Phylogenetic placement and reassessment of *Asperisporium pongamiae* as *Pedrocrousiella pongamiae gen. et comb. nov.* (*Mycosphaerellaceae*)

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Abstract: The leaf spot disease of *Pongamia pinnata* caused by an asperisporium-like asexual morph, which is usually referred to as *Asperisporium pongamiae*, is quite common during monsoon seasons in India. Phylogenetic analyses, based on LSU and *rpb2* sequence data, and blast searches using ITS sequence data, revealed that this ascomycete forms a lineage within *Mycosphaerellaceae* distant from all other generic lineages. *Pedrocrousiella gen. nov.*, with *P. pongamiae comb. nov.*, based on *Fusicladium pongamiae* (≡ *A. pongamiae*), as type species is introduced for this lineage. This species has been considered the asexual morph of *Mycosphaerella pongamiae* (≡ *Stigmatea pongamiae*). However, this connection is unproven and was just based on the occasional association of the two taxa in some collections. Several attempts to induce the formation of a sexual morph in culture failed, therefore the putative connection between these morphs could not be confirmed. *Asperisporium pongamiae-pinnatae* is reduced to synonymy with *P. pongamiae*. *Asperisporium pongamiae-pinnatae* was introduced because of the wrong assumption that *F. pongamiae* had been described on another host, *Pongamia globosa*. But *Fusicladium pongamiae* was actually described in India on *Pongamia glabra*, which is a synonym of *P. pinnata*, and hence on the same host as *Asperisporium pongamiae-pinnatae*. *Pedrocrousiella pongamiae* clusters in a clade containing *Distocercospora*, *Clypeosphaerella*, and "*Pseudocercospora*" *nephrolepidicola*, a species which is not congeneric with *Pseudocercospora*. Phylogenetically, *Pedrocrousiella* is distant from the *Asperisporium s. str.* clade (type species *A. caricae*), which is more closely related to *Amycosphaerella*, *Pseudocercosporella*, *Distomycovellosiella* and *Nothopassalora*.

INTRODUCTION

Most cercosporoid genera with and without mycosphaerella-like sexual morphs belong in the *Mycosphaerellaceae* (*Mycosphaerellales*, *Dothideomycetes*, *Ascomycota;* Abdollahzadeh *et al*. 2020), and based on phylogenetic data about 120 genera are now accepted within this family (Videira *et al.* 2017). The genus name *Mycosphaerella*, which has previously been applied to cercosporoid fungi as sexual genus, is now, on the basis of the current Code (ICNafp; Turland *et al*. 2018), a synonym of *Ramularia*, which is included in a list of protected names (Wijayawardene *et al*. 2014, Rossman *et al*. 2015, Videira *et al.* 2015a, b, 2017). The species-rich family *Mycosphaerellaceae* is characterised by having a considerable morphological and genetical diversity. Most of the included species are biotrophic and encompass numerous economically important plant pathogens worldwide (Crous *et al.* 2015). The phylogeny and

taxonomy of cercosporoid fungi is complex, challenging, and far from being completely examined (Baker *et al.* 2000, Crous & Braun 2003, Crous *et al.* 2007, 2009a, 2019, Videira *et al.* 2017, to name but a few).

Maublanc (1913) introduced *Asperisporium* with *A. caricae* (≡ *Cercospora caricae*) as type species for a foliicolous, leaf-spotting hyphomycete on papaya, characterised by forming well-developed stromata giving rise to densely fasciculate, cicatrised conidiophores which produce verruculose amero- to phragmosporous conidia singly. A detailed description of the genus *Asperisporium* was published in Braun *et al.* (2013). Sydow & Sydow (1913) described *Fusicladium pongamiae* on living leaves of *Pongamia pinnata* from Tamil Nadu, India. Subramanian (1971) introduced the combination *Passalora pongamiae*, and Deighton (Ellis 1976) transferred *F. pongamiae* to *Asperisporium*. *Asperisporium pongamiae* has been reported from Bangladesh, India, Sri Lanka (Mohanan 1988) and North

Queensland, Australia (Shivas & Alcorn 1996). Sivanesan (1985) described and illustrated *Mycosphaerella pongamiae*, based on *Stigmatea pongamiae* (Raciborski 1900), which he considered the sexual morph of *Asperisporium pongamiae*. However, this assumption was just premised on the close association between the caespituli of *A. pongamiae* and ascomata of *M. pongamiae* in some collections (*in vivo*, but not *in vitro*). An additional *Asperisporium* species, *A. pongamiae-pinnatae* (Kharwar *et al.* 2012), described on living leaves of *P. pinnata* from Uttar Pradesh, India, has to be taken into consideration as well.

Pongamia pinnata is a medium-sized evergreen Indo-Malaysian tree species, common in alluvial and coastal habitats from India to Fiji, from sea level to 1 200 m alt. (Yadav *et al.* 2011, Pavithra *et al.* 2014), but also widely planted in other regions of the world, such as Kenya, Seychelles, South Africa, Tanzania, Uganda and Zimbabwe in Africa, Australia, New Zealand, and the USA (Florida, Hawaii) (https://www.cabi.org/isc/datasheet/428 35#todistributionDatabaseTable, Yadav *et al.* 2011). *Pongamia pinnata* is widely used as ornamental, windbreaker and shade tree, and the seeds contain pongam oil, which is applied for pharmaceutical purposes and as therapeutic product to treat various human diseases, also in the traditional medicine in India, such as skin diseases, piles, ulcers, diabetes, rheumatism, tumors, and wounds (see, https://hort.purdue.edu/newcrop/duke_energy/Pongamia_pinnata.html; Yadav *et al.* 2011). *Pongamia pinnata* is mainly appreciated for its oils, such as Karanjin (flavone) and pongamol (chalcone), extracted from roots, bark and seeds (Al-Muqarrabun *et al.* 2013), whereas the leaves are used only as fodder (Arote & Yeole 2010). Therefore, the leaf blight disease caused by *A. pongamiae* is not known to cause any major economic losses. Nevertheless, *A. pongamiae* causes a disease of an important widely used tree species, which underlines the importance to clarify the phylogeny and taxonomy of this leaf-spotting fungus.

The true generic affinity of *A. pongamiae* is so far quite unclear and unproven. In view of the complexity of cercosporoid fungi within the *Mycosphaerellaceae* and the limitation of using morphological traits for the elucidation of generic affiliations (Videira *et al.* 2017), phylogenetic examinations of the foliar pathogen causing a severe leaf blotch disease of *Pongamia pinnata* were performed, based on specimens collected during the monsoon season of 2018, 2019 and 2020 in the Kothrud area of Pune, India. Samples were subjected to *in vitro* culturing and molecular studies were performed to clarify the correct position of this cercosporoid ascomycete within the *Mycosphaerellaceae*.

MATERIALS AND METHODS

Isolates

Leaves with visible disease symptoms were collected during the monsoon 2018 and post-monsoon seasons of 2019 and 2020 from wild stands growing in a Shiva temple property in the Kothrud area of Pune, India. Conidia were directly isolated from infected leaves observed through a Nikon SMZ1500 dissecting microscope with a digital camera control unit DS-Vi1 (Tokyo, Japan). Single conidial cultures were established on 2 % malt extract agar (MEA; HiMedia, Mumbai, India) plates. Needles made of micro-dissecting pins (stainless steel headless pins D) were used to pick the conidial mass from sporodochia and to transfer it to a single-cavity microscopic slide containing 20 μL

double-distilled water. The conidial suspension was thoroughly mixed using a micropipette, and 10 μL volume was dropped over a 2 % MEA plate and trailed by tilting the Petri dish at a 90° angle. Trails were further marked on the lower lid of Petri dishes using a marker pen, and single conidia were spotted through a Olympus (Model CX-41, Japan) compound microscope (4× objective). Conidial germination was observed after 6, 24, and 30 h after inoculation. Germinated conidia were further transferred to fresh MEA plates and incubated at 25 ± 2 °C, and observations were noted after 3, 5, 7, 9, and 15 d. Fungarium specimens were deposited in the Ajrekar Mycological Fungarium (AMH), and the derived cultures were accessioned and preserved in the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India.

DNA extraction, amplification, and phylogenetic analyses

Colonies were grown on MEA plates, and genomic DNA extraction was done following the modified protocols of the rapid salt extraction method by Aljanabi & Martinez (1997). The ITS region was amplified using the primer pair ITS5 and ITS4 (White *et al.* 1990). The first part of the large subunit nuclear ribosomal DNA (LSU) gene was amplified using the primer pairs LROR (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990). For the amplification of the partial DNA-directed RNA polymerase II second largest subunit (*rpb2*) gene, the primer pairs RPB2-5F and RPB2-7cR (Liu *et al.* 1999) were used with touch-up PCR conditions: nine cycles with denaturing temperature 95 °C for 1 min followed by 50 °C for 30 s, 72 °C for 90 s; 30 cycles with 95 °C for 1 min, 52 °C for 30 s, 72 °C for 90 s; nine cycles of 95 °C for 1 min, 55 °C for 30 s, 72 °C for 90 s and a final elongation at 72 °C for 10 min. The PCR products were purified with a StrataPrep PCR Purification Kit (Agilent Technologies, TX, USA), and sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing reactions were run on ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA).

Sequence alignments, Bayesian phylogenetic analyses and tree layout followed the protocols of Crous *et al.* (2020). The NCBI GenBank nucleotide sequence database was queried using megablast searches to identify closest matching sequences in the database. To create the combined LSU-*rpb2* alignment, the novel sequences generated in this study were manually added to the alignment of Videira *et al.* (2017) downloaded from TreeBASE (study 21537), as well as any close sequences from the blast searches not included in that alignment (Table 1). An initial tree was calculated from this alignment and used as basis for the reduced set tree shown in this study. The final Bayesian posterior probability analysis was performed using MrBayes v. 3.2.7a (Ronquist *et al.* 2012), using the parameter settings of two parallel runs of four chains each, run for 100 M generations but with the stop value set at 0.01, the temperature set at 0.35 and the sample frequency every 100th generation. The model identified in Videira *et al.* (2017) as the best model for both partitions was also used in this study (dirichlet base frequencies and the GTR+I+G model). The 50 % majority rule consensus tree was created after the first 25 % of saved trees were discarded as burn-in. In addition, maximum likelihood branch support values (ML-BS) were obtained with the ultrafast bootstrap (Hoang *et al.* 2018) method implemented in the IQ-TREE v. 2.1.2 software (Nguyen *et al.* 2015) and parsimony bootstrap support values (MP-BS; 1 M fast bootstrap replicates) using PAUP v. 4.0b10 (Swofford 2003). DNA sequences newly generated in this study

Table 1. Collection details and GenBank accession numbers of additional sequences added to the alignment of Videira *et al.* (2017). For details of all other sequences, see Videira *et al.* (2017). The ITS GenBank numbers are provided for completeness and the taxonomic novelty is highlighted with bold text.

Species	Culture accession number(s)[1]	Country	Substrate	Collector(s)	GenBank accession number[2]			Sequence references
					ITS	LSU	*rpb2*	
Cercospora pseudochenopodii	CBS 136022 = CCTU 1038, ex-type	Iran	*Chenopodium* sp.	M. Bakhshi	KJ88516.1	–	MH511954.1	Bakhshi *et al.* (2015), Bakhshi *et al.* (2018)
Clypeosphaerella sticheri	CPC 24733	Brazil	*Sticherus bifidus*, fronds	E. Guatimosim	KT037536.1	KT037577.1	–	Guatimosim *et al.* (2016)
Collapsinycopappus styracis	HH 30067, ex-type	Japan	*Styrax obassia*, living leaves	K. Tanaka and Y. Harada	NR_158348.1	NG_064448.1	LC333042.1	Hashimoto *et al.* (2018)
	KT 2939	Japan	*Styrax obassia*, living leaves	K. Tanaka	LC333028.1	LC333034.1	LC333040.1	Hashimoto *et al.* (2018)
Neosonderhenia eucalypti	CBS 145081 = CPC 34405, ex-type	Australia	*Eucalyptus costata*	B.A. Summerell	NR_165602.1	MN162191.1	MN162578.1	Crous *et al.* (2019)
	CBS 145082 = CPC 34395	Australia	*Eucalyptus costata*	B.A. Summerell	MN161928.1	MN162192.1	MN162579.1	Crous *et al.* (2019)
Nothoseptoria caraganae	CPC 36563 = CBS 145993	Russia	*Caragana arborescens*, living leaves	T.S. Bulgakov	MT223825.1	MT223917.1	MT223693.1	Crous *et al.* (2020)
	CPC 36565	Russia	*Caragana arborescens*, living leaves	T.S. Bulgakov	MT223826.1	MT223918.1	MT223694.1	Crous *et al.* (2020)
Pedrocrousiella pongamiae	NFCCI 4881, ex-epitype	India	*Pongamia pinnata*, leaves	K.C. Rajeshkumar	MW327548.1	MW327593.1	MW363496.1	Present study
Pseudocercospora nephrolepidicola	CBS 128211 = CPC 17049, ex-type	Australia	*Nephrolepis falcata*, leaves	P.W. Crous and R.G. Shivas	HQ599590.1	HQ599591.1	KX462646.1	Crous *et al.* (2010), Nakashima *et al.* (2016)
Pseudozasmidium parkii	CBS 387.92 = CPC 353, ex-type	Brazil	*Eucalyptus grandis*	M.J. Wingfield	KF901785.1	GU214448.1	–	Crous *et al.* (2009b), Quaedvlieg *et al.* (2014)
Zasmidium musicola	CBS 122479, ex-type	India	*Musa acuminata* AAA Group cv. Cavendish	I.W. Buddenhagen	NR_156516.1	NG_069906.1	MF951717.1	Arzanlou *et al.* (2008), Videira *et al.* (2017), Vu *et al.* (2019)

[1] CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CCTU: Culture Collection of Tabriz University, Iran; CPC: Culture Collection of Pedro Crous, housed at CBS; HH: Culture collection at Hiroshaki University, Japan; KT: Culture collection of Kazuaki Tanaka, Hirosaki University, Japan; NFCCI: National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India.

[2] ITS: internal transcribed spacers and intervening 5.8S nrDNA; LSU: large subunit (28S) of the nrRNA gene operon; *rpb2*: partial DNA-directed RNA polymerase II second largest subunit gene.

were deposited in GenBank (Table 1), the alignment and trees in TreeBASE (study 27395) and the taxonomic novelty in MycoBank (www.MycoBank.org; Crous *et al*. 2004).

Morphology

For morphological studies and photomicrographs, a ZEISS Axio Imager 2 compound microscope (Carl Zeiss, Oberkochen, Germany) and a Nikon SMZ1500 stereomicroscope with a digital camera control unit DS-Vi1 (Tokyo, Japan) were used. Conidia and conidiophores were mounted in lactic acid cotton blue and measured using the AxioVision v. 4.8 software, with 30 measurements per structure. Culture characteristics were studied on MEA. Colony colours were determined using the Methuen Handbook of Colour (Kornerup & Wanscher 1978). Induced sporulation studies were performed (in a biomulti incubator at 25 ± 2 °C) in an attempt to verify putative asexual-sexual morph associations on MEA and Cornmeal Agar (CMA) media (HiMedia, Mumbai, India) along with host tissue or carnation leaves. Symptomatic leaves from field collections were further subjected to Scanning Electron Microscopy (SEM) to verify the sporodochial development and conidial ornamentation using a Carl Zeiss EVO 50 Scanning Electron Microscope (Carl Zeiss, Oberkochen, Germany) at Agharkar Research Institute, Pune.

RESULTS

Phylogeny

Based on megablast and blastn searches of the NCBI's GenBank nucleotide database, only distant hits were obtained using the ITS sequence, such as *Pseudocercosporella bakeri* CBS 119488 [GenBank KX287306.1; identities = 354/397 (89 %), 12 gaps (3 %)], *Neosonderhenia eucalypti* CBS 145081 [GenBank NR_165602; identities = 405/463 (87 %), 18 gaps 3 %)], *Xenosonderhenia eucalypti* CBS 138858 [GenBank NR_137937; identities = 406/467 (87 %), 19 gaps (4 %)], and "*Passalora*" *nattrassii* z3 [GenBank KF863691.1; identities = 399/455 (88 %), 8 gaps (1 %); only ITS was available and could therefore not be included in the phylogenetic tree]. Based on megablast and blastn searches of the NCBI's GenBank nucleotide database, the closest hits were obtained using the LSU sequence, such as "*Pseudocercospora*" *nephrolepidicola* CBS 128211 [GenBank HQ599591.1; identities = 807/834 (97 %), 2 gaps (0 %)], *Sonderhenia_eucalyptorum* CBS 120220 [as *Mycosphaerella swartii*; GenBank DQ923536.1; identities = 806/835 (97 %), 3 gaps (0 %)], *Sonderhenia eucalypticola* CMW 20333 [as *Mycosphaerella walkeri*; GenBank DQ267574.1; identities = 806/835 (97 %), 3 gaps (0 %)], and *Clypeosphaerella quasiparkii* CBS 123243 [GenBank MH874811.1; identities = 807/836 (97 %), 4 gaps (0 %)]. Based on megablast and blastn searches of the NCBI's GenBank nucleotide database using the *rpb2* sequence (NFCCI 4881), only distant hits were obtained, such as *Distocercospora pachyderma* CBS 138247 [GenBank MF951486; identities = 712/866 (82 %), no gaps], "*Pseudocercospora*" *nephrolepidicola* CBS 128211 [GenBank KX462646.1; identities = 561/685 (82 %), no gaps)], *Clypeosphaerella calotropidis* CBS 129.30 [GenBank MF951477.1; identities = 874/1 073 (81 %), no gaps], and *Zasmidium musicola* CBS 122479 [GenBank MF951717.1; identities = 714/922 (77 %), 16 gaps (1 %)].

The final combined LSU-*rpb2* dataset comprised a total of 1 449 characters (including five question marks which were used to separate the two loci but were excluded from the actual analysis and including all alignment gaps) for 101 strains (including the outgroup sequence). The data partitions contained 203 and 457 unique site patterns for LSU and *rpb2*, respectively. The analysis ran for 2 M generations after which it stopped as the average standard deviation of split frequencies reached 0.009815. In total, 40 002 trees were saved after which 30 002 were sampled to calculate the posterior probability (PP) values and the 50 % majority rule consensus tree (Fig. 1). Support values from the maximum likelihood and parsimony analyses are also plotted on the tree (Fig. 1). The sister relationship between *Pedrocrousiella pongamiae* (NFCCI 4881) and *Distocercospora pachyderma* (CBS 138247) was fully to highly supported in all analyses (PP = 1.00 / ML-BS = 98 % / MP-BS = 90 %). The newly sequenced strain was found not to be congeneric with *Asperisporium* (located in the bottom clade of Fig. 1) or any other genus for which sequence data are available, hence a new genus is established below to accommodate it. All three phylogenetic analyses calculated the same clustering for *Pedrocrousiella pongamiae*; only in the parsimony analysis was the relationship between "*Pseudocercospora*" *nephrolepidicola* and *Clypeosphaerella sticheri* unresolved but this is most likely a result of the missing *rpb2* data for the latter species.

Taxonomy

Pedrocrousiella Rajeshkumar, U. Braun & J.Z. Groenew., ***gen. nov.*** MycoBank MB838146.

Etymology: Named after Pedro Crous, director of the Westerdjik Fungal Biodiversity Institute and researcher who established the modern taxonomy and backbone of *Mycosphaerellaceae.*

Classification: *Mycosphaerellaceae, Mycosphaerellales*, Dothideomycetes.

Diagnosis: *Pedrocrousiella* is morphologically indistinguishable from *Asperisporium s. lat.*, but it differs phylogenetically from *Asperisporium s. str.*, determined by its type species, *A. caricae*, by forming a distant lineage.

Conidiomata foliicolous, sporodochial, scattered, olive brown to dark brown, erumpent. *Conidiophores* arising from stromata, densely fasciculate, aseptate or septate, macronematous, mononematous, simple, straight to slightly sinuous, almost smooth to verruculose-rugose, pale brown, wall thin to somewhat thickened. *Conidiogenous cells* integrated, terminal, or conidiophores reduced to conidiogenous cells, cylindrical (geniculation caused by sympodial proliferation not evident), polyblastic, often with numerous conidiogenous loci thickened and darkened (cicatrized). *Conidia* formed singly, broad ellipsoid, ovate or obclavate, 0–2-septate, wall thin, pale olivaceous to olivaceous brown, verruculose, apices obtuse, bases truncated, basal hilum barely to somewhat thickened and darkened, schizolytic.

Type species: *Pedrocrousiella pongamiae* (Syd. & P. Syd.) Rajeshkumar, U. Braun & J.Z. Groenew. (≡ *Fusicladium pongamiae* Syd. & P. Syd.).

Cylindroseptoria ceratoniae CBS 477.69[KF251655.1/KF951419.1]

1/98/100
Xenoramularia arxii CBS 342.49[KX287258.1/KX288720.1]
Xenoramularia polygonicola CBS 141102[GU214695.1/KX288723.1]
Xenoramularia neerlandica CBS 141101[KX287260.1/KX288722.1]
1/100/-
Zymoseptoria passerinii CBS 120382[JQ739843.2/KP894763.1]
Zymoseptoria brevis CBS 128853[JQ739833.1/KX348109.1]
1/100/98
Zymoseptoria tritici CBS 115943[GU214436.1/KX348112.1]
1/100/98
Ramularia endophylla CBS 113265[AY490776.2/KP894673.1]
0.87/87/-
Ramularia nyssicola CBS 127665[KJ504724.1/KJ504636.1]
Ramularia pusilla CBS 124973[KP894141.1/KP894687.1]

1/100/-
Cercospora zeina CBS 118820[MF951147.1/MF951469.1]
Cercospora fagopyri CBS 132623[MF951143.1/MF951463.1]
Cercospora sojina CPC 11353[KX286969.1/KX288419.1]
1/100/99
Cercospora pseudochenopodii CCTU 1038[-/MH511954.1]
1/100/-
Passalora bacilligera CBS 131547[MF951210.1/MF951585.1]
Nothoseptoria caraganae CPC 36563[MT223917.1/MT223693.1]
Nothoseptoria caraganae CPC 36565[MT223918.1/MT223694.1]
0.92/89/-
1/99/-
Pseudophaeophleospora stonei CPC 13330[FJ493210.1/MF951636.1]
Pseudophaeophleospora atkinsonii CBS 124565[MF951236.1/MF951635.1]
1/99/-
Sonderhenia eucalyptorum CBS 120220[DQ923536.1/MF951673.1]
Sonderhenia eucalypticola CBS 112502[KF902019.1/MF951672.1]
Neosonderhenia eucalypti CBS 145081[MN162191.1/MN162578.1]
Neosonderhenia eucalypti CBS 145082[MN162192.1/MN162579.1]
-/94/-
Trochophora simplex CBS 124744[GU253880.1/MF951684.1]
Parapallidocercospora colombiensis CBS 110968[KF901969.1/MF951581.1]
Parapallidocercospora thailandica CBS 120723[KF442667.1/MF951582.1]
0.96/93/-
Scolecostigmina mangiferae CBS 125467[GU253877.1/MF951660.1]
0.96/94/-
Nothophaeocryptopus gaeumannii CBS 244.38[MF951191.1/GU357766.1]
1/100/92
Pallidocercospora heimioides CBS 111190[GQ852607.1/MF951555.1]
0.97/99/-
Pallidocercospora acaciigena CBS 112515[KF902166.1/KX348062.1]
0.93/-/-
Pallidocercospora heimii CBS 110682[GQ852604.1/MF951554.1]
Uwemyces elaeidis CPUwZC-01[KX228356.1/KX228371.1]
1/100/-
Coremiopassalora eucalypti CBS 111306[GU253860.1/MF951481.1]
Coremiopassalora leptophlebae CBS 129524[KF901939.1/MF951483.1]
-/96/-
Pseudocercospora zambiae CBS 136423[KF777228.1/MF951630.1]
Pseudocercospora vitis CBS 132012[GU214483.1/KX348076.1]
0.98/90/-
Pseudocercospora pistacina CPC 23118[KF442674.1/KX348074.1]
Pseudocercospora catappae MUCC 1109[MF951225.1/MF951616.1]
Pseudocercospora robusta CBS 111175[KF442539.1/MF951623.1]
1/98/90
"*Pseudocercospora*" *nephrolepidicola* CBS 128211[HQ599591.1/KX462646.1]
Pedrocrousiella pongamiae gen. et comb. nov.
1/100/82
Distocercospora pachyderma CBS 138247[MF951156.1/MF951486.1]
-/95/-
Clypeosphaerella sticheri CPC 24733[KT037577.1/–]
Clypeosphaerella calotropidis CBS 129.30[MF951153.1/MF951477.1]
0.88/-/92
Clypeosphaerella quasiparki CBS 123243[KF902128.1/MF951478.1]
1/99/-
Xenosonderhenia eucalypti CBS 138858[KP004485.1/MF951688.1]
0.99/81/-
Collapsimycopappus styracis HHUF 30067[NG_064448.1/LC333042.1]
0.99/-/-
Collapsimycopappus styracis KT 2939[LC333034.1/LC333040.1]
0.99/-/-
Paramycosphaerella marksii CBS 110750[DQ303075.1/MF951573.1]
Paramycosphaerella brachystegia CPC 21136[KF777230.1/MF951567.1]
Paramycosphaerella wachendorfiae CBS 129579[JF951163.1/MF951578.1]
1/100/80
Pseudozasmidium eucalypti CBS 121101[KF901931.1/MF951637.1]
0.95/92/-
Pseudozasmidium vietnamense CBS 119974[JF700944.1/MF951639.1]
1/99/-
Pseudozasmidium parkii CBS 387.92[GU214448.1/–]
1/99/-
Mycosphaerelloides madeirae CBS 112895[KF902017.1/KX348057.1]
Mycosphaerelloides madeirae CBS 116066[KX286989.1/KX288444.1]
0.99/-/-
Epicoleosporium ramularioides CPC 10672[GU214688.1/KX288433.1]
1/99/81
Epicoleosporium ramularioides CPC 10673[MF951160.1/KX288434.1]

1/100/-
Zasmidium arcuata CBS 113477[EU041836.1/MF951692.1]
Zasmidium musicola CBS 122479[NG_069906.1/MF951717.1]
1/100/-
Zasmidium cellare CBS 892.85[MF951262.1/KT356875.1]
-/82/-
Zasmidium eucalyptorum CBS 118500[MF951266.1/MF951702.1]
Exutisphaerella laricina CBS 326.52[GU253693.1/MF951496.1]
1/100/90
Rosisphaerella rosicola CBS 138.35[MF951252.1/MF951658.1]
1/100/88
Phaeocercospora colophospermi CBS 132687[JX069854.1/MF951586.1]
0.99/99/-
Pleopassalora sp. CBS 122466[MF951221.1/MF951608.1]
Pleopassalora perplexa CBS 116363[MF951220.1/MF951606.1]
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Phaeoramularia capsicicola CBS 113384[MF951214.1/MF951597.1]
1/100/91
Phaeoramularia gomphrenicola CBS 142182[MF951216.1/MF951599.1]
0.99/98/-
Ragnhildiana perfoliati CBS 125419[GU214453.1/MF951647.1]
1/100/92
Ragnhildiana pseudotithoniae CBS 136442[KF777231.1/MF951652.1]
1/100/97
Ragnhildiana diffusa CBS 106.14[MF951239.1/MF951642.1]
-/99/88
Ragnhildiana ampelopsidis CBS 249.67[MF951238.1/MF951641.1]
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Ragnhildiana ferruginea CBS 546.71[MF951242.1/MF951645.1]
0.93/91/-
Fulvia fulva CBS 142314[MF951163.1/MF951498.1]
1/100/95
Stromatoseptoria castaneicola CBS 102322[KF251774.1/MF951681.1]
1/98/-
Hyalocercosporidium desmodii CBS 142179[MF951168.1/MF951503.1]
1/100/-
Dothistroma pini CBS 116486[JX901823.1/KX348053.1]
Dothistroma septosporum CBS 128782[JX901829.1/KX348054.1]
1/98/-
Neophloeospora maculans CBS 115123[GU214670.1/MF951547.1]
1/100/82
Micronematomyces caribensis CBS 113380[MF951175.1/MF951517.1]
Rhachisphaerella mozambica CBS 122464[MF951237.1/MF951640.1]
1/96/-
Paramycovellosiella passaloroides CPC 10770[MF951209.1/MF951580.1]
0.99/100/-
Neocercosporidium smilacis CBS 556.71[KJ633269.1/MF951535.1]
0.99/95/-
Sultanimyces vitiphyllus CBS 206.48[MF951260.1/MF951683.1]
0.84/-/-
Paracercosporidium microsorum CBS 100352[EU167599.1/MF951561.1]
"*Sirosporium*" *celtidis* CBS 158.25[MF951253.1/MF951669.1]
1/99/-
Collarispora valgourgensis CBS 129531[JF951175.1/MF951479.1]
1/100/97
Cercosporidium californicum CBS 128857[MF951148.1/MF951470.1]
Cercosporidium miurae CPC 14628[MF951150.1/MF951472.1]
Pluripassalora bougainvilleae CBS 142237[MF951224.1/MF951612.1]
0.97/99/-
Nothopassalora personata CBS 222.38[MF951234.1/MF951631.1]
Asperisporium caricae CBS 130298[MF951128.1/MF951437.1]
Asperisporium caricicola CBS 139998[KR611891.1/MF951439.1]
1/100/-
0.85/90/-
Pantospora guazumae CBS 130299[MF951196.1/MF951556.1]
Paracercospora egenula CBS 132030[GU253738.1/MF951557.1]
-/92/-
Pseudocercosporella bakeri CBS 119488[KX287005.1/KX288462.1]
-/96/-
Distomycovellosiella brachycarpa CBS 115124[GU214664.1/MF951492.1]
Amycosphaerella africana CBS 680.95[KF902048.1/MF951426.1]
1/100/93
Amycosphaerella keniensis CBS 111001[GQ852610.1/MF951433.1]
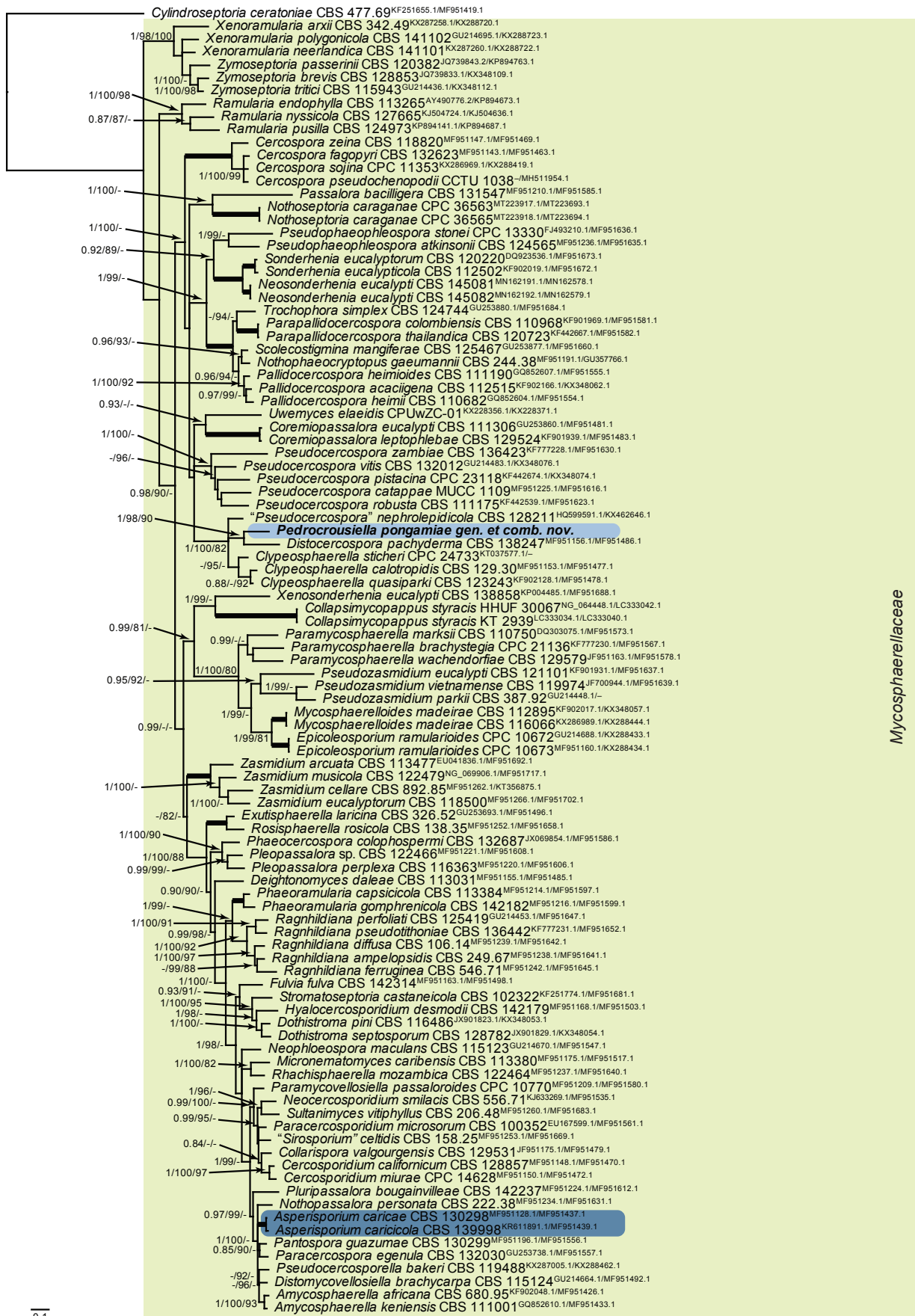
Mycosphaerellaceae

0.1

Fig. 1. Consensus phylogram (50 % majority rule) resulting from a Bayesian analysis of the combined LSU and *rpb2* sequence data of *Mycosphaerellaceae* (reference sequences based on Videira *et al.* 2017). Bayesian Posterior probability (PP) values > 0.79, maximum likelihood branch support values (ML-BS) > 79 % and parsimony bootstrap support values (MP-BS) > 79 % are given at the nodes and thickened branches are fully supported (PP = 1.00 / ML-BS = 100 % / MP-BS = 100 %). The tree is rooted to *Cylindroseptoria ceratoniae* CBS 477.69. The tree is drawn to scale, with branch lengths measured in the expected changes per site. The family is indicated with a large coloured block and the two shades of blue represent the taxonomic novelty described here and the originally reported genus, respectively.

Pedrocrousiella pongamiae (Syd. & P. Syd.) Rajeshkumar, U. Braun & J.Z. Groenew., ***comb. nov.*** MycoBank MB838147. Figs 2–5.
Basionym: *Fusicladium pongamiae* Syd. & P. Syd., *Ann. Mycol.* **11**(4): 328. 1913.
Synonyms: *Passalora pongamiae* (Syd. & P. Syd.) Subram., *Hyphomycetes*: 237. 1971.
Asperisporium pongamiae (Syd. & P. Syd.) Deighton, In Ellis, *More Dematiaceous Hyphomycetes*: 241. 1976.
Asperisporium pongamiae-pinnatae Khawar, A. Kumar, Bhat & C. Nakash., *Vegetos* **25**(1): 336. 2012.

Typus: **India**, Tamil Nadu, Coimbatore, Iruttu Palam, on leaves of *Pongamia pinnata* (= *P. glabra*), Dec. 1909, *C.E.C. Fischer* (**lectotype** designated here, S-F45748, MycoBank MBT395139). **India**, Maharashtra, Pune, Kothrud, on leaves of *Pongamia pinnata*, 23 Jun. 2018, *K.C. Rajeshkumar* (**epitype** designated here AMH 10302, MycoBank MBT395140); culture ex-epitype NFCCI 4881. Ex-epitype sequences: MW327548 (ITS), MW327593 (LSU), MW363496 (*rpb2*).

In vivo: Phytopathogenic, causing leaf spots, amphigenous, colour, shape and size variable, subcircular to irregular, sometimes diffuse, 2–20 mm diam or confluent and larger, sometimes large leaf segments or almost entire leaves discoloured, yellowish green, ochraceous to brownish, reddish brown, later becoming dark brown to blackish brown by the development of abundant sporodochia. *Mycelium* internal. *Stromata* immersed, well-developed, large, to 120 µm diam, medium to dark brown, composed of swollen hyphal cells, 2–7 µm diam, rounded in outline to irregularly shaped. *Conidiomata* sporodochial, mostly hypophyllous, scattered to usually dense, olive brown, dark brown to blackish, erumpent, 50–250 µm diam. *Conidiophores* densely fasciculate, very numerous, arising from stromata, macronematous, mononematous, septate below or conidiophores reduced to conidiogenous cells, unbranched, straight to slightly sinuous, but not geniculate, smooth to verruculose-rugose, pale brown, wall thin or somewhat thick-walled, 35–65 × 2.5–5 µm. *Conidiogenous cells* integrated, terminal, up to about 40 µm long, cylindrical, proliferation sympodial, but not causing any trace of geniculation, polyblastic, usually with numerous, often densely arranged thickened and darkened conidiogenous loci, 1–2 µm wide. *Conidia* formed singly, broad ellipsoid, ovoid, short subcylindrical or obclavate, apices obtuse, bases truncate, 15–30 × 4.5–7 µm, young conidia 10–12.5 × 5–5.5 µm, 0–1(–2)-septate, wall pale olivaceous to olivaceous brown, thin, verruculose, basal hilum slightly thickened and darkened or almost undifferentiated, 0.5–1.5 µm wide, schizolytic.

Colonies on MEA at 25 ± 2 °C after 15 d slow growing, 20–28 mm (40–48 mm after 45 d) diam, dark brown to black, velutinous with umbonate centre, reverse black. Colonies on CMA at 25 ± 2 °C after 15 d 20–25 mm, velutinous, blackish, reverse black.

Additional materials examined: **India**, Tamil Nadu, former Madras Presidency, Malabar District, Chalisseri, on leaves of *Pongamia pinnata*, 10 Jul. 1912, *W. McRay* (S-F45747, syntype); Maharashtra, Pune, Kothrud, on leaves of *Pongamia pinnata*, 9 Sep. 2020, *K.C. Rajeshkumar* RKC-2020; cultures RKC-2020.1 and RKC-2020.2. **Sri Lanka**, Peradeniya, on leaves of *Pongamia pinnata*, Dec. 1913, *T. Petch* [Petrak, Mycoth. Gen. 736] (M); *ibid*. [Syd., Fungi Exot. Exs. 441] (M).

Notes: Scanning Electron Microscopy studies confirmed the erumpent sporodochial nature of the conidiomata, the densely fasciculate conidiophores and ovoid to obclavate conidia having verruculose conidial ornamentation as observed by light microscopy. *Asperisporium pongamiae-pinnatae* (Khawar *et al.* 2012) is undoubted a heterotypic synonym of *A. pongamiae*. Type material of *A. pongamiae-pinnatae* was not available for re-examination, but this species shares *Pongamia pinnata* as type host with *A. pongamiae* and, based on its original description, it is morphologically indistinguishable from *A. pongamiae*.

The fungus on *Pongamia* studied here is not congeneric with any of the genera known from sequence data. The blast results presented here do not provide any conclusive placement for this species, while the phylogenetic study places it in a clade (PP = 1.00 / ML-BS = 100 % / MP-BS = 82 %) containing *Clypeosphaerella*, *Distocercospora*, and "*Pseudocercospora*" *nephrolepidicola*, a species which is not congeneric with *Pseudocercospora*. One can choose to apply a single generic name to this whole clade. *Distocercospora* is a genus from 1988 (Pons & Sutton 1988), *i.e.*, much older than *Clypeosphaerella* (Guatimosim *et al*. 2016), and would therefore be a candidate genus name for the whole clade, including "*Pseudocercospora*" *nephrolepidicola*. In this study, we follow the generic concepts of Videira *et al*. (2017) who recognized *Clypeosphaerella* and *Distocercospora* as different genera such as the sufficient phylogenetic distance between these genera and strong morphological differences of the asexual morphs. *Distocercospora* is phylogenetically much closer to *Pedrocrousiella* than *Clypeosphaerella*, and could therefore be a candidate genus for the fungus on *Pongamia* studied here. The ITS, LSU and *rpb2* sequences of *Pedrocrousiella* and *Distocercospora* are only 83 % (372/449, including 25 gaps; GenBank NR_156369.1), 96 % (694/724, including one gap; GenBank NG_059178.1) and 82 % (712/866, no gaps; GenBank MF951486) similar. However, given that the morphology of *Distocercospora* is very different from the pongam fungus (non-sporodochial, loosely fasciculate, frequently branched, long conidiophores, distoseptation of conidia, *etc.*), and that they are (phylo)genetically different, we believe the introduction of a new genus for this species is warranted. Numerous examples where strains are either considered to belong to the same or different genera exist across the phylogenetic tree presented here (Fig. 1) and thus branch length alone is not a good criterion to judge generic affinity in *Mycosphaerellaceae*.

Induced sporulation studies on MEA and CMA media using asymptomatic host tissues (*Pongamia pinnata*) and carnation leaves were unsuccessful to prove the putative asexual-sexual morph connection between *Pedrocrousiella pongamiae* and *Mycosphaerella pongamiae* postulated by Sivanesan (1985). Cultures did not sporulate even after 45 d of incubation at 25 ± 2 °C. For this reason, we prefer to maintain *M. pongamiae* as a separate species as indicated below for now:

Stigmatea pongamiae Racib., *Parasit. Alg. Pilze Java's* **3**: 36. 1900.
Synonyms: *Spilosticta pongamiae* (Racib.) Bat. & Peres, *Portug. Acta Biol., Sér. B*, **7**(1): 26. 1960.
Mycosphaerella pongamiae (Racib.) Sivan., *Trans. Brit. Mycol. Soc*. **84**(3): 363. 1985.

Syntypes: **Indonesia**, Java, Noesa, Kanbangan, on *Pongamia pinnata* (= *P. glabra*), 1899 (KRA-F-1899-124, KRA-F-1899-125); *ibid*., 1900 (KRA-F-1900-45); *ibid.*, undated (KRA-F-0-2103).
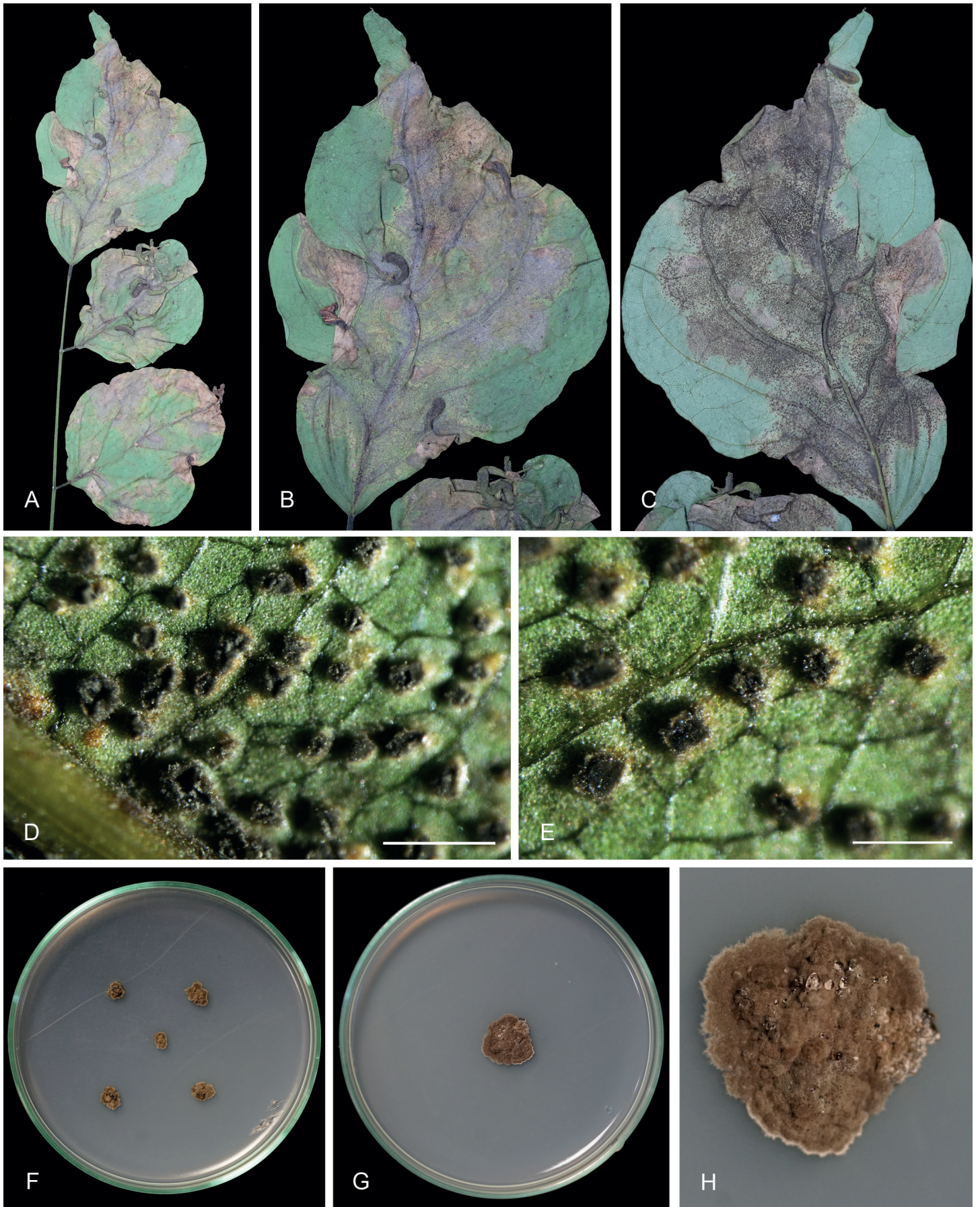
Fig. 2. *Pedrocrousiella pongamiae* (AMH 10302 – epitype). **A–C.** Symptoms on the upper and lower surface of the host leaves, *Pongamia pinnata*. **D, E.** Sporodochial development on abaxial surface. **F.** Colonies on MEA after 15 d. **G, H.** Colonies on MEA after 45 d. Scale bars = 500 μm.
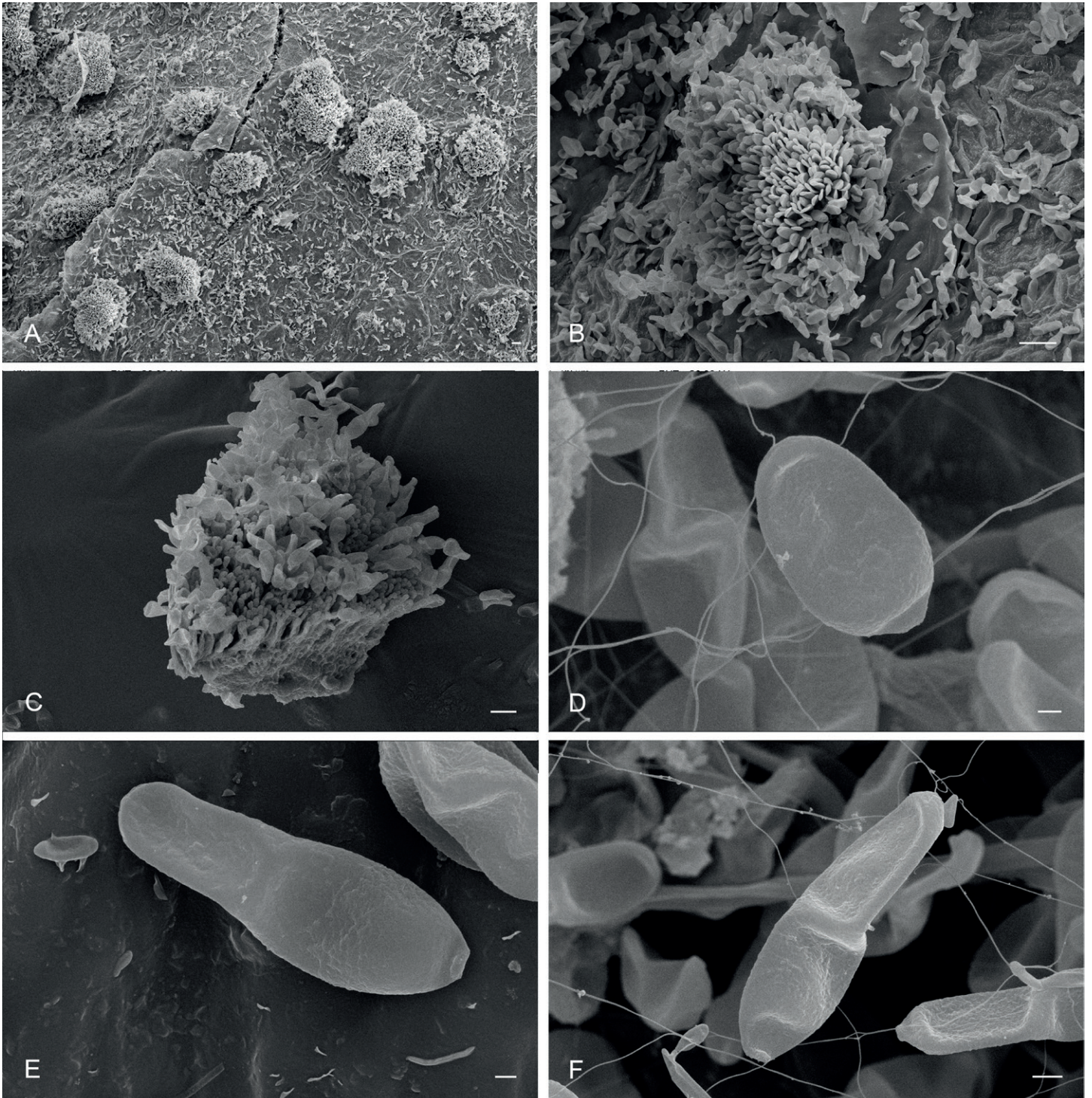
Fig. 3. *Pedrocrousiella pongamiae* (AMH 10302 – epitype). **A, B.** Scanning Electron Micrograph of sporodochia on abaxial surface of leaves. **C.** Section through sporodochia showing conidiophores. **D.** Young ovoid detached conidium with truncate base. **E, F.** Mature obclavate conidia. Scale bars: A, B = 20 µm, C = 10 µm, D–F = 1 µm.

DISCUSSION

There are 24 species names assigned to *Asperisporium* (https://www.mycobank.org, queried 23 November 2020). *Asperisporium caricae*, the type species of this genus, is responsible for an important leaf and fruit spot disease of *Carica papaya* [papaw or papaya] (Stevens 1939) that is commonly referred to as black spot, blight or 'rust' of pawpaw (Ellis & Holliday 1972, Minnis *et al.* 2011). A comprehensive treatment of *A. caricae*, including lecto- and epitypification with ex-epitype sequences (ITS and LSU), was published by Minnis *et al.* (2011). Currently, the type

species of *Asperisporium* is placed in a well-supported clade, closely related to *Amycosphaerella* and *Paramycovellosiella* (clade 13 *sensu* Videira *et al.* 2017). Videira *et al.* (2017) also pointed out the necessity of reassessing every species assigned to *Asperisporium*.

Phylogenetic analyses based on the LSU and *rpb2* sequence data retrieved from the ex-epitype culture of *Asperisporium pongamiae*, a leaf-spotting cercosporoid ascomycete on the economically important, variously utilised pongam oil tree, revealed that this fungus constitutes a lineage closely allied to *Distocercospora*, *Clypeosphaerella* and "*Pseudocercospora*"

Fig. 4. *Pedrocrousiella pongamiae* (AMH 10302 – epitype). **A, B.** Sporodochia. **C, D.** Conidiophores with cicatrised conidiogenous cells and attached conidia. **E.** Densely fasciculate conidiophores. **F.** Young broad, ellipsoid, aseptate conidium. **G, H.** Mature, obclavate, 1–2-septate conidia. **I–K.** Conidial variation. Scale bars = 10 μm.
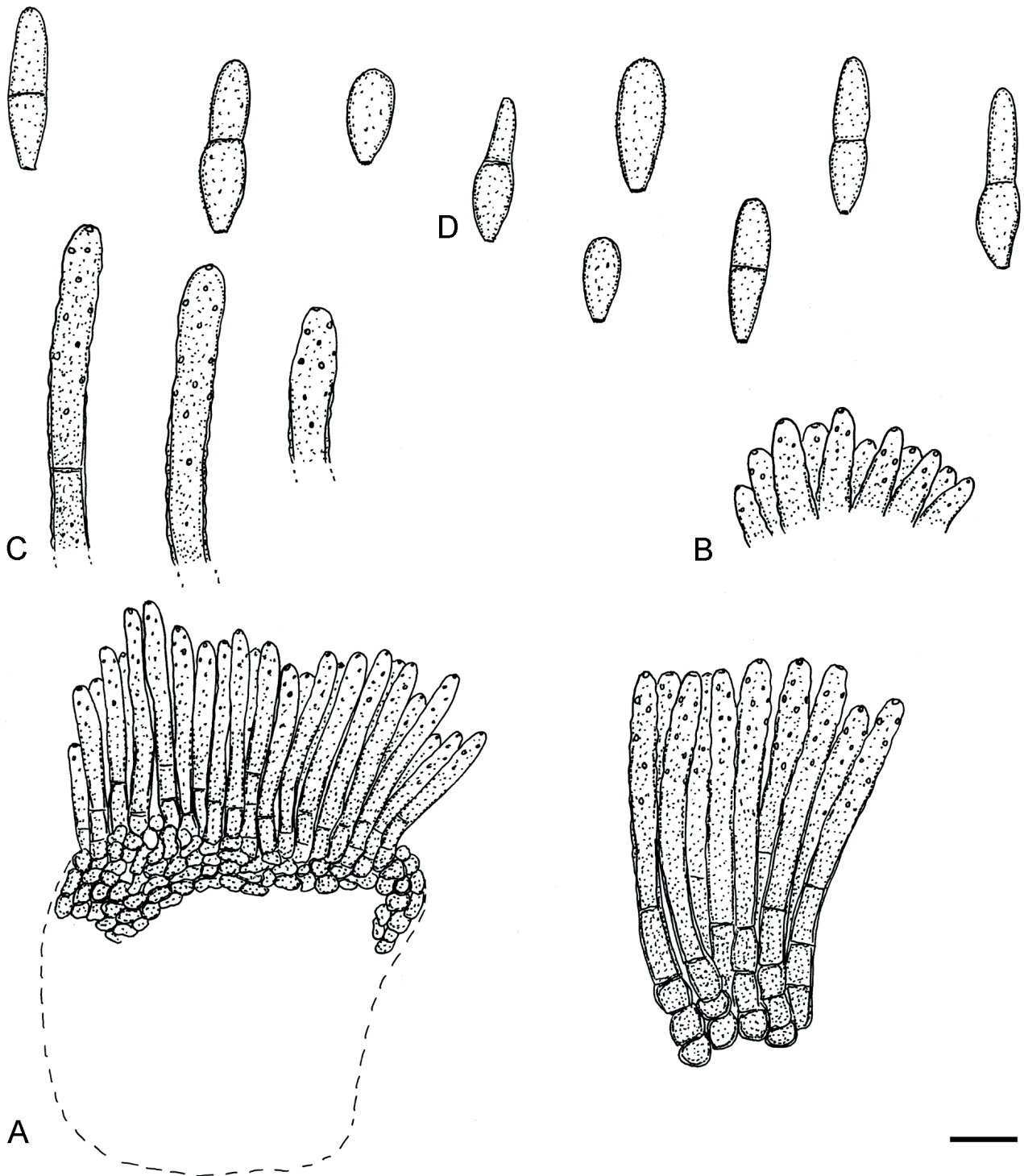
Fig. 5. *Pedrocrousiella pongamiae* (Syd., Fungi Exot. Exs. 441 – M). **A.** Section through a sporodochium**. B.** Fasciculate conidiophores. **C.** Apical part of conidiophores. **D.** Conidia. Scale bar = 10 μm. U. Braun *del*.

nephrolepidicola, a species which does not pertain to *Pseudocercospora* as currently circumscribed on the basis of phylogenetic data (also see discussion in Nakashima *et al.* 2016), but quite distant from the *Asperisporium* (*s. str.*) clade. *Asperisporium pongamiae* is morphologically indistinguishable from previous broad concepts of *Asperisporium s. lat.*, just based on morphology. However, the phylogenetic position of *A. pongamiae*, quite distant from the *Asperisporium s. str.* clade, does now allow to maintain this species in the latter genus. These results justify the introduction of a new genus for this lineage, *viz.*, *Pedrocrousiella*. The reasons for this decision, above all the differentiation against *Distocercospora*, is discussed above in

the taxonomy section under notes. *Fusicladium pongamiae* (≡ *Asperisporium pongamiae*), the name of a common cercosporoid ascomycete on pongam oil tree, is available for the leaf spot disease examined and used as type species of the new genus. *Asperisporium pongamiae-pinnatae*, described from India on *Pongamia pinnata*, is morphologically indistinguishable from *A. pongamiae* and was erroneously introduced on the basis of the wrong assumption that *A. pongamiae* was originally described on another host, *Pongamia globosa*.

A special still unresolved problem concerns the relation between *Asperisporium pongamiae* and *Mycosphaerella pongamiae*. Sivanesan (1985) considered *A. pongamiae* the

asexual morph of *M. pongamiae*. However, this putative connection was just based on the occurrence of the two morphs together on leaf lesions in some collections, but it has never been verified in culture or by molecular data. All attempts to induce the formation of a sexual morph in cultures of *A. pongamiae* during the course of the present examinations failed. It remains unconfirmed whether there is any relation between the two morphs. A connection can currently not be completely ruled out. However, even in the event that the two morphs would be part of the life cycle of a single species, there is no automatism to use the older sexual morph-typified name for the naming of the species concerned. The ICNafp provides sufficient tools to maintain more appropriate, commonly used names, particularly in case of pleomorphic fungi. Above all in the phylogeny and taxonomy of cercosporoid fungi, the asexual morphs play a much greater role compared to the less significant sexual morphs (Crous *et al*. 2019). For the time being, based on the unproven connection between the two morphs, we prefer to retain *M. pongamiae* as a separate species.

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