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ORIGINAL ARTICLE

Fungi from leaves of lotus (*Nelumbo nucifera*)

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Abstract In spite of the self-cleaning property of its leaves called the lotus effect, leaves of lotus (*Nelumbo nucifera*) provide a habitat for an unknown fungal diversity. The aim of this study was to detect and identify fungi from leaves of *N. nucifera*, including ectophytic, parasitic and endophytic fungi, in Taiwan using different collection strategies, as well as morphological and diverse molecular markers established in the different systematic groups of fungi. Among ectophytic and parasitic fungi, a new species of *Dissoconium* and of *Pseudocercospora* are described, respectively. *Phyllosticta nelumbonis* Sawada is transferred to *Diaporthe*. Among plant parasitic fungi, *Erysiphe takamatsui* and *Ps. nymphaeacea* are recorded in Taiwan for the first time. *Euryale* is recorded as a new host genus for *Ps. nymphaeacea*. The basidiomycetous yeast *Fereydounia khargensis* is recorded for the first time from living plants and in East Asia. Endophytic fungi from lotus were studied for the first time. From 1002 plant segments, 476 endophytic isolates were produced in culture, comprising 33 typical terrestrial species mainly belonging to the genera *Colletotrichum* (mainly *C. siamense*), *Diaporthe* (*D. tulliensis* and *D. ueckerae*) and *Fusarium* (*F. solani* species 6, hitherto known from clinical samples), as well as to Xylariaceae, but no Ingoldian fungi. Most isolates were from leaf laminas (71%) compared to those from petioles (29%). From this observation, we conclude that the fungi of the aquatic lotus plant appear to have terrestrial origin and, after dispersal by wind and in spite of the lotus effect, may enter the plant from the lamina. Only three species isolated as endophytes were also found as ectophytic or parasitic fungi.

Keywords Aquatic fungi · Capnodiales · Diaporthales · Erysiphales · Hypocreales

Introduction

Lotus (*Nelumbo nucifera* Gaertn.) is an ornamental and edible plant in East Asia. The rhizome is used as a vegetable; its starch is isolated and used as a food ingredient. Parts of the leaves, flowers and fruits are also made into food and medicinal products, such as tea and noodles. Lotus is an economically important plant being used for thousands of years (Zhao 2010) and was introduced to Taiwan in the seventeenth century (Hsueh and Yang 2016). The lotus plant is the symbol of purity in several Asian countries. The major reason for this symbolism might be the self-cleaning property of its leaves. The lotus effect means that the adaxial side of lotus leaf lamina has a very high water repellence and clean surface. Due to a complex microstructure made of papillate epidermis cells and nanostructure of waxy material on the surface, water on the leaf surface turns into distinct water droplets running off from the leaf and removing dirt particles and cells of microorganisms (Barthlott and Neinhuis 1997). Bionic application of the lotus effect led to the development of new coatings, paints and roof tiles with water-repellant time- and cost-saving

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properties, and cleaning agents become almost redundant (Forbes 2005).

While in previous botanical classifications *Nelumbo* was united with other aquatic plants into the Nymphaeaceae (Nymphaeales), *Nelumbo* has since been separated into an own family Nelumbonaceae (Proteales). The similar morphologies in both plant families are now considered convergent adaptations within distantly related groups of angiosperms in the case of Nymphaeaceae belonging to magnoliids ("primitive dicots") and of Nelumbonaceae to eudicots (Simpson 2010).

About 25 species of mainly plant parasitic fungi have been recorded to successfully colonise not only the rhizome but also the leaves of lotus (Table 1). These records show clear ecological separation of fungi from the persisting rhizome submersed in mud [*Fusarium oxysporum* species complex, *F. tricinctum*(Corda) Sacc., *Ilyonectria radicicola* (Gerlach & L. Nilsson) P. Chaverri & Salgado, species of Pythiaceae] and from the annual leaves that are exposed to wind and sun (almost all other species in Table 1). Some of these species records, however, need to be revised according to modern standards.

Compared to much more than 100 fungi known to be associated with the aquatic weeds *Eichhornia* spp. (Pontederiaceae), water hyacinths, only about 25 fungi are known from lotus (Farr and Rossman 2017). In contrast to water hyacinths (Almeida et al. 2015), lotus has not been subjected to the study of endophytic fungi. The purpose of this study was to detect and identify fungi from leaves of *N. nucifera*, including ectophytic, parasitic and endophytic fungi, in order to address the fungal biodiversity associated with leaves of this plant. The term "endophytic" is applied as in other recent publications (Delaye et al. 2013; Ibrahim et al. 2016; Kirschner 2017; Lledó et al. 2015; de Oliveira et al. 2016; Su et al. 2016; Tateno et al. 2015). Since the parasitic nature of many fungi associated with leaf lesions has not been proven and in order to distinguish them linguistically from endo- and ectophytic fungi, the term "ectendophytic" may be more appropriate (Kirschner 2017).

Materials and methods

Collection

Collections of lotus leaves (leaf blade with petiole) were made in northern, central and southern Taiwan, covering the subtropical and tropical areas of the island. Collection places were Taipei Botanical Garden in Taipei City, Jhongli, Guanyin and Taoyuan Districts in Taoyuan City, Taichung Botanical Garden and Nantun District in Taichung City, Baihe District in Tainan City and Kaohsiung Museum of Fine Arts in Kaohsiung City. The specimens from Tainan were collected by Chee-Jen Chen and some from Taichung by Siou-Zhen Chen and sent by post. Because of the annual leaf development with mature leaves available mainly in summer and autumn, collections were sampled using an opportunistic strategy rather than at regular intervals; particularly parasitic fungi were most conspicuous at the end of the growing season in October and November. Samples were individually placed in bags, returned to the laboratory and kept at ca. 8 °C until further processing.

Isolation and cultivation of fungal strains

Leaf blades were directly and randomly observed for the presence of ectophytic and parasitic fungi with a dissecting microscope, particularly areas with obvious lesions. Spores were transferred from sporulating structures with a flamed acupuncture needle to 1.3% malt extract agar (MEA, Fluka) or corn meal agar (CMA, Fluka) with 0.2% chloramphenicol (Sigma). Healthy leaf fragments were fixed downwards beneath the lid of Petri dishes containing the same medium in order to allow forcibly discharged spores to fall onto the agar surface. For one yeast species (*Fereydounia khargensis*), ballistospore production was tested using 2-day-old colonies on CMA inverted above empty CMA plates for 7 days at room temperature (Kurtzman et al. 2011).

For investigating leaf endophytic fungi, the lamina and petiole were separated. The ca. 70 cm long petiole was divided into three fragments (upper, middle and lower parts). The lamina was cut into four radial sections, followed by cutting off three fragments from each radial section along the radius. Then, the cut margins of leaf lamina (5 × 5 cm) and petiole (5 cm) fragments were sealed with molten wax in order to prevent penetration of disinfection agents into the aerenchyma. These sealed margins were cut off and discarded after surface sterilisation. For isolation of endophytic fungi, samples were processed as in Yeh and Kirschner (2014), including use of the imprint technique in order to verify the effectiveness of the surface sterilisation. Localisation and size of fragments, as well as the procedures for surface sterilisations of leaf blades and petioles, are shown in Fig. 1. All isolates obtained from each plant sample on MEA were classified according to their morphological appearance into morphotypes. For identification of *Alternaria* strains, the method of culturing on potato carrot agar (PCA) as recommended by Simmons (2007) was used. Representative isolates were deposited at the Bioresource Collection and Research Center, Hsinchu, Taiwan (BCRC).

Morphology

Fungi were directly observed from preparations from the leaves by transversal leaf sections made by hand and by using transparent tape fixed onto the plant surface and transferred to

Table 1 Revised list of fungal species on *Nelumbo nucifera* from different locations reported in the literature (with reference) and in this study (in **bold**)

Species	Growth	Location	Reference
***Arthrinium rasikravindrae* Shiv M. Singh et al.**	**end**	**Taiwan**	**This study**
***Alternaria alternata* (Fr.) Keissl.**	**end**	**Taiwan**	**This study**
	ectend	India	Farr and Rossman (2017)
Alternaria caudata (Cooke & Ellis) E.G. Simmons (*A. nelumbicola* T.Y. Zhang)	ectend	China	Zhang and Gao (2000)
Botrytis cinerea Pers.	ectend	U.S.A.	Farr and Rossman (2017)
	ectend	Japan	Katumoto (2010)
Calonectria hawksworthii (Peerally) L. Lombard et al. (= *Cylindrocladium hawksworthii* Peerally)	ectend	Mauritius	Crous (2002)
Calonectria reteaudii (Bugnic.) C. Booth	ectend	Mauritius	Crous (2002)
Cercospora apii Fresen. s. lat. (= *C. californiensis* Chupp)	ectend	China	Guo et al. (2005)
Choanephora infundibulifera (Curr.) D.D. Cunn.	ectend	Japan	Katumoto (2010)
Cladosporium oxysporum Berk. & M.A. Curtis	ectend	Hong Kong	Farr and Rossman (2017)
	ectend/end	**Taiwan**	**This study**
Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. s. lat. (= as *Gloeosporium nelumbii* Tassi)	ectend	China	Farr and Rossman (2017)
	ectend	Hong Kong	Farr and Rossman (2017)
	ectend	Japan	Katumoto (2010)
	ectend	Italy	Saccardo (1902)
	ectend	Japan	Nisikado and Watanabe (1953)
	ectend	Korea	Farr and Rossman (2017)
***Colletotrichum siamense* Prihast., L. Cai & K.D. Hyde**	**ectend/end**	**Taiwan**	**This study**
***Colletotrichum tropicale* E.I. Rojas, S.A. Rehner & Samuels**	**end**	**Taiwan**	**This study**
Corynespora cassiicola (Berk. & M.A. Curtis) C.T. Wei	ectend	Japan	Katumoto (2010)
Curvularia lunata (Wakker) Boedijn	ectend	China	Cui and Sun (2012)
Diaporthe nelumbonis Sawada ex R. Kirschner (*Phyllosticta nelumbonis* Sawada)	ectend	Taiwan	Sawada (1959), **this study**
***Diaporthe ueckerae* Udayanga & Castl.**	**end**	**Taiwan**	**This study**
***Dissoconium nelumbonis* R. Kirschner & Kuan L. Chen**	**ect**	**Taiwan**	**This study**
Dothiorella nelumbii Ellis & H.W. Anderson	ectend	USA	Saccardo (1892)
Dothiorella woronowii Petr. (*Macrophoma nelumbii* Siemaszko)	ectend	Georgia (Caucasus)	Siemaszko (1923)
Erysiphe magnifica (U. Braun) U. Braun & S. Takam.	ect	Germany	Kirschner (2010)
Erysiphe takamatsui Y. Nomura	ect	Japan	Meebon and Takamatsu (2015)
	ect	**Taiwan**	**This study**
Fereydounia khargensis S. Nasr, M.R. Soudi, H.D.T. Nguyen, M. Lutz & Piątek	**ect**	**Taiwan**	**This study**
Fusarium oxysporum species complex	ectend; rh	Japan	Nisikado and Watanabe (1953)
***Fusarium solani* species complex, species 6**	**end**	**Taiwan**	**This study**
Fusarium tricinctum (Corda) Sacc.	ectend; rh	China	Li et al. (2016)
Globisporangium proliferum (Cornu) P.M. Kirk	ectend; rh	Malaysia	Farr and Rossman (2017)
Globisporangium spinosum (Sawada) Uzuhashi, Tojo & Kakish.	ectend; rh	Japan	

Table 1 (continued)

Species	Growth	Location	Reference
			Takahashi et al. (1965)
***Golubevia pallescens* (Gokhale) Q.M. Wang, F.Y. Bai, Begerow & Boekhout**	**ect**	**Taiwan**	**This study**
Ilyonectria radicicola (Gerlach and L. Nilsson) P. Chaverri & Salgado	ectend; rh	Japan	Nisikado and Watanabe (1953)
Thelonectria mammoidea (W. Phillips & Plowr.) C. Salgado & R.M. Sanchez (*Nectria nelumbicola* Henn.)	ectend	Germany	Hennings (1899)
***Nigrospora oryzae* (Berk. & Broome) Petch**	**end**	**Taiwan**	**This study**
Ovulariopsis eliadei Negru	ect	Romania	Braun and Cook (2012)
Pestalotiopsis nelumbonis Y.X. Chen & G. Wei	ectend	China	Ge et al. (2009)
Phoma nelumbii Cooke & Massee	ectend	Taiwan	Sawada (1959)
	ectend	UK	Cooke (1888)
Physoderma nelumbii Mishra & Thirum.	ectend	India	Thirumalachar and Mishra (1953)
Phytopythium helicoides (Drechsler) Abad et al.	ectend; rh	China	Yin et al. (2016)
Phytopythium ostracodes (Drechsler) Abad et al.	ectend; rh	Japan	Takahashi et al. (1965)
Pseudocercospora nelumbonis R. Kirschner & Kuan L. Chen	ectend	China?	Chi (1994, *Ps. nymphaeacea*)
	ectend	**Taiwan**	**This study**
	ectend	Thailand	Meeboon (2009, *Ps. nymphaeacea*)
Pythium afertile Kanouse & T. Humphrey	ectend; rh	Japan	Katumoto (2010)
Pythium nelumbii M. Takah. & Ouchi	ectend; rh	Japan	Takahashi et al. (1965)
***Rhodosporidiobolus odoratus* (J.P. Samp. A. Fonseca & E. Valério) Q.M. Wang et al.**	**ect**	**Taiwan**	**This study**
Sclerotium hydrophilum Sacc.	ectend	Japan	Katumoto (2010)

Growth in the plant indicated as: ect = ectophytic, end = endophytic, ectend = ectendophytic/parasitic (rh = in the rhizome, submersed; otherwise in emersed plant organs)

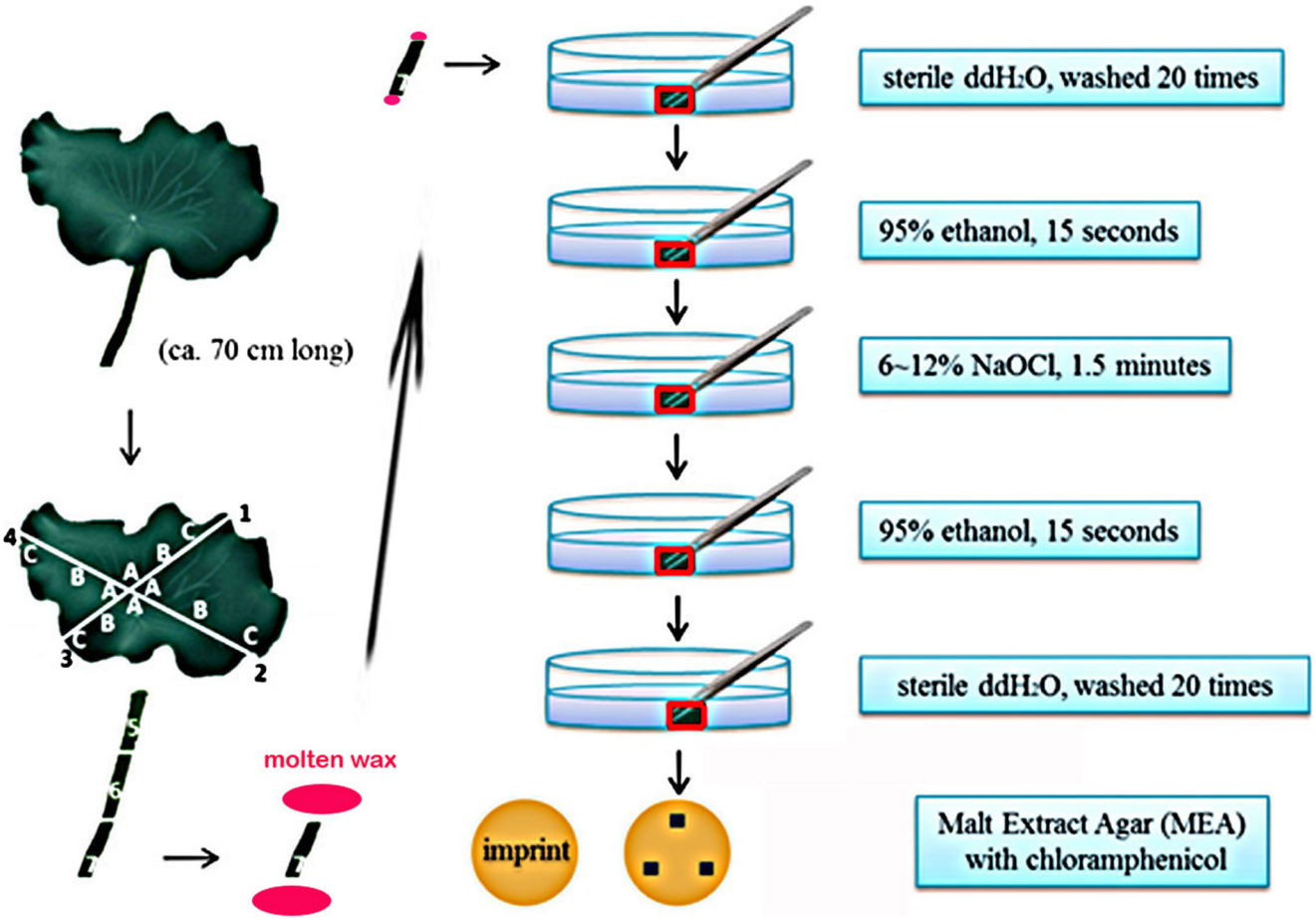

Fig. 1 Diagrammatic representation of the process of dividing and surface sterilisation of leaf lamina and petiole of leaves of *Nelumbo nucifera*

5–10% (w/v) aqueous KOH solution with or without 1% phloxine for observation with light microscopy. The same mounting medium was used for specimens from cultures. Statistical treatment of measurements was based on *n* replicates and given as the mean value ± standard deviation, with extreme values in brackets. Dried specimens were deposited at the herbarium of the National Museum of Natural Science, Taichung, Taiwan (TNM).

DNA analysis

For DNA isolation, polymerase chain reaction (PCR), sequencing and sequence editing, the methods described by Yeh and Kirschner (2014), were used. DNA sequences of the internal transcribed spacer regions (ITS)and/or ribosomal large subunit RNA gene (LSU rDNA) were generated for a preliminary identification. Additionally, the primer pair TUB2Fd/TUB4Rd was used for amplification of the beta-tubulin gene (*TUB*; Groenewald et al. 2013), particularly for the identification of *Colletotrichum* species (Damm et al. 2012; Weir et al. 2012) and *Diaporthe* species (Gomes et al. 2013; Udayanga et al. 2015). For the *Fusarium solani* species complex, partial sequences of the gene coding for the elongation translation factor 1 alpha (*TEF1A*) were generated according to Bills et al. (2009). For *Pseudocercospora* species, sequences of the gene for the DNA-directed RNA polymerase II second largest subunit (*RPB2*) were applied (Nakashima et al. 2016). Sequences of the ITS, the LSU rDNA and the protein genes were searched using the BLAST function of GenBank. For phylogenetic analysis of selected strains, nucleotide sequences were selected according to the BLAST search results and recent publications dealing with particular genera and aligned using the default options of MUSCLE implemented in MEGA6 without manual editing except for truncating the ends. The sequences in the alignments of *Diaporthe* were from Gomes et al. (2013), Shivas et al. (2015) and Udayanga et al. (2015), of *Dissoconium* from Crous et al. (2008) and Li et al. (2012), of *Fusarium* species mostly from O'Donnell et al. (2008), with additional sequences from Schroers et al. (2016) and Short et al. (2011), and of *Pseudocercospora* with *Ps. vitis* (Lév.) Speg. [= *Phaeoisariopsis vitis* (Lév.) Sawada] as the outgroup, all from Nakashima et al. (2016). The evolutionary history was inferred by using the maximum likelihood method with the default options of MEGA6 (Tamura et al. 2013). In *Diaporthe* and *Dissoconium*, the Tamura 3-

parameter(Gamma) model was used, and in the *Fusarium solani* species complex and *Pseudocercospora*, the Kimura 2-parameter(Gamma) model was used. Node support was estimated with 1000 bootstrap replications. GenBank numbers are given after the species names in the unrooted trees shown in Figs. 3, 5 and 9 and Supplemental Material 2. DNA sequence accession numbers of strains/specimens are also given in the text below and in Supplemental Material 1.

Results

The 476 endophytic isolates could be divided into 33 morphospecies (Supplemental Material 1) based on colony macromorphology and ITS sequence comparison. Critical species were further identified using protein gene sequences. Most isolates were from leaf blades (71%) compared to those from leaf petioles (29%). The four most frequently isolated species were represented by more than five strains (1% of 476 strains): *Fusarium solani* (Mart.) Sacc. species complex (46 strains, 9.7%, represented by species 6, see Supplemental Material 2), *Nigrospora oryzae* (Berk. & Broome) Petch (22 strains, 4.6%), *Xylaria* cf. *curta* Fr. (16 strains, 3.4%) and *Annulohypoxylon* cf. *stygium* (Lév.) Y.M. Ju, J.D. Rogers & H.M. Hsieh (10 strains, 2.1%). Species of *Diaporthe* were also comparatively frequent, but because they could not be unequivocally separated from each other by morphology and ITS sequences, they are not included in this list of the most common species. The majority of strains remained sterile in culture, but were identified as far as possible according to DNA barcodes. Three species represented by seven endophytic strains were assigned to the *Bipolaris–Curvularia* complex, but were lost before they could be identified further. The same failure happened with two *Penicillium* species represented by four endophytic strains and few other species each represented by a single strain, which are, therefore, not included in Supplemental Material 1.

Eleven species of ectophytic and parasitic (ectendophytic) fungi were identified as *Alternaria alternata* (Fr.) Keissl., *Cladosporium oxysporum* Berk. & M.A. Curtis, *Colletotrichum siamense* Prihast., L. Cai & K.D. Hyde, *Co. tropicale* E.I. Rojas, S.A. Rehner & Samuels, new species of *Diaporthe* and *Dissoconium*, *Erysiphe takamatsui* Y. Nomura, *Fereydounia khargensis* S. Nasr, M.R. Soudi, H.D.T. Nguyen, M. Lutz & Piątek, a new species of *Pseudocercospora*, *Rhodosporidiobolus odoratus* (J.P. Samp. A. Fonseca & E. Valério) Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout and *Golubevia pallescens*(Gokhale) Q.M. Wang, F.Y. Bai, Begerow & Boekhout (Table 1). Three of these species were also found as endophytic fungi (*Alternaria alternata*, *Cladosporium oxysporum* and *Colletotrichum siamense*). New species as well as new records are arranged according to their growth habitus (ecto-, endophytic or parasitic hyphal fungi or yeasts) and characterised in detail below. All species are listed in Table 1 and Supplemental Material 1.

Taxonomy of new and newly recorded taxa

Parasitic and endophytic *Diaporthe* species

Diaporthe nelumbonis Sawada ex R. Kirschner, **sp. nov.** Fig. 2.

MycoBank: MB821926.

≡ *Phyllosticta nelumbonis* Sawada, Descriptive Catalogue of Taiwan (Formosan) Fungi 11, Special Publication College of Agriculture, National Taiwan University 8: 140 (1959), nom. inval.

Leaf spots irregular-circular, 1–2 cm diam. (0.5–5 cm; Sawada 1959), pale brown with 3–5 darkly bordered zones being more conspicuous on the adaxial than on the abaxial leaf side. Pycnidia immersed in the upper layer of mesophyll, separate, slightly applanate, unilocular, ca. 55–87 μm high and 80–125 μm wide. Pycnidial wall brown, pseudoparenchymatous, ca. 10 μm thick, at the base thickened to ca. 20 μm, composed of ca. 3 rows of 2–6 μm wide cells, at the centre of the base more than 3 rows. Ostiole single, non-papillate, circular, ca. 20–30 μm wide. Paraphyses absent. Conidiophores formed by the inner cells of the pycnidial wall, reduced to the conidiogenous cell or with a separate basal cell that often turns into an intercalary conidiogenous cell, mostly simple, rarely two conidiogenous cells arising from the same basal cell of the conidiophore. Conidiogenous cells terminal or intercalary, pyriform to obclavate or lageniform, apex conspicuously narrowed, sometimes with minute periclinal thickening, without collarette, (3–)4.5–7.5(−9) × 2–3 μm (n = 30) in the type, in R. Kirschner 4114 (6–)6.5–10(−11) × (1.5–)2–3 (n = 20). Alpha-conidia hyaline, aseptate, oblong-ellipsoidal, straight or slightly curved, rounded at the apex, attenuated towards the base, mostly with two large guttules which, in some cases, appear to be divided into smaller guttules, (6–)6.5–8(−9) × 2–2.5 μm (n = 30) in the type, in R. Kirschner 4114 (5–)6–7 × (1.5–)2 μm (n = 30), β-conidia not observed. Teleomorph not observed. Only sterile mycelium produced in culture.

Specimens examined: On leaves of *Nelumbo nucifera*, TAIWAN: Taipei, 8 Jul. 1920, E. Kurozawa (as *Phyllosticta nelumbonis* Sawada; BPI 352726, **holotype designated here for*D. nelumbonis***; PPMH, **isotype designated here for*D. nelumbonis***), Taoyuan, Jhongli, National Central University campus, 27 Oct. 2014, R. Kirschner 4114 (TNM, **reference specimen designated here**; culture BCRC FU30382, **reference strain designated here**, ITS KT821501, *TUB* LC069368), same place, 14 Nov. 2014, R. Kirschner 4126 (TNM); Taoyuan District, Taoyuan stadium, 7 Feb. 2015, R. Kirschner 4162 (TNM, *TUB* LC086652).

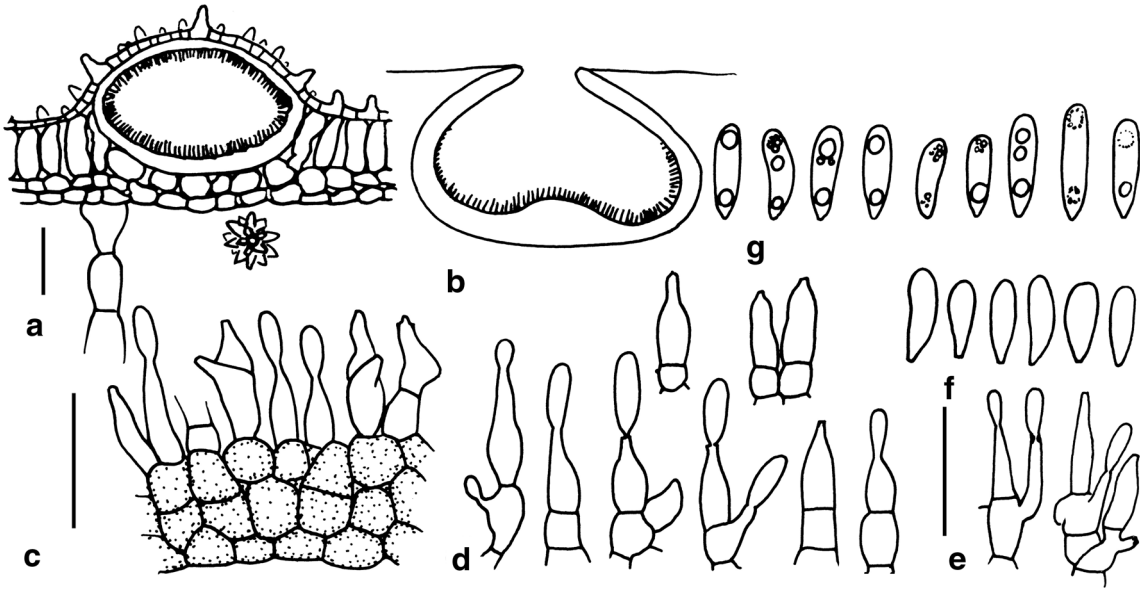

Fig. 2 *Diaporthe nelumbonis* (**b**–**d**, **g** type, BPI 353736; **a**, **e**, **f** R. Kirschner 4114): **a**, **b** habitus of conidiomata in transversal leaf sections, **a** showing the adaxial epidermis and cells and a crystal of the mesophyll, **b** schematically indicating the outline of an opened subepidermal conidioma and arrangement of conidiophores, **c** detail of conidioma wall with conidiophores, **d**, **e** conidiophores, **f**, **g** conidia. Scale bars: **a**, **b** = 25 μm, **c** = 10 μm, **d**–**g** = 10 μm

Notes: The sizes of pycnidia, ostioles and conidia conform to those given in the brief description of *Phyllosticta nelumbonis* by Sawada (1959). van der Aa and Vanev (2002) stated that the morphology described by Sawada (1959) does not agree with the concept of *Phyllosticta* and that, by the lack of description of conidiophores, a placement in another genus was not possible. With more complete knowledge of the morphology, the fungus can be accommodated in *Diaporthe*. Its applanate, unilocular pycnidia without papillate ostiole, and particularly the lack of β-conidia distinguishes this species from most other species of the genus. This combination of characteristics, however, also occurs in other *Diaporthe* species, particularly in leaves (e.g. Chi 1994; Punithalingam 1975; Udayanga et al. 2015). Since we merely validate the taxon described by Sawada, we refer to his specimens as types. Ariyawansa et al. (2014) challenged the widespread misapplication of epitypification in the mycological literature, proposed strict guidelines (such as multigene analyses) and suggested designating "reference specimens/strains" for cases when these guidelines cannot be applied. We follow these suggestions by designating specimen/strain R. Kirschner 4114 as the reference.

The ITS sequences of *D. nelumbonis* differed at least for 6 positions from sequences of other *Diaporthe* species available in GenBank. The most similar matches with BLAST search exceeding a length of 540 positions of the ITS sequences were *Phomopsis liquidambaris* C.Q. Chang, Z.D. Jiang & P.K. Chi with a similarity of 99% (544 or 546/552 bp), *D. hongkongensis* R.R. Gomes, C. Glienke & Crous with a similarity of 99% (541/552 bp) and *D. ceratozamiae* Crous & R.G. Shivas (GenBank JQ044420) with a similarity of 98% (543/552 bp). Among these species, only *D. ceratozamiae* also lacks β-conidia. It produces paraphyses and long, branched conidiophores and can, thus, be distinguished from *D. nelumbonis*(Chang et al. 2005; Crous and Shivas 2011). In the phylogenetic estimate derived from *TUB* sequences (Fig. 3), *D. nelumbonis* formed a well-supported subclade within a clade containing *D. fraxini-angustifoliae* R.G. Shivas, Jacq.

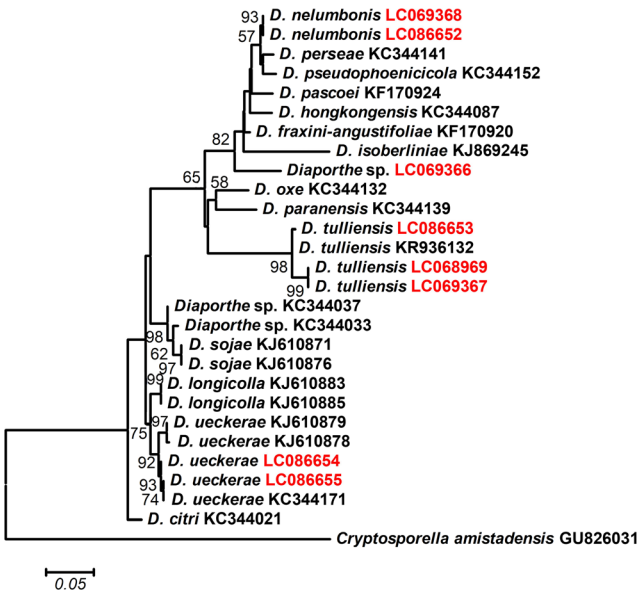

Fig. 3 Phylogenetic hypothesis derived from a maximum likelihood analysis of *TUB* sequences of *Diaporthe* species. Own sequences in *red*. *Cryptosporella amistadensis* L.C. Mejía (Gnomoniaceae, Diaporthales) was included in the unrooted tree as the outgroup

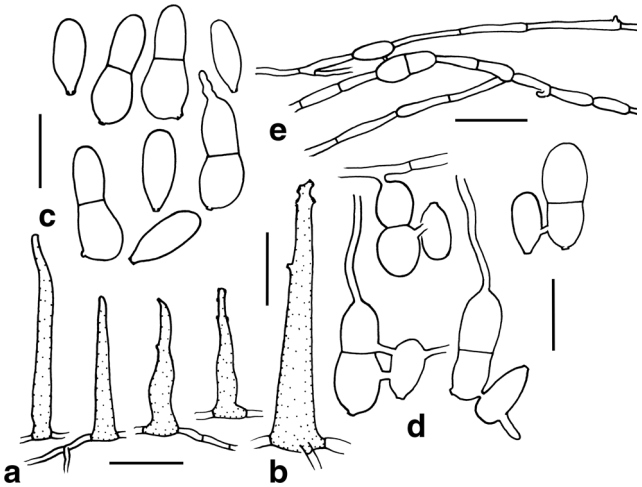

Fig. 4 *Dissoconium nelumbonis* (R. Kirschner 3925): **a**–**c** conidiophores and conidia from in situ on the lotus leaf: **a**, **b** conidiophores, **c** conidia, **d**, **e** germinating conidia in culture. Scale bars: **a**, **e** = 20 μm, **b**–**d** = 10 μm

Edwards & Y.P. Tan, *D. hongkongensis* and *D. pascoei* R.G. Shivas, Jacq. Edwards & Y.P. Tan. In the analysis of Du et al. (2016), these three species also occur together in a well-supported clade clearly separated from the sister clade which contains the *D. eres* Nitschke complex and *D. citri* (H.S. Fawc.) F.A. Wolf. *Diaporthe citri*, the single species of this clade in our analysis, appears basal in our topology. Hitherto, no other species of *Diaporthe* has been described from lotus.

The *Diaporthe* species isolated from lotus leaves as endophytes in our study were located in clades other than *D. nelumbonis*, which was associated with leaf spots. Among the endophytic *Diaporthe* isolates, *D. ueckerae* Udayanga & Castl. could be identified with some certainty according to Udayanga et al. (2015), who considered *Diaporthe* sp. 1. (strain LGMF937 from *Glycine max*, Brazil) in Gomes et al. (2013) as conspecific with their new taxon *D. ueckerae*. This species (including *D. miriciae* R.G. Shivas et al. as the synonym, Gao et al. 2016) was isolated from Asteraceae, Cucurbitaceae, Fabaceae and Theaceae and a human source in America, Asia, Australia and Europe (Gao et al. 2016; Gomes et al. 2013; Udayanga et al. 2015).

Another endophytic species from lotus leaves clustered with *D. tulliensis* R.G. Shivas, Vawdrey & Y.P. Tan, which had been described based on a single strain from a cacao fruit in Australia (Shivas et al. 2015). Recent further isolates of *D. tulliensis* from kiwifruit plants in China were identified based on *TEF1A* sequences without providing *TUB* sequences (Bai et al. 2017).

Ectophytic *Dissoconium* and powdery mildew fungi

Dissoconium nelumbonis R. Kirschner & Kuan L. Chen, **sp. nov.** Fig. 4.

MycoBank: MB821927.

Characteristics in situ (R. Kirschner & K.-L. Chen 3925): Hyphae external on the leaf, undulate, hyaline, smooth, 1–3 μm wide. Conidiophores arising solitarily from hyphae, erect, subulate, straight or slightly bent or geniculate in the apical half, pale brown, smooth, aseptate, consisting only of conidiogenous cell, (30–)31–46(−56) × (4–)4.5–6(−7) μm (n = 30), with 1 to several conidiogenous loci at the top or upper third of the conidiogenous cell. Conidiogenous loci flat to slightly denticulate, slightly blackened, particularly on older conidiophores, 1–1.5 μm wide. Conidia dimorphic, usually a pair of a 2-celled and a 1-celled conidium discharged together from the conidiophore apex, 2-celled conidia with asymmetrical base, basal cell conspicuously wider than the apical one, (11–)13–16 × (4.5–)5–6(−6.5) μm (n = 17), 1-celled conidia fusiform-oblong with broadly rounded apex and gradually narrowing to a truncate base, (7–)8–11 × 3–4.5(−5) μm (n = 15), hila in both kinds of conidia slightly blackened, 1–1.5 μm wide.

Cultural characteristics on CMA: Colonies spreading, with sparse aerial mycelium and irregular margins; surface sienna, with patches of white and cinnamon; reaching 20 mm diam. after 7 days. Hyphae smooth, hyaline to pale brown, 1–5 μm wide. Conidiophores solitary, arising from hyphae, subcylindrical, subulate or lageniform, tapering to a bluntly rounded or truncate apex, straight to gently curved, smooth, hyaline, becoming medium brown with age, aseptate, (18–)24–40(−44) × (3–)4–5(−6) μm (n = 30). Conidia dimorphic, 2-celled conidia straight to somewhat curved, hyaline to pale olivaceous, ellipsoid, not or slightly constricted at median septum, apex obtuse, base obconic-truncate, tapering at somewhat protruding hilum, unthickened, not darkened, 2-celled conidia (12–)12.8–15(−16.5) × (4–)5–6(−6) μm (n = 15, R. Kirschner) or (11–)12–14(−14.5) × 5–6 μm (n = 15, K.-L. Chen), one-celled conidia shaped as in situ, 8.5–10(−10.5) × (3–)3.5–4.5(−5) μm (n = 15, R. Kirschner) or 7–8(−9) × 3–4 (n = 15, K.-L. Chen), germination hyphae of discharged conidium pairs anastomosing with each other.

Specimens examined: On living leaves of *Nelumbo nucifera*, TAIWAN: Taoyuan City, Jhongli District, National Central University, 26 Aug. 2013, R. Kirschner & K.-L. Chen

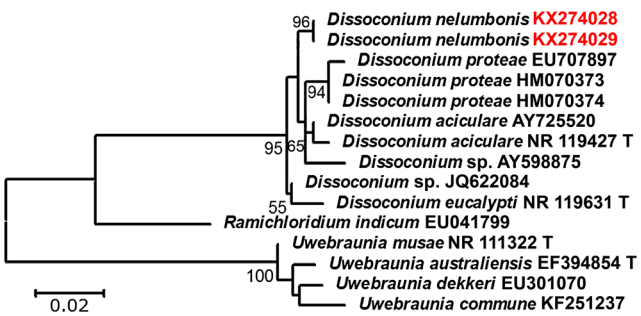

Fig. 5 Phylogenetic hypothesis derived from a maximum likelihood analysis of ITS sequences of *Dissoconium* and related species. Own sequences in *red*. *T* indicates sequence derived from ex-type strain

Table 2 Morphological characteristics of species classified in *Dissoconium*, including former *Dissoconium* species now classified in *Uwebraunia*

Species	Hyphae	Conidiophores	Septate conidia	Aseptate conidia	Reference
D. aciculare	1.5–3 μm	75–100 × 7.5–12 μm	12–25 × 5–8 μm	7.5–12 × 3.5–6 μm	de Hoog et al. (1983)
D. eucalypti	2–3 μm wide	10–30 × 4–8 μm	(8–)10–12(–14) × (4.5–)5–6 μm	4–7 × 2.5–3 μm	Crous et al. (2007)
D. proteae	Smooth, hyaline to pale brown hyphae, 1.5–2 μm wide	10–30 × 3–5 μm	(9–)10–11(–12) × (3–)3.5(–4) μm	7–8(–10) × (3–)3.5(–4) μm	Crous et al. (2008)
D. subuliferum	0.7–2 μm	50–100 × 4.5–5 μm	11–16 × 3.8–4.5(–5) μm	–	Matsushima (1975)
U. australiensis	2–3 μm wide	20–27 × 4–5 μm	(20–)23–25(–27) × (3–)4(–5) μm	–	Crous et al. (2007)
U. communis	Pale brown to olivaceous, 1.5–3 μm wide	0–1-septate, simple or branched, 15–30 × 4–6 μm	20–30 × 4–5 μm	4–5 × 3–4 μm	Crous et al. (2004)
U. dekkeri	1–6 μm wide	3.5–4.5 μm wide (length not given)	22–-30 × 3.8–4.5 μm	3.3 (“33”) μm	De Hoog et al. (1991)
U. musae	2–3 μm wide	(10–)19–25(–53) × (2.5–)3–5 μm	(11–)22–26(–35) × (3–)4–5 μm	4–5 × 3–4 μm	Arzanlou et al. (2008)

3925 (TNM, **holotype**; ITS KX274029), same place, 8 Dec. 2014, R. Kirschner 4135 (BCRC FU30415), same place, 8 Oct. 2015, R. Kirschner 4204 (TNM), Taoyuan City, Guanyin District, Aug. 2013, K.-L. Chen 016 (not preserved, ITS KX274028).

Notes: Cultures of the species soon after isolation ceased to grow or were quickly overgrown by other fungi so that it was difficult to deposit a living culture, although the fungus was repeatedly collected in the years 2013, 2014 and 2015. In *Dissoconium*, four species are now accepted, after some species have been transferred to *Uwebraunia*(Table 2). A species originally described as *D. mali* is not a species of *Dissoconium*, but represents two species of *Ramichloridium*(Li et al. 2012). Analysis of ITS sequences of *Dissoconium* and *Uwebraunia* species (Crous et al. 2008) showed that the *Dissoconium* strains from lotus formed a strongly supported clade within *Dissoconium*(Fig. 5).

Since the hitherto described other species of *Dissoconium* were only described from culture, we compared the morphologies in situ and in vitro. In culture on CMA, characteristics were basically identical to those from in situ, but hyphae were wider, up to 5 μm wide, and conidiophores and conidia slightly smaller. The new species is most similar to *D. eucalypti* Crous & Carnegie, but even in culture has slightly larger dimensions of conidiophores and conidia (Crous et al. 2007). In spite of investigating several other host plants in Taiwan (e.g. Kirschner 2014), the *Dissoconium* species was only found on lotus leaves, which might indicate substrate specificity.

Erysiphe takamatsui Y. Nomura, Taxonomical Study of Erysiphaceae of Japan (Tokyo): 208 (1997) Fig. 6.

Mycelium epiphyllous, sometimes also hypophyllous, forming small patches that can become confluent and cover the whole leaf. Hyphae hyaline, smooth or verruculose, cells ca. 40–74 μm long, 4–6 μm wide, with nipple-shaped to lobed, up to 6 μm wide, mostly single, rarely opposite appressoria. Haustoria globose, intraepidermal, only found in epidermal cells neighbouring guard cells. Conidiophores erect, straight, in some cases slightly bent at the base, smooth or at the apex with fine longitudinal striations, basal septum almost at the same level as the surface of the supporting hypha, exceptionally raised to 17 μm above the surface, foot cell (25–)29–44(−50) μm long (n = 20), followed by 1–3 shorter cells, entire conidiophores (51–)66–97(−112) μm long (n = 30), 5–8 μm wide at the narrowest part, widening towards the apex to 8–11 μm. Conidia solitary, oblong-cylindrical, doliiform, in some cases (primary conidia?) apex slightly pointed, finely longitudinally striated, (25–)28–34(−36) × (11–)12–15 μm (n = 30), germination hyphae formed subterminally, short, with lobed appressoria. Above measurements based on specimen R. Kirschner 3805. Sizes in R. Kirschner 4163: foot cell (28–)29–42(−51) × (6–)7.5–9.5(−11) μm (n = 20), conidia (26–)28–38(−47) × (9–)13–17 μm (n = 30).

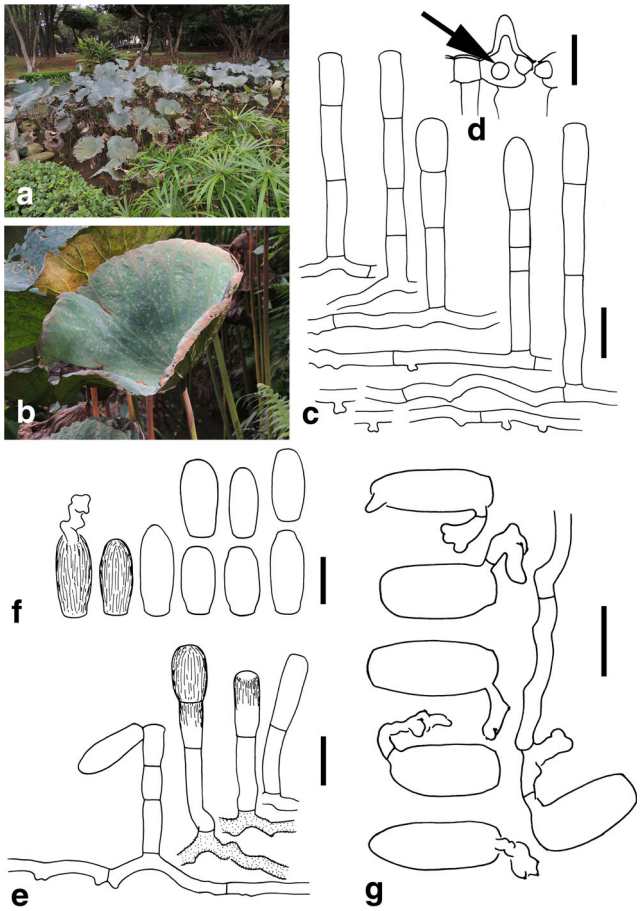

Fig. 6 *Erysiphe takamatsui*: **a** silvery discolouration of lotus leaves due to mass infection by powdery mildew (National Central University, 19 Nov. 2014), **b** distinct colonies of powdery mildew (R. Kirschner 4113), **c**–**g** microscopic characteristics of fresh material (R. Kirschner 3805), **c** hyphae, hyphal appressoria and conidiophores, **d** haustorium (*arrow*) in cell next to a guard cell in transversal leaf section, **e** surface structures of cell walls exemplarily shown for the two hyphae and conidiophores in the middle, **f** conidia, surface structure indicated in the two left ones, **g** germinating conidia with conidial appressoria. Scale bars = 20 μm

Specimens examined: On living leaves of *Nelumbo nucifera*, TAIWAN: Taoyuan City, Jhongli District, National Central University, lotus pond, 22 Nov. 2012, R. Kirschner 3805 (TNM), same place, 23 Oct. 2013, W.A. Liu LWA26A (not preserved, ITS KY400008), same place, 27 Oct. 2014, R. Kirschner 4113 (TNM); Taoyuan City, Taoyuan District, Taoyuan Stadium, 7 Feb. 2015, R. Kirschner 4163 (TNM).

Notes: On lotus, *Erysiphe magnifica* (U. Braun) U. Braun & S. Takam. and *Oidium* sp. were recorded from Germany (Braun et al. 2006; Kirschner 2010; Kruse et al. 2014), and *E. takamatsui* from Japan (Meeboon and Takamatsu 2015). *Ovulariopsis eliadei* Negru is considered a doubtful species, probably not even being a powdery mildew (Braun and Cook 2012). Conidium sizes of the material from Taiwan were slightly smaller than those from Japan. Recently, Meeboon and Takamatsu (2015) provided detailed descriptions and ITS sequence analyses based on rediscovered *E. takamatsui* from lotus in Japan. They stated that this species could not be distinguished from *E. aquilegiae* DC., *E. catalpae* Simonyan and *E. macleayae* R.Y. Zheng & G.Q. Chen using ITS sequences and that these species are morphologically similar to each other, so further study is required for clarifying the species boundaries. Using BLAST search with our ITS sequence, we also found 100% identity with those of *E. takamatsui* (GenBank AB916688) and *E. macleayae*, and only a single different position when compared to certain sequences of *E. aquilegiae*, *E. catalpae* and *E. macleayae*. Similarly to the observation by Meeboon and Takamatsu (2015), our sequence differed from a second sequence of *E. takamatsui* (GenBank AB916689) for two positions. *Erysiphe magnifica* is not closely related, because its ITS sequences differ for at least 20 positions from those of *E. takamatsui*. Because of the same host and ITS sequence, the specimens from Taiwan are identified as *E. takamatsui*.

Fereydounia and other basidiomycetous yeasts

Fereydounia khargensis S. Nasr, M.R. Soudi, H.D.T. Nguyen, M. Lutz & M. Piątek, Mycol. Progr. 13(4): 1223 (2014) Fig. 7.

Growth on the adaxial side of leaf lamina inconspicuous, only represented by budding yeast cells without pseudohyphae, on the abaxial side forming conspicuous powdery colonies composed of pseudohyphae and conidia extending from the substrate into the air. Cells at least 4 μm long, 1.5–3 μm wide, no hyphal-like cells, stalk formation rarely observed.

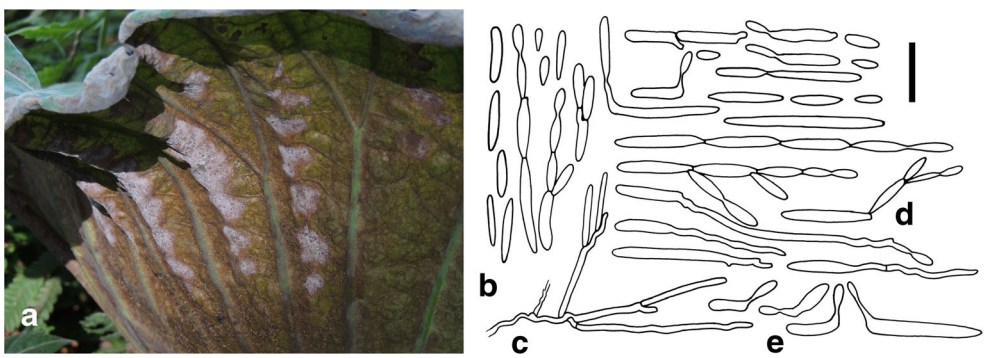

Fig. 7 *Fereydounia khargensis*: **a**, **b** pseudomycelium and conidia on abaxial side of living lotus leaf (R. Kirschner 4109), **c**, **d** pseudohyphae, polar budding and stalk formation on CMA (R. Kirschner 4117), **e** stalk formation on MEA (R. Kirschner 4117). Scale bar = 10 μm

Colony surface on CMA white and powdery, with fringed margin, formed by pseudohyphae, or cream, smooth, with defined margin, formed by budding cells; usually both growth forms occurring in different areas of the same colony, reverse pale pink. Cells 3 to less than 100 μm long and 1.5–2.5 μm wide. Daughter cells produced by polar budding, subpolar budding from a short, sympodial elongation, sometimes by several repetitions resulting in a zig-zag shape of the elongated cell, and by budding from a short subpolar stalk. Elongated cells becoming hyphal-like, but only form retraction septa. Ballistospores not observed within seven days with the mirror plate technique.

Specimens examined: On adaxial leaf surface of *Nelumbo nucifera*, TAIWAN: Taichung City, National Museum of Natural Science, lotus pond, 19 Oct. 2014, R. Kirschner 4108 (TNM: dried culture; ITS KY400009, LSU rDNA KY498534); on abaxial leaf surface of *N. nucifera*, same place, 4 Nov. 2014, S.-Z. Chen, R. Kirschner 4117 (TNM, BCRC FU30380, ITS KY400010); on abaxial leaf surface of *N. nucifera*, Taoyuan City, Jhongli District, National Central University, lotus pond, 27 Oct. 2014, R. Kirschner 4109 (TNM).

Notes: The ITS sequences of our two strains differed from the sequences in GenBank by a single position at the 5′ end which might be related to sequencing errors, and by a further position in strain R. Kirschner 4108. The LSU rDNA sequence was 100% identical to the five sequences available at GenBank, whereas the similarity with sequences from other species was 93% or lower. Phylogenetic analysis indicates a relationship with smut fungi belonging to the order Urocystidales, where the monotypic Fereydouniaceae and the monogeneric Doassansiopsidaceae form the two most basal clades (Nasr et al. 2014). Doassansiopsidaceae are known as spore ball-forming smut fungi infecting aquatic plants. Although we found yeast cells of *F. khargensis* covering epidermal cells on the adaxial lotus leaf epidermis, the fungus was more conspicuous in its powdery growth formed by pseudomycelium along lesions of the abaxial epidermis. Preparations of these infection sites did not reveal any smut spores or other kind of fungal growth. Its association with leaves appear secondary and saprobic rather than parasitic, but is the first recorded association with living plants. Since lotus plants are aquatic similar to the hosts of Doassansiopsidaceae, the common substrate may indicate a closer relationship between the two fungal groups than can presently be inferred by insufficient taxon sampling in phylogenetic analyses. A recent study of *F. khargensis* from blood samples in Malaysia indicated that the fungus may be widespread in Asia and is resistant against certain antifungals (Tap et al. 2016). These findings might be important for farmers who cultivate lotus fields.

The morphology of colonies and cells agree with that recently described from unidentified plant remains in Iran, except for the proposed formation of ballistospores (Nasr et al. 2014). Since stalk-borne daughter cells and the stalk formation of the yeast cells itself were symmetrical, discontinuous spread on the agar plate was not observed, and no mirror image was produced within seven days with the mirror plate technique, there is no formation of ballistoconidia, but only of conidia from stalks. The other basidiomycetous yeasts found in this study were *Rhodosporidiobolus odoratus* and *Golubevia pallescens*. Their formation of ballistoconidia can be deduced not only from the disjunct spread of colonies in the Petri dish, but also from their asymmetric conidia with laterally displaced apiculus (Kirschner 2017), which are quite different from the symmetric daughter cells developing on stalks in *F. khargensis*. In contrast to *F. khargensis* observed mainly on the lower side of leaves, the two ballistosporic yeasts were not found by direct observation, but as contamination observed during use of the spore-fall method for isolating *Dissoconium nelumbonis* from the upper leaf surface.

Pseudocercospora species on Nelumbonaceae and Nymphaeaceae

Pseudocercospora nelumbonicola R. Kirschner & Kuan L. Chen, **sp. nov.** Fig. 8.

MycoBank: MB821928.

Leaf spots adaxially pale brown to purplish, with a diffuse, irregular margin, abaxially marked by an irregular pale brown contour line surrounding not distinctly discoloured tissues, 2–12 mm diam., without shot-hole symptoms. Hyphae intercellular, pale brown, smooth, 2–3.5 μm wide. Stromata composed of few brown cells or well-developed, subglobular, brown, 5–15 μm high and 3–10 μm broad (n = 15). Conidiophores epiphyllous, penetrating through stomata, 9–17 per fascicle, unbranched, rarely branched, pale brown, smooth, straight to slightly curved, 0–2-septate, (20–)23.5–30(−34) × (2.5–)3–4(−4.5) μm (n = 30). Conidiogenous cells terminal, straight or curved, almost not geniculate, up to the same size as conidiophores, conidiogenous loci 1–2, apical or subapical, 1–2 μm wide. Conidia solitary, pale brown, cylindric to cylindro-obclavate, straight to curved, 1–4-septate, (22–)28.5–50(−60) × (1–)2(−3) μm (n = 30), hila 1–2 μm.

Specimens examined: On living leaves of *Nelumbo nucifera*, TAIWAN: Taoyuan City, Guanyin District, Lanpu Village, 4 Aug. 2012, R. Kirschner 3706 (TNM, **paratype**; ITS sequence KY304489), 1 Sep. 2013, K.L. Chen KL007, 3 October 2013, K.L. Chen KL008 and KL009, Jhongli District, National Central University campus, 27 Oct. 2014, R. Kirschner 4111 (TNM, **holotype**; ex-type culture: BCRC FU30367; ITS sequence KY304492, *TUB* LC200982, *RPB2* LC199940), same place, 14 Nov. 2014, R. Kirschner 4125 (TNM, **paratype**; ITS sequence LC200980).

Additional specimen examined: *Cercospora nelumbinis* Tharp, on leaf of *Nelumbo lutea* Willd., USA: Texas,

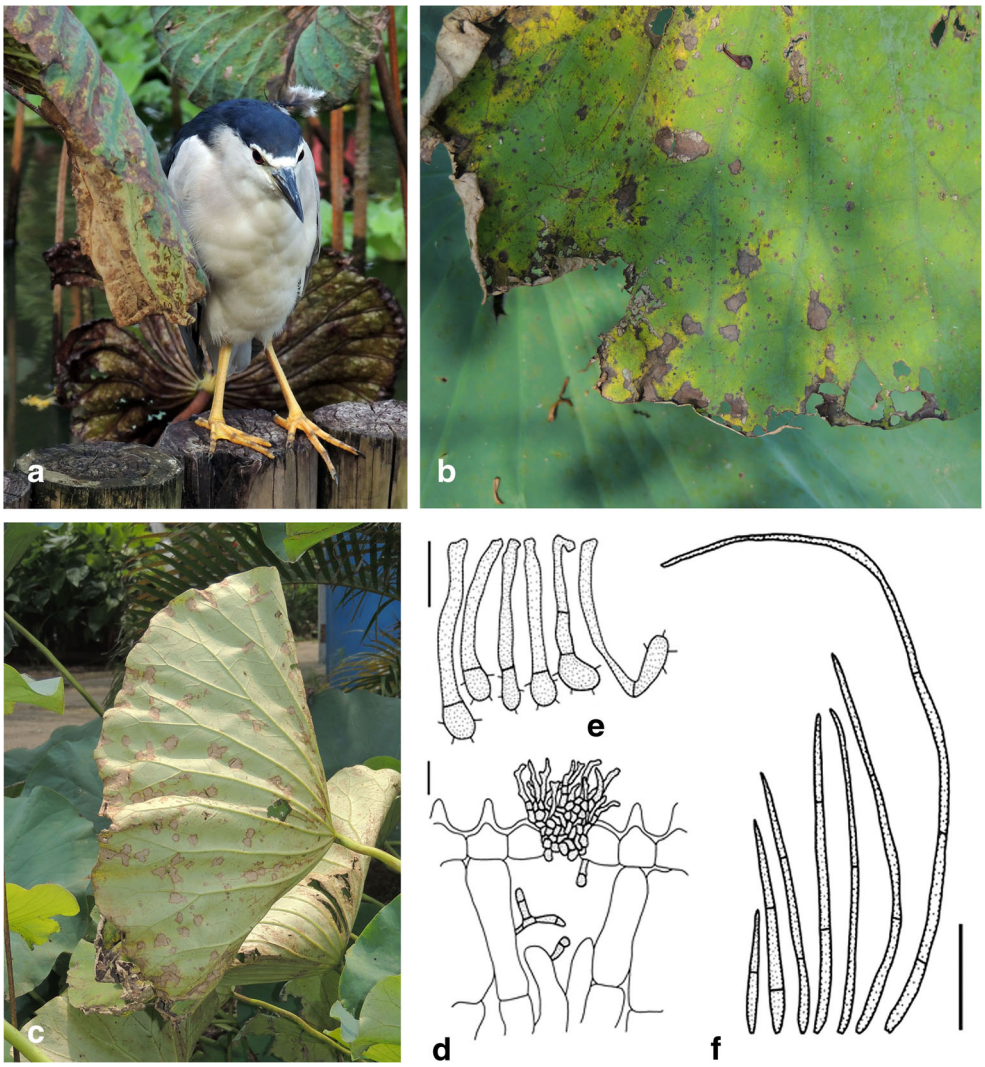

Fig. 8 *Pseudocercospora nelumbonicola*: **a** leaf spots in the Botanical Garden Taipei, 10 Oct. 2012, with night heron (*Nycticorax nycticorax*), **b** lesions on upper side of leaf (R. Kirschner 3706), **c** lesions on lower side of leaf (R. Kirschner 3706), **d** epiphyllous fascicle of conidiophores penetrating through ruptured stoma in transversal leaf section (K.-L. Chen KL007), **e** conidiophores (K.-L. Chen KL007), **f** conidia (K.-L. Chen KL007). Scale bars = 10 μm

Palestine, 31 Oct. 1914, Lewis & Tharp (BPI 438779, holotype).

Notes: No data of *Pseudocercospora* spp. on Nelumbonaceae exist in GenBank, but recently, sequences of *Ps. nymphaeacea* have been entered (Park et al. 2015). Comparison of the ITS sequence derived from R. Kirschner 3706 with an own ITS sequence from *Ps. nymphaeacea* (R. Kirschner 3760) revealed 100% identity (587/587 bp). Since the ITS sequences of different *Pseudocercospora* and related *Mycosphaerella* species from only distantly related host plants are often 100% identical (Park et al. 2015), 100% identity between ITS sequences of *Ps. nelumbonicola* and *Ps. nymphaeacea* does not mean conspecificity. After testing different DNA regions (ITS, *TUB*), only *RPB2* sequences

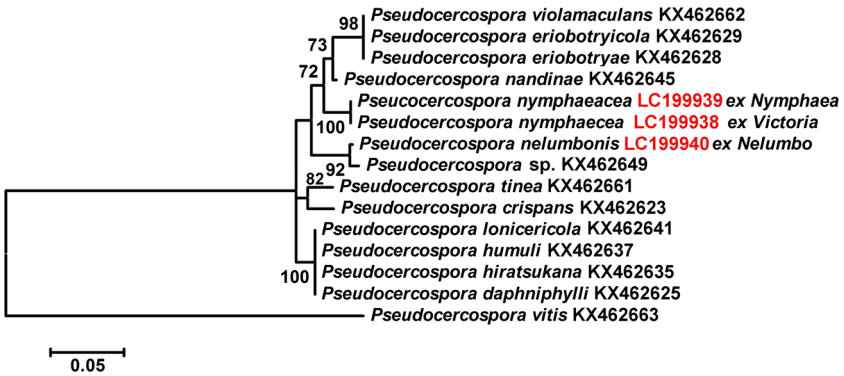

Fig. 9 Phylogenetic hypothesis derived from a maximum likelihood analysis of *RPB2* sequences of *Pseudocercospora nelumbonicola*, *Ps. nymphaeacea* (in *red*) and most similar sequences from Nakashima et al. (2016) according to BLAST searches. Own sequences in *red*. *Pseudocercospora* (*Phaeoisariopsis*) *vitis* was used as the outgroup in the unrooted tree

allowed conclusive separation of the species on *Nelumbo* (R. Kirschner 4111) and Nymphaeaceae (R. Kirschner 4342 ex *Nymphaea*, R. Kirschner & S.-Z. Chen 3760 ex *Victoria*, Fig. 9).

Since *Pseudocercospora* species are considered to be highly specific to host families or even closely related genera within a single family of host plants, the species on Nelumbonaceae and on Nymphaeaceae are not likely to be conspecific, since Nymphaeaceae belong to the Nymphaeales within the magnoliids and Nelumbonaceae to the Proteales within the eudicots with a huge phylogenetic distance (Simpson 2010).

The symptoms of *Pseudocercospora* leaf spots differ on Nelumbonaceae and Nymphaeaceae because the leaf spots on Nymphaeaceae are always distinctly circular and transform into shot-hole symptoms (see below and Park et al. 2015), but those on Nelumbonaceae are irregular and do not form shot-hole symptoms. The sizes of conidiophores and conidia in *Ps. nelumbonicola* are generally somewhat smaller than those of *Ps. nymphaeacea* (Meeboon 2009: on lotus, Park et al. 2015: on *Nymphaea* spp.). On *Nelumbo nucifera* in mainland China, however, *Ps. nymphaeacea* was described from circular, 2–5 mm wide leaf spots, with small stromata (15–36 μm diam.) and conidia measuring 62–106 × 2–3.5 μm (Chi 1994). The small circular leaf spots and wide conidia might indicate a confusion of *Nymphaea* with *Nelumbo*. The description from Chi (1994) was reproduced by Guo et al. (1998), who additionally provided a drawing showing a strongly reduced stroma. Records on *Nelumbo nucifera* in mainland China are considered to be *Ps. nelumbonicola*, but need re-examination.

Cercospora nelumbonis Tharp from *Nelumbo lutea* Willd. in North America has been considered a synonym of *Ps. nymphaeacea* described from *Nymphaea odorata* Aiton. Our study of the holotype of *C. nelumbonis* confirmed the host genus, but conidiophores and conidia were not found. Conidia were described as hyaline and 3–4 μm wide for *C. nelumbonis* by Tharp (1917), whereas conidium widths and colours were clearly distinguished in other species described by him under *Cercospora*. It is, therefore, probably not a species of *Pseudocercospora* but another genus. Because no material of *C. nelumbonis* is available for comparison, the problem of whether this doubtful taxon from North American lotus is conspecific with the East Asian *Ps. nelumbonicola* or a further species cannot be solved. For this reason, a new species is described instead of proposing a new combination.

Pseudocercospora nymphaeacea (Cooke & Ellis) Deighton, Trans. Br. Mycol. Soc. 88(3): 390 (1987) Fig. 10.

Leaf spots amphigenous, distinctly circular, pale brown, with a darker brown margin (0.5–5 mm), eventually forming shot-hole symptoms. Hyphae intercellular, pale brown, smooth, 2–4 μm wide. Stromata composed of few brown cells or well-developed, subglobose, brown, 20–30 μm high and 24–35 μm broad (n = 15). Conidiophores epiphyllous, penetrating through stomata, 18–25 per fascicle, unbranched, rarely branched, pale brown, smooth, 0–4-septate, (20–)25–36(−45) × (2–)3 μm (n = 30). Conidiogenous cells terminal, straight, rarely curved, almost not geniculate, (9–)10–17(−23) × 2–3 μm (n = 30), conidiogenous loci 1(−2), apical or subapical, 1–2 μm wide. Conidia solitary, pale brown, smooth, cylindrical, straight to slightly curved, 3–8-septate, (41–)52–75(−95) × (2–)2.5–3(−3.5) μm (n = 30), hila 1.5–2 μm.

Specimens examined: On living leaves of *Euryale ferox* Salisb. ex K.D. Koenig & Sims, TAIWAN: Taichung City, National Museum of Natural Science, Botanical Garden, 5 Oct. 2012, R. Kirschner & S.-Z. Chen 3771 (TNM); on living leaves of *Nymphaea* sp., TAIWAN: Jiayi County, National Chiayi University, 1 Mar. 2011, R. Kirschner 3453 (TNM, ITS KY304490); on living leaves of *Nymphaea lotus* L., TAIWAN: Taoyuan City, Guanyin District, 8 Oct. 2013, K.L. Chen KL010 and KL011 (not preserved), Taoyuan City, Jhongli District, National Central University, 20 Jun. 2012, R. Kirschner 3668 (TNM, living culture BCRC FU30002, ITS KY304491), same place, 29 Sep. 2016, R. Kirschner 4342 (TNM, *RPB2* LC199939); on living leaves of *Victoria cruziana* Orb., TAIWAN: Taichung City, National Museum of Natural Science, Botanical Garden, 5 Oct. 2012, R. Kirschner & S.-Z. Chen 3760 (TNM, living culture BCRC FU30022, *TUB* LC200981, *RPB2* LC199938).

Notes: For comparison with *Pseudocercospora* specimens on lotus, see above. *RPB2* sequences of *Ps. nymphaeacea* from *Nymphaea* and *Victoria* form a clade separate from the *Pseudocercospora* sequence from lotus. The fungus is a new record for Taiwan, *Euryale* is a newly recorded host genus. *Euryale ferox* is native to Taiwan, whereas species of *Nymphaea* and *Victoria* were introduced to Taiwan in the twentieth century (Hsueh and Yang 2016).

Discussion

In spite of the self-cleaning properties of the leaf lamina considered as protection against microbial colonisation, our first systematic study of fungi associated with lotus leaves indicates a previously unknown fungal diversity. The ectophytic and parasitic fungi contained newly described species and new records, whereas the endophytic isolates were identified as known species based on ITS, LSU rDNA, *TUB* and *TEF1A* sequences. For several species, ITS sequences were sufficient markers for species identification and confirming the novelty of *Dissoconium nelumbonis*. Following the recommendation for *Colletotrichum* species (Damm et al. 2012; Weir et al. 2012) and *Diaporthe* species (Gomes et al. 2013; Udayanga et al. 2015), *TUB* was a good additional marker for discriminating between closely related species. For example, the

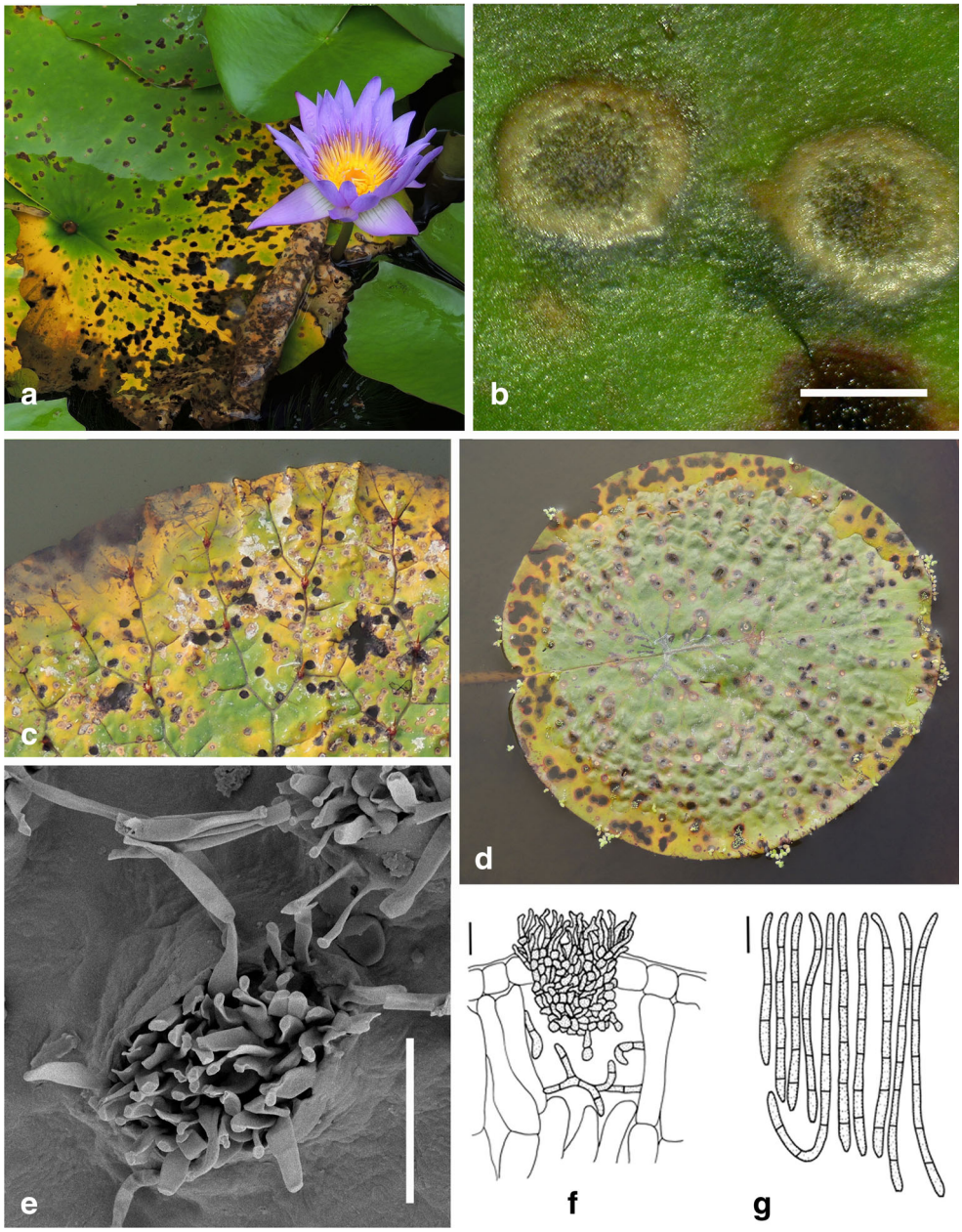

Fig. 10 *Pseudocercospora nymphaeacea*: **a** pale brown leaf spots and dark brown shot-hole symptoms of *Nymphaea lotus* (Taiwan, Jhongli, 5 Jun. 2013), **b** two leaf spots of *N. lotus* (R. Kirschner 3668), **c** leaf spots and shot-hole symptoms of *Euryale ferox* (R. Kirschner & S.-Z. Chen 3771), **d** leaf spots and shot-hole symptoms of *Victoria cruziana* (R. Kirschner & S.-Z. Chen 3760), **e** scanning electron microscopy of conidiophores emerging through a stoma of *N. lotus* (R. Kirschner 3453), **f** stroma and conidiophores in transversal section of leaf of *N. lotus* (K.-L. Chen KL010), **g** conidia (K.-L. Chen KL010). Scale bars: **b** = 2 mm, **e** = 20 μm, **f**, **g** = 10 μm

single *Colletotrichum* species found as ecto- and endophytic fungus on lotus in our study, *C. siamense*, was identified by 99–100% ITS sequence similarity between our sequences and those represented in GenBank (Weir et al. 2012), i.e. with 0–1 different positions, whereas the second most similar sequence of a reliably identified species was from *Co. fructicola* with three different positions (Weir et al. 2012). A *TUB* sequence (414 bp from R. Kirschner 4118) also revealed 100% identity with over 30 sequences of *Co. siamense* (including the synonymous *Co. hymenocallidis* Yan L. Yang et al.) from GenBank, whereas sequences from other species differed at least for one position (similarity 99% or lower).

For identification of the approx. 60 biological species within the *Fusarium solani* species complex (FSSC), phylogenetic analysis of the translation elongation factor 1α gene (*TEF1A*) sequences is recommended (O'Donnell et al. 2008). For three randomly selected endophytic strains, *TEF1A* sequences revealed that they all belonged to the FSSC species 6 within clade 3 (Supplemental Material 2).

Pseudocercospora species are mostly distinguished based on their specificity for host families or even genera and morphology. Morphology as well as ITS and *TUB* sequences were not suitable for discriminating between the *Pseudocercospora* species on Nelumbonaceae and Nymphaeaceae. Following

Nakashima et al. (2016), who, for the first time, successfully applied *RPB2* sequences for revealing terminal clades of *Pseudocercospora* species, our strains from the two host families could be separated.

Among the seven species characterised as ectophytic in our study, two species may rarely occur in substrates other than leaf surfaces, namely *Dissoconium nelumbonis*(Ascomycota) and *Rhodosporidiobolus odoratus* (Basidiomycota). Although both species are not closely related phylogenetically, they share the strategy of exclusively superficial growth on the substrate and asexual reproduction by forcibly discharged conidia, which might be considered a strategy of quick colonisation of leaf surfaces under the adverse condition of the lotus effect. *Dissoconium nelumbonis* was found by direct microscopic observation of leaf fragments, but *Rh. odoratus* only by cultivation. Both species were isolated by allowing spores to fall off from the same leaf fragments attached beneath the lid of a Petri dish containing an agar medium. Attempts to isolate *D. nelumbonis* from samples collected on four different dates were hampered by the rapid spread of contaminating *Rh. odoratus*. This accidental observation in vitro may indicate some antagonism between the two species also on the leaf. Since the yeast could not be confirmed by direct observation in contrast to *D. nelumbonis*, the occurrence and role of *Rh. odoratus* on lotus leaves need more investigation. The basidiomycetous yeast *Rh. odoratus* belongs to the most common yeast species isolated from phylloplanes of a broad range of plants, including aquatic plants like *Typha latifolia* L. (Pugh and Mulder 1971).

For detecting endophytic fungi, the cultivation approach had to be adjusted to the particular anatomy of lotus leaves. The imprint technique ensured that the surface sterilisation method worked efficiently, and sealing the cut margins with molten wax before surface sterilisation prevented the sterilisation agents from entering into the aerenchymatic tissues and killing internal hyphae. Among the 33 morphospecies (Supplemental Material 1) found as endophytes in lotus leaves, the four most frequently isolated species were *Fusarium solani* species complex (46 strains, 9.7%, mainly species 6, Supplemental Material 2), *Nigrospora oryzae* (22 strains, 4.6%), *Xylaria* cf. *curta* (16 strains, 3.4%) and *Annulohypoxylon* cf. *stygium* (10 strains, 2.1%). Species of *Fusarium*, *Nigrospora* and Xylariaceae have also been frequently recorded as endophytes from numerous other plant species (Lawson et al. 2014; Tateno et al. 2015). Species of *Fusarium* are not only widespread plant pathogens, but also often recorded as endophytes and may even protect plants in different stress or be the basis of disease suppression. Rodriguez et al. (2008) demonstrated that *Fusarium culmorum* (Wm.G. Sm.) Sacc., isolated as an endophyte from *Leymus mollis* (Trin.) Pilg. on a coastal beach, can confer salt stress tolerance to the host. In Xylariaceae, although species identification is often problematic due to the lack of reliable data, the diverse biologically active compounds found in this group of fungi appear to predestinate numerous interactions with the host plants (Stadler 2011). Since our strains of *Annulohypoxylon* were identified with a LSU rDNA sequence, but for some of the recently revised *Annulohypoxylon* taxa (Kuhnert et al. 2016) LSU rDNA sequences are not available, our species identification is tentative. Species of *Fusarium* and *Nigrospora* can also act as pathogens on living plants and disperse by conidia on dead herbaceous plant parts so that there might be a continuum from endophytes to parasites and saprobes on the same plant. Asexual and sexual morphs of Xylariaceae, however, in nature, are more commonly found to sporulate on dead wood than on herbaceous plant parts so that their behaviour as endophytes in herbaceous plants appears to be more markedly separated from their growth and sporulation on wood.

All fungi isolated from lotus leaves were terrestrial species, with no typical aquatic Ingoldian hyphomycetes or zoosporic fungi. The majority of fungi recovered from *N. nucifera* are members of the Ascomycota, with very few Basidiomycota. The aquatic plants *Myriophyllum verticillatum* L. and *Ottelia acuminata* (Gagnep.) Dandy were found to have high numbers of Eurotiomycetes and Dothideomycetes, with the most predominant fungal taxa from *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium* and *Trichoderma*(Li et al. 2010). A similar mycobiota was recorded for the semi-aquatic plant *Typha latifolia*(Pugh and Mulder 1971).

Aquatic hyphomycetes (Ingold 1975) are important members of the mycota decomposing submerged leaf and wood litter in various types of freshwater. Aquatic hyphomycetes were also often found as endophytes in roots of terrestrial plants (Raviraja et al. 1996; Sati et al. 2009; Selosse et al. 2008). A possible reason for the lack of aquatic endophytes in lotus may be that aquatic hyphomycetes occur more commonly in natural streams or soil. These habitats might be exposed to more aeration than the artificial lotus ponds.

The colonisation rate of fungal species isolated from different plant organs may indicate how endophytic fungi of terrestrial origin enter into the plants. Most isolates were from leaf blades (71%) compared to those from leaf petioles (29%). One way of entering into the plant might be from air to leaf by airborne spores postulated as inocula for the majority of leaf endophytes (Petrini et al. 1992). Another way might be from the root and rhizome into the leaf. In this case, the colonisation rate of the petioles would be expected to be at least as high as in the lamina. We also isolated two endophytic fungi from an underground rhizome collected in Taoyuan belonging to *Alternaria* sp. (data not shown). Further study of rhizomes might provide further proof of the occurrence of several Pythiaceae and *Fusarium* species outside FSSC being restricted to the rhizome of lotus (Li et al. 2016; Takahashi et al. 1965; Yin et al. 2016). In our study, *Fusarium* was represented in the leaves only by FSSC species 6 within clade 3

(Supplemental Material 2) and no Pythiaceae were found. In several other plant species, endophytic fungi in the rhizomes were also different to isolates from aerial parts (Petrini et al. 1992). Among fungi associated with fruit receptacle and fruit of lotus, only the poorly characterised species *Dothiorella nelumbii* Ellis & H.W. Anderson is known to us (Saccardo 1892).

The relationship between endophytism and parasitism has been elucidated in a pioneering approach by Delaye et al. (2013). Compared to frequent switches between saprobic or endophytic lifestyle and a necrotrophic one, a biotrophic-parasitic lifestyle is considered a stable evolutionarily advanced trait. A somewhat similar conclusion may be supported from our observation on *Diaporthe* species, because the species associated with leaf spots (*D. nelumbonis*) was not found among the species isolated as endophytes.

Sampling is also important to evaluate evolutionary traits in fungi with comparatively many clinical reports. Since lotus is widely grown and consumed as a vegetable in Asia, the new records of potentially human-pathogenic fungi associated with this plant are of some medical significance, such as *Fereydounia khargensis*(Tap et al. 2016) and *Fusarium solani* species 6 (Mehl and Epstein 2007; O'Donnell et al. 2008). All 14 strains assigned to this species group by O'Donnell et al. (2008) were isolated from humans in the USA. Virulent plant pathogens have not yet been found in FSSC species 6 (Aoki et al. 2014). A similar case exists for FSSC species 1, since its members are also associated with cultivated plants (mainly cucurbits), as well as with humans (Mehl and Epstein 2007). The recent description of an endophytic *Phialemoniopsis* species indicated a plant–fungus relationship in a genus whose species hitherto did not show such a relationship with living plants, but were often associated with human disease (Su et al. 2016). Clinical samples appear sometimes overrepresented compared to sampling from natural substrates, simply because of the better funding of clinical studies.

Compared to the widespread and unspecific endophytes, ectophytic and parasitic fungi included comparatively more substrate-specific and rarely recorded species. Species epitheta such as "*nelumbii*", "*nelumbinis*" and "*nelumbicola*" might suggest some host specificity, but, after re-study, revealed the facultative occurrence of rather unspecific fungi on lotus leaves. In cases where these species are maintained, some epitheta have to be corrected to "*nelumbonis*" and "*nelumbonicola*" according to the Code (Art. 18, Ex. 2, McNeill et al. 2012). Several of these species listed in Table 1 need re-examination with modern standards. *Gloeosporium nelumbii* Tassi was recognised as a synonym of *Co. gloeosporioides* in the broad sense of von Arx (1970), but has hitherto not been included in modern revisions. The records of *G. nelumbii* from lotus leaves in Italy and Japan seem to refer to two different species, because conidia in the record from Japan (Nisikado and Watanabe 1955) differ from those described from Italy as being somewhat larger and often slightly curved. Because of the curved conidia, the species recorded from Japan is probably not conspecific with *Co. siamense* with straight conidia, which was the most common *Colletotrichum* species in our study. Further study is necessary in order to clarify the relationships of *G. nelumbii* with other species of *Colletotrichum*.

Compared to field collection of plant-associated fungi, methods to detect endophytic fungi can be more easily standardised and allow quick identification of a high diversity of fungi associated with a given plant species. The detection of ectophytic and parasitic fungi requires particular experience. The few overlaps between endophytic, parasitic and ectophytic species in our study might be partly based on the basically different methods and partly on really divergent ecology of the fungi. Only a combination of direct observation of fungi on the substrate and isolation of strains from the plant can give a more reliable impression about the diversity of mycobiota associated with specific plants. Some fungi are first discovered as endophytic isolates, as indicated by slightly divergent DNA sequences, but because of the lack of sporulation in culture, meaningful taxonomic conclusions could often be based only after subsequent discovery of sporulation on the natural substrate found by field collection (Ibrahim et al. 2016; Yang et al. 2016).

In some genera, solely routinely applying a 97% ITS sequence similarity threshold for addressing fungal biodiversity leads to wrong species identification and needs to be complemented with additional methods. Further study of fungi associated with lotus, particularly in other geographic regions and from the rhizome of lotus plants, might reveal additional new insights into the fungal diversity associated with this plant.

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