DNA barcoding of *Mycosphaerella* species of quarantine importance to Europe

W. Quaedvlieg[1,2], J.Z. Groenewald[1], M. de Jesús Yáñez-Morales[3], P.W. Crous[1,2,4]

Key words

EPPO
Lecanosticta
Q-bank
QBOL

Abstract The EU 7th Framework Program provided funds for Quarantine Barcoding of Life (QBOL) to develop a quick, reliable and accurate DNA barcode-based diagnostic tool for selected species on the European and Mediterranean Plant Protection Organization (EPPO) A1/A2 quarantine lists. Seven nuclear genomic loci were evaluated to determine those best suited for identifying species of *Mycosphaerella* and/or its associated anamorphs. These genes included β-tubulin (Btub), internal transcribed spacer regions of the nrDNA operon (ITS), 28S nrDNA (LSU), Actin (Act), Calmodulin (Cal), Translation elongation factor 1-alpha (EF-1α) and RNA polymerase II second largest subunit (RPB2). Loci were tested on their Kimura-2-parameter-based inter- and intraspecific variation, PCR amplification success rate and ability to distinguish between quarantine species and closely related taxa. Results showed that none of these loci was solely suited as a reliable barcoding locus for the tested fungi. A combination of a primary and secondary barcoding locus was found to compensate for individual weaknesses and provide reliable identification. A combination of ITS with either EF-1α or Btub was reliable as barcoding loci for EPPO A1/A2-listed *Mycosphaerella* species. Furthermore, *Lecanosticta acicola* was shown to represent a species complex, revealing two novel species described here, namely *L. brevispora* sp. nov. on *Pinus* sp. from Mexico and *L. guatemalensis* sp. nov. on *Pinus oocarpa* from Guatemala. Epitypes were also designated for *L. acicola* and *L. longispora* to resolve the genetic application of these names.

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INTRODUCTION

In order to manage phytosanitary risks in an ever growing and increasingly dynamic import and export market, the EU 7th Framework Program funded the Quarantine Barcoding of Life project to develop a quick, reliable and accurate DNA barcode-based diagnostic tool for selected species on the EPPO A1/A2 lists and EU Council Directive 2000/29/EC (www.QBOL.org). There are currently almost 350 pest and quarantine organisms, covering bacteria, phytoplasmas, fungi, parasitic plants, insects and mites, nematodes, virus and virus-like organisms on the EPPO A1 (currently absent from the EPPO region) and A2 (locally present but controlled in the EPPO region) lists of organisms that require standardised protocols against introduction into, and spread within, the EPPO region. Under QBOL, informative loci from the selected quarantine species and their taxonomically related species were subjected to DNA barcoding from voucher specimens in order to produce reliable DNA barcode sequences that are made publicly available through an online and searchable database called Q-bank (www.q-bank.eu) (Bonants et al. 2010). Within the QBOL project, the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands), was tasked with barcoding the *Mycosphaerella* complex (order *Capnodiales*, class *Dothideomycetes*) on the EPPO A1/A2 lists and their taxonomically related closest sister species (Table 1).

A major problem with correctly identifying many of the EPPO A1/A2-listed fungi is the fact that individual species are often named for their particular morphs in separate publications. Dual nomenclature makes effective cooperation between scientists and the individual quarantine authorities very confused and complicated. The dual nomenclatural system was recently abandoned at the International Botanical Congress in Melbourne (Hawksworth et al. 2011, Wingfield et al. 2012). In accordance with this decision, the concept 'one fungus = one name' will be applied in this paper.

The *Mycosphaerella* generic complex comprises one of the largest families within the phylum *Ascomycota*, whose species have evolved as either endophytes, saprophytes and symbionts. Mostly, *Mycosphaerella* s.l. consists of foliicolous plant pathogens which are the cause of significant economical losses in both temperate and tropical crops worldwide (Crous et al. 2001). The *Mycosphaerella* teleomorph morphology is relatively conserved, but is linked to more than 30 anamorph genera (Crous 2009). Although originally assumed to be monophyletic (Crous et al. 2001), phylogenetic analyses of numerous *Mycosphaerella* species and their anamorphs by Hunter et al. (2006) and Crous et al. (2007) have shown that the *Mycosphaerella* complex is in fact polyphyletic. This has since led to taxonomic redistribution of most of the phylogenetic clades within the complex, although several clades remain unresolved due to limited sampling (Crous 2009, Crous et al. 2009a, c).

During the 2011 Fungal DNA Barcoding Workshop in Amsterdam, The Netherlands, it was decided that the internal transcribed spacers region (ITS) of the nrDNA operon was to become the official primary fungal barcoding gene (Schoch et al. 2012). The ITS locus is easily amplified and gives a good species resolution in many fungal groups. Lack of sufficient ITS interspecies variation within some genera of *Mycosphaerella*-like fungi (e.g. *Septoria*, *Cercospora* and *Pseudocercospora*)

[1] CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands;
corresponding author e-mail: w.quaedvlieg@cbs.knaw.nl.
[2] Microbiology, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.
[3] Colegio de Postgraduados, Km. 36.5 Carr, Mexico-Texcoco, Montecillo, Mpio. de Texcoco, Edo. de Mexico 56230, Mexico.
[4] Wageningen University and Research Centre (WUR), Laboratory of Phytopathology, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands.

Table 1 Isolates used during this study. Isolates marked with an asterisk (*) are type. Isolates marked in grey are quarantine species. Isolates used for the subset trees (Fig.1) are marked with a delta (Δ).

					GenBank Accession no[2]						
Species	Isolate no[1]	Host	Location	Collected by	ACT	CAL	EF-1α	Btub	RPB2	ITS	LSU
Cercosporella virgaureae	CBS 113304	*Erigeron annueus*	–	H.D. Shin	JX902067	JX901506	JX901618	JX902189	JX901944	GU214658	JX901820
Dothistroma pini	CBS 121011	*Pinus pallasiana*	Russia	A.C. Usichenko	JX902068	JX901507	JX901619	JX902190	JX901945	JX901734	JX901821
	CBS 116487	*Pinus nigra*	USA	G. Adams	JX902069	JX901508	JX901620	JX902191	JX901946	GU214532	JX901822
	CBS 116486	*Pinus nigra*	USA	G. Adams	JX902070	JX901509	JX901621	JX902192	JX901947	JX901735	JX901823
	CBS 116484	*Pinus nigra*	USA	G. Adams	JX902071	JX901510	JX901622	JX902193	JX901948	JX901736	JX901824
	CBS 116483	*Pinus nigra*	USA	G. Adams	JX902072	JX901511	JX901623	JX902194	JX901949	JX901737	JX901825
	CBS 117609	*Pinus pallasiana*	Russia	A.C. Usichenko	JX902073	JX901512	JX901624	JX902195	JX901950	JX901738	JX901826
	CBS 116485	*Pinus nigra*	USA	G. Adams	JX902074	JX901513	JX901625	JX902196	JX901951	JX901739	JX901827
	CBS 121005 Δ	*Pinus pallasiana*	Russia	T.S. Bulgakov	JX902075	JX901514	JX901626	JX902197	JX901952	JX901740	JX901828
D. septosporum	CBS 128782	*Pinus mugo*	The Netherlands	W. Quaedvlieg	JX902076	JX901515	JX901627	JX902198	JX901953	JX901741	JX901829
	CPC 16799	*Pinus mugo*	The Netherlands	W. Quaedvlieg	JX902077	JX901516	JX901628	JX902199	JX901954	JX901742	JX901830
	CBS 543.74	*Pinus pinaster*	Brazil	T. Namekata	JX902078	JX901517	JX901629	JX902200	JX901955	JX901743	JX901831
	CBS 383.74	*Pinus coulteri*	France	M. Morelet	JX902079	JX901518	JX901630	JX902201	JX901956	EU167578	JX901832
	CBS 112498 Δ	*Pinus radiata*	Ecuador	P.W. Crous	JX902080	JX901519	JX901631	JX902202	JX901957	JX901744	JX901833
Lecanosticta acicola	LNPV241	*Pinus radiata*	France	P. Chandelier	JX902081	JX901520	JX901632	JX902203	JX901958	JX901745	JX901834
	LNPV242	*Pinus muricata*	France	P. Chandelier	JX902082	JX901521	JX901633	JX902204	JX901959	JX901746	JX901835
	WPF4.12 *	*Pinus strobum*	USA	B. Ostrofsky	KC013004	KC013010	KC013001	KC013007	KC013013	KC012998	KC013016
	CBS 133791 = WPF13.12	*Pinus strobum*	USA	B. Ostrofsky	KC013005	KC013011	KC013002	KC013008	KC013014	KC012999	KC013017
	WPF73.12	*Pinus strobum*	USA	J. Weiner	KC013006	KC013012	KC013003	KC013009	KC013015	KC013000	KC013018
	LNPV244	*P. attenuata* × *radiata*	France	P. Chandelier	JX902083	JX901522	–	JX902205	JX901960	JX901747	JX901836
	LNPV245	*P. attenuata* × *radiata*	France	P. Chandelier	JX902084	JX901523	–	JX902206	JX901961	JX901748	JX901837
	LNPV246	*P. attenuata* × *radiata*	France	P. Chandelier	JX902085	JX901524	JX901634	JX902207	JX901962	JX901749	JX901838
	LNPV247	*P. radiata*	France	P. Chandelier	JX902086	JX901525	JX901635	JX902208	JX901963	JX901750	JX901839
	LNPV248	*P. attenuata* × *radiata*	France	P. Chandelier	JX902087	JX901526	JX901636	JX902209	JX901964	JX901751	JX901840
	LNPV249	*P. attenuata* × *radiata*	France	P. Chandelier	JX902088	JX901527	JX901637	JX902210	JX901965	JX901752	JX901841
	LNPV250	*Pinus sp.*	France	P. Chandelier	JX902089	JX901528	JX901638	JX902211	JX901966	JX901753	JX901842
	LNPV251	*P. attenuata* × *radiata*	France	P. Chandelier	JX902090	JX901529	–	JX902212	JX901967	JX901754	JX901843
	LNPV252	*P. attenuata* × *radiata*	France	P. Chandelier	JX902091	JX901530	JX901639	JX902213	JX901968	JX901755	JX901844
	LNPV253	*P. palustris*	USA	C. Affeltranger	JX902092	JX901531	JX901640	JX902214	JX901969	JX901756	JX901845
	LNPV254	*Pinus* sp.	France	P. Chandelier	JX902093	JX901532	JX901641	JX902215	JX901970	JX901757	JX901846
	LNPV255	*Pinus* sp.	France	P. Chandelier	JX902094	JX901533	JX901642	JX902216	JX901971	JX901758	JX901847
	LNPV256	*Pinus* sp.	France	P. Chandelier	JX902095	JX901534	JX901643	JX902217	JX901972	JX901759	JX901848
	LNPV257	*Pinus radiata*	France	P. Chandelier	JX902096	JX901535	JX901644	JX902218	JX901973	JX901760	JX901849
	CBS 133790 = LA773A	*Pinus mugo*	Lithuania	S. Markovskaja, A. Kačergius & A. Treigienė	JX902097	JX901536	JX901645	JX902219	JX901974	HM367708	JX901850
	LA773B	*Pinus mugo*	Lithuania	S. Markovskaja, A. Kačergius & A. Treigienė	JX902098	JX901537	JX901646	JX902220	JX901975	HM367707	JX901851
	LNPV243 Δ	*P. pinaster*	France	P. Chandelier	JX902099	JX901538	JX901647	JX902221	JX901976	JX901761	JX901852
	CBS 871.95 Δ	*Pinus radiata*	France	M. Morelet	JX902100	JX901539	–	JX902222	JX901977	GU214663	JX901853
	CBS 133789 = CPC 17822 Δ	*Pinus* sp.	Mexico	J.Y. Morales	JX902101	JX901540	JX901648	JX902223	JX901978	JX901762	JX901854
L. brevispora	CBS 133601 = CPC 18092 * Δ	*Pinus* sp.	Mexico	J.Y. Morales	JX902102	JX901541	JX901649	JX902224	JX901979	JX901763	JX901855
L. guatamalensis	IMI 281598 * Δ	*Pinus oocarpa*	Guatemala	H.C. Evans	JX902103	JX901542	JX901650	JX902225	JX901980	JX901764	JX901856
L. longispora	CPC 17940 Δ	*Pinus* sp.	Mexico	J.Y. Morales	JX902104	JX901543	JX901652	JX902226	JX901981	JX901765	JX901857
	CBS 133602 = CPC 17941 * Δ	*Pinus* sp.	Mexico	J.Y. Morales	JX902105	JX901544	JX901651	JX902227	JX901982	JX901766	JX901858
Mycosphaerella ellipsoidea	CBS 110843 * Δ	*Eucalyptus cladocalyx*	South Africa	P.W. Crous	JX902106	JX901545	JX901653	JX902228	JX901983	AY725545	JX901859
M. endophytica	CBS 114662 *	*Eucalyptus* sp.	South Africa	P.W. Crous	JX902107	JX901546	JX901654	JX902229	JX901984	DQ302953	JX901860
	CBS 111519 *	*Eucalyptus* sp.	South Africa	P.W. Crous	JX902108	JX901547	JX901655	JX902230	JX901985	DQ302952	JX901861
M. laricis-leptolepidis	MAFF 410081	*Larix leptolepis*	Japan	K. Ito	JX902109	JX901548	JX901656	JX902231	JX901986	JX901767	JX901862
	MAFF 410632	*Larix leptolepis*	Japan	T. Yokota	JX902110	JX901549	JX901657	JX902232	JX901987	JX901768	JX901863
	MAFF 410633	*Larix leptolepis*	Japan	T. Yokota	JX902111	JX901550	JX901658	JX902233	JX901988	JX901769	JX901864
	MAFF 410234 Δ	*Larix leptolepis*	Japan	N. Ota	JX902112	JX901551	JX901659	JX902234	JX901989	JX901770	JX901865

M. latebrosa	CBS 687.94	*Acer pseudoplatanus*	The Netherlands	G. Verkley	JX902113	JX901552	JX901660	JX902235	JX901990	JX901771	JX901866
	CBS 183.97	*Acer pseudoplatanus*	The Netherlands	H.A. van der Aa	JX902114	JX901553	JX901661	JX902236	JX901991	AF362051	JX901867
	CBS 652.85	*Acer pseudoplatanus*	The Netherlands	H.A. van der Aa	JX902115	JX901554	JX901662	JX902237	JX901992	AF362067	JX901868
M. populicola	CBS 100042 Δ	*Populus trichocarpa*	USA	G. Newcombe	JX902116	JX901555	JX901663	JX902238	–	JX901772	JX901869
Mycosphaerella sp.	CBS 111166	*Eucalyptus cladocalyx*	South Africa	P.W. Crous	JX902117	JX901556	JX901664	JX902239	JX901993	JX901773	JX901870
	CBS 110501 Δ	*Eucalyptus globulus*	Australia	A. Maxwell	JX902118	JX901557	JX901665	JX902240	JX901994	EU167580	JX901871
M. sumatrensis	CBS 118501	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	JX902119	JX901558	–	JX902241	JX901995	JX901774	JX901872
	CBS 118502	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	JX902120	JX901559	–	JX902242	JX901996	JX901775	JX901873
	CBS 118499 * Δ	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	JX902121	JX901560	–	JX902243	JX901997	JX901776	JX901874
Phaeophleospora eugeniae	CPC 15143	*Eugenia uniflora*	Brazil	Alfenas	JX902122	JX901561	JX901666	JX902244	JX901998	FJ493188	JX901875
	CPC 15159 Δ	*Eugenia uniflora*	Brazil	Alfenas	JX902123	JX901562	JX901667	JX902245	JX901999	FJ493189	JX901876
Pseudocercospora angolensis	CBS 244.94	*Citrus* sp.	Zimbabwe	P.W. Crous	JX902124	JX901563	JX901668	JX902246	JX902000	JX901777	JX901877
	CBS 112748	*Citrus* sp.	Zimbabwe	P.W. Crous	JX902125	JX901564	JX901669	JX902247	JX902001	JX901778	JX901878
	CBS 112933	*Citrus* sp.	Zimbabwe	M.C. Pretorius	JX902126	JX901565	JX901670	JX902248	JX902002	AY260063	JX901879
	CBS 115645	*Citrus* sp.	Zimbabwe	P.W. Crous	JX902127	JX901566	JX901671	JX902249	JX902003	JX901779	JX901880
	CBS 149.53 * Δ	*Citrus sinensis*	Angola	T. de Carvalho & O. Mendes	JX902128	JX901567	JX901672	JX902250	JX902004	JQ324941	JX901881
P. assamensis	CBS 122467	*Musa* sp.	India	I.W. Buddenhagen	JX902129	JX901568	JX901673	JX902251	JX902005	EU514281	JX901882
P. atromarginalis	CPC 11372	*Solanun nigrum*	Republic of Korea	H.D. Shin	JX902130	–	JX901674	JX902252	JX902006	JX901780	JX901883
P. cercidis-chinensis	CPC 14481	*Cercis chinensis*	Republic of Korea	H.D. Shin	JX902131	–	JX901675	JX902253	JX902007	DQ267602	JX901884
P. chiangmaiensis	CBS 123244 *	*Eucalyptus camaldurensis*	Thailand	R. Cheewangkoon	JX902132	–	JX901676	JX902254	JX902008	JX901781	JX901885
P. clematidis	CPC 11657 Δ	*Clematis* sp.	USA	M. Palm	JX902133	JX901569	JX901677	JX902255	JX902009	DQ303072	JX901886
P. flavomarginata	CBS 118824 *	*Eucalyptus camaldulensis*	China	M.J. Wingfield	JX902134	–	JX901678	JX902256	JX902010	JX901782	JX901887
P. gracilis	CPC 11144	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	JX902135	JX901570	JX901679	JX902257	JX902011	DQ302961	JX901888
	CPC 11181	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	JX902136	JX901571	JX901680	JX902258	JX902012	DQ302962	JX901889
	CBS 111189	*Eucalyptus urophylla*	–	M.J. Wingfield	JX902137	JX901572	JX901681	JX902259	JX902013	DQ302960	JX901890
P. humuli-japonici	CPC 11315	*Humulus japonicus*	Republic of Korea	H.D. Shin	JX902138	JX901573	JX901682	JX902260	JX902014	JX901783	JX901891
	CPC 11462	*Plectranthus*	Republic of Korea	H.D. Shin	JX902139	JX901574	JX901682	JX902261	JX902015	JX901784	JX901892
P. madagascariensis	CBS 124155 *	*Eucalyptus camaldulensis*	Madagascar	M.J. Wingfield	JX902140	–	JX901683	JX902262	JX902016	FJ790268	JX901893
P. norchiensis	CBS 120738 *	*Eucalyptus* sp.	Italy	W. Gams	JX902141	JX901575	JX901684	JX902263	JX902017	JX901785	JX901894
P. paraguayensis	CBS 111286 Δ	*Eucalyptus nitens*	Brazil	P.W. Crous	JX902142	–	JX901685	JX902264	JX902018	DQ267602	JX901895
P. pini-densiflorae	CBS 125139	*Pinus thunbergii*	Japan	Sung-Oui Suh	JX902143	–	JX901686	JX902265	JX902019	JX901786	JX901896
	CBS 125140	*Pinus kesiya*	Japan	Sung-Oui Suh	JX902144	–	JX901687	JX902266	JX902020	JX901787	JX901897
	CBS 125138 Δ	*Pinus* sp.	Japan	Sung-Oui Suh	JX902145	–	JX901688	JX902267	JX902021	JX901788	JX901898
P. pseudoeucalyptorum	CPC 12802	*Eucalyptus globulus*	Portugal	A. Phillips	JX902146	JX901576	JX901689	JX902268	JX902022	GQ852759	JX901899
	CPC 13769	*Eucalyptus punctata*	South Africa	P.W. Crous	JX902147	JX901577	JX901690	JX902269	JX902023	GQ852762	JX901900
	CPC 12568	*Eucalyptus nitens*	Tasmania	C. Mohammed	JX902148	JX901578	JX901691	JX902270	JX902024	GQ852758	JX901901
P. pyracanthigena	CPC 10808 Δ	*Pyracantha angustifolia*	Republic of Korea	H.D. Shin	JX902149	–	JX901692	JX902271	JX902025	JX901789	JX901902
P. rhoina	CPC 11464	*Rhus chinensis*	Republic of Korea	H.D. Shin	–	–	JX901693	JX902272	JX902026	JX901790	JX901903
P. robusta	CBS 111175 * Δ	*Eucalyptus robur*	Malaysia	M.J. Wingfield	JX902150	JX901579	JX901694	JX902273	JX902027	DQ303081	JX901904
P. schizolobii	CBS 124990	*Eucalyptus camaldulensis*	Thailand	W. Himaman	JX902151	–	JX901695	JX902274	JX902028	GQ852765	JX901905
P. sphaerulinae	CBS 112621 *	*Eucalyptus* sp.	–	P.W. Crous	–	JX901580	JX901696	JX902275	JX902029	JX901791	JX901906
P. subulata	CBS 118489	*Eucalyptus botryoides*	New Zealand	M. Dick	JX902152	JX901581	JX901697	JX902276	JX902030	JX901792	JX901907
P. tereticornis	CPC 13008	*Eucalyptus tereticornus*	Australia	A.J. Carnegie	JX902153	JX901582	JX901698	JX902277	JX902031	GQ852769	JX901908
	CPC 13315	*Eucalyptus tereticornus*	Australia	P.W. Crous	JX902154	JX901583	JX901699	JX902278	JX902032	GQ852771	JX901909
	CBS 124996 Δ	*Eucalyptus nitens*	Australia	A.J. Carnegie	JX902155	JX901584	JX901700	JX902279	JX902033	GQ852768	JX901910
	CPC 13299 *	*Eucalyptus tereticornus*	Australia	A.J. Carnegie	JX902156	JX901585	JX901701	JX902280	JX902034	GQ852770	JX901911
P. vitis	CPC 11595	*Vitis vinifera*	Republic of Korea	H.D. Shin	JX902157	JX901586	JX901702	JX902281	JX902035	DQ073923	JX901912
Septoria abeliceae	CBS 128591	*Zelkova serrata*	Republic of Korea	S.B. Hong	JX902158	JX901587	JX901703	JX902282	JX902036	JX901793	JX901913
S. cf. chrysanthemella	CBS 483.63	*Chrysanthemum* sp.	The Netherlands	H.A. van der Aa	JX902159	JX901588	JX901704	JX902283	JX902037	JX901794	JX901914
	CBS 351.58	*Chrysanthemum indicum*	Germany	R. Schneider	JX902160	JX901589	JX901705	JX902284	JX902038	JX901795	JX901915
S. citri	CBS 315.37 Δ	–	–	L.L. Huillier	JX902161	JX901590	JX901706	JX902285	JX902039	DQ897650	JX901916
S. cucurbitacearum	CBS 178.77 Δ	*Cucurbita maxima*	–	H.J. Boesewinkel	JX902162	JX901591	JX901707	JX902286	JX902040	JX901796	JX901917
S. lycopersici	CBS 353.49	–	–	S.P. Doolittle	JX902163	JX901592	JX901708	JX902287	JX902041	DQ841155	JX901918
	CBS 128654 Δ	*Lycopersicon esculentum*	Republic of Korea	S.B. Hong	JX902164	JX901593	JX901709	JX902288	JX902042	JX901797	JX901919
S. malagutii	CBS 106.80 Δ	*Solanum* sp.	Peru	L.J. Turkensteen	JX902165	JX901594	JX901710	JX902289	JX902043	DQ841154	JX901920
S. matricariae	CBS 109000	*Matricaria discoidea*	The Netherlands	G. Verkley	JX902166	JX901595	JX901711	JX902290	JX902044	JX901798	JX901921
	CBS 109001	*Matricaria discoidea*	The Netherlands	G. Verkley	JX902167	JX901596	JX901712	JX902291	JX902045	JX901799	JX901922

Table 1 (cont.)

Species	Isolate no[1]	Host	Location	Collected by	GenBank Accession no[2]							
					ACT	CAL	EF-1α	Btub	RPB2	ITS	LSU
S. musiva	CBS 130559	Hybrid poplar	Canada	J. LeBoldus	JX902168	JX901597	JX901713	JX902292	JX902046	JX901800	JX901923
	D7L2 #	*P. deltoides* × *P. balsamifera*	Canada	J. LeBoldus	JX902169	JX901598	JX901714	JX902293	JX902047	JX901801	JX901924
	CBS 130560	Hybrid poplar	Canada	J. LeBoldus	JX902170	JX901599	JX901715	JX902294	JX902048	JX901802	JX901925
	CBS 130561	*P. deltoides* × *P. balsamifera*	Canada	J. LeBoldus	JX902171	JX901600	JX901716	JX902295	JX902049	JX901803	JX901926
	CBS 130562	Hybrid poplar	Canada	J. LeBoldus	JX902172	JX901601	JX901717	JX902296	JX902050	JX901804	JX901927
	CBS 130563	*P. deltoides* × *P. balsamifera*	Canada	J. LeBoldus	JX902173	JX901602	JX901718	JX902297	JX902051	JX901805	JX901928
	CBS 130564	*Populus deltoides*	Canada	J. LeBoldus	JX902174	JX901603	JX901719	JX902298	JX902052	JX901806	JX901929
	CBS 130565	*Populus deltoides*	Canada	J. LeBoldus	JX902175	JX901604	JX901720	JX902299	JX902053	JX901807	JX901930
	CBS 130566	*Populus deltoides*	Canada	J. LeBoldus	JX902176	JX901605	JX901721	JX902300	JX902054	JX901808	JX901931
	CBS 130567	*Populus deltoides*	Canada	J. LeBoldus	JX902177	JX901606	JX901722	JX902301	JX902055	JX901809	JX901932
	CBS 130568	*Populus deltoides*	Canada	J. LeBoldus	JX902178	JX901607	JX901723	JX902302	JX902056	JX901810	JX901933
	CBS 130569	*Populus deltoides*	Canada	J. LeBoldus	JX902179	JX901608	JX901724	JX902303	JX902057	JX901811	JX901934
	CBS 130570	*Populus deltoides*	Canada	J. LeBoldus	JX902180	JX901609	JX901725	JX902304	JX902058	JX901812	JX901935
	CBS 130571	*Populus deltoides*	Canada	J. LeBoldus	JX902181	JX901610	JX901726	JX902305	JX902059	JX901813	JX901936
	CBS 130558 Δ	*P. deltoides* × *P. balsamifera*	Canada	J. LeBoldus	JX902182	JX901611	JX901727	JX902306	JX902060	JX901814	JX901937
S. obesa	CBS 354.58	*Chrysanthemum indicum*	Germany	R. Schneider	JX902183	JX901612	JX901728	JX902307	JX902061	AY489285	JX901938
	CBS 128759	*Chrysanthemum morifolium*	Republic of Korea	S.B. Hong	JX902184	JX901613	JX901729	JX902308	JX902062	JX901815	JX901939
	CBS 128623	*Chrysanthemum indicum*	Republic of Korea	H.D. Shin	JX902185	JX901614	JX901730	JX902309	JX902063	JX901816	JX901940
	CBS 128588	*Artemisia lavandulaefolia*	Republic of Korea	S.B. Hong	JX902186	JX901615	JX901731	JX902310	JX902064	JX901817	JX901941
S. populi	CBS 391.59 Δ	*Populus pyramidalis*	Germany	R. Schneider	JX902187	JX901616	JX901732	JX902311	JX902065	JX901818	JX901942
Teratosphaeria nubilosa	CPC 12243 Δ	*Eucalyptus globulus*	Portugal	A.J.L. Phillips	JX902188	JX901617	JX901733	JX902312	JX902066	JX901819	JX901943

[1] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Pedro Crous working collection housed at CBS, LNPV: laboratoire national de la protection des végétaux, Angers, France. MAFF:Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan.

[2] ACT = Actin, Btub = β-tubulin, CAL = Calmodulin, LSU = 28S large subunit of the nrRNA gene, RPB2= RNA polymerase II second largest subunit, ITS= Internal transcribed spacer and EF-1α = Translation elongation factor 1-alpha. # isolate provided by J. LeBoldus.

might make this locus less than ideal for resolving some anamorph genera or cryptic species complexes within these genera (Verkley et al. 2004, Hunter et al. 2006, Schoch et al. 2012). To compensate for this perceived lack of resolution within the ITS locus of *Mycosphaerella*-like species, seven loci were screened, which have individually or in combination been used in the past to successfully identify *Mycosphaerella*-like species. These include β-tubulin (Btub) (Feau et al. (2006)), internal transcribed spacer (ITS), Actin (Act) (Schubert et al. 2007, Crous et al. In press), Translation elongation factor 1-alpha (EF-1α) (Schubert et al. 2007, Crous et al. In press) and 28S nrDNA (LSU) (Hunter et al. 2006), Calmodulin (Cal) (Groenewald et al. 2005) and RNA polymerase II second largest subunit (RPB2) (Quaedvlieg et al. (2011)).

The aims of this study were to 1) identify the closest neighbours of seven *Mycosphaerella*-like species of quarantine importance using sequences of both the internal transcribed spacer regions and 5.8S nrRNA gene of the nrDNA operon (ITS). These isolates were then 2) screened with the seven previously mentioned test loci to determine the most optimal DNA barcode region(s) based on PCR efficiency, the size of the K2P barcode gaps and the molecular phylogenetic resolution of the individual loci. Based on the obtained results and existing literature, 3) the taxonomic status of these quarantine species was then revised employing the one fungus one name principle as stated by Hawksworth et al. (2011).

MATERIALS AND METHODS

Isolates and morphology

Most of the DNA used during this study were isolated from pure cultures that were either available at, or were made available to, the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS). Reference strains were either maintained in the culture collection of CBS, the Ministry of Agriculture, Forestry and Fisheries of Japan culture collection (MAFF) and/or at the LNPV – Mycologie, Malzéville, France (LNPV) (Table 1). Fresh collections were made from leaves of diverse hosts by placing material in damp chambers for 1–2 d. Single conidial colonies were established from sporulating conidiomata on Petri dishes containing 2 % malt extract agar (MEA) as described earlier by Crous et al. (1991). Colonies were sub-cultured onto potato-dextrose agar (PDA), oatmeal agar (OA), MEA (Crous et al. 2009b), and pine needle agar (PNA) (Lewis 1998), and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation. Morphological descriptions are based on slide preparations mounted in clear lactic acid from colonies sporulating on PNA. Observations were made with a Zeiss V20 Discovery stereo-microscope, and with a Zeiss Axio Imager 2 light microscope using differential interference contrast (DIC) illumination and an AxioCam MRc5 camera and software. Colony characters and pigment production were noted after 1 mo of growth on MEA, PDA and OA (Crous et al. 2009b) incubated at 25 °C. Colony colours (surface and reverse) were rated according to the colour charts of Rayner (1970). Sequences derived in this study were lodged with GenBank, the alignments in TreeBASE (www.treebase.org), and taxonomic novelties in MycoBank (www.MycoBank.org) (Crous et al. 2004a).

Multi-locus DNA screening

Genomic DNA was extracted from mycelium growing on MEA (Table 1), using the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). These strains were screened for seven loci (ITS, LSU, Act, Cal, EF-1α, RPB2 and Btub) using the primer sets and conditions listed in Table 2. The PCR amplifications were performed in a total volume of 12.5 µL solution containing 10–20 ng of template DNA,

Table 2 Primers used in this study for generic amplification and sequencing.

Locus	Primer	Primer sequence 5' to 3':	Annealing temperature (°C)	Orientation	Reference
Actin	ACT-512F	ATGTGCAAGGCCGGTTTCGC	52	Forward	Carbone & Kohn (1999)
Actin	ACT2Rd	ARRTCRCGDCCRGCCATGTC	52	Reverse	Groenewald et al. (In press)
Calmodulin	CAL-235F	TTCAAGGAGGCCTTCTCCCTCTT	50	Forward	Present study
Calmodulin	CAL2Rd	TGRTCNGCCTCDCGGATCATCTC	50	Reverse	Groenewald et al. (In press)
Translation elongation factor-1α	EF1-728F	CAT CGA GAA GTT CGA GAA GG	52	Forward	Carbone & Kohn (1999)
Translation elongation factor-1α	EF-2	GGA RGT ACC AGT SAT CAT GTT	52	Reverse	O'Donnell et al. (1998)
β-tubulin	T1	AACATGCGTGAGATTGTAAGT	52	Forward	O'Donnell & Cigelnik (1997)
β-tubulin	β-Sandy-R	GCRCGNGGVACRTACTTGTT	52	Reverse	Stukenbrock et al. (2012)
RNA polymerase II second largest subunit	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	49	Forward	Liu et al. (1999)
RNA polymerase II second largest subunit	fRPB2-414R	ACMANNCCCCARTGNGWRTTRTG	49	Reverse	Quaedvlieg et al. (2011)
LSU	LSU1Fd	GRATCAGGTAGGRATACCCG	52	Forward	Crous et al. (2009a)
LSU	LR5	TCCTGAGGGAAACTTCG	52	Reverse	Vilgalys & Hester (1990)
ITS	ITS1	GAAGTAAAAGTCGTAACAAGG	52	Forward	White et al. (1990)
ITS	ITS4	TCC TCC GCT TAT TGA TAT GC	52	Reverse	White et al. (1990)

Table 3 Amplification success, phylogenetic data and the substitution models used in the phylogenetic analysis, per locus.

Locus	Act	Cal	EF1	RPB2	Btub	ITS	LSU
Amplification succes (%)	98	90	97	99	100	100	100
Q-amplification succes (%)	100	86	100	100	100	100	100
Number of characters	615	385	800	337	430	658	751
Unique site patterns	235	228	551	165	290	214	120
Sampled trees	198	686	716	148	238	728	406
Number of generations (×1000)	150	642	857	123	168	433	272
Substitution model used	GTR-I-gamma	HKY-I-gamma	HKY-I-gamma	GTR-I-gamma	HKY-I-gamma	GTR-I-gamma	GTR-I-gamma

1 × PCR buffer, 0.7 µL DMSO (99.9 %), 2 mM $MgCl_2$, 0.4 µM of each primer, 25 µM of each dNTP and 1.0 U BioTaq DNA polymerase (Bioline GmbH, Luckenwalde, Germany). PCR amplification conditions were set as follows: an initial denaturation temperature of 96 °C for 2 min, followed by 40 cycles of denaturation temperature of 96 °C for 45 s, primer annealing at the temperature stipulated in Table 3, primer extension at 72 °C for 90 s and a final extension step at 72 °C for 2 min. The resulting fragments were sequenced using the PCR primers together with a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA). Sequencing reactions were performed as described by Cheewangkoon et al. (2008).

Phylogenetic analysis

A basic alignment of the obtained sequence data was first done using MAFFT v. 6 (http://mafft.cbrc.jp/alignment/server/index.html (Katoh et al. 2002) and if necessary, manually improved in BioEdit v. 7.0.5.2 (Hall 1999). Bayesian analyses (critical value for the topological convergence diagnostic set to 0.01) were performed on the individual loci using MrBayes v. 3.2.1 (Huelsenbeck & Ronquist 2001) as described by Crous et al. (2006b). Suitable models were first selected using Models of nucleotide substitution for each gene as determined using MrModeltest (Nylander 2004), and included for each gene partition. The substitution models for each locus are shown in Table 3. *Teratosphaeria nubilosa* (CPC 12243) was used as outgroup for all phylogenetic analyses.

Kimura-2-parameter values

Inter- and intraspecific distances for each individual dataset were calculated using MEGA v. 4.0 (Tamura et al. 2007) using the Kimura-2-parameter (pair-wise deletion) model.

RESULTS

Identification of the ideal DNA barcode

The dataset of the seven test loci was individually tested for three factors, namely amplification success, Kimura-2-parameter values (barcode gap) and molecular phylogenetic resolution.

Amplification success

The amplification success scores of the seven test loci on the 118 strains varied from 100 % amplification success for both ITS and LSU to only 90 % for Cal. The other four test loci (EF-1α, Act, RPB2 and Btub) gave amplification success scores of respectively 97, 98, 99 and 100 % (Table 3). The tested Cal primers failed to amplify the quarantine species *Pseudocercospora pini-densiflorae* and several other associated *Pseudocercospora* species. Consequently, Cal is considered unsuitable as a barcoding locus for this quarantine dataset.

Although it had a very high overall amplification success rate (99 %), RPB2 failed to amplify in *M. populicola*. Although *M. populicola* is not a quarantine species, it is very closely related and morphologically similar to the quarantine species *Septoria musiva*. This deficit, combined with the fact that RPB2 amplification within the dataset was not robust (often multiple PCR and/or sequencing runs were needed to get good sequencing reads), makes RPB2 unsuitable to serve as a barcoding locus for the quarantine dataset. The remaining five test loci successfully amplified all quarantine species.

Molecular phylogenies

General information per locus for the analysis, such as the number of characters used per dataset and the selected model are displayed in Table 3. The trees resulting from the Bayesian analyses of the seven individual loci showed that most loci have difficulty discriminating between closely related *Septoria* and *Pseudocercospora* species. Deciding the sequence difference

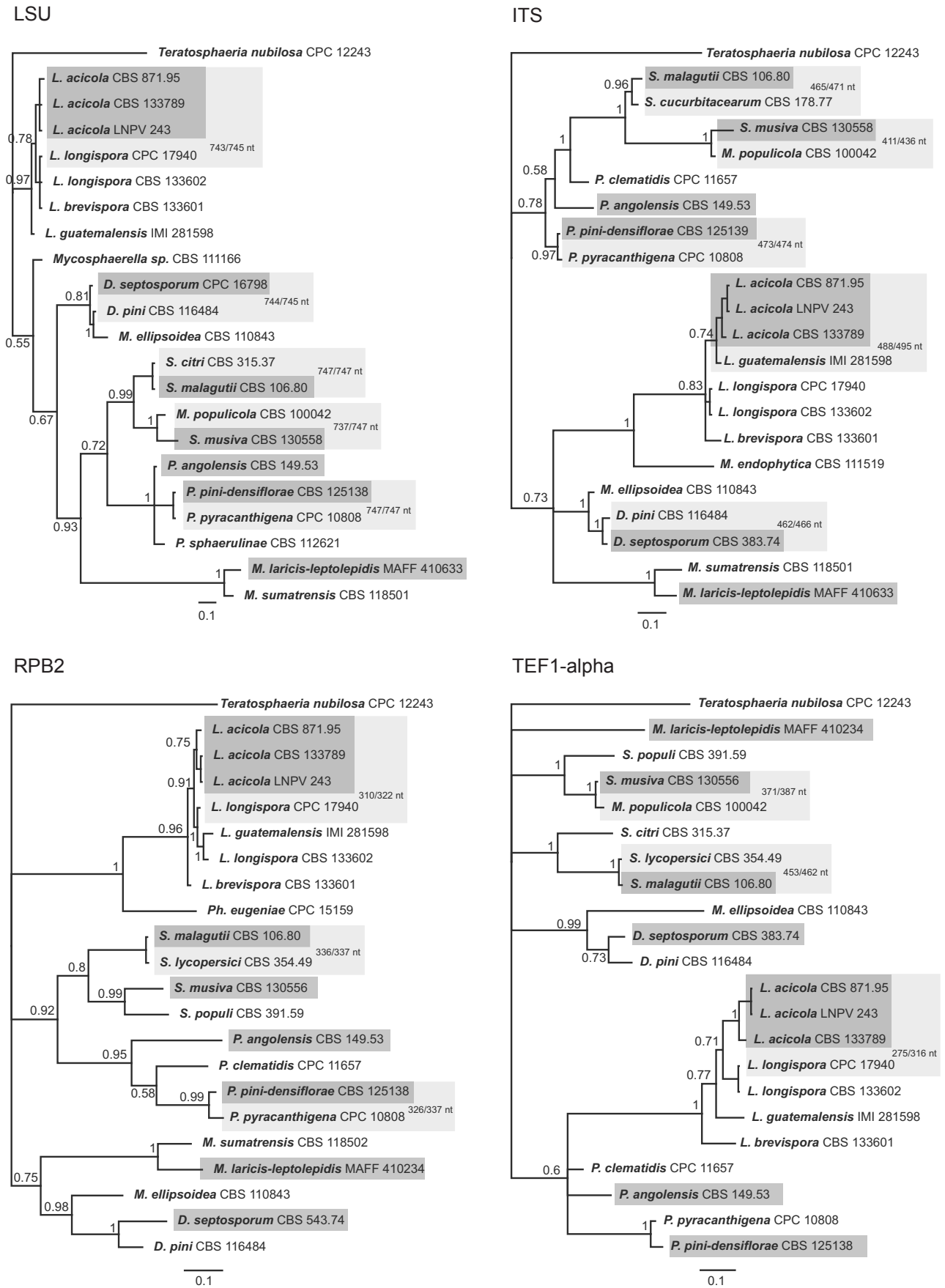

Fig. 1 Subset of Bayesian 50 % majority rule consensus trees of the individual test loci incorporating all *Mycosphaerellaceae* quarantine species (marked in grey) and their closest neighbour species as determined from the full-scale individual loci trees containing the complete dataset (available as supplementary data in TreeBASE). The following abbreviations were used for the genera: *T* = *Teratosphaeria*, *M* = *Mycosphaerella*, *Ph* = *Phaeophleospora*, *P* = *Pseudocercospora*, *D* = *Dothistroma* and *S* = *Septoria*. A stop rule (set to 0.01) for the critical value for the topological convergence diagnostic was used for the Bayesian analyses. The trees were all rooted to *Teratosphaeria nubilosa* (CPC 12243). The scale bar indicates 0.1 expected changes per site.

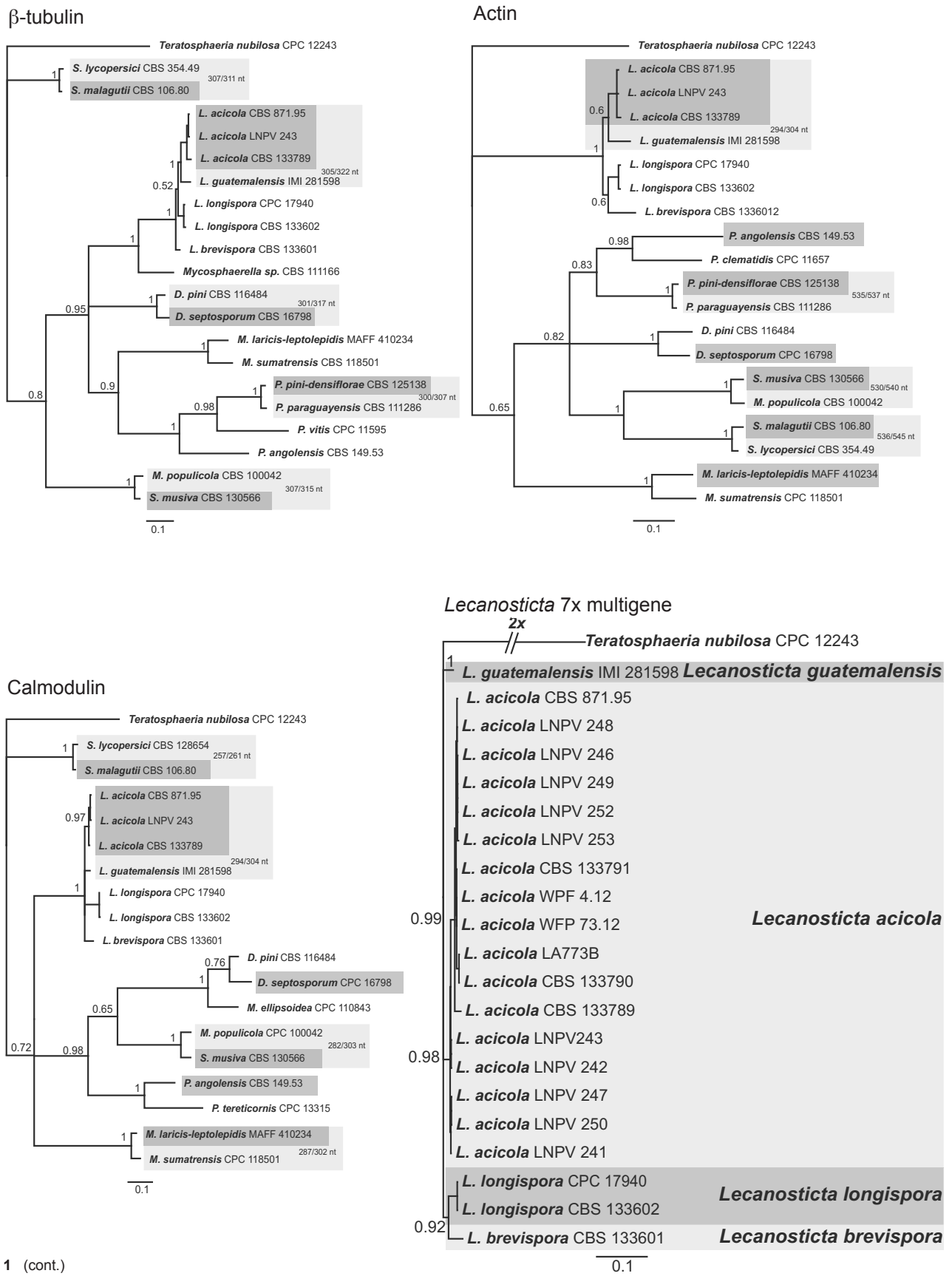

Fig. 1 (cont.)

that constitutes a positive discrimination threshold between species is arbitrary. If a threshold value of at least five base pairs difference is accepted as successfully discriminating between species, then only EF-1α discriminated between all tested Q-species (Fig. 1). If we set the threshold value to four base pairs difference, then Cal, EF-1α and Btub successfully discriminated between all tested species (Fig. 1). The ITS, LSU, Act and RPB2 loci were unable to discriminate among the various Q-species and closely related neighbours.

Kimura-2-parameter values

The Kimura-2-parameter distribution graphs (Fig. 2) visualise the inter- and intraspecific distances per locus corresponding to the barcoding gap (Hebert et al. 2003). A good barcoding locus

should not overlap between inter- and intraspecific Kimura-2-parameter distances.

The individual test loci showed varying degrees of overlap in their Kimura-2-parameter distribution graphs. For example, Act, ITS and LSU had much higher overlap than RPB2, EF-1α, Cal and Btub, which had minimal overlap. The primary cause for the existing Kimura-2-parameter overlap within the test loci is the low interspecific variation between the *Pseudocercospora* species used in this dataset. Excluding the *Pseudocercospora* species from the analyses (data not shown) removed the existing Kimura-2 overlap for RPB2, EF-1α and Btub, while reducing it significantly in Act. Excluding these *Pseudocercospora* species had only negligible effect on the ITS and LSU Kimura-2-parameter overlap (i.e. their lack of variation is more universal). Because Cal had a very low amplification success rate within the negatively affecting *Pseudocercospora* species used in this dataset, its Kimura-2-parameter graph is subsequently much less negatively affected (i.e. no Kimura-2-parameter

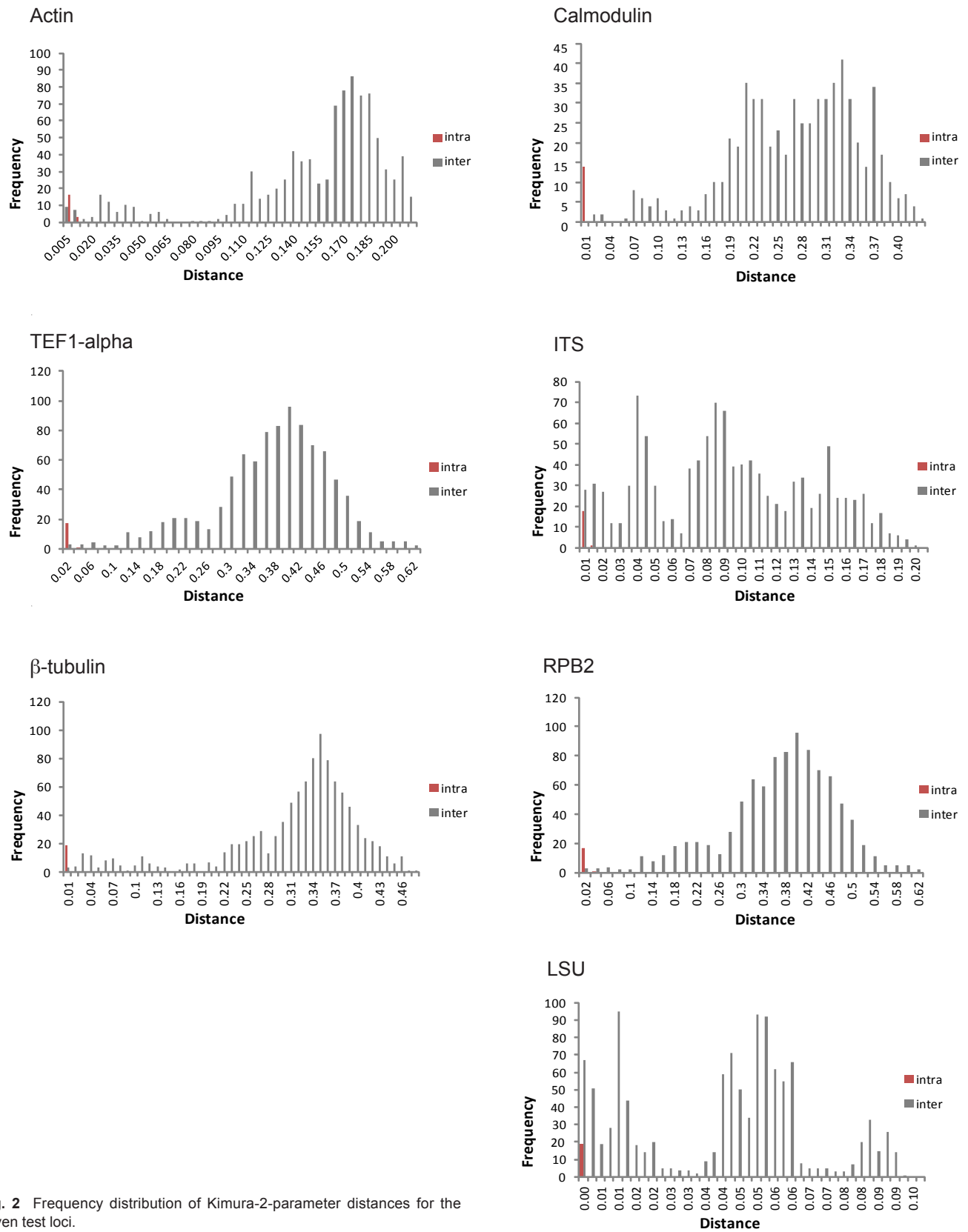

Fig. 2 Frequency distribution of Kimura-2-parameter distances for the seven test loci.

overlap) than the other four protein-coding test loci. The ITS and LSU loci, either with or without the *Pseudocercospora* dataset, showed a generally large Kimura-2-parameter overlap. Based on Kimura-2-parameter values, the RPB2, Btub, Act, Cal and EF-1α loci are not ideally suited for identifying *Pseudocercospora* species, but have a sufficient barcoding gap to successfully serve as the barcoding locus for the other species in this dataset. Both ITS and LSU are not suitable to serve as barcoding loci for this dataset.

Taxonomy

Dothistroma septosporum (Dorog.) M. Morelet (as '*septospora*'), Bull. Soc. Sci. Nat. Archéol. Toulon Var. 177: 9. 1968

Basionym. *Cytosporina septospora* Dorog., Bull. Trimestriel Soc. Mycol. France 27: 106. 1911.

≡ *Septoriella septospora* (Dorog.) Sacc. apud Trotter, Syll. Fung. 25: 480. 1931.

≡ *Septoria septospora* (Dorog.) Arx, Proc. Kon. Ned. Akad. Wetensch. C 86, 1: 33. 1983.

≡ *Dothistroma septosporum* var. *keniense* (M.H. Ivory) B. Sutton, in Sutton, The coelomycetes. Fungi imperfecti with pycnidia acervuli and stromata (Kew): 174. 1980.

= *Actinothyrium marginatum* Sacc., Nuovo Giorn. Bot. Ital. 27: 83. 1920.

= *Dothistroma pini* var. *lineare* Thyr & C.G. Shaw, Mycologia 56: 107. 1964.

= *Dothistroma pini* var. *keniense* M.H. Ivory (as '*keniensis*'), Trans. Brit. Mycol. Soc. 50: 294. 1967.

= *Mycosphaerella pini* Rostr., in Munk, Dansk Bot. Ark. 17, 1: 312. 1957.

≡ *Eruptio pini* (Rostr.) M.E. Barr, Mycotaxon 60: 438. 1996.

= *Scirrhia pini* A. Funk & A.K. Parker, Canad. J. Bot. 44: 1171. 1966.

≡ *Mycosphaerella pini* (A. Funk & A.K. Parker) Arx, Proc. Kon. Ned. Akad. Wetensch. C 86, 1: 33. 1983 (nom. illegit., Art. 53).

Specimens examined. Brazil, São Paulo, Santo Antonio do Pinhal, on needles of *Pinus pinaster*, 1974, *T. Namekata*, CBS 543.74. – Ecuador, on needles of *P. radiata*, CPC 3779 = CBS 112498. – France, Meurthe et Moselle, Arboretum d'Amance, on needles of *P. coulteri*, 27 Feb. 1970, CBS 383.74. – The Netherlands, Lunteren, Pinetum Dennenhorst, on needles of *Pinus mugo* '*Rostrata*', 1 June 2009, *W. Quaedvlieg*, CPC 16799, CPC 16798 = CBS 128782.

Notes — *Dothistroma septosporum* is the causal agent of Dothistroma needle blight (Red band disease of pine). This disease is endemic to virtually all continents and occurs on a small number of *Pinus* and *Larix* spp. where it can cause varying degrees of needle blight depending on humidity and temperature. Periods of higher humidity and temperature lead to more severe symptoms (Evans 1984, Barnes et al. 2004, EPPO 2012). Based on LSU data, isolates of *M. pini* cluster with *D. pini* and *M. africana* (Crous et al. 2009c, 2011b) and a large number of *Passalora*-like species (Videira et al. unpubl. data). Because the genus *Mycosphaerella* is linked to *Ramularia* (Verkley et al. 2004, Crous et al. 2009c), the name *Dothistroma* should be used for this clade, and *D. septosporum* for this species.

Lecanosticta acicola (Thüm.) Syd., Ann. Mycol. 22: 400. 1924. — Fig. 3

Basionym. *Cryptosporium acicola* Thüm., Flora 178. 1878.

≡ *Septoria acicola* (Thüm.) Sacc., Syll. Fung. 3: 507. 1884.

≡ *Dothistroma acicola* (Thüm.) Schischkina & Tzanava, Novosti Sist. Nizsh. Rast. 1967: 277. 1967.

= *Lecanosticta pini* Syd., Ann. Mycol. 20: 211. 1922.

= *Oligostroma acicola* Dearn., Mycologia 18: 251. 1926.

≡ *Scirrhia acicola* (Dearn.) Sigg., Phytopathology 29: 1076. 1939.

= *Systremma acicola* (Dearn.) F.A. Wolf & Barbour, Phytopathology 31: 70. 1941.

= *Mycosphaerella dearnessii* M.E. Barr, Contr. Univ. Michigan Herb. 9: 587. 1972.

On PNA: *Conidiomata* acervular, erumpent, brown, up to 600 µm diam, opening by means of longitudinal slit. *Conidiophores* subcylindrical, densely aggregated, dark brown, verruculose, unbranched or branched at base, 1–3-septate, 20–60 × 4–6 µm. *Conidiogenous cells* terminal, integrated, subcylindrical, brown, verruculose, 8–20 × 3–4.5 µm; proliferating several times percurrently near apex. *Conidia* solitary, straight to curved, subcylindrical with obtusely rounded apex, base truncate, brown, guttulate, verruculose, (0–)3(–8)-septate, base 2.5–3.5 µm diam, with minute marginal frill, (17–)30–45(–55) × (3–)4(–4.5) µm.

Culture characteristics — Colonies erumpent, spreading, with sparse aerial mycelium, surface folded, with smooth, lobate margin; colonies reaching 7 mm diam after 2 wk at 25 °C. On MEA surface olivaceous-grey to iron-grey, reverse olivaceous-grey. On PDA surface olivaceous-grey with diffuse umber pigment in agar, reverse pale olivaceous-grey. On OA surface olivaceous-grey with diffuse umber pigment.

Specimens examined. France, Gironde, Le Teich, on needles of *Pinus radiata*, Apr. 1995, *M. Morelet*, CBS H-21114, culture CBS 871.95. – Lithuania, on needles of *Pinus mugo*, 2009, *S. Markovskaja*, *A. Kačergius & A. Treigienė*, CBS H-21109, cultures LA773A & LA773B = CBS 133790. – Mexico, on needles of a *Pinus* sp., 30 Nov. 2009, *M. de Jesús Yáñez-Morales*, CBS H-21112, cultures CPC 17822 = CBS 133789. – USA, South Carolina, Aiken, needles of *Pinus caribaea*, 1876, *H.W. Ravenel*, IMI 91340, isotype of *Cryptosporium acicula* ex Padova No 1484; Arkansas, Pike City, alt. 700 ft, needles of *Pinus* (*palustris* or *taeda*), 24 Apr. 1918, coll. *J.A. Hughes*, det. Sydow, syntype of *Lecanosticta pini*, BPI 393329, BPI 393331; Florida, Silver Spring, needles of *Pinus palustris*, 27 Feb. 1919, coll. *Geo G. Hedgcock*, det. *J. Dearness*, type of *Oligostroma acicola*, BPI 643015; Maine, Bethel, on needles of *P. strobus*, 14 June 2011, coll. *B. Ostrofsky*, det. *K. Broders*, WPF4.12; ibid., on needles of *P. strobus*, 15 June 2011, coll. *B. Ostrofsky*, det. *K. Broders*, WPF13.12; New Hampshire, Blackwater, on needles of *P. strobus*, 25 June 2011, coll. *J. Weimer*, det. *K. Broders*, WPF13.12, epitype designated here CBS H-21113, culture ex-epitype CBS 133791.

Notes — *Lecanosticta acicola* is the causal agent of brown spot needle blight on *Pinus* spp. This disease is endemic to North and Central America, the central EPPO region and Eastern Asia where it causes yellowish, resin-soaked lesions with a prominent orange border on infected needles. As the disease progresses, lesions coalesce and cause defoliation and dieback. Over several years this may lead to branch and tree death (Evans 1984, Barnes et al. 2004, EPPO 2012). Based on LSU data, *L. acicola* clusters in a unique clade within the *Mycosphaerellaceae*, for which Crous et al. (2009c) chose the generic name *Lecanosticta* (based on *L. acicola*). The name *Mycosphaerella dearnessii* is no longer applicable, as *Mycosphaerella* s.str. is linked to the genus *Ramularia* (Verkley et al. 2004, Crous et al. 2009c). The correct name for this species should therefore be *Lecanosticta acicola*.

Lecanosticta brevispora Quaedvlieg & Crous, *sp. nov.* — MycoBank MB801940; Fig. 4

Etymology. Named after its relatively short conidia.

On PNA: *Conidiomata* acervular, erumpent, brown, up to 500 µm diam, opening by means of longitudinal slit. *Conidiophores* subcylindrical, densely aggregated, dark brown, verruculose, unbranched or branched at base, 0–2-septate, 10–25 × 3–4 µm. *Conidiogenous cells* terminal, integrated, subcylindrical, brown, verruculose, 5–8 × 2–3 µm; proliferating several times percurrently near apex. *Conidia* solitary, subcylindrical to narrowly fusoid-ellipsoidal, with subobtusely rounded apex, base truncate, brown, verruculose, frequently with mucoid sheath, (0–)1-septate, base 2 µm diam, with minute marginal frill, (11–)13–15(–18) × 3(–4) µm.

Culture characteristics — Colonies flat to somewhat erumpent, spreading, with sparse aerial mycelium, surface folded,

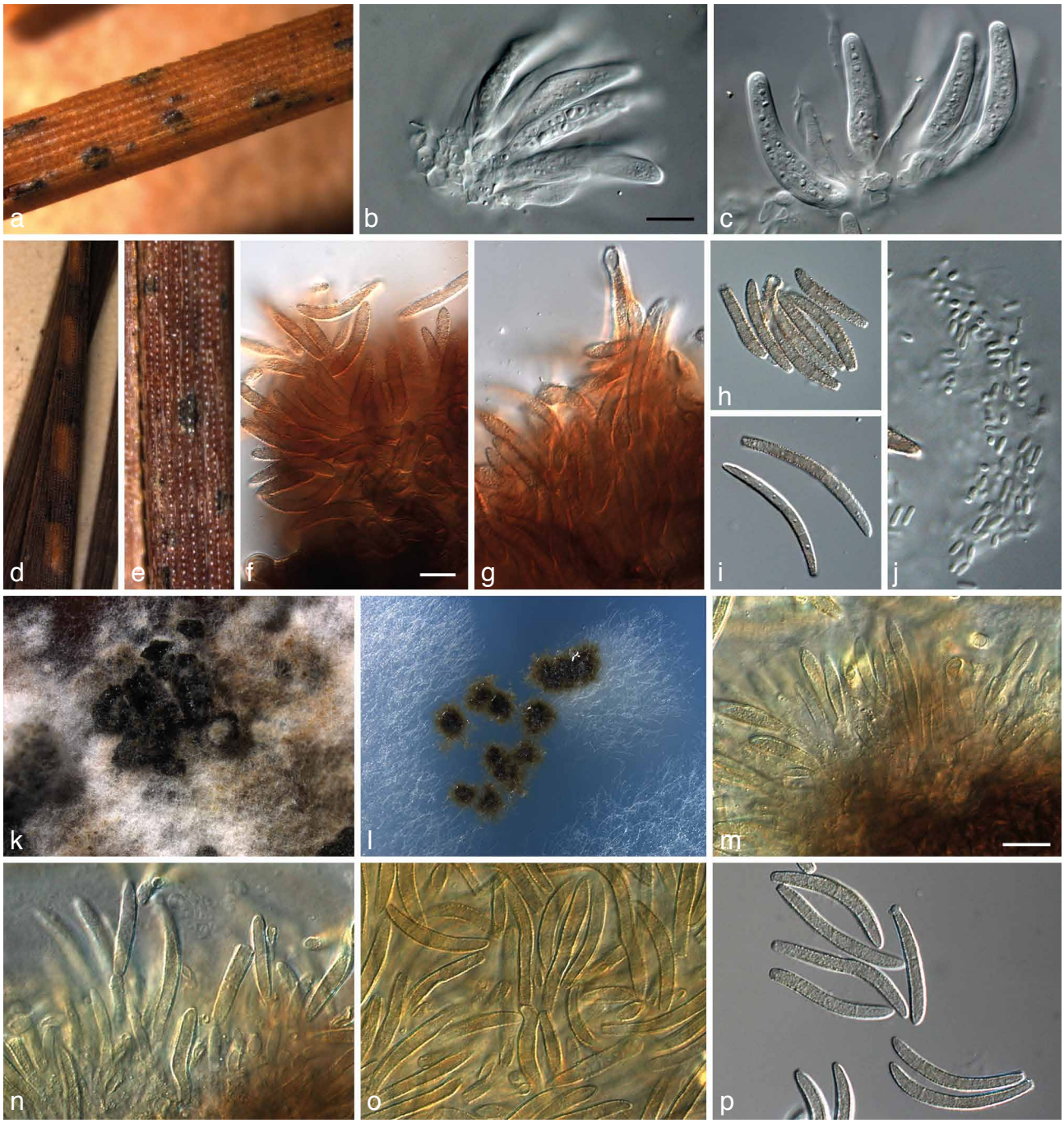

Fig. 3 *Lecanosticta acicola*. a–c. Needles with ascomata, asci and ascospores (BPI 643015); d–j. needles with acervuli, conidia and spermatia (BPI 39329); k. colony on PDA; l. colony on SNA; m–p. conidia formed on PNA (k–p = CPC 12822). — Scale bars = 10 µm.

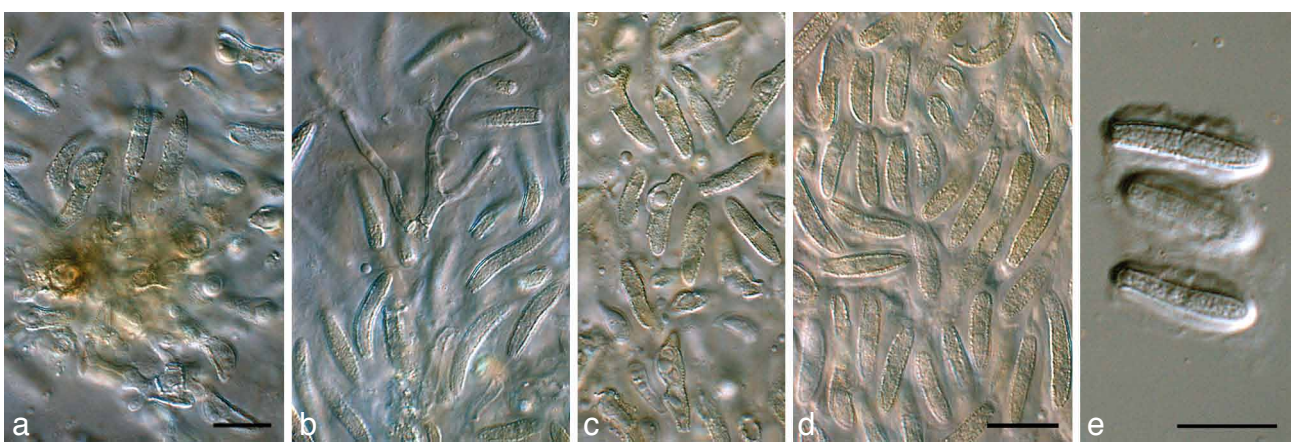

Fig. 4 *Lecanosticta brevispora* (CPC 18092). a, b. Conidiogenous cells giving rise to conidia; c–e. conidia (note mucoid sheath). — Scale bars = 10 µm.

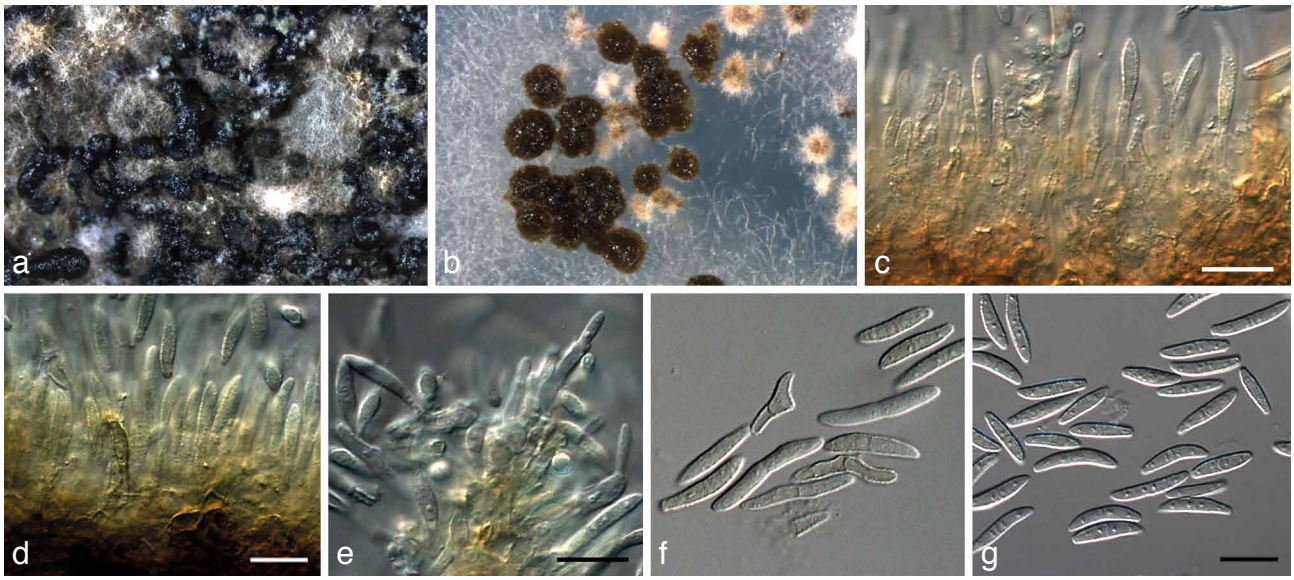

Fig. 5 *Lecanosticta guatemalensis* (IMI 281598). a. Colony sporulating on PDA; b. colony sporulating on SNA; c–e. conidiogenous cells giving rise to conidia; f, g. conidia. — Scale bars = 10 µm.

with smooth, lobate margin; colonies reaching 15 mm diam after 2 wk at 25 °C. On MEA surface dirty white with patches of pale olivaceous-grey, reverse olivaceous-grey in centre, luteous in outer region. On PDA surface dirty white in centre, isabelline in outer region, and isabelline in reverse. On OA surface dirty white with diffuse umber outer region.

Specimen examined. Mexico, on needles of a *Pinus* sp., 24 Oct. 2009, *M. de Jesús Yáñez-Morales*, holotype CBS H-21110, cultures ex-type CPC 18092 = CBS 133601.

Notes — *Lecanosticta brevispora* is distinguished from the other taxa within the genus by either Btub or EF-1α. Morphologically it is distinct in having much smaller conidia than *L. acicola*; with narrower and less septate conidia than *L. cinereum* (1–3-septate, (12–)14–18(–20) × (3.5–)4–5 µm, with obtuse apices), and *L. gloeospora* (1–3-septate, (9.5–)10.5–14.5(–17) × 3.5–4.5 µm, with obtuse apices) (Evans 1984).

Lecanosticta guatemalensis Quaedvlieg & Crous, *sp. nov.* — MycoBank MB801941; Fig. 5

Etymology. Named after the country where it was collected, Guatemala.

On PNA: *Conidiomata* acervular, erumpent, brown, up to 500 µm diam, opening by means of longitudinal slit. *Conidiophores* subcylindrical, densely aggregated, brown, verruculose, unbranched or branched at base, 0–3-septate, 15–25 × 3–4 µm. *Conidiogenous cells* terminal, integrated, pale brown, finely verruculose, subcylindrical to narrowly ampulliform, 6–15 × 2.5–3.5 µm; proliferating several times percurrently near apex. *Conidia* solitary, straight to curved, subcylindrical with subobtusely rounded apex, tapering towards truncate base, pale brown, finely verruculose, (0–)1(–2)-septate, base 2–2.5 µm diam, with minute marginal frill, (12–)15–20(–23) × 3(–3.5) µm.

Culture characteristics — Colonies erumpent, spreading, with sparse aerial mycelium, surface folded, with smooth, lobate margin, except on PDA, where margin is feathery; colonies reaching 30 mm diam after 2 wk at 25 °C. On MEA surface dirty white, reverse cinnamon with patches of isabelline, olivaceous-grey to iron-grey, reverse olivaceous-grey. On PDA surface and reverse olivaceous-grey. On OA surface buff.

Specimen examined. Guatemala, on needles of *Pinus oocarpa*, 28 Apr. 1983, *H.C. Evans*, holotype CBS H-21108, culture ex-type IMI 281598.

Notes — *Lecanosticta guatemalensis* can easily be distinguished from the other taxa presently known within the genus by either Btub or EF-1α. Morphologically it is distinguished by having conidia that are smaller than those of *L. acicola*, but larger than those of *L. brevispora*.

Lecanosticta longispora Marm., Mycotaxon 76: 395. 2000. — Fig. 6

On PNA: *Conidiomata* acervular, erumpent, brown, up to 600 µm diam, opening by means of longitudinal slit. *Conidiophores* subcylindrical, densely aggregated, brown, verruculose, unbranched or branched at base, 0–4-septate, 15–55 × 3–4 µm. *Conidiogenous cells* terminal, integrated, subcylindrical, brown, verruculose, 10–15 × 2–3.5 µm; proliferating several times percurrently near apex. *Conidia* solitary, subcylindrical with subobtusely rounded apex, base truncate, brown, guttulate, verruculose, 1–3-septate, base 2 µm diam, with minute marginal frill, (16–)30–45(–50) × 3(–4) µm.

Culture characteristics — Colonies flat, somewhat erumpent, spreading, with sparse aerial mycelium, surface folded, with smooth, lobate margin on MEA, but feathery on PDA and OA; colonies reaching 20 mm diam after 2 wk at 25 °C. On MEA surface pale olivaceous-grey with patches of olivaceous-grey. On PDA surface olivaceous-grey, reverse iron-grey. On OA surface dirty white in centre, with patches of pale olivaceous-grey and olivaceous-grey.

Specimens examined. Mexico, Nuevo León, Galeana, Cerro del Potosí, on *Pinus culminicola*, *J.G. Marmolejo*, 6 June 1993, holotype CFNL; Michoacan State, Zinapecuaro area, on needles of a *Pinus* sp., 24 Oct. 2009, *M. de Jesús Yáñez-Morales & C. Méndez-Inocencio*, epitype designated here CBS H-21111, cultures ex-epitype CPC 17941, CPC 17940 = CBS 133602.

Notes — *Lecanosticta longispora* is distinguished from the other taxa within the genus by either Btub or EF-1α. Morphologically it is similar to *L. acicola* in conidial length, but distinct in that conidia have 1–3 septa (Marmolejo 2000).

Mycosphaerella laricis-leptolepidis Kaz. Itô, K. Satô & M. Ota (as '*larici-leptolepis*'), Bull. Gov. Forest Exp. Sta. 96: 84. 1957

Specimens examined. Japan, Yamagata, on needles of *Larix leptolepis*, 1954–1955, *K. Itô*, MAFF 410081; Hokkaidou, on needles of *L. leptolepis*, 1954–1955, *T. Yokota*, MAFF 410632, MAFF 410633; Yamagata, on needles of *L. leptolepis*, May 1954, *N. Ota*, MAFF 410234.

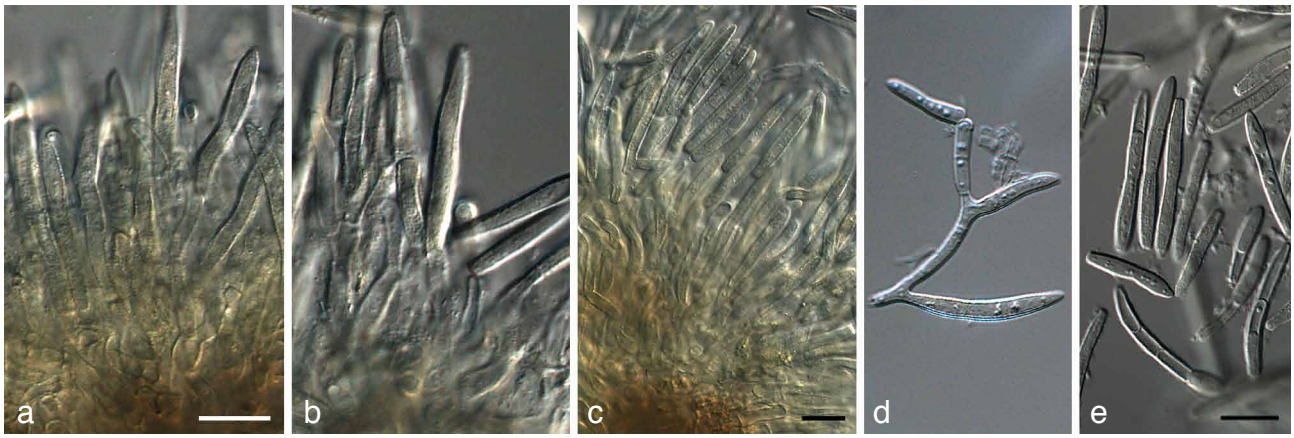

Fig. 6 *Lecanosticta longispora* (CPC 17940). a–d. Conidiogenous cells giving rise to conidia; e. conidia. — Scale bars = 10 µm

Notes — *Mycosphaerella laricis-leptolepidis* is the causal agent of needle cast of Japanese larch. This disease is endemic to East Asia and Japan where it occurs on indigenous *Larix* species. It causes brown necrotic lesions on the needles that coalesce, leading to defoliation, stunted growth and even host plant death (Kobayashi 1980, EPPO 2012). Based on LSU data, *M. laricis-leptolepidis* clusters in a clade described as '*Polythrincium*' by Crous et al. (2009c). Although the genus *Mycosphaerella* s.str. is distinct from the '*Polythrincium*' clade, the name *M. laricis-leptolepidis* is retained until more data becomes available.

Pseudocercospora angolensis (T. Carvalho & O. Mendes) Crous & U. Braun, Sydowia 55: 301. 2003

Basionym. *Cercospora angolensis* T. Carvalho & O. Mendes, Bol. Soc. Brot. 27: 201. 1953.

≡ *Phaeoramularia angolensis* (T. Carvalho & O. Mendes) P.M. Kirk, Mycopathologia 94: 177. 1986.

≡ *Pseudophaeoramularia angolensis* (T. Carvalho & O. Mendes) U. Braun, Cryptog. Mycol. 20: 171. 1999.

Specimens examined. Angola, Bié, from *Citrus sinensis*, Dec. 1953, *T. de Carvalho & O. Mendes*, holotype IMI 56597, ex-type CBS 149.53. – Zimbabwe, from *Citrus* sp., March 1993, *P.W. Crous*, CPC 751 = CBS 244.94; ibid., from *Citrus* sp., 2002, *P.W. Crous*, CPC 4111 = CBS 112748; ibid., from *Citrus* sp., Sept. 2002, *M.C. Pretorius*, CBS H-20851, CPC 4118 = CBS 112933; ibid., from *Citrus* sp., 2002, *P.W. Crous*, CPC 4117 = CBS 115645.

Notes — *Pseudocercospora angolensis* is the causal agent of Citrus leaf spot (Citrus fruit spot) and is endemic to sub-Saharan Africa, where it occurs on all major *Citrus* species. It causes greenish yellow lesions on leaves and fruit that coalesce and turn necrotic, leading to defoliation or abscission of young fruit (Timmer et al. 2000, Crous & Braun 2003, EPPO 2012). Based on LSU data, *P. angolensis* clusters within the *Pseudocercospora* clade (Pretorius et al. 2003, Crous et al. 2009c, In press). As the genus *Pseudocercospora* is taxonomically correct and in current use, *Pseudocercospora angolensis* is the correct name for the causal agent of Citrus fruit leaf spot.

Pseudocercospora pini-densiflorae (Hori & Nambu) Deighton, Trans. Brit. Mycol. Soc. 88: 390. 1987

Basionym. *Cercospora pini-densiflorae* Hori & Nambu, Tokyo J. Plant Protection 4: 353. 1917.

≡ *Cercoseptoria pini-densiflorae* (Hori & Nambu) Deighton, Mycol. Pap. 140: 167. 1976.

= *Mycosphaerella gibsonii* H.C. Evans, Mycol. Pap. 153: 61. 1984.

Specimens examined. Japan, from needles of *Pinus thunbergii*, 1971, *Sung-Oui Suh*, CBS 125139; from needles of *Pinus kesiya*, 1971, *Sung-Oui Suh*, CBS 125140; from needles of a *Pinus* sp., 1971, *Sung-Oui Suh*, CBS 125138.

Notes — *Pseudocercospora pini-densiflorae* is the causal agent of brown needle blight of pine (Cercospora pine blight). This disease is mostly endemic to the tropics and subtropics in Brazil, sub-Saharan Africa, India, Southeast and East Asia, where it may infect indigenous *Pinus* spp. It causes brown necrotic lesions on the needles leading to defoliation and is especially damaging on young saplings, on which defoliation leads to stunted growth and host plant death (Deighton 1987, Lewis 1998, EPPO 2012). Based on LSU data, isolates of *P. pini-densiflorae* cluster within the *Pseudocercospora* clade (Crous et al. In press), confirming its generic placement as reported by Deighton (1987). The generic name *Mycosphaerella* is considered a synonym of the genus *Ramularia* (Verkley et al. 2004, Crous et al. 2009c), and therefore *Mycosphaerella* should not be used for the pathogen associated with brown needle blight of pine. The application of the name *Pseudocercospora pini-densiflorae* is therefore correct.

Septoria malagutii E.T. Cline, Mycotaxon 98: 132. 2006

= *Septoria lycopersici* var. *malagutii* Ciccar. & Boerema, Phytopathol. Medit. 17: 87. 1978; nom. inval., Art. 37.1

Specimen examined. Peru, Dep. Junin, Huasahuasi, from a *Solanum* spp., 1975, *L.J. Turkensteen*, holotype CBS H-18113, culture ex-type CBS 106.80.

Notes — *Septoria malagutii* is the causal agent of Septoria leaf spot (angular leaf spot) of potato, and is endemic to Central and South America, where it occurs on leaves of potato and other tuber-bearing *Solanum* species. It causes leaf lesions that coalesce until the leaves turn necrotic, leading to defoliation and severe losses in crop production (Stevenson 2001, EPPO 2012). Based on LSU data, *S. malagutii* clusters within *Septoria* s.str. as defined by Quaedvlieg et al. (2011). The correct name for this species is therefore *Septoria malagutii* (Cline & Rossman 2006).

Septoria musiva Peck, Ann. Rep. New York State Mus. Nat. Hist. 35: 138. 1884

= *Mycosphaerella populorum* G.E. Thomps., Phytopathology 31: 246. 1941.

≡ *Davidiella populorum* (G.E. Thomps.) Aptroot, in Aptroot, Mycosphaerella and its anamorphs: 2. Conspectus of Mycosphaerella: 164. 2006.

Specimens examined. Canada, Quebec City, from leaf of *Populus deltoides*, *J. LeBoldus*, MAC = CBS 130564, LP3 = CBS 130565, PPP = CBS 130566, PP = CBS 130567, LPR = CBS 130568, RCL = CBS 130569, SA = CBS 130570, RPN = CBS 130571, D2L2 = CBS 130558; Alberta, from leaves of *P. deltoides* × *P. balsamifera*, *J. LeBoldus*, D2L2 = CBS 130558, NW3L1 = CBS 130563, NW2L2 = CBS 130561, D7L2; Alberta, from leaves of hybrid *Populus* spp., *J. LeBoldus*, APC = CBS 130559, APH1 = CBS 130560, APH3 = CBS 130562.

Notes — *Septoria musiva* is the causal agent of Septoria canker of poplar and is endemic to North America and Argentina, where it occurs on all native *Populus* spp. It causes severe cankering and die-back and is especially damaging to hybrid *Populus* species (Bier 1939, Waterman 1954, Ostry 1987, Dickmann 2001, EPPO 2012). Based on LSU data, *S. musiva* clusters within *Septoria* s.str. as defined by Quaedvlieg et al. (2011). However, ongoing work by Quaedvlieg and Verkley (unpubl. data) revealed that *S. musiva* is located in a cryptic phylogenetic lineage sister to *Septoria* s.str., and therefore the genus name of this clade might change in the future.

DISCUSSION

Current EPPO protocols for identifying A1/A2 listed *Mycosphaerella* species are based either on ITS-RFLP or fungal morphology (Table 4). These approaches each have limitations that make them ill-suited as identification tools for plant protection policy enforcement officers.

Morphology-based techniques are heavily dependent on highly skilled personnel that need to perform time-consuming identifications of mature, sporulating cultures that often need to be grown on specific media and under specific conditions. The rapid advance of molecular techniques in recent years has underlined the limitations of identifications based solely on morphology and/or ITS sequencing. Examples of this are the new *Lecanosticta* species that have been described during this study. These isolates had previously been identified as *Lecanosticta acicola* based both on morphology and limited ITS sequencing. The sequencing of additional loci revealed that *L. acicola* actually represented a species complex rather than a single species. This is yet another example of the tenet of Crous & Groenewald (2005) which states “Show me a plant pathogen, and I will show you a species complex”. Another example was the *Cercospora apii* complex, which was considered to be a single species based on morphology (Crous & Braun 2003), but which was found to represent several species when DNA sequencing techniques where employed (Crous et al. 2004b, 2006a, In press, Groenewald et al. 2005, In press). This inability to discriminate between cryptic species and their dependency on mature, sporulating cultures make morphology-based techniques poorly suited for the rapid and reliable identification of *Mycosphaerella* species on trade goods.

PCR-RFLP-based methods work on a ‘hit or miss’ principle, and work well for identifying small groups of well-characterised fungal species with little genetic variation. Unfortunately these methods lack the inherent ability to cope with expanding natural variation. Point mutations, insertion or deletion events can lead to the loss of restriction sites, making isolates unrecognizable for PCR-RFLP based methods (Majer et al. 1996). Species of *Mycosphaerella* also co-colonize lesions, increasing the chance of having a mixed DNA sample if single-spored or hyphal-tipped colonies are not used in the assay (Crous & Groenewald 2005).

The use of a DNA barcode or the combination of sequence data from two or more discriminatory loci (multi-locus sequence typing), for the recognition of species of quarantine importance has numerous advantages over previously used techniques. It does not require fruiting bodies or a mature life stage, it is fast, (relatively) cheap, and can be performed by moderately skilled personnel and has a high probability of yielding a result, even with unknown species. But the single most important aspect of DNA barcoding is its ability to identify species (even cryptic species) with almost no margin of error, on condition that a large, validated, reference database library is available.

One of the main goals of this project was to determine the most suitable barcoding locus/loci by which to identify *Mycosphaerella*-like spp. on the EPPO A1/A2 lists. Hebert et al. (2003) proposed that a good barcoding locus should show a clear separation between the distributions of the mean intra- and interspecific distances (the so-called ‘Kimura-2-parameter barcoding gap’). The authors proposed that a locus should have a mean inter- / intraspecific distance ratio of at least 10, to be suitable as a barcoding locus. The loci tested in this study all had mean inter-, intraspecific distance ratios that were much higher than 10. Mean distribution ratios varied from 486 for LSU to 69 for ITS (Fig. 2). By these criteria alone, these loci should all be suitable barcoding loci. Almost all loci showed a Kimura-2-parameter overlap between their absolute inter- and intraspecific distribution frequencies. When the *Pseudocercospora* isolates were included in the dataset, the size of this absolute inter- and intraspecific distribution frequencies data overlap varied from 12 % (LSU), 16 % (ITS), 3.4 % (Act), 1.2 % (EF-1α), 0.6 % (RPB2), 0.5 % (Btub) and 0 % (Cal), respectively. Calmodulin did not overlap simply because this locus failed to amplify most of the *Pseudocercospora* spp. that are mostly responsible for this Kimura-2-parameter inter- and intraspecific distribution overlap in the other loci.

The relatively high Kimura-2-parameter distribution overlap in the two nuclear ribosomal DNA loci (ITS and LSU) is caused by the low natural variation that exists within these loci between species of certain genera (in this dataset *Septoria* spp. and *Pseudocercospora* spp. had very low variability between species). This difference within the natural variation present within the different genera in the complete dataset can clearly be seen in the ITS and LSU Kimura-2-parameter distribution graphs (Fig. 2). These two graphs clearly show multiple ‘peaks’ that represent the difference in natural variation within the varying genera used in this dataset.

Table 4 EPPO and EU Council Directive-listed *Mycosphaerella* species of quarantine importance, their currently advised identification method(s) and their valid taxonomic names. Taxonomic names marked in grey have yet to be resolved, therefore the *Mycosphaerella* name for this species should still be used.

Name on EPPO A1 and A2 lists	Name in EU Council Directive	Valid taxonomic name	EPPO-listed identification method	Reference
Mycosphaerella populorum / *Septoria musiva*	*Mycosphaerella populorum*	*Septoria musiva*	Fruiting body morphology	Bier (1939), Peace (1962), Waterman (1954)
Mycosphaerella gibsonii / *Cercoseptoria pini-densiflorae*	*Cercoseptoria pini-densiflorae*	*Pseudocercospora pini-densiflorae*	Fruiting body morphology	Deighton (1987)
Mycosphaerella laricis-leptolepidis / *Phyllosticta laricis*	*Mycosphaerella larici-leptolepis*	*Mycosphaerella larici-leptolepis*	Fruiting body morphology	Peace (1962)
Phaeoramularia angolensis	*Cercospora angolensis*	*Pseudocercospora angolensis*	Fruiting body morphology	Kirk (1986)
Septoria lycopersici / *Spegazzini* var. *malagutii*	*Septoria lycopersici* / *Spegazzini* var. *malagutii*	*Septoria malagutii*	Fruiting body morphology	Cline & Rossman (2006)
Mycosphaerella dearnessii / *Lecanosticta acicola*	*Scirrhia acicola*	*Lecanosticta acicola*	Fruiting body morphology / ITS-RFLP	Barnes et al. (2004)
Mycosphaerella pini / *Dothistroma septospora*	*Scirrhia pini*	*Dothistroma septosporum*	Fruiting body morphology / ITS-RFLP	Evans (1984), Barnes et al. (2004)

From the three independent barcode suitability tests we can conclude that, based on a threshold of at least five base pairs difference, EF-1α is the best locus to use for DNA barcoding of the isolates within this dataset. If we use a threshold of four base pairs, then Btub is also suited to serve as DNA barcoding locus for this dataset. The other tested loci either have a clear amplification problem (Cal) or do not have sufficient resolution (Δ ≥ 4nt) (ITS, LSU, Act and RPB2) to discriminate between some of the quarantine species and their closest relative species (Fig. 1).

Although the EF-1α and Btub loci have the highest species discrimination levels for the species used in this dataset, these loci have the disadvantage that there is not much reference data concerning these loci available in online databases which can help identify isolates not used in this dataset. To compensate for this lack of reference data, we recommend using a combination of a primary and a secondary locus to give more reliable identification results.

The ITS locus is the prime candidate for the primary locus. ITS has recently been proposed as one of the primary fungal barcoding loci (Schoch et al. 2012). ITS sequencing data is easily obtained and a good starting point to rapidly identify genera and sometimes species. If an unknown genus or species is not represented in a curated database such as Q-bank, a GenBank blast could be used to supplement these curated databases. Mycology has a long history of using ITS data to identify fungal species and GenBank would thus be a good supplementary (although not completely curated) database. The use of ITS as the primary locus, and if necessary using a secondary locus following a molecular decision protocol, would be the most stable approach for a reliable identification. This is also the identification protocol as it is currently implemented in Q-bank.

As a secondary barcoding locus to supplement the ITS sequence data, either Btub or EF-1α would suffice for this dataset. Both loci are easily amplifiable and have a high amplification rate (100 % and 97 %, respectively), posses only minimal Kimura-2-parameter inter- and intraspecific distribution overlap (0.5 % and 1.2 %, respectively) and both have 100 % species discrimination success rate within the tested dataset (Δ ≥ 4nt). The use of either Btub or EF-1α may complement each other if amplification problems with either locus occur, thus leading to a successful identification of an unknown *Mycosphaerella* species of possible quarantine importance.

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