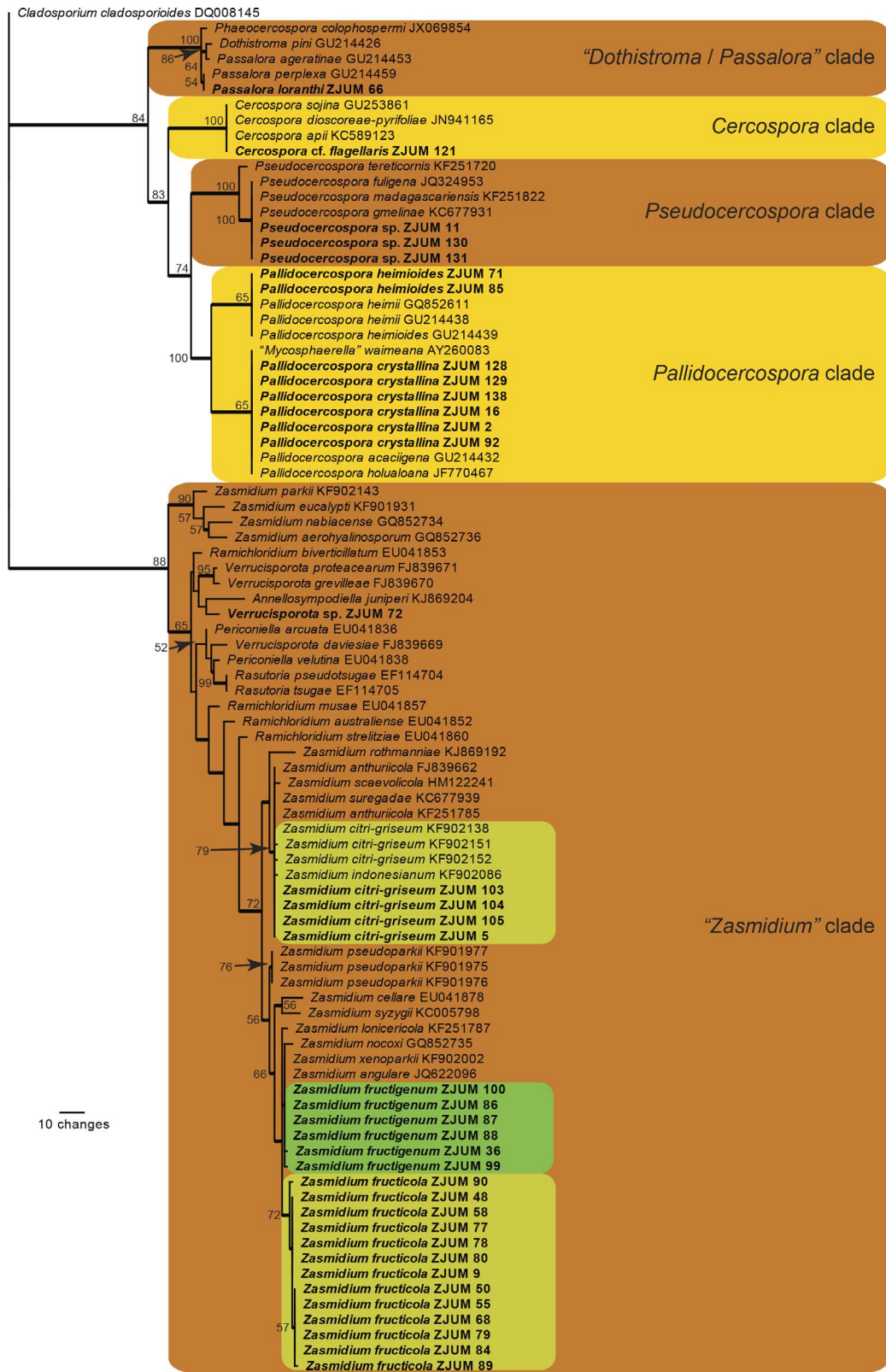


FIG. 2. The first of 20 equally most parsimonious trees (TL = 372, CI = 0.543, RI = 0.920, RC = 0.500) from a parsimony analysis of the 28S rDNA alignment representing cercosporoid genera in the *Mycosphaerellaceae*. Bootstrap support values are indicated at the nodes, and the bar represents the number of changes. Thickened branches reflect those branches present in the strict consensus tree. Major lineages are indicated with colored blocks and the *Zasmidium* species of interest in the present study with internal blocks. Isolates from China are in boldface text. The tree was rooted to *Cladosporium cladosporioides* (GenBank DQ008145).

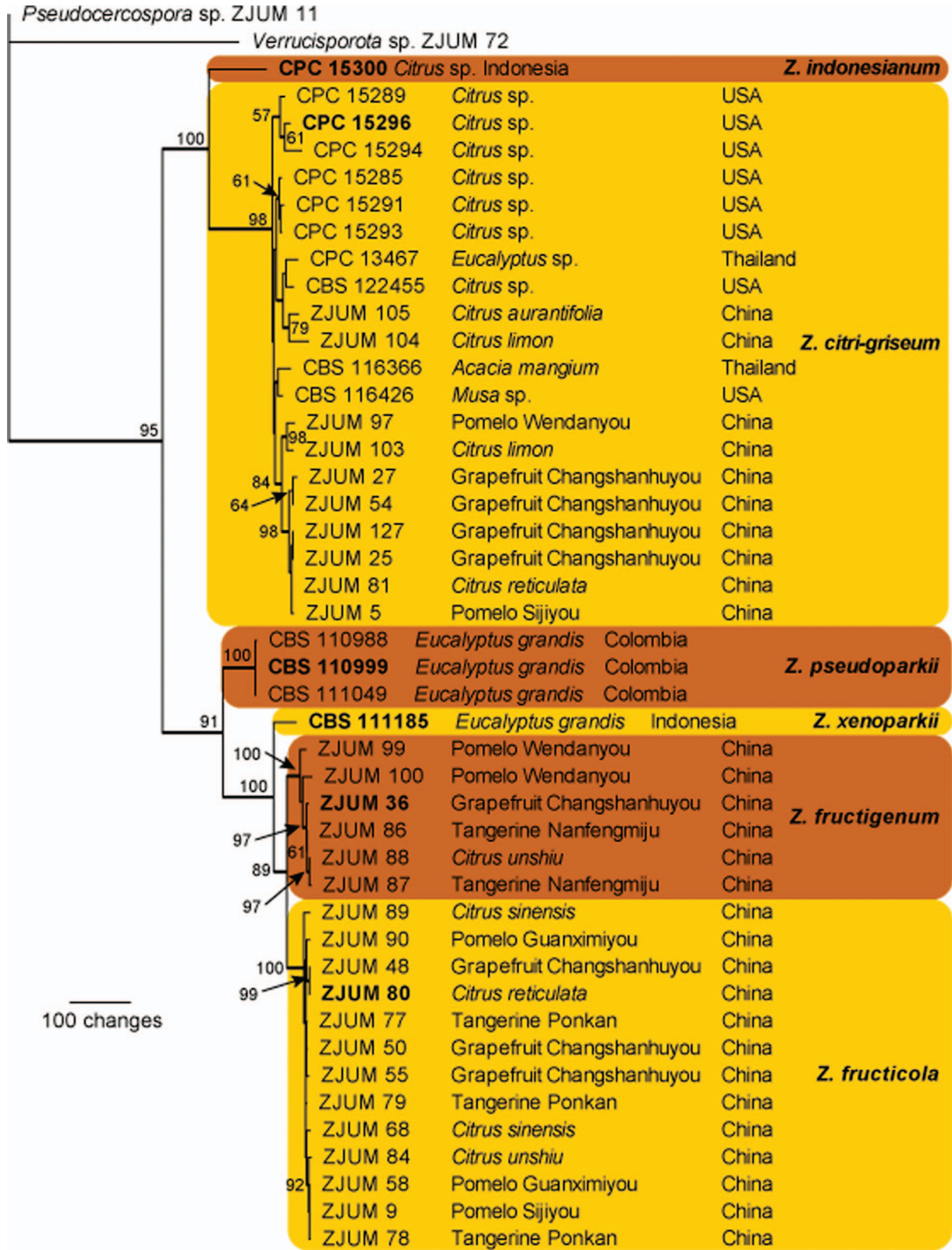


FIG. 3. The first of 1000 equally most parsimonious trees (TL = 1250, CI = 0.675, RI = 0.907, RC = 0.612) from a parsimony analysis of the combined ITS, *tef1*, 28S rDNA and *tub2* alignment representing species of *Zasmidium*. Bootstrap support values are indicated at the nodes, and the bar represents the number of changes. Thickened branches reflect those present in the strict consensus tree. Species are indicated with colored blocks, and ex-type cultures are in boldface text. The substrate and country of origin are next to culture collection number. The tree was rooted to *Pseudocercospora* sp. ZJUM 11 and *Verrucisporota* sp. ZJUM 72 (GenBank accession numbers listed in TABLE II).

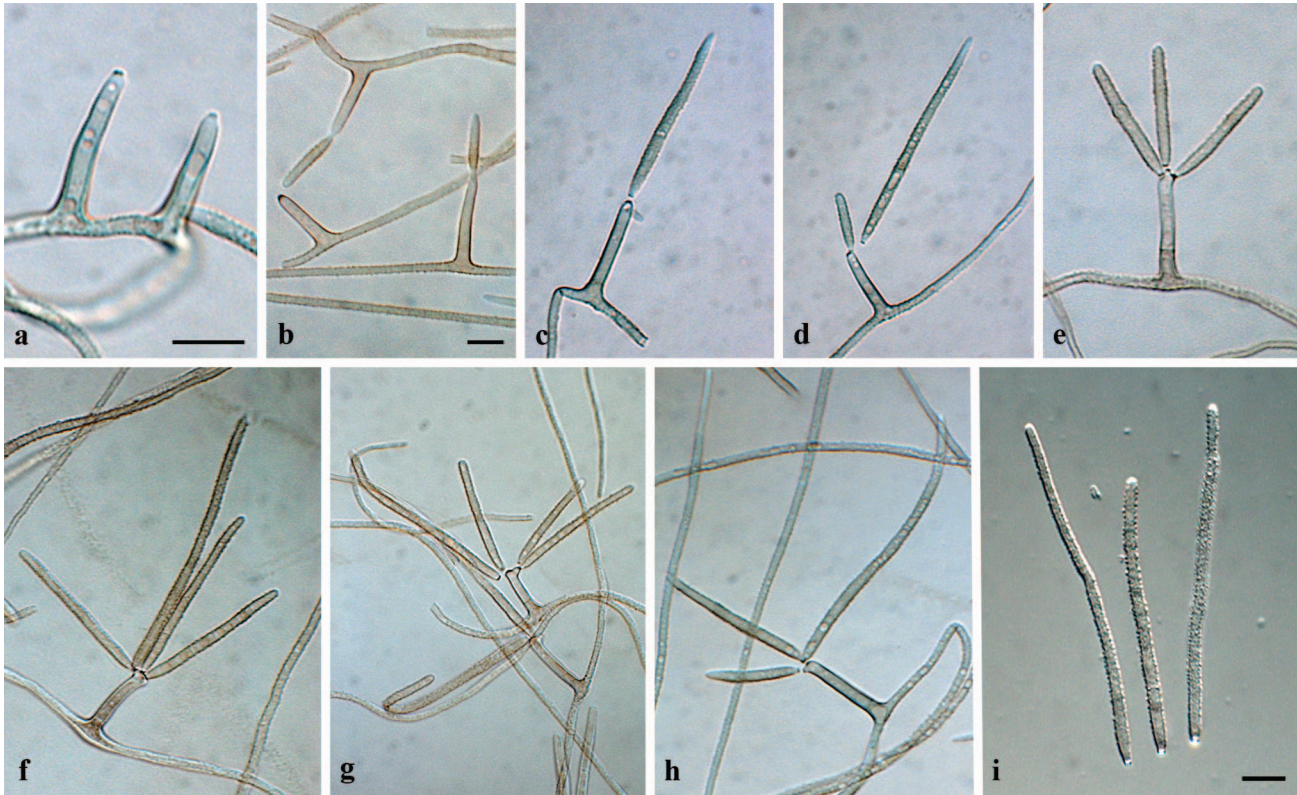


FIG. 4. *Zasmidium citri-griseum* (CPC 15296). a–i. Solitary conidiophores giving rise to chains of conidia on SNA. Bars = 10 μ m (b applies to c–h).

Zasmidium could be induced to sporulate. These taxa are treated below.

Zasmidium citri-griseum (F.E. Fisher) U. Braun & Crous, IMA Fungus 5:337. 2014. FIG. 4.

Basionym. *Cercospora grisea* F.E. Fisher, Phytopathology 51:300. 1961.

\equiv *Stenella citri-grisea* (F.E. Fisher) Sivan., Bitunicate Ascomycetes and their Anamorphs: 226. 1984.

$=$ *Mycosphaerella citri* Whiteside, Phytopathology 62:263. 1972.

\equiv *Zasmidium citri* (Whiteside) Crous, Persoonia 23:105. 2009.

Typification: USA, FLORIDA: Lake Alfred & Haines City, on *Citrus* sp., May 1970, F.E. Fisher (NEOTYPEK [M] IMI 148810); single ascospore isolates, associated with citrus greasy leaf spot disease symptoms, *Citrus* sp., 2003, S.N. Mondal (EPITYPE designated here CBS H-22176, MBT201426, culture ex-epitype CBS 139467 = CPC 15296).

Description: See Braun et al. (2014).

Additional materials examined: CHINA, YUNNAN PROVINCE: Mengdian, on leaves with yellow spot of *Citrus limon* (Lemon), Jul 2011, L. Zhu (cultures ZJUM 103 = CPC 24500, ZJUM 104 = CPC 24501); on leaf with yellow spot of *C. aurantifolia* (Key lime), Jul 2011,

L. Zhu (culture ZJUM 105 = CPC 24502); ZHEJIANG PROVINCE: Cangnan, on leaf with yellow spot of *C. grandis* (Pomelo Sijiyou), Dec 2009, L. Zhu (culture ZJUM 5 = CPC 24464); Changshan, on leaves of *C. paradisi* \times *Citrus* sp. (Grapefruit Changshanhuoyou), May 2009, L. Zhu (culture ZJUM 25 = CPC 24468, ZJUM 27 = CPC 24469); Nov 2011, L. Zhu (culture ZJUM 54 = CPC 24474); Huangyan, on leaf with big round spot of *C. reticulata* (Tangerine), Apr 2010, L. Zhu (culture ZJUM 81 = CPC 24488); Yuhuan, on leaf with greasy spot of *C. grandis* (Pomelo Wendanyou), Nov 2011, L. Zhu (culture ZJUM 97 = CPC 24497); Jiangshan, on leaf with brown small round spot of *C. paradisi* \times *Citrus* sp. (Grapefruit Changshanhuoyou), Apr 2013, F. Huang (culture ZJUM 127 = CPC 24504). — THAILAND, on living leaves of *Eucalyptus* sp., Jan 2006, W. Himaman (culture CPC 13467); Chonburi, on living leaves of seedlings of *Acacia mangium*, 19 Nov 2002, M.J. Wingfield (culture CPC 10522 = CBS 116366). — USA, FLORIDA: Polk County, Babson Park, on *Citrus limon*, 15 Jan 1958, F.E. Fisher (presumably lost); single ascospore isolates, associated with citrus greasy leaf spot disease symptoms, *Citrus* sp., 2003, S.N. Mondal (cultures CPC 15289, CPC 15290 = CBS 122455, CPC 15294, CPC 15285, CPC 15291,

CPC 15293); on leaves of *Musa* sp., 2003, *J. Cavaletto* (culture CBS 116426).

Host range and distribution: See Braun et al. (2014).

Notes: When Whiteside (1972) introduced the name *Mycosphaerella citri* he questioned the conspecificity with *Cercospora citri-grisea* (Fisher 1961). Fisher (1961) reported conidia to be 0–multiseptate, 25–200 × 1.5–3 µm, whereas Whiteside reported conidia as being 0–9-septate, 6–50 × 2–3.5 µm. In their examination of the neotype, Braun et al. (2014) reported conidia to be (0–)1–6(–10)-septate, 6–70(–120) × 2–4.5 µm, also questioning the synonymy between the Fisher and Whiteside taxa but choosing the older name, *Z. citri-griseum*.

Based on an examination of several specimens, Pretorius et al. (2003) regarded this as a single species with wider host range, while Crous et al. (2004) provided molecular data to show that this taxon also could occur on *Acacia mangium* (Thailand) and *Musa* sp. (USA). Furthermore, Arzanlou et al. (2008) added an isolate from *Musa* sp. (Tonga) while Maxwell et al. (2005) reported this species on *Eucalyptus camaldulensis* (Vietnam) and Crous et al. (2009b) on *Eucalyptus* sp. (Thailand).

In the present study we added several single-ascospore isolates from Florida in USA (Mondal et al. 2004) to the molecular analyses, which reveals conidia of this species to be morphologically variable, with those of the neotype being solitary or catenulate, 0–multiseptate, (6–)45–70(–120) × 2–4.5 µm. Isolate CPC 24500 from citrus in China had conidia that occurred in branched chains, 0–multiseptate, 20–200 × 2.5–3 µm. Isolate CBS 116366 from *Acacia* in Thailand had conidia that were 0–multiseptate, occurring mostly in unbranched chains, (15–)45–65(–120) × (2–)2.5–3 µm. Although there appears to be some morphological variation in *Z. citri-griseum*, the observed sexual nature of this taxon could account for this and we presently choose to retain it as a single taxon with a cosmopolitan distribution and wide host range until such time as more robust data is presented to prove otherwise. Furthermore, in this study we also found *Z. citri-griseum* to be associated with a range of disease symptoms, including greasy spot, yellow spot and small brown spot.

Zasmidium fructicola Crous, F. Huang & Hong Y. Li, sp. nov. FIG. 5

Mycobank MB811381

Typification: CHINA, ZHEJIANG PROVINCE: Huangyan, on fruit of *Citrus reticulata* (Tangerine), Jan 2010, X.H. Wang (HOLOTYPE CBS H-22177, culture ex-type ZJUM 80 = CPC 24487 = CBS 139625).

Etymology: Named after its occurrence on *Citrus* fruit.

Description: Colonies sporulating on PNA. *Mycelium* consisting of pale brown to brown, verruculose,

branched, septate, 1.5–2 µm diam hyphae. *Conidiphores* solitary, erect, subcylindrical, medium brown, straight to once geniculate, 0–2-septate, 15–25 × 2.5–3 µm. *Conidiogenous cells* integrated, terminal, medium brown, verruculose, subcylindrical, straight to once geniculate, 8–12 × 2.5–3 µm; loci sympodial, forming a short rachis with darkened, thickened, refractive, 0.5 µm diam scars. *Conidia* in branched chains, brown, verruculose, subcylindrical to fusoid-ellipsoid, guttulate; ramoconidia 0–1-septate, 10–15 × 3–4 µm; conidia aseptate, brown, verruculose, fusoid-ellipsoid, apex subobtuse, base obconically truncate, darkened, refractive, thickened, 0.5 µm diam, (5–)7–9(–12) × (2–)3 µm.

Culture characteristics: Colonies flat, spreading with sparse to moderate aerial mycelium and smooth, lobate margins, reaching 40 mm diam after 2 wk on OA and PDA; on OA surface iron gray (due to collapsed aerial mycelium); on PDA surface and reverse iron gray.

Additional materials examined: CHINA, ZHEJIANG PROVINCE: Huangyan, on fruit with citrus black spot of *C. unshiu* (Mandarin), Jan 2010, X.H. Wang (culture ZJUM 84 = CPC 24489); Cangnan, on fruit with greasy spot of *C. grandis* (Pomelo Sijiyou), Oct 2010, L. Zhu (culture ZJUM 9 = CPC 24465); Changshan, on fruit with yellow spot of *C. paradisi* × *Citrus* sp. (Grapefruit Changshanhuoyou), Nov 2010, L. Zhu (cultures ZJUM 48 = CPC 24472, ZJUM 50 = CPC 24473); on fruit with black dot of *C. paradisi* × *Citrus* sp. (Grapefruit Changshanhuoyou), Dec 2010, L. Zhu (culture ZJUM 55 = CPC 24475); Linhai, on fruit with black dot of *C. sinensis* (Orange), Nov 2010, G.Q. Chen (culture ZJUM 89 = CPC 24494); FUJIAN PROVINCE: on fruit with greasy spot of *C. grandis* (Pomelo Guanximiyou), Nov 2010, L. Zhu (culture ZJUM 58 = CPC 24477); Nanjing, on fruit with greasy spot of *C. grandis* (Pomelo Guanximiyou), Nov 2009, L. Zhu (culture ZJUM 90 = CPC 24495); GUANGDONG PROVINCE: Pingyuan, on fruit with citrus black spot of *C. sinensis* (Orange), Nov 2009, X.H. Wang (culture ZJUM 68 = CPC 24479); HUNAN PROVINCE: Jishou, on fruits of *C. reticulata* (Tangerine Ponkan), Nov 2011, X.H. Wang (cultures ZJUM 77 = CPC 24484, ZJUM 78 = CPC 24485, ZJUM 79 = CPC 24486).

Zasmidium fructigenum Crous, F. Huang & Hong Y. Li, sp. nov. FIG. 6

Mycobank MB811382

Typification: CHINA, ZHEJIANG PROVINCE: Changshan, on fruit with greasy spot of *Citrus paradise* × *Citrus* sp. (Grapefruit Changshanhuoyou), Nov 2009, L. Zhu (HOLOTYPE CBS H-22178, culture ex-type ZJUM 36 = CPC 24471 = CBS 139 626).

Etymology: Named after its occurrence on *Citrus* fruit.

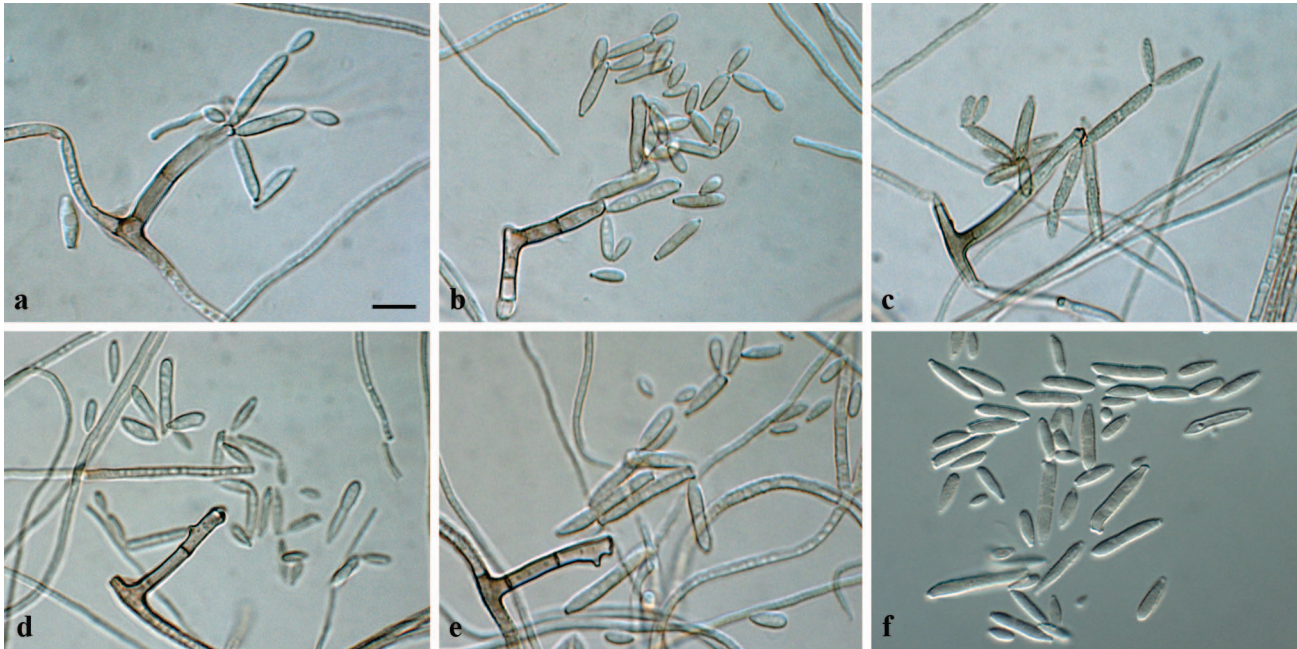


FIG. 5. *Zasmidium fructicola* (CPC 24487). a–f. Solitary conidiophores giving rise to chains of conidia on SNA. Bars = 10 μm (a applies to all photos).

Description: Colonies sporulating on PNA. Mycelium consisting of pale brown, verruculose, branched, septate, 1.5–2 μm diam hyphae. Conidiophores erect, subcylindrical, medium brown, straight, 1–2-septate, 15–25 \times 2.5–3 μm . Conidiogenous cells integrated, terminal, medium brown, verruculose, subcylindrical, 15–20 \times 2.5–3 μm ; loci sympodial, forming a short rachis with darkened, thickened, refractive, 0.5 μm diam scars. Conidia in branched chains, pale brown, verruculose, subcylindrical, guttulate; ramoconidia 0–1-septate, 10–22 \times 2 μm ; conidia 0–1-septate, subcylindrical to narrowly obclavate, apex subobtuse, base obconically truncate, darkened, refractive, thickened, 0.5 μm diam, (5–)8–12(–15) \times 2 μm .

Culture characteristics: Colonies flat, spreading with moderate aerial mycelium and smooth, lobate margins, reaching 40 mm diam after 2 wk on OA and PDA; on OA surface smoke gray in center, olivaceous gray in outer region; on PDA surface and reverse iron gray.

Additional materials examined: CHINA, ZHEJIANG PROVINCE: Yuhuan, on fruits with greasy spot of *C. grandis* (Pomelo Wendanyou), Nov 2010, L. Zhu (cultures ZJUM 99 = CPC 24498, ZJUM 100 = CPC 24499); Linhai, on fruit with black dot of *C. unshiu* (Mandarin), Nov 2010, G.Q. Chen (culture ZJUM 88 = CPC 24493); JIANGXI PROVINCE: on fruit with citrus black spot of *C. reticulata* (Tangerine Nanfengmiju), Nov 2010, X.H. Wang (cultures ZJUM 86 = CPC 24491, ZJUM 87 = CPC 24492).

Note: *Zasmidium fructicola* differs from *Z. fructigenum* by having conidiophores that are more irregular in appearance (geniculate) and conidia that are brown rather than pale brown, wider and more fusoid-ellipsoid.

Zasmidium indonesianum Crous, F. Huang & Hong Y. Li, sp. nov. FIG. 7

Mycobank MB811383

Typification: INDONESIA: on leaf spots of *Citrus* sp., 2004, M. Arzanlou (HOLOTYPE CBS H-22179, culture ex-type CPC 15300 = CBS 139627); additional collections from same host and locality (CPC 15301, CPC 15302).

Etymology: Named after the country from where it was collected, Indonesia.

Description: Colonies sporulating on PNA. Mycelium consisting of pale brown, verruculose, branched, septate, 1.5–2 μm diam hyphae. Conidiophores erect, subcylindrical, medium brown, straight, 0–2-septate, 15–25 \times 2.5–3 μm . Conidiogenous cells integrated, terminal, medium brown, verruculose, subcylindrical, 12–15 \times 2.5–3 μm ; loci sympodial, 1–3 at apex, with darkened, thickened, refractive, 0.5–1 μm diam scars. *Conidia* mostly solitary, pale brown, verruculose, guttulate, (0–)3–6(–multiseptate), subcylindrical to narrowly obclavate, apex subobtuse, base obconically truncate, darkened, refractive, thickened, 0.5 μm diam, (10–)25–45(–70) \times (2–)2.5–3 μm .

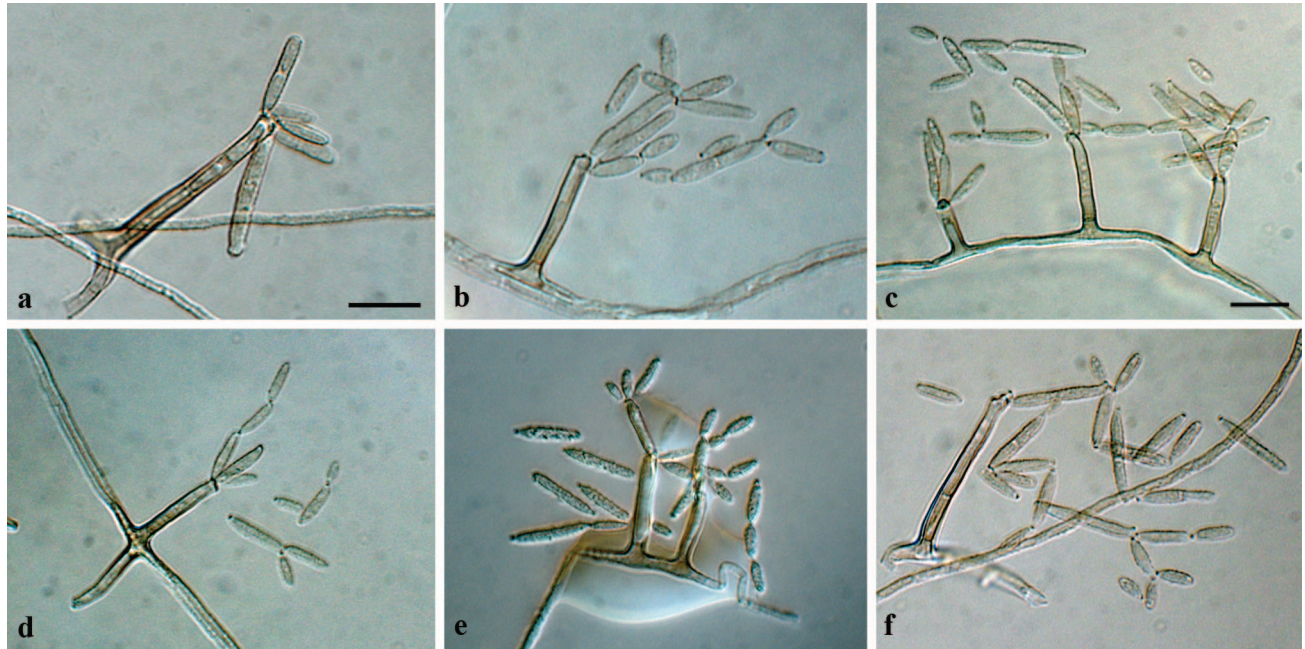


FIG. 6. *Zasmidium fructigenum* (CPC 24471). a–f. Solitary conidiophores giving rise to chains of conidia on SNA. Bars = 10 µm (a applies to b, c applies to d–f).

Culture characteristics: Colonies flat, spreading with moderate aerial mycelium and smooth, lobate margins, reaching 30 mm diam after 2 wk on MEA; on MEA surface olivaceous gray, reverse iron gray.

Notes: *Zasmidium indonesianum* differs from *Z. fructicola* and *Z. fructigenum* based on its larger conidia, being up to 70 µm long, mostly formed singly. *Zasmidium citri-griseum* differs from all three species by having wider scars and conidial hila and long, curved, multiseptate conidia, up to 120 µm long or sometimes even longer.

A KEY TO *ZASMIIDIUM* SPECIES ON *CITRUS*

- 1. Conidia short, generally shorter than 25 µm 2
- 1'. Conidia long, generally longer than 25 µm 3
- 2. Conidia in branched chains, pale brown, subcylindrical, 10–22 × 2 µm. *Z. fructigenum*
- 2'. Conidia in branched chains, brown, subcylindrical to fusoid-ellipsoid, 10–15 × 3–4 µm. *Z. fructicola*
- 3. Conidia mostly solitary, pale brown, (0–)3–6(–multi) septate, (10–)25–45(–70) × (2–)2.5–3 µm, up to 70 µm long. *Z. indonesianum*
- 3'. Conidia solitary or catenate, long, curved, multiseptate, up to 120 µm long or sometimes even longer. *Z. citri-griseum*

DISCUSSION

Because the recognition that *Mycosphaerella* represents a polyphyletic assemblage of taxa (Crous et al. 2007), the

taxonomy of fungi formerly accommodated under this name underwent significant changes and presently is thought to represent more than 40 different genera in the *Capnodiales* (Crous 2009, Crous et al. 2009a, Quaedvlieg et al. 2014). Although these genera are chiefly delineated based on their DNA sequence data, in most cases they also correlate with the distinct morphology of their asexual morphs (Crous et al. 2009b). Much of the research on this complex in recent years has been based on revising the mycosphaerella-like fungi associated with specific plant hosts like *Acacia* (Crous et al. 2004c), *Eucalyptus* (Crous et al. 2004b, Cheewangkoon et al. 2008), *Citrus* (Crous and Braun 2003, Pretorius et al. 2003), *Musa* (Arzanlou et al. 2008), *Proteaceae* (Crous et al. 2008) and *Myrtaceae* (Hunter et al. 2006, Perez et al. 2013). In the present study we aimed to determine which cercosporoid species occur on *Citrus*, focusing primarily on those isolated from various *Citrus* plantations in China.

Based on the phylogenetic analyses of the 28S rDNA sequence data, all strains from this study could be placed in *Cercospora*, *Pallidocercospora*, *Passalora*, *Pseudocercospora*, *Verrucisporota* and *Zasmidium*. In addition, four species of *Zasmidium* could be distinguished on *Citrus*, namely *Z. citri-griseum*, *Z. fructicola*, *Z. fructigenum* and *Z. indonesianum*.

Of these species *Z. citri-griseum* (= *Mycosphaerella citri*) is recognized as the causal agent of citrus greasy spot and has greatly affected the citrus industry worldwide (Fisher 1961, Braun et al. 2014). Based on our results, however, this pathogen is not only restricted

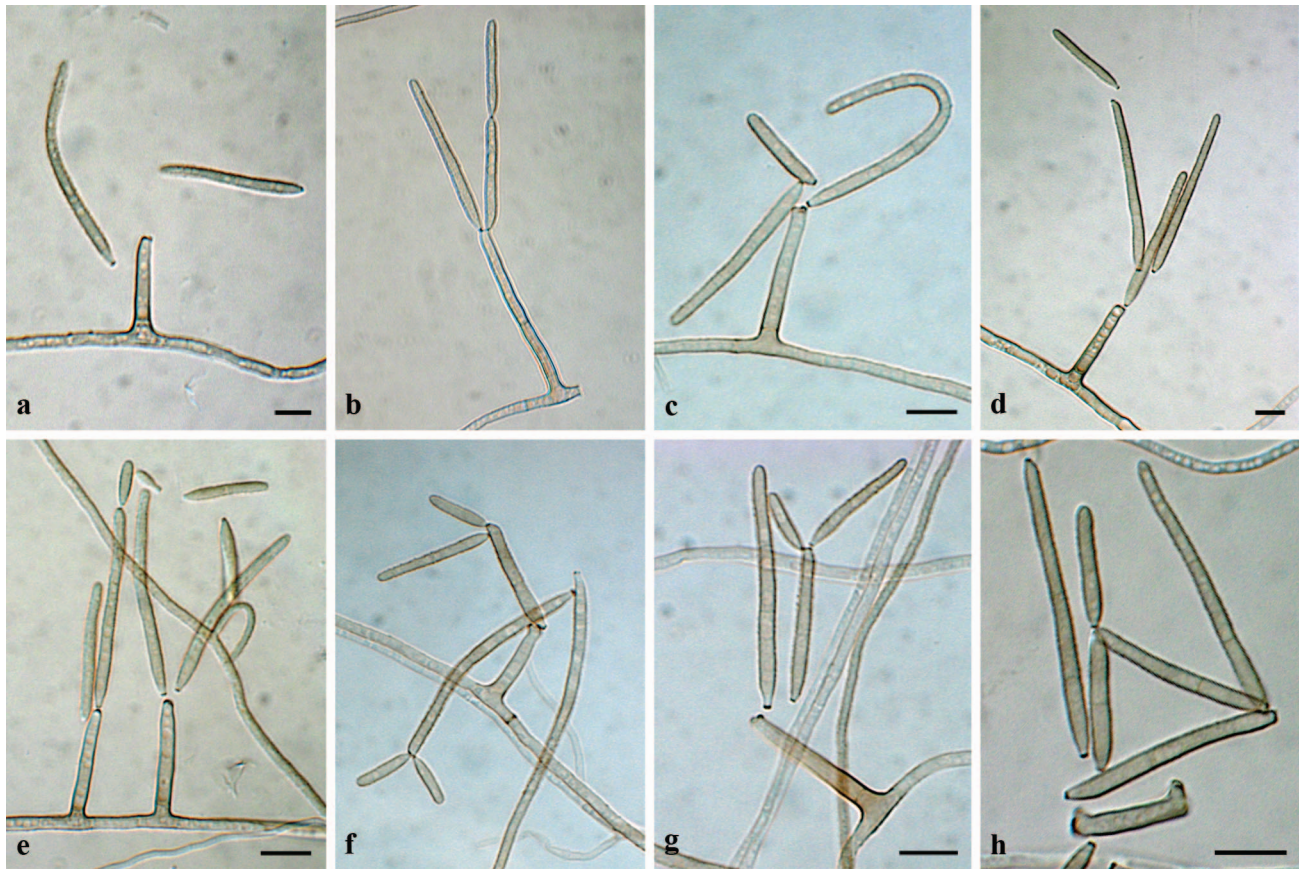


FIG. 7. *Zasmidium indonesianum* (CPC 15300). a–f. Solitary conidiophores giving rise to chains of conidia on SNA. Bars = 10 µm (a applies to b, e applies to f).

to *Citrus* but also occurs on other plant hosts such as *Eucalyptus* sp., *Acacia mangium* and *Musa* sp. (Crous et al. 2004c, Maxwell et al. 2005, Arzanlou et al. 2008, Crous et al. 2009b).

Other than *Z. citri-griseum*, *Z. fructicola* was represented by 13 isolates, which were isolated from citrus fruits exhibiting greasy spot and yellow spot symptoms but not small brown spot symptoms. *Zasmidium fructigenum* was represented by six isolates that also were obtained from citrus fruits exhibiting greasy spot symptoms but not yellow spot or small brown spot. *Zasmidium indonesianum* occurred on leaf spots of *Citrus* sp. and thus far is known only from this host in Indonesia.

The complexity of the disease symptomatology associated with multiple fungal species made it difficult to clearly link species to symptom types. Disease symptoms on a specific host might often be associated with and caused by several cercosporoid pathogens (Crous et al. 2004b, 2013; Groenewald et al. 2013). Furthermore, the symptoms of a specific disease, caused by the same agent, could vary depending on development period of disease, species or cultivars of the host,

maturational stage of the infected tissue, as well as the environment (Timmer and Gottwald 2000, Timmer et al. 2000).

In total 15 isolates were obtained from greasy spot symptoms, including two isolates of *Z. citri-griseum*, three of *Z. fructicola*, three of *Z. fructigenum*, three of *Pseudocercospora* sp. and four of *Pallidocercospora crystallina*, suggesting that this kind of symptom on *Citrus* is associated with multiple cercosporoid fungi. However, four of the 10 isolates obtained from yellow spot symptoms were *Z. citri-griseum*, as were one of the two isolates obtained from small brown spot symptoms. These results therefore suggest that the latter two disease symptoms are prone to be caused by infections of *Z. citri-griseum* and that this species is not solely associated with greasy spot disease symptoms.

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