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Entomopathogenic fungi in Portuguese vineyards soils: suggesting a '*Galleria-Tenebrio*-bait method' as bait-insects *Galleria* and *Tenebrio* significantly underestimate the respective recoveries of *Metarhizium* (*robertsii*) and *Beauveria* (*bassiana*)

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Abstract

Entomopathogenic fungi (EPF) are the natural enemies of insect-pests. However, EPF recoveries can be influenced by the soil habitat-type(s) incorporated and/or the bait-insect(s) used. *Galleria mellonella* (GM) as bait-insect, i.e. '*Galleria*-bait', is arguably the most common methodology, which is sometimes used solely, to isolate EPF from soils. Insect baiting using *Tenebrio molitor* (TM) has also been employed occasionally. Here 183 soils were used to estimate the functional diversity of EPF in Portuguese Douro vineyards (cultivated habitat) and adjacent hedgerows (semi-natural habitat), using the TM bait method. Moreover, to study the effect of insect baiting on EPF recovery, 81 of these 183 soil samples were also tested for EPF occurrences using the GM bait method. Twelve species were found in 44.26% ± 3.67% of the total of 183 soils. *Clonostachys rosea* f. *rosea* was found in maximum soils (30.05% ± 3.38%), followed by *Beauveria bassiana* (12.57% ± 2.37%), *Purpureocillium lilacinum* (9.29% ± 2.14%) and *Metarhizium robertsii* (6.01% ± 1.75%). *Beauveria pseudobassiana* ($P < 0.001$), *C. rosea* f. *rosea* ($P = 0.006$) and

Cordyceps cicadae (*P*=0.023) were isolated significantly more from hedgerows, highlighting their sensitivities towards agricultural disturbances. *Beauveria bassiana* (*P* = 0.038) and *M. robertsii* (*P* = 0.003) were isolated significantly more using GM and TM, respectively. Principal component analysis revealed that *M. robertsii* was associated both with TM baiting and cultivated habitats, however, *B. bassiana* was slightly linked with GM baiting only. Ecological profiles of *B. bassiana* and *P. lilacinum* were quite similar while *M. robertsii* and *C. rosea* f. *rosea* were relatively distant and distinct. To us, this is the first report on (a) *C. cicadae* isolation from Mediterranean soils, (b) *Purpureocillium lavendulum* as an EPF worldwide; and (c) significant recoveries of *M. robertsii* using TM over GM. Overall, a '*Galleria-Tenebrio*-bait method' is advocated to study the functional diversity of EPF in agroecosystems.

Keywords
Biocontrol fungi; Functional diversity; Host-pathogen interaction; Hypocreales; Soil ecology; Vineyards

Introduction

Grape production and winemaking contribute significantly in many economies worldwide. However, vineyards attract many primary, secondary or tertiary insect pests (Gonçalves et al. 2017, Sharma et al. 2018). For example, one of the key insect-pest in vineyards is the European Grapevine Moth, *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae). It exhibits polyphagy and is distributed across Asia, Central Europe and the Mediterranean basin, USA, Chile and Argentina. It can reduce the total crop yield by 50% at the time of harvest in countries such as Portugal (Carlos et al. 2013). Finding strategies to control vineyards' pests is of utmost importance especially from an economic point of view (Sharma et al. 2018).

With increased awareness towards the environment, biological methods to control crop pests such as biopesticides based on entomopathogenic fungi (EPF) have been receiving greater attention as alternatives to chemicals pesticides (Jaronski 2010). Many fungal species belonging to Hypocreales (Ascomycota) have shown insect pathogenicity and dwell in the soil for a significant part of their life cycle, outside the host. Protection from UV radiation and numerous adverse biotic and abiotic influences have made soil an excellent environmental reservoir for EPF (Keller and Zimmermann 1989). Therefore, studying soils for EPF diversity has been a common practice (Meyling and Eilenberg 2006, Quesada-Moraga et al. 2007, Goble et al. 2010, Rudeen et al. 2013, Muñiz-Reyes et al. 2014, Clifton et al. 2015, 2018).

Interestingly, the distribution of EPF in crop cultivated and semi-natural habitats, such as hedgerows, is always arguable. While some studies showed a higher abundance of *Beauveria bassiana* (Balsamo) Vuillemin in soils from hedgerows and *Metarhizium anisopliae* (Metschnikoff) Sorokin in soils from cultivated fields (Meyling and Eilenberg 2006), others reported a higher abundance of *M. anisopliae* in marginal soils (Clifton et al. 2015). Habitat-specific preferences have also been noticed in the case of some EPF (Bidochka et al. 1998, Quesada-Moraga et al. 2007, Medo and Cagáň 2011, Medo et al. 2016). Knowing the differences in EPF abundances within different habitat-types is important in understanding which fungal species is suitable to and would proliferate in a particular habitat-type (Quesada-Moraga et al. 2007).

Insect baiting by *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae) or the '*Galleria*-bait method' (Zimmermann 1986), is a renowned methodology for the isolation of EPF. The main advantage of the insect baiting method is that only entomopathogens are obtained selectively amongst other soil microbes (Vega et al. 2012). Studies in the past find insect baiting as an effective methodology for EPF isolation over culturing soil suspensions on selective media (Keller et al. 2003, Enkerli et al. 2004, Imoulan et al. 2011, Keyser et al. 2015). A selective medium can only be viewed as a semi-quantitative method for EPF isolation as they may provide a false picture of fungal diversity and density, leading to a biased view of many microbial systems (Scheepmaker and Butt 2010). The approach of using bait-insects *G. mellonella* along-with *T. molitor* for EPF isolations, instead of a selective media, has been previously employed (Vänninen 1996, Oddsdottir et al. 2010, Meyling et al. 2012).

Using different bait-insects sometimes may result in an occasional occurrence of a different, not so common EPF (Goble et al. 2010), however, to isolate the known EPF from soils, such as *Beauveria* and *Metarhizium*, the bait-insect *G. mellonella* has been the first choice as a bait-insect for the last three decades (Zimmermann 1986). Numerous investigations have relied only on this method of EPF isolation (Chandler et al. 1997, Bidochka et al. 1998, Ali-Shtayeh et al. 2003, Meyling and Eilenberg 2006, Quesada-Moraga et al. 2007, Sun and Liu 2008, Sun et al. 2008, Sevim et al. 2009, Fisher et al. 2011, Muñiz-Reyes et al. 2014, Pérez-González et al. 2014, Fernández-Salas et al. 2017, Gan and Wickings 2017, Kirubakaran et al. 2018). The bait-insect *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) has also been used solely in some studies (Sánchez-Peña et al. 2011, Aguilera Sammaritano et al. 2016).

Fewer studies used these two bait-insects in parts or throughout their investigations (Hughes et al. 2004, Oddsdottir et al. 2010, Meyling et al. 2012). Hughes et al. (2004) noticed increased isolations of *Beauveria* and *Metarhizium* when bait-insects *G. mellonella* and *T. molitor*, respectively were used. This raised a question whether *Beauveria* and *Metarhizium* have preferences for the two common bait-insects *G. mellonella* and *T. molitor*? The main objectives of the above-mentioned and noteworthy studies were different. Hence, the observations of any insect species-specific differences remained obscure especially as no significant differences were observed.

Due to the lack of any study which focuses primarily on the differences of *Beauveria* and *Metarhizium* occurrences from soils while using *G. mellonella* and *T. molitor* bait-insects, some of the most recent and noteworthy studies, even those reported in the last few months, still use the *Galleria*-bait method as the standard (only) methodology to recover EPF from soils (Fernández-Salas et al. 2017, Gan and Wickings 2017, Kirubakaran et al. 2018). Keyser et al. (2015) compared the use of *T. molitor* against culturing soil samples over selective medium and a found a drastic contrast where the former was found highly effective over the latter. Although *T. molitor* has been used previously, still some very recent and interesting studies have, however, used *G. mellonella* and neglected the use of *T. molitor* even when the main objective was to understand the ecology of *Metarhizium* (Hernández-Domínguez and Guzmán-Franco 2017).

The influence of the use of *T. molitor* as a bait-insect to isolate EPF such as *Beauveria* and *Metarhizium*, if any, when compared with *G. mellonella*, remains an important question, especially after the observations of Hughes et al. (2004), as described earlier. Moreover, as different fungal entomopathogens are susceptible to different bait-insects as well as habitat-types, another important question, that might be of interest, is to understand what is the major factor(s), if any, that governs the recovery of common EPF such as *Beauveria* and *Metarhizium*.

Although there are previous reports on the EPF from different agroecosystems, the information on the functional diversity of EPF in vineyards is, however, very limited. The landscape of the Douro Wine Region (DWR) provides a good opportunity to understand the differences in EPF abundance and diversity amongst vineyards and adjacent hedgerows. Hence, the objectives of the work were to elucidate the effects of (1) habitat-types, i.e. cultivated soils of vineyards and semi-natural soils of nearby hedgerows and (2) bait-insects, i.e. *T. molitor* and *G. mellonella* on EPF while exploring (a) their recoveries, (b) ecological proximities and (c) the principal factors governing their presence in the soils of the vineyards of the DWR of Portugal. The focus of the investigation was to understand the functional fungal entomopathogenicity of soils.

Methods

Soil sampling

Soil samples were collected from six different farms of Portuguese DWR in September and October 2012, i.e. Arnozelo, Aciprestes, Carvalhas, Cidrô, Granja and S. Luiz. Details of geographic coordinates and altitudes of these farms are given in Fig. 1A. The sampling strategy was adapted from Klingen et al. (2002) and Goble (2010) and presented in Fig. 1B and the authors find it quite similar to that undertaken by Clifton et al. (2015). In brief, at each site, the surface litter was removed and the soil was dug to a depth of 20 cm with a soil core borer (width = 20 mm) at five places within 0.25 m^2 area. All five sub-samples from one site were put in the same polyethylene bag and sealed with a rubber band. This mix of five subsamples was considered as one soil sample from a site. The next sampling site was at 20 m away and the soil borer was washed with 5% sodium hypochlorite (NaOCl) between the sites. In total, 183 soil samples were collected, out of which 155 were from vineyards and 28 were from adjacent hedgerows. Hedgerows were mainly composed of oaks (*Quercus* spp. L., Fagaceae) and pine (*Pinus* spp. L., Pinaceae) trees. Soil samples were brought inside the laboratory and were spread on a tray and left overnight for the moisture to be equilibrated with the room temperature. This was done to avoid infestation with entomopathogenic nematodes (EPN), if any, as suggested by Quesada-Moraga et al. (2007). Soil samples were always processed within 24 hours of spreading on to the trays. The number of soil samples collected from each farm is provided in Table 1.

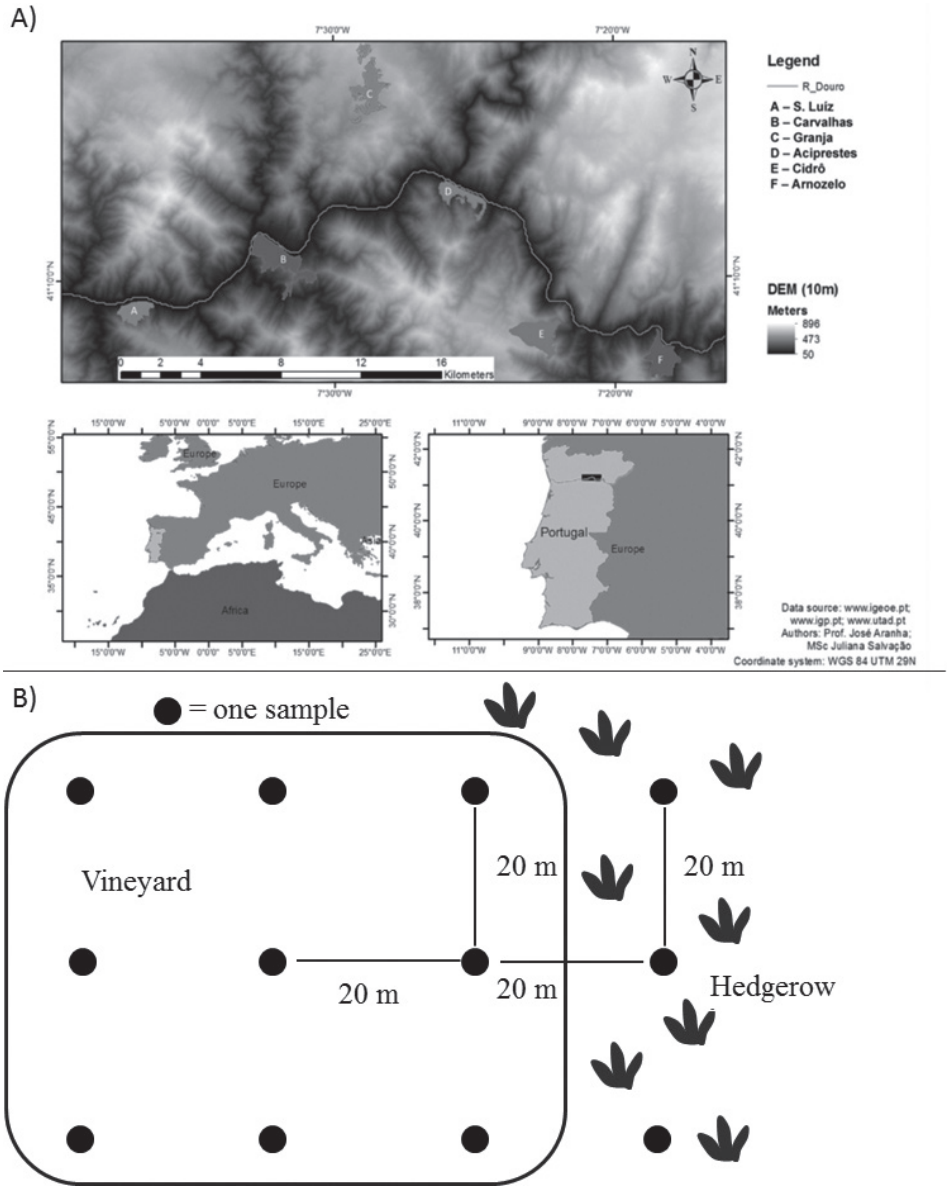

Figure 1. Geographic coordinates and altitudes of the farms and details of the soil sampling strategy adopted. **a** Details of the six farms of the Douro Wine Region, Portugal, which were considered in this study **b** Details of the soil sampling strategy from vineyards and adjacent hedgerows.

Insect baiting

Two hundred and fifty grams (g) of sieved soil was put in a plastic bowl with small holes on the cap for ventilation. A total of 183 soil samples were used to compare the effect of habitat-type on fungal isolations. For each soil sampling site, four such bowls,

Table 1. Occurrence frequency (% of positive samples) of entomopathogenic fungi Douro vineyards' soils and adjacent hedgerows.

Species	Species occurrence in the whole farm (*F*wf)						%*F*v	%*F*h	%*F*overall	Previous reports
	S. Luiz (*N* = 51)	Carvalhas (*N* = 44)	Granja (*N* = 26)	Arnozelo (*N* = 20)	Acipreses (*N* = 20)	Cidrô (*N* = 22)				
All species*	37.25	59.09	61.54	45	30	22.73	39.35	71.43	44.26	
Beauveria bassiana	15.69	11.36	15.38	10	15	4.55	12.26	14.29	12.57	Several
Beauveria pseudobassiana	1.96	6.82	–	10	–	–	–	21.43	3.28	Several
Beauveria varroae	–	–	–	5	–	–	–	3.57	0.55	Several
Clonostachys rosea f. *rosea*	19.61	45.45	42.31	25	20	22.73	25.81	53.57	30.05	Several
Cordyceps sp.	3.92	2.27	–	–	–	–	1.94	–	1.64	Several
Cordyceps cicadae	3.92	–	–	–	–	–	–	7.14	1.1	Several
Lecanicillium aphanocladii	3.92	–	–	–	–	–	1.29	–	1.1	Several
Lecanicillium dimorphum	3.92	2.27	–	–	–	–	1.94	–	1.64	Several
Metarhizium robertsii	3.92	2.27	30.77	–	–	–	7.1	–	6.01	Several
Metarhizium guizhouense	1.96	–	3.85	–	–	–	1.29	–	1.1	Several
Purpureocillium lavendulum	–	2.27	–	–	–	–	0.65	–	0.55	This study
Purpureocillium lilacinum	9.8	13.64	15.38	10	–	–	10.32	3.57	9.29	Several

*, 12 different fungal species in total.

N: Number of soil samples.

%*F*v: Percentage frequency of the number of soil samples harbouring a particular fungal species isolated from 155 soil samples from vineyards' soils of six farms.

%*F*h: Percentage frequency of the number of soil samples harbouring a particular fungal species isolated from 28 soil samples from hedgerows' soils of six farms.

%*F*overall: Percentage frequency of the number of soil samples harbouring a particular fungal species isolated from all 183 soil samples from six farms.

*F*wf: Percentage frequency of the number of soil samples harbouring a particular fungal species isolated from total number of soil samples collected from that respective farm.

i.e. 1 kg of the soil was analysed in total and four late instar *T. molitor* larvae were put in each of these bowls, i.e. the total number of larvae used (*n*) = 16. To study the effect of insect baiting, 81 of the total 183 soil samples were baited with late instar larvae of *G. mellonella* (*n* = 8) and *T. molitor* (*n* = 8) similarly, such that the total number of larvae, irrespective of the bait-insect type, remained same, i.e. *n* = 16. These 81 soil samples were from the three farms with a relatively diverse landscape, i.e. S. Luis, Carvalhas and Granja, as reported by Carlos et al. (2013). Hence, these farms were chosen to enhance the fungal diversity, in theory. This would facilitate studying the effect of insect baiting on a rather diverse group of EPF. *Galleria mellonella* was given heat shock by immersing in 56 °C water prior to baiting, to reduce the tendency of silk web formation within soil samples as suggested by Meyling and Eilenberg (2006). Bowls

were kept in an environmental chamber (Panasonic MLR-352H-PE) at a temperature of 22 °C and relative humidity of 85%, in the dark. Bowls were frequently inverted, shaken gently and kept upside down for the total incubation period of three weeks as per Meyling and Eilenberg (2006).

Fungal isolation and screening

The presence of insect cadavers was observed every day for the first week and every second day for the remaining two weeks. Everyday monitoring was necessary for the first week as death by EPN, if any, generally was caused within the first three days of larvae incubation in soils, although slightly delayed infection cannot be neglected. The schedules were monitored rigorously and the insect cadavers were observed quite carefully. Any cadavers with a foul smell were constantly discarded. Obtained cadavers were washed with 1% NaOCl for three minutes, followed by three distinct washes of 100 ml sterilised distilled water for three minutes each. It was done to isolate only the fungi which have penetrated the insect cuticles and proliferated within the insect haemocoel or have been ingested into the haemocoel. The cadavers were subsequently cultured on to potato dextrose agar (PDA) (Liofilchem) plates supplemented with 0.1 g/l streptomycin (Acros) and 0.05 g/l tetracycline (Acros). In cases of mixed infections or inhibited fungal growth, cadavers were cultured on to oatmeal agar (OA) supplemented with 0.5 g/l chloramphenicol (Acros) and 0.6 g/l cetyl trimethyl ammonium bromide (CTAB) (Sigma) as described in Posadas et al. (2012). Repeated culturing on OA or/and Sabouraud dextrose agar (SDA) (Prolabo) was undertaken until the pure culture of fungus was obtained. Plates were repeatedly observed through a low magnifying stereomicroscope (Olympus SZX9, 40X magnification) and, if any emergence of nematodes were observed, they were discarded no matter if a fungal growth was present or absent. Any possibility of cross-contamination or external contamination was carefully monitored as described by Steinwender et al. (2014). No colony forming units (CFUs) were observed in any of the tests for contaminations. To confirm Koch's postulates, all the obtained fungi were tested using bioassays for pathogenicity against the larvae from which they were isolated. The method was initially described by Ali-Shtayeh et al. (2003), however, a modified protocol was used as described in Sun and Liu (2008) and Goble et al. (2010). The fungi found pathogenic to insect larvae were considered further in this study.

Fungal identification and DNA extraction

The appearance on the infected larvae and morphological characteristics were used as the preliminary identification of fungi. Morphological characteristics that were used for identification are described in a taxonomic key (Domsch et al. 2007). For molecular identification, DNA was extracted from fungal mycelium as described earlier by

Möller et al. (1992). Moreover, the protocol was optimised for hard-to-crush mycelium and spores as in Sharma et al (2018). The fungal internal transcribed spacer (ITS) region was amplified using the forward primer ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns 1993). The PCR reaction was performed as described in Yurkov et al. (2015). Primers used for PCR reactions were also used for amplicon sequencing. Sequences were edited using BioEdit 7.2.1 (Hall 1999) and further aligned using MAFFT version 7 (Katoh and Standley 2013) to validate polymorphisms amongst sequences. Obtained ITS sequences from EPF were aligned with those from the respective type strain sequences using BLASTn and the identity results are shown in Suppl. material 1: Table S4. Newly generated sequences were submitted to EMBL nucleotide sequence database and the accession numbers are provided in the Suppl. material 1: Table S4.

Data analyses

Fungal species richness (*S*) was compared in terms of habitat-types and bait-insects used for isolation. Jaccard's similarity coefficients (*J*) for fungal species shared between different habitats and bait-insects were measured as described in Garrido-Jurado et al. (2015). $J = a/(a+b+c)$, where "*a*" represents the number of species occurring in both variables, "*b*" represents the number of species occurring only in variable 1 and "*c*" represents the number of species occurring only in variable 2. *J* can range between 0 (no shared species) to 1 (all shared species). Software IBM SPSS Statistics 22 was used to perform statistical data processing. Infections were counted qualitatively per site, i.e. whether a particular fungus infected one or several insect larvae of the same bait-insect, it was registered as one infection for that fungal species, as described in Klingen et al. (2002) and Goble et al. (2010). Therefore, effects of soil habitat-types and bait-insects are counted in accordance with the number of soil samples found harbouring a fungal species as in Klingen et al. (2002), Goble et al. (2010) and Clifton et al. (2015). Data were treated using Fisher's exact test as it gives the exact *P* value for a 2×2 contingency table (https://www.graphpad.com/). Besides, farm type variations could only be analysed using the χ^2 (chi-square) test and Monte Carlo simulations were used in case the cells have the expected count of less than 5. Data used for different analyses, i.e. (1) effect of bait-insect type on the occurrence of EPF; (2) effect of habitat-type (hedgerows vs. vineyards) on EPF occurrence; and (3) effect of farm type on EPF occurrence, are provided in detail within the Suppl. material 1: Tables S1, S2 and S3, respectively. To compare possible factors which may influence fungal recoveries, a principal component analysis (PCA) was performed. The PCA was conducted on the mean-centred and scaled data in order to investigate the discriminations of the obtained fungal species. For the PCA plots, only those soils samples were considered where both the bait-insects, i.e. *T. molitor* and *G. mellonella* were used, i.e. soils from the farms S. Luis, Carvalhas and Granja (Suppl. material 1: Table S1). Fungi with isola-

tion frequencies of <10% from either vineyards or hedgerows were considered as rare EPF. Hierarchical clustering was then employed to investigate the degree of similarities of fungal isolations based on their ecological proximities, i.e. in terms of habitat-type and bait-insect type. The resulting dendrogram was obtained based on the Euclidean distance and Ward aggregation method as in Sharma et al. (2018). Software R 3.4.2 was used to generate PCA plots and hierarchical clustering.

Results

Overall fungal species abundance

The total numbers of soil samples used were 183 and the number of soil samples found positive (*N*) with any EPF were 81, i.e. 44.26% ± 3.67% soils. A total of 12 different species were observed (Table 1). *Clonostachys rosea* f. *rosea* (Link) Schroers, Samuels, Seifert & Gams was found in the maximum number of soil samples i.e. 30.05% ± 3.38% (*N* = 55), followed by *B. bassiana* (12.57% ± 2.37% (*N* = 23)), *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (9.29% ± 2.14% (*N* = 17)) and *Metarhizium robertsii* Bischoff, Rehner & Humber (6.01% ± 1.75% (*N* = 11)).

Isolations of *Beauveria pseudobassiana* Rehner & Humber (3.38% ± 1.31% (*N* = 6)), *Cordyceps* sp. Fries (1.64% ± 0.94% (*N* = 3)), *Lecanicillium dimorphum* (Chen) Zare & Gams (1.64% ± 0.94% (*N* = 3)), *Cordyceps cicadae* (Miq.) Massee (1.10% ± 0.77% (*N* = 2)), *Lecanicillium aphanocladii* Zare & Gams (1.10% ± 0.77% (*N* = 2)), *Metarhizium guizhouense* Chen & Guo (1.10% ± 0.77% (*N* = 2)), *Beauveria varroae* Rehner & Humber (0.55% ± 0.54% (*N* = 1)) and *Purpureocillium lavendulum* Perdomo, García, Gené, Cano & Guarro (0.55% ± 0.54% (*N* = 1)) were also observed (Table 1). The fungal occurrence was the highest in the farm Granja, i.e. 61.54% ± 9.54% (*N* = 16), followed by Carvalhas (59.09% ± 7.4% (*N* = 26)), Arnozelo (45% ± 11.12% (*N* = 9)), S. Luiz (37.25% ± 6.77% (*N* = 19)), Aciprestes (30% ± 10.24% (*N* = 6)) and Cidrô (22.73% ± 8.93% (*N* = 6)) (Table 1).

Effect of insect baiting on fungal isolation

To test the effect of insect baiting on EPF recoveries, bait-insects *G. mellonella* (*n* = 8) and *T. molitor* (*n* = 8) were employed on 81 soil samples from the three farms which had quite diverse landscapes, i.e. S Luiz, Carvalhas and Granja. Hence, in total, 16 larvae from two different bait-insects were used. Eleven EPF species were observed amongst the three farms and a few significant differences were detected within fungal recoveries (Fig. 2A, Suppl. material 1: Table S1). Significantly more soil samples were found positive for *B. bassiana* when *G. mellonella* was used as a bait-insect, i.e. 15 isolates (18.52% ± 4.31%) than *T. molitor,* i.e. 4 isolates (4.94% ± 2.4%) (*P* = 0.038). On the contrary,

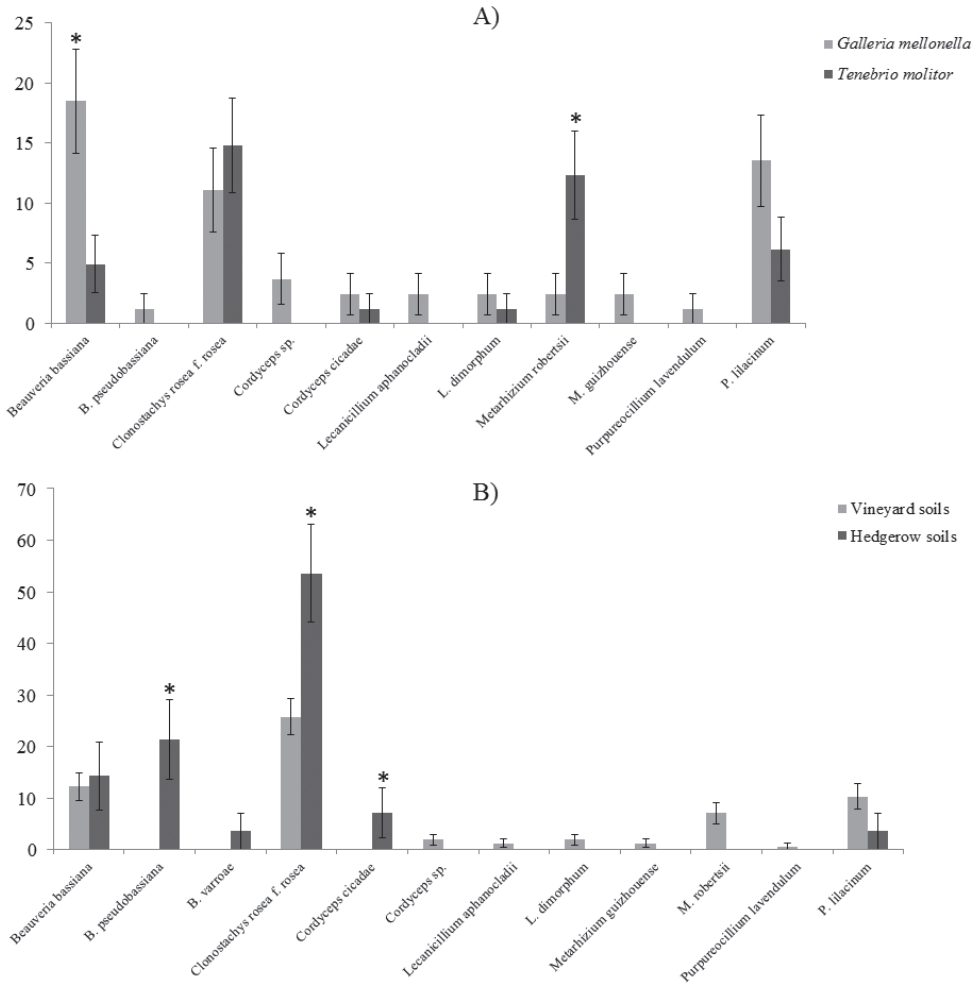

Figure 2. Effect of insect baiting and habitat-type on the isolation of the entomopathogenic fungi. **a** Occurrence (% of soil samples ± SE) of entomopathogenic fungi when different bait-insects were incorporated **b** Occurrence (% of soil samples ± SE) of entomopathogenic fungi when soils were collected from different habitat-types. Bars with asterisk (*) show significant isolations, i.e. (P<0.05).

isolation of *M. robertsii* was increased significantly by *T. molitor*, i.e. 10 isolates (12.35% ± 3.65%) compared to *G. mellonella*, i.e. 2 isolates (2.47% ± 1.72%) (P = 0.003).

Clonostachys rosea f. *rosea* was isolated more often by *T. molitor*, i.e. (14.81% ± 3.94% (N = 12)) than by *G. mellonella*, i.e. (11.11% ± 3.49% (N = 9)). Moreover, *T. molitor* specific isolations were noticed for *M. guizhouense*, i.e. 2.47% ± 1.72% (N = 2). However, *G. mellonella* recovered more *C. cicadae* and *L. dimorphum*, i.e. 2.47% ± 1.72% (N = 2) than 1.23% ± 1.22% (N = 1) by *T. molitor*, in cases of both the fungi. *Galleria mellonella* specific isolations for *Cordyceps* sp. (3.79% ± 2.09% (N = 3)), *L. dimorphum* (2.47% ± 1.72% (N = 2)) and *P. lavendulum* (1.23% ± 1.22% (N = 1)) were also recorded (Fig. 2A, Suppl. material 1: Table S1). Overall, using *G. mellonella* yielded slightly more fungal species (i.e. S = 10) than *T. molitor* (i.e. S = 7) (Table 2).

Effect of habitat-types on fungal isolation

To study the habitat type variation, 183 soil samples from all the six farms were considered, i.e. 155 from vineyards and 28 from hedgerows. As two different bait-insects, *G. mellonella* and *T. molitor*, were used in the three farms, i.e. S. Luiz, Carvalhas and Granja and only one bait-insect *T. molitor* was used in the other farms, i.e. Aciprestes, Arnozelo and Cidrô, the numbers of bait-insects larvae used to study the habitat-type variations in each farm were kept constant, i.e. $n = 16$.

Out of 155 soil samples from vineyards, a total of nine EPF species were observed in 61 vineyards' soils, i.e. 39.35% ± 3.81% soils were found harbouring at least one EPF. Six fungal species were observed solely from vineyards, i.e. *Cordyceps* sp. (1.94% ± 1.1% ($N = 3$)), *L. aphanocladii* (1.29% ± 0.9% ($N = 2$)), *L. dimorphum* (1.94% ± 1.1% ($N = 3$)), *M. robertsii* (7.10% ± 2.06% ($N = 11$)), *M. guizhouense* (1.29% ± 0.9% ($N = 2$)) and *P. lavendulum* (0.65% ± 0.64% ($N = 1$)). Although *M. robertsii* was isolated only from vineyards, however, recoveries were not significant ($P = 0.220$). Three species, i.e. *P. lilacinum*, *C. rosea* f. *rosea* and *B. bassiana* were shared amongst both habitat-types. *Purpureocillium lilacinum* was isolated more frequently from vineyard soils i.e. 16 isolates (10.32% ± 2.44%) than hedgerows, i.e. 1 isolate (3.57% ± 3.50%), however, non-significantly ($P = 0.228$) (Fig. 2B, Table 1).

Beauveria bassiana was slightly more abundant in hedgerows, i.e. 4 isolates in 28 samples (14.29% ± 6.61%) than in vineyards, i.e. 19 isolates in 155 samples (12.26% ± 2.63%), although differences were not significant ($P = 0.759$) (Table 1), (Fig. 2B). *Clonostachys rosea* f. *rosea* was also more frequent in hedgerows, i.e. in 15 of the 28 samples (53.57% ± 9.42%) than in vineyards i.e. 40 of the 155 samples (25.81% ± 3.51%) ($P = 0.006$). Moreover, *B. pseudobassiana* only occurred in hedgerows, i.e. 6 isolates (21.43% ± 7.75%) ($P<0.001$). *Beauveria varroae* (3.57% ± 3.50% ($N = 1$)) and *C. cicadae* (7.14% ± 4.86% ($N = 2$)) ($P = 0.023$) were also noticed in hedgerows' soils only (Fig. 2B). Overall, significantly higher number of soil samples were found positive for EPF in hedgerows, i.e. 20 isolates in 28 samples (71.43% ± 8.53%), than in vineyards, i.e. 61 isolates in 155 samples (39.35% ± 3.92%) ($P<0.001$) (Table 1). However, fungal species richness (S) was higher in soils from vineyards, i.e. $S = 9$ than from hedgerows, i.e. $S = 6$ (Table 2). Additional information on the habitat-types variations is shown in Suppl. material 1: Table S2.

Farm type variation

Those EPF which were recovered from all six farms using *T. molitor* larvae ($n = 16$) only, were considered to study the farm type variations. This was done to avoid any bias as *T. molitor* was the bait-insect used in all six farms. Nine EPF species were recovered and *C. rosea* f. *rosea* was isolated significantly more from Carvalhas, i.e. from 18 of the total of 48 soil samples collected from the respective farm ($N = 18/48$), (37.5% ± 6.98%) ($\chi^2 = 12.981$, df = 5, $P = 0.0024$). *Metarhizium robertsii* was isolated more frequently from Granja ($N = 8/11$) (72.72% ± 13.4%) ($\chi^2 = 33.657$, df = 5, $P<0.001$). *Beauveria*

Table 2. Entomopathogenic fungal species richness and similarities amongst isolations from different habitat-types and bait-insects.

	Observed species (*S*, richness)		**Jaccard coefficient (*J*)**
	Vineyards	Hedgerows	*J*(habitat)
Soil(GM)	8	5	0.435
Soil(TM)	6	4	0.41
Soil*	9	6	0.44
	Galleria mellonella	*Tenebrio molitor*	*J*(bait-insect)
Soil(V)	8	6	0.39
Soil(H)	5	4	0.35
Soil#	10	7	0.39

Soil(GM): soil samples baited by *Galleria mellonella* larvae; Soil(TM): soil samples baited with *Tenebrio molitor* larvae; Soil(V): soil samples collected from vineyards; Soil(H): soil samples collected from vineyards.

*, overall samples irrespective of bait-insect type.

#, overall samples irrespective of habitat-type.

Note: Jaccard coefficient for similarity amongst habitat types, *J*(habitat) = a/(a + b + c), where "a" is the number of species occurring in both habitats, "b" is the number of species specific to vineyards and "c" is the number of species specific to hedgerows. *J* ranges from 0 (no shared species amongst habitats) to 1 (all species are shared amongst habitats). Similar calculations were done for *J*(bait-insect), where values corresponded to observed fungal species when different bait-insects were used.

bassiana was found distributed throughout all farms, i.e. Aciprestes (*N* = 3/20) (15% ± 7.98%); Arnozelo (*N* = 2/20) (10% ± 6.7%), S. Luiz (*N* = 3/51) (5.88% ± 3.29%), Carvalhas (*N* = 2/44) (4.55% ± 3.14%), Cidrô (*N* = 1/22) (4.55% ± 4.44%) and Granja (*N* = 1/26) (3.85% ± 3.77%). *Purpureocillium lilacinum* was found in four of the six farms, i.e. Arnozelo (*N* = 2/20) (10% ± 6.7%), Carvalhas (*N* = 2/44) (4.55% ± 3.14%), S. Luiz (*N* = 2/51) (3.92% ± 2.71%) and Granja (*N* = 1/26) (3.85% ± 3.77%). More details about other fungi are in the supplementary information (Suppl. material 1: Table S3).

Ecological proximities based dendrogram and principal recovery factors

A PCA was performed on the EPF recovery data from the 81 soils of the three farms, i.e. S. Luis, Carvalhas and Granja, where both habitat-types and bait-insects were incorporated. This kind of analysis was done to understand which element(s), i.e. bait-insect(s) and/or habitat-type(s), governs the recovery of the EPF. Using PCA, 89.9% of the variance among fungal recoveries could be described by the three principal components, i.e. PC1 (55%), PC2 (21.7%) and PC3 (13.2%) (Fig. 3A, B, C). Second principal component (PC2) was slightly dominated by the type of bait-insect used (Fig. 3A, C). The occurrences of *B. bassiana* and *P. lilacinum* were slightly and marginally governed by insect baiting using *G. mellonella*, respectively. However, the isolations of *C. rosea* f. *rosea* and *M. robertsii* were slightly and mainly governed by baiting using *T. molitor*, respectively (Fig. 3A–D). Third principal component (PC3)

could distinctly separate the two habitat-types (Fig. 3B, C). The isolations of *C. rosea* f. *rosea* were mostly governed by semi-natural habitats. However, *M. robertsii* and *P. lilacinum* were highly and slightly influenced also by cultivated habitats, respectively. *Codyceps cicadae* recovery was governed only by hedgerows (Fig. 3A–D). Hierarchical clustering dendrogram of the ecological proximities of fungi, after profiling their recoveries from bait-insects and habitat-types, placed *B. bassiana* and *P. lilacinum* closer, while *C. rosea* f. *rosea* and *M. robertsii* were quite different and distinct (Fig. 3E). Moreover, the dendrogram also separated rare EPF, i.e. those with an isolation frequency of <10% from either of the habitat-types (cluster 1), from relatively more frequent EPF (cluster 2) (Fig. 3E).

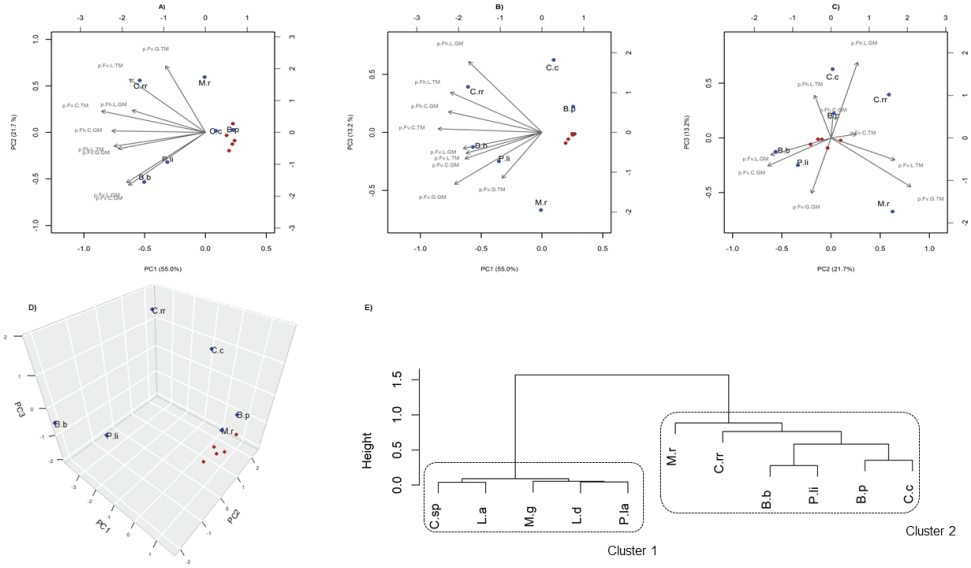

Figure 3. Principal component analysis (PCA) and hierarchical clustering of the observations based on the fungal isolations. **a** PC1 vs. PC2. **b** PC1 vs. PC3. **c** PC2 vs. PC3. **d** PCA 3D plot **e** Hierarchical clustering dendrogram to access the ecological proximities of obtained fungi based on their respective isolation profiles. Software R 4.3.2 was used to obtain the PCA plots and the hierarchical clustering. There was no fungal isolation from hedgerows from the farm Granja when bait-insect *T. molitor* was used and hence, it could not be included in any of the analysis which relies on proportions, i.e. PCA plots, hierarchical clustering. To reduce any bias, the authors also discarded the soil samples (*N*=1) which yielded the fungal isolations, when *G. mellonella* was used, from the hedgerows of the farm Granja. The blue balls represent relatively more frequent EPF, i.e. *Beauveria bassiana*, *Beauveria pseudobassiana*, *Clonostachys rosea* f. *rosea*, *Cordyceps cicadae*, *Purpureocillium lilacinum* and *Metarhizium robertsii*. The red balls represent other fungi such as *Cordyceps* sp., *Lecanicillium aphanocladii*, *Lecanicillium dimorphum*, *Metarhizium guizhouense* and *Purpureocillium lavendulum*. Hierarchical clustering based dendrogram classified isolated EPF into two clusters, i.e. rarely occurring EPF (cluster 1) and relatively more frequent EPF (cluster 2). Abbreviations used are: *Beauveria bassiana* (B.b), *Beauveria pseudobassiana* (B.p), *Cordyceps cicadae* (C.c), *Cordyceps* sp. (C.sp), *Lecanicillium aphanocladii* (L.a), *Lecanicillium dimorphum* (L.d), *Metarhizium guizhouense* (M.g), *Purpureocillium lavendulum* (P.la), *Purpureocillium lilacinum* (P.l), *Clonostachys rosea* f. *rosea* (C.rr) and *Metarhizium robertsii* (M.r).

Discussion

Insects baiting of soils for EPF recovery

Considering the number of soil samples and the objectives, this study was comparable with others on EPF occurrence and diversity (Tarasco et al. 1997, Klingen et al. 2002, Ali-Shtayeh et al. 2003, Quesada-Moraga et al. 2007, Sun et al. 2008, Imoulan et al. 2011, Schneider et al. 2012). The '*Galleria*-bait method', i.e. using *G. mellonella* for EPF recovery from soils, was described by Zimmermann in the year 1986 (Zimmermann 1986). Since then it has been used quite often in numerous studies as the only method for EPF isolations, in the past three decades (Chandler et al. 1997, Bidochka et al. 1998, Ali-Shtayeh et al. 2003, Meyling and Eilenberg 2006, Quesada-Moraga et al. 2007, Sun and Liu 2008, Sun et al. 2008, Sevim et al. 2009, Fisher et al. 2011, Muñiz-Reyes et al. 2014, Pérez-González et al. 2014, Fernández-Salas et al. 2017, Gan and Wickings 2017, Kirubakaran et al. 2018). Similarly, in few other studies, insect baiting using *T. molitor* is the only method used for the EPF recovery (Sánchez-Peña et al. 2011, Steinwender et al. 2014).

Fungal recovery using *Galleria mellonella* bait-insect

Beauveria bassiana was isolated significantly more from *G. mellonella* (P = 0.038) (Fig. 2A) as in South Africa by Goble et al. (2010). Klingen et al. (2002) found insect-specific isolations of *B. bassiana* by *G. mellonella* in Norway. Studies in Iceland and Greenland also concluded that *B. bassiana* was isolated more often by *G. mellonella* (Oddsdottir et al. 2010, Meyling et al. 2012). Many previous reports are available on the recovery of different fungi from *G. mellonella*, for example, *C. cicadae* (Barker and Barker 1998), *P. lilacinum* (Imoulan et al. 2011), *Lecanicillium* spp. (Hypocreales: Cordycipitaceae) (Asensio et al. 2003, Meyling and Eilenberg 2006), as in the present study. To our knowledge, this study reports the first isolation of *P. lavendulum* from an insect.

Fungal recovery using *Tenebrio molitor* bait-insect

In the present study, insect-specific isolation of *M. guizhouense* and significant isolation of *M. robertsii* was reported from *T. molitor* (P = 0.003) (Fig. 2A) (Suppl. material 1: Table S1). Comparing *G. mellonella* and *T. molitor*, insect-specific isolation of *Metarhizium* has been reported using the latter (Oddsdottir et al. 2010). Hughes et al. (2004) found that, out of the 20 soils sampled, 15 harboured *Metarhizium* when *T. molitor* was used as bait-insect, compared with just four when *G. mellonella* was used. *Metarhizium* was found to be the most abundant EPF in the soils from the tropical forests of Panama, although the soils were collected within 5 m from the nest of leaf-cutting ants (insect host) which possibly increased EPF recovery. Nonetheless, the major drawback of the

study was that a very limited number of soil samples were used and the results were not analysed statistically (Hughes et al. 2004). In the present study, 81 soil samples were used to study the effect of insect baiting on EPF recovery. Moreover, a random selection of soil samples was promoted to reduce any bias for an enhanced EPF recovery and to maintain a practical scenario where no prior information on the presence of insect-host is necessary.

To our knowledge, this is the first report on the significantly higher recovery of *M. robertsii* by *T. molitor* when compared with that from *G. mellonella*. *Galleria*-bait is still a widely used method to isolate EPF from soils. Even the most recent reports, i.e. those reported in the past few months, overlook the use of *T. molitor* while studying with ecologies of EPF such as *Metarhizium* (Fernández-Salas et al. 2017, Gan and Wickings 2017, Hernández-Domínguez and Guzmán-Franco 2017, Kirubakaran et al. 2018). This study signifies that the use of both of the bait-insects is more important than considered before and *T. molitor* should always be used along with *G. mellonella*, especially when *Metarhizium* is being isolated from soils. Enhanced recovery of *Metarhizium* from *T. molitor* could be due to the higher sensitivity of the insect towards this fungus. Vänninen et al. (2000) found that even after three years post application, *M. anisopliae* could kill over 80% of the *T. molitor* baited in soils from different places.

Entomopathogenic fungal communities within hedgerows' soils (semi-natural habitat)

In this study, 15.3% of the total soil samples were from hedgerows, which were comparable with 20.5% of the soil samples from hedgerows examined by Meyling and Eilenberg (2006). *Beauveria bassiana* was slightly more abundant in hedgerows than in vineyards (Table 1), (Fig. 2B). Some previous studies also did not report any significant habitat preference for *B. bassiana* (Klingen et al. 2002, Quesada-Moraga et al. 2007). Only the soils from hedgerows could lead to the isolation of *B. pseudobassiana* and it was significant (P<0.001) (Fig. 2B). This finding agreed with Meyling and Eilenberg (2007), who found *B. pseudobassiana* only in hedgerows. *Cordyceps cicadae* was also isolated in significant amounts from hedgerows (P = 0.023) (Fig. 2B). Barker and Barker (1998) reported that *C. cicadae* isolations were restricted to forest soils (i.e. less disturbed soils). To our knowledge, this is the first report on the isolation of *C. cicadae* from Mediterranean soils. *Clonostachys rosea* f. *rosea* was isolated more from less disturbed (i.e. orchard) soils than intensively disturbed (i.e. field crops) soils in this study as in Sun et al. (2008).

A possible reason of higher occurrence of *B. bassiana* and the habitat-specific occurrence of *B. pseudobassiana* and *B. varroae* in hedgerows could be the relatively higher dependence of *Beauveria* on secondary infections on insect hosts, as hedgerows are expected to host rather diverse insect communities (Goble et al. 2010). Besides, factors such as reduced ultra-violet radiation and temperatures, increased humidity and long-term environmental stability could also lead to an increased viability of these fungal spores (Meyling et al. 2009). Mycoparasitism, a characteristic of *B. bassiana* (Vega et al. 2009) and *C. rosea* (Keyser et al. 2016), could provide dominance amongst opportunistic saprophytes in hedgerows.

Entomopathogenic fungal communities in vineyards (cultivated habitat)

Although *Purpureocillium lilacinum* and *M. robertsii* were isolated more from vineyards' soils, the results were, however, non-significant, i.e. $P = 0.228$ and $P = 0.220$ (Fig. 2B). Moreover, two strains of *M. guizhouense* were also isolated only from vineyards (Table 1). *Purpureocillium lilacinum* could tolerate a wide range of temperatures, from 8 °C to 38 °C and pH (Roumpos 2005). As these properties provide robustness against agricultural disturbances, according to Wei et al. (2009), *P. lilacinum* is the most widely tested fungus under field conditions. Higher isolations of *Metarhizium* spp. from crop cultivated lands in Spain and Mexico have been reported (Quesada-Moraga et al. 2007, Sánchez-Peña et al. 2011). Tillage seemed to distribute *Metarhizium* CFUs evenly throughout the field which subsequently increases chances of fungal recovery from different sites (Kepler et al. 2015).

Fungal species richness (*S*) was higher in soils from vineyards, i.e. $S = 9$ than hedgerows, i.e. $S = 6$ (Table 2). Few genera mentioned in Table 1 were previously reported to be isolated more often from relatively more disturbed soils, for example, *Lecanicillium* (Meyling and Eilenberg 2006). Moreover, Sun et al. (2008) found higher species richness in soils of crop fields than from orchards soils (i.e. less disturbed soils), as in the present study.

More diverse fungal species in cultivated soils is not surprising. Practices such as ploughing, reseeding and fertilising increase environmental patches and niche availability for EPF and subsequently increase fungal diversity (Sun et al. 2008). The higher organic matter also increases biological activity in the soil which positively affects the presence of saprophytic fungi which lead to lesser organic resources for EPF and therefore, reduced survivability (Goble et al. 2010).

Factors, ecological proximities and hierarchical clustering dendrogram of fungi

Studies on the EPF ecology in soils consider either different bait-insects or habitat-types or both, as discussed earlier. Principal component analysis was done to understand the most important factor, if any, that governs the recoveries of EPF. It was found that isolations of *B. bassiana* were slightly governed by baiting with *G. mellonella*, irrespective of the habitat-type incorporated (Fig. 3A, C, D). However, the isolations of *M. robertsii* were influenced both by the cultivated habitat-type as well as by baiting with *T. molitor* (Fig. 3A–D). The ecological proximities of *B. bassiana* and *P. lilacinum* could be explained as *P. lilacinum* was isolated more frequently from vineyard soils than from hedgerows and *B. bassiana* isolations were almost equal from vineyards to those from hedgerows (Figs 2B, 3D, E). Moreover, the bait-insect *G. mellonella* favoured *P. lilacinum* and *B. bassiana* isolations (Fig. 2A). Distinct profiles of *C. rosea* f. *rosea* and *M. robertsii* suggest their unique ecologies in terms of habitat-type and bait-insect preferences (Fig. 3D, E). The main advantage of fungal profiling by hierarchical clustering based dendrogram is that those EPF which were not isolated in this study can also be investigated for their roles in the biological control of interest pests in agroecosystems, if they exhibit similar ecological profiles (Sharma et al. 2018).

Fungal abundance and diversity

Entomopathogenic fungi was observed in 44.26% ± 3.67% of the soil samples and it was comparable to previous studies in Finland (38.6%) (Vänninen 1996), Palestine (33.6%) (Ali-Shtayeh et al. 2003), Alicante province, Spain (32.8%) (Asensio et al. 2003), South Africa (21.53%) (Goble et al. 2010), UK (17.6%) (Chandler et al. 1997) and southern Italy (14.9%) (Tarasco et al. 1997). More diverse fungal species were found in the present study when compared with the other studies in Mediterranean regions, for example, in Italy (Tarasco et al. 1997), Spain (Asensio et al. 2003, Quesada-Moraga et al. 2007, Garrido-Jurado et al. 2015), Turkey (Sevim et al. 2009) and Morocco (Imoulan et al. 2011). Different studies suggest that *Metarhizium* spp. are either absent (Ali-Shtayeh et al. 2003, Oliveira et al. 2012) or less prevalent in the Mediterranean region (Tarasco et al. 1997, Asensio et al. 2003, Quesada-Moraga et al. 2007, Garrido-Jurado et al. 2015). Surprisingly, Garrido-Jurado et al. (2015) reported just four isolates of *M. robertsii* from 270 soil samples in Spain which was quite a small number compared with the 11 isolates from 183 soil samples found in the present study. Occasional isolations of many species were noticed in the present study and, according to our knowledge, this is the first isolation of entomopathogenic strains of *B. varroae*, *L. aphanocladii*, *L. dimorphum*, *M. robertsii* and *M. guizhouense* in Portugal.

Conclusion

Entomopathogenic fungi have been known for their potential as insect biocontrol agents and recent studies focus on their use for conservation biological control. However, the information about their ecology in vineyards is very limited. The main aim of the research was to analyse functional fungal entomopathogenicity of the soils of DWR in Portugal. It was found that different habitat-types and bait-insects have significant effects on the isolation of certain EPF species. Species richness and abundance differed amongst soil habitats. *Clonostachys rosea* f. *rosea* is a renowned mycoparasite and, recently, it has been tested positive for endophytism and entomopathogenicity. The higher recovery of *C. rosea* f. *rosea* from semi-natural habitats suggests its use in less disturbed soils. Moreover, hedgerow-specific isolation of *B. pseudobassiana* points to its inability to withstand harsher conditions in cultivated soils. The first isolation of *C. cicadae* as an EPF from Mediterranean soils supports its biocontrol potential in this climate, at least in less-disturbed habitats. Therefore, these properties should be capitalised accordingly. Principal component analysis could decipher that baiting, using *G. mellonella*, influence the isolations of *B. bassiana*, irrespective of the habitat-type incorporated. However, *M. robertsii* isolations were highly governed by the cultivated habitat-type as well as by the use of *T. molitor* as bait-insect. Overall, it was observed that DWR harbour various EPF which could be used as potential biocontrol agents for vineyard pests such as the European Grapevine Moth and understanding the functional ecology of EPF could help in using them more efficiently.

Although *T. molitor* has been used previously on a few occasions, still many of the recent studies, even those conducted in the past few months, overlook the use of *T. molitor* when dealing with EPF and especially *Metarhizium* ecology. While these studies bring a significant advancement to our knowledge in EPF ecology, they suffer from the lack of any concrete study which highlights the significant limitations of using the '*Galleria*-bait method' alone to isolate *Metarhizium* from soils. As *G. mellonella* was a significantly better bait-insect for isolating *B. bassiana*, therefore, the combined use of *G. mellonella* and *T. molitor* is indispensable for a more complete understanding of EPF diversity and distribution within a region. In this study, the authors modify the existing '*Galleria*-bait method' and propose the use of the '*Galleria-Tenebrio*-bait method' for future studies in this area.

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Supplementary material 1

Supplementary tables

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Data type: species data

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RESEARCH ARTICLE

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A new species of *Lecidea* (Lecanorales, Ascomycota) from Pakistan

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Abstract

We describe here a new species, *Lecidea aptrootii*, in *Lecidea* sensu stricto from Swat Valley, Pakistan. It is most similar to *L. fuscoatra* in having an areolate thallus and black, lecideine apothecia with a persistent margin. However, *L. aptrootii* can be readily distinguished by having smaller ascospores (average length 8-10 µm). In phylogenetic analyses, using ITS and nuLSU rDNA sequences, *L. aptrootii* forms a sister-group relationship to *L. grisella*, which differs in having a rimose thallus.

Keywords

Asia, Lecideaceae, lichenised fungi, new species, taxonomy

Introduction

Pakistan is a country with a broad altitudinal range from sea level at the Arabian Sea to the second highest point of the world (K-2) at 8,611 m (Khan 1991). This variation in altitude is associated with diverse ecosystems, constituting 18 distinct ecoregions (IUCN 2006). Consequently, the lichen flora of this region is expected to be rich but

so far largely unknown due to lack of detailed surveys (Aptroot and Iqbal 2012). Swat is the focal point of the Hindu Kush Himalayan region of Pakistan and its lichen flora is currently being studied by the first author (MK). The area is primarily montane and spreads over 8220 km^2 of land with altitudinal variation ranging from 600 m in the south to more than 6000 m in northern high peaks (Ahmad et al. 2015). The known lichen flora of Swat represents roughly 26% of the total lichen flora of the country but almost all reports are from small localities and easily approachable picnic spots (Aptroot and Iqbal 2012). In our studies of the lichen flora of this region, an interesting, crustose lichen of the genus *Lecidea* was found.

The genus *Lecidea* Ach. (Lecideaceae) that was originally described by Acharius (1803), underwent many systematic changes and its traditional wide circumscription (Zahlbruckner 1926) dramatically changed over the last decades (Hafellner 1984; Hertel 1967, 1977, 2006). Of the 427 species included in *Lecidea* sensu lato (Kirk et al. 2008), only about 100 belong to *Lecidea* sensu stricto based on, amongst other characters, the presence of a *Lecidea*-type ascus (Hertel 2006). The taxonomy of *Lecidea* species is complex because of the morphological variation within and amongst species. The species circumscription in *Lecidea* needs revision and molecular data are helpful in interpreting subtle morphological differences that have been considered as intraspecific variability (Schmull et al. 2011). From Pakistan, so far five *Lecidea* spp. have been reported, viz. *L. atrobrunnea* (Ram.) Schaer., *L. atroviridis* (Arnold) Th.Fr., *L. bohlinii* H. Magn, *L. portensis* Nádv. and *L. tessellata* Flörke (Aptroot and Iqbal 2012). The new record of a saxicolous lichen in the Swat district adds a sixth species of the genus. Below, this species is described morphologically and chemically and molecular evidence identified that it represents a new taxon in *Lecidea* sensu stricto.

Material and methods

Morphological and chemical studies

Collections were made in August 2016 during a mycological survey of Gabin Jabba and Malam Jabba (Swat Valley) where altitude varies from 600 m to 2500 m. These areas have a moist temperate climate and remain under snow cover during winter while the summer season is accompanied by a significant amount of rainfall. Standard microscopy and spot tests (Hale 1979) were used for identification. Measurements were made from free-hand sections mounted in water. Amyloid reactions were tested with Lugol's solution with and without pretreatment with 10% KOH. High performance thin layer chromatography (HPTLC) was performed using solvent C following standard methods (Arup et al. 1993, Lumbsch 2002, Orange et al. 2001).

DNA extraction, PCR amplification and sequencing

We used apothecia to extract DNA with Fungal/Bacterial DNA Miniprep Kit (Zymo Research Corp., Irvine, CA) following the manufacturer's instructions. Molecular data were generated for two loci: the internal transcribed spacer (ITS) and the nuclear large subunit (nuLSU) ribosomal DNA. The primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) were used to amplify the ITS region; and primer pair AL2R (Mangold et al. 2008) and LR6 (Vilgalys and Hester 1990) were used to amplify the nuLSU region. Polymerase chain reactions (PCR) were performed in 12.5 µl volume per reaction using MyTaq™ Red Mix (Bioline International, Toronto, Canada). PCR protocol for the ITS region consisted of initial denaturation of 5 min at 94 °C, 40 cycles of 30 sec at 94 °C, 30 sec at 48 °C, 1.5 min at 72 °C and a final extension of 5 min at 72 °C. PCR protocol for LSU consisted of initial denaturation of 75 sec at 94.5 °C, 35 cycles of 35 sec at 95 °C, 55 sec at 55 °C, 42 sec at 72 °C and a final extension of 10 min at 72 °C. PCR products were visualised on 1% agarose gel and cleaned using Exo SAP-IT (USB, Cleveland, Ohio, USA) following the manufacturer's instructions. Cycle sequencing reactions were performed using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) with the same primers as used for the PCR amplification. The sequencing reactions were run on an ABI 3730 48-capillary electrophoresis DNA analyser sequencer according to established protocols (Applied Biosystems) at the Pritzker Laboratory for Molecular Systematics at the Field Museum, Chicago, IL, USA.

Phylogenetic analyses

The ITS locus for two specimens and nuLSU gene for one specimen were successfully amplified and sequenced. Sequences of other *Lecidea* spp. based on initial BLAST searches and those used in a recent study on *Lecidea* by McCune et al. (2017) were used in phylogenetic analyses (Table 1). *Bellemerea cinereorufescens* (Ach.) Clauzade & Roux was used as the outgroup. Multiple sequence alignments for each individual locus were performed using programme MAFFT v7 with all parameters set to default values (Kotah and Standley 2013). The ends of alignments were trimmed to nearly an equal number of sites for all sequences. All gaps were treated as missing data. ITS and nuLSU sequences were concatenated in Bioedit v7.2.5 (Hall 1999) using the append file option. Maximum likelihood analyses were performed via CIPRES Science Gateway (Miller et al. 2010) employing RAxML-HPC2 on XSEDE (Stamatakis 2014). For bootstrapping, the GTRCAT model was selected. One thousand rapid bootstrap replicates were run. Two molecular analyses (one with ITS and another with combined ITS & nuLSU rDNA) were performed since only a limited amount of nuLSU data were available, whereas a larger number of ITS sequences, available in GenBank, allow for in-depth inference of the phylogenetic position of the new species.

Table 1. GenBank accession numbers of sequences used in phylogenetic analyses.

Species	ITS	nuLSU
Bellemerea cinereorufescens	KY800500	-
Lecidea andersonii 1	EU257685	-
Lecidea andersonii 2	EU257683	-
Lecidea andersonii 3	EU257684	-
***Lecidea aptrootii* 1** Gabin Jabba (GB-1)	**MH618901**	**-**
***Lecidea aptrootii* 2** Malam Jabba (MJ-3)	**MH594348**	**MH594349**
Lecidea atrobrunnea 1	EU259897	HQ660535
Lecidea atrobrunnea 2	EU259898	AY532993
Lecidea cancriformis 1	EU357674	-
Lecidea cancriformis 2	EU257671	-
Lecidea cancriformis 3	EU257672	-
Lecidea fuscoatra 1	HQ605929	HQ660541
Lecidea fuscoatra 2	HQ605926	AY756339
Lecidea fuscoatra var grisella 1	HQ605931	HQ660542
Lecidea fuscoatra var grisella 2	HQ605928	-
Lecidea laboriosa 1	EU259902	KJ766586
Lecidea laboriosa 2	EU259901	DQ986882

Results and discussion

Taxonomy

***Lecidea aptrootii* M. Khan, A.N. Khalid, H. T. Lumbsch, sp. nov.**
MycoBank no: MB825562
Figures 3–4

Type. PAKISTAN. Khyber Pakhtunkhwa Province, Swat district, Gabin Jabba valley, 1600 m alt., 37.1706°N, 72.3711°E, 18 Aug 2016, AN Khalid, GB-1 (Holotype LAH-35505).

Diagnosis. Saxicolous, thallus irregularly areolate, apothecia epruinose, lecideine with persistent margin, asci with tholus, I+ blue, ascospores simple, ellipsoid with average size of 8–10 × 4.5–5.5 µm.

Description. Thallus crustose, irregularly areolate, subcontiguous; prothallus usually indistinct, black when present; areoles flat, up to 1.2 mm in diameter and 300 µm thick, uniformity in colour from centre to edge; surface rough, not shiny, greenish-grey to light brown; Cortex not clearly differentiated, up to 31.5 µm in thickness; medulla I-, medullary hyphae thin walled, compactly arranged, 2.4–3.2 µm in diameter; photobiont layer up to 63 µm thick, algal cells 12.8–14.4 µm; apothecia black, round to irregular in outline, up to 1.5 mm in diameter, lecideine, epruinose with slightly raised, black, thin and persistent margin, frequently present, disc black, flat to slightly convex, proper exciple thin, dark brown to black; epihymenium light brown to dark

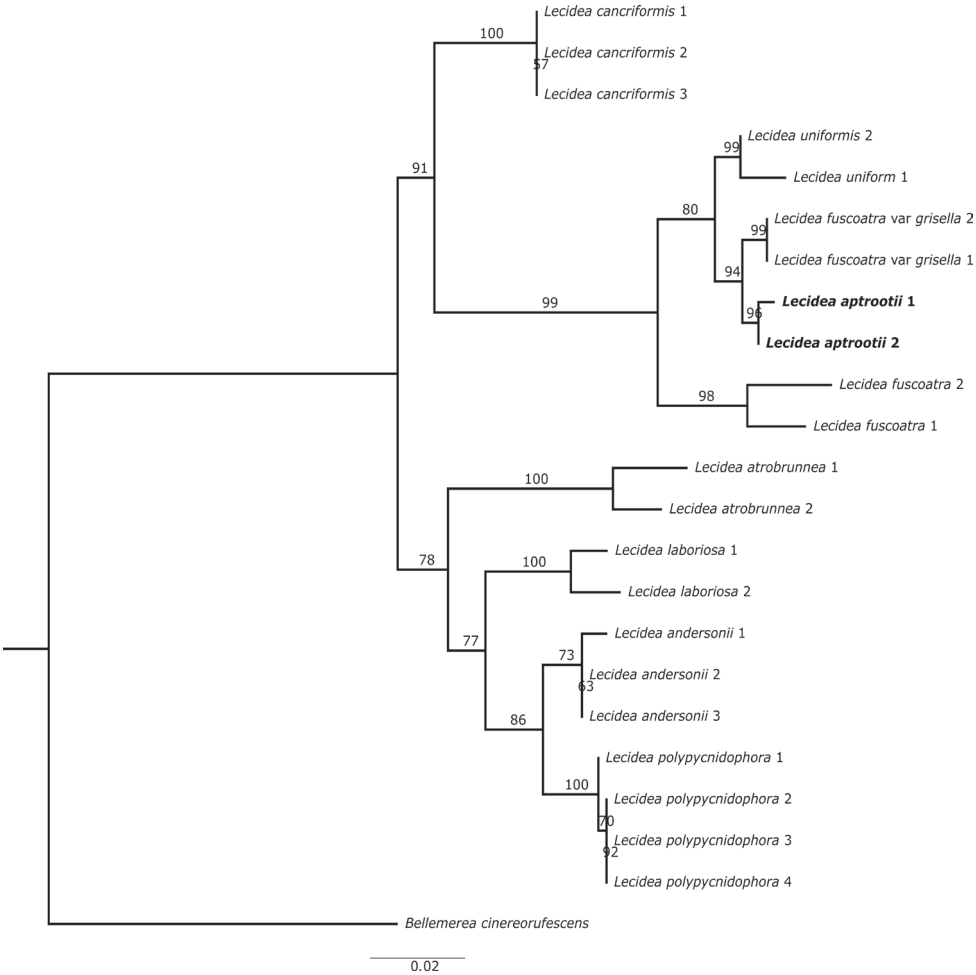

Figure 1. Most likely phylogenetic relationship of *Lecidea aptrootii* and associated taxa inferred with ITS data based on rooting with *Bellemerea cinereorufescens* as outgroup. Branch lengths are based on the estimated number of substitutions per site.

brown, 8–16 µm; hymenium hyaline to olivaceous brown, (60)-70–98 µm tall; subhymenium light brown to dark brown, Hypothecium darkly pigmented throughout, Asci clavate with distinct tholus, the tip I+ blue, 8-spored, 50–68 × 12.30–16.70 µm; Ascospores simple, ellipsoid, (7)8–10(11) × (4)4.5–5.5(6) µm; paraphyses branched and net-like, 1.6–2.4 µm wide, not expanded at tips; vegetative propagules and conidiomata not seen.

Chemistry. Thallus K-, KC+, C+ Red, P-, UV-. Gyrophoric acid, schizopeltic acid and 2'-*O*-methylperlatolic acid were detected with HPTLC.

Distribution and ecology. The species is so far only known from two collections in the Swat district in Pakistan, where the species occurs on exposed siliceous rocks between 1600 and 1900 m altitude.

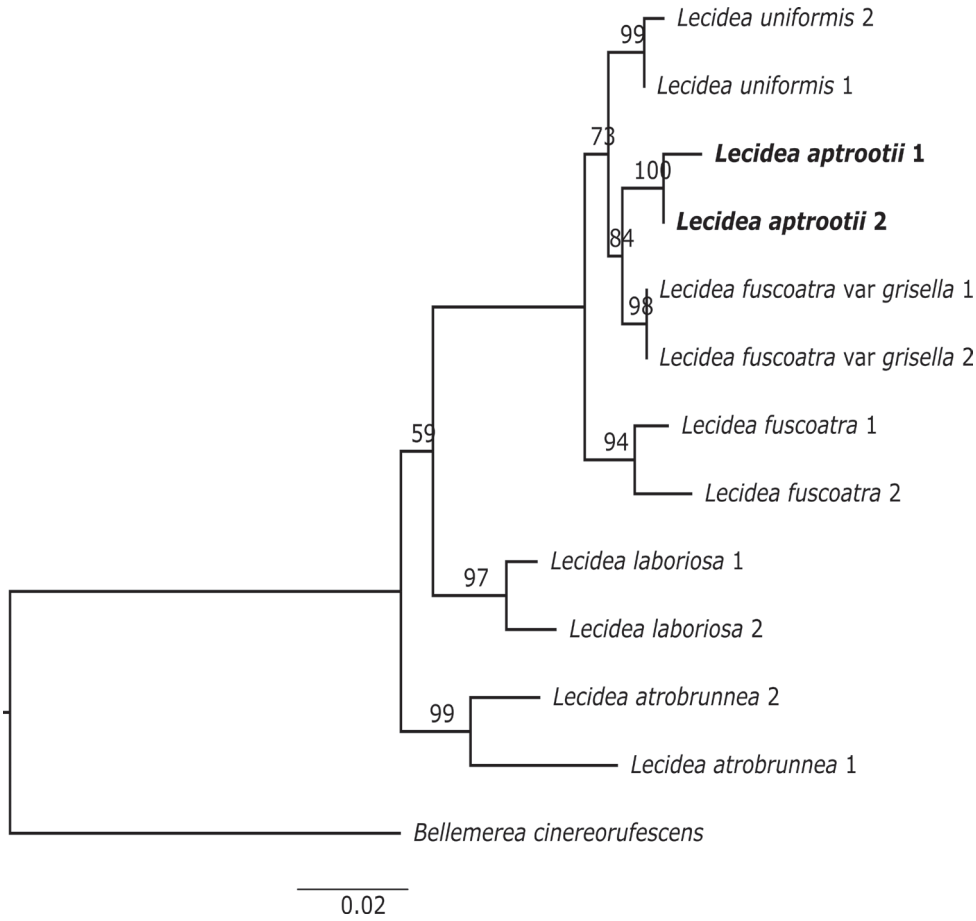

Figure 2. Phylogenetic relationships of *Lecidea aptrootii* and associated taxa inferred from ITS & nuLSU rDNA data under maximum likelihood. *Bellemerea cinereorufescens* was used as outgroup. Bootstrap values indicated at nodes.

Etymology. The epithet "aptrootii" refers to the lichenologist André Aptroot who has contributed to the knowledge of lichen diversity in Pakistan (e.g. Aptroot and Iqbal 2012) and has indicated to the first author that the material from Pakistan might represent an undescribed species.

Notes. *Lecidea aptrootii* belongs to *Lecidea* sensu stricto (Hertel 2006). The new species is a member of *Lecidea* subgen. *Lecidea*, according to the generic sub-classification as suggested by Rambold (1989). In the field, it looks like *L. fuscoatra* with an areolate thallus and black apothecia. A microscopic study revealed it differs from that species in having smaller ascospores. Another similar species in the complex is *L. grisella*, which, however, is readily distinguished by having a rimose rather than areolate thallus (Aptroot and Van Herk 2007). Recently, *Lecidea grisella* has been reported from China, which might belong to *L. aptrootii* and has ascospores 8-12(13) µm in

Figure 3. *Lecidea aptrootii* in natural habitat.

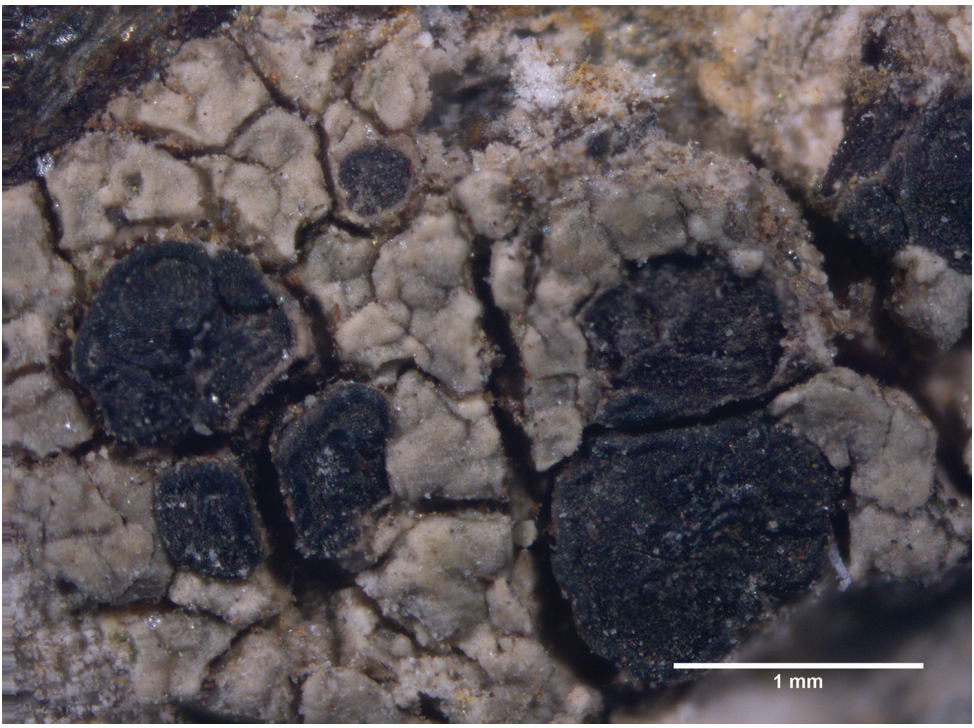

Figure 4. *Lecidea aptrootii*. Thallus and apothecia of the holotype.

length (Zhao et al. 2017) that overlap with an average spore length for *L. aptrootii* i.e. 8-10 µm. Additional studies are necessary to determine whether the Chinese material belongs to *L. aptrootii* or represents an additional lineage in this complex. Molecular data in *Lecidea* are helpful to interpret morphological features previously considered as intraspecific variation (Schmull 2011).

The areoles of *L. fuscoatra* have a differentiated black or grey margin, in contrast to the black cortex, whereas in *L. aptrootii*, the margins of areoles are concolorous with the areoles. This is a common feature of *L. aptrootii* and the recently described *L. uniformis* from North America (McCune et al. 2017). However, the two species differ in the branching of paraphyses and presence of tholus in the asci. Further, molecular data support that they represent distinct lineages (Figs 1–2).

In addition, *L. oreophila* K. Knudsen & Kocourk. with irregularly areolate thallus, light to dark grey upper surface and epruinose apothecia, might be confused with *L. aptrootii* but the former has apothecia that are usually higher than areoles and rarely branched paraphyses with expanded apices up to 5 µm (Knudsen and Kocourková 2014).

Molecular analyses of ITS and two ribosomal loci (ITS & nuLSU) dataset (605 and 1433 unambiguously aligned positions in ITS and two loci dataset, respectively) support the fact that the Pakistan collections are phylogenetically distinct from the morphologically similar *L. fuscoatra* and *L. uniformis* (Figs 1–2). In fact, the morphologically different *L. grisella* forms a well-supported sister-group relationship with *L. aptrootii*.

Additional specimen examined. Pakistan, Khyber Pakhtunkhwa province, Swat district, Malam Jabba valley, 1900 m alt., on rock, 20 Aug 2016, AN Khalid, MJ-3 (LAH-35506).

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RESEARCH ARTICLE

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Sulcispora supratumida sp. nov. (Phaeosphaeriaceae, Pleosporales) on *Anthoxanthum odoratum* from Italy

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Abstract

Sulcispora is typified by *S. pleurospora*. We collected a sulcispora-like taxon on leaves of *Anthoxanthum odoratum* L. in Italy and obtained single ascospore isolates. Combined ITS, LSU, SSU and tef1 sequence analyses suggested that *Sulcispora* is placed in the family Phaeosphaeriaceae and a newly collected *Sulcispora* species is introduced here as *S. supratumida* sp. nov. Detailed descriptions and illustrations are provided for *Sulcispora supratumida* and it is compared with the type species, *S. pleurospora*.

Keywords

Combined gene analysis, Dothideomycetes, graminicolous fungi, new species, spore septation

Introduction

Phaeosphaeriaceae is a highly diverse and large family in the order Pleosporales (Hyde et al. 2013) with more than 42 accepted genera (Hyde et al. 2017; Karunarathna et al. 2017; Wanasinghe et al. 2018). Members of Phaeosphaeriaceae are pathogens or hyper-parasites on living plants and humans and saprobes of decaying plant matter (Tennakoon et al. 2016; Ahmed et al. 2017).

Sulcispora was proposed by Shoemaker and Babcock (1989) as a monotypic genus to accommodate *Sulcispora pleurospora* (≡ *Phaeosphaeria pleurospora* Niessl). Some morphological characters of *Phaeosphaeria pleurospora* did not fit within species concepts of *Phaeosphaeria* and Shoemaker and Babcock (1989), therefore, introduced the genus *Sulcispora*. The genus name refers to the numerous furrows on the ascospore wall (Shoemaker and Babcock 1989). *Sulcispora pleurospora* has been reported on monocotyledonous hosts in genera such as *Anthoxanthum*, *Carex*, *Deschampsia*, *Sesleria* and *Tofieldia* (Leuchtmann 1984; Shoemaker and Babcock 1989).

In this study, we collected sulcispora-like species associated with leaf spots of *Anthoxanthum odoratum* in Italy. We compared the morphological characters of our collection with the isotype of *Sulcispora pleurospora*. Morphologically, our collection differs from the type species of *Sulcispora*, *S. pleurospora.* Therefore, we introduce our collection as a new species. Combined ITS, LSU, SSU and tef1 sequence analysis including taxa in Phaeosphaeriaceae indicates that the here-studied fungus grouped with "*Phaeosphaeria pleurospora*" (CBS 460.84) with high support value.

Methods

Sample collection, specimen examination and single spore isolation

Specimens were collected from *Anthoxanthum odoratum* L. from Italy in 2013. They were examined and photographed using a Carl Zeiss Discovery V8 stereo-microscope fitted with Axiocam. Sections of ascomata were taken by hand under a stereo-microscope. Sections and other micro-morphological characters were photographed using a Nikon Eclipse 80i compound microscope fitted with a Canon 450D digital camera. All microscopic measurements were made with Tarosoft image framework (v. 0.9.0.7). Colony characteristics were recorded from cultures grown on Malt Extract Agar (MEA).

Single spore isolation was carried out following the method described by Chomnunti et al. (2014). Germinated ascospores were aseptically transferred into fresh MEA plates and incubated at 20 °C to obtain pure cultures and later transferred to MEA slants and stored at 4 °C for further study. The holotype and paratype specimens were deposited at the Mae Fah Luang University (MFLU) fungaria and the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (HKAS), respectively. Living cultures were deposited at the Mae Fah Luang Culture Collection (MFLUCC).

MycoBank (http://www.mycobank.org/) and Facesoffungi (Jayasiri et al. 2015) numbers were obtained for the new strain. The new species was established based on recommendations outlined by Jeewon and Hyde (2016).

DNA extraction, PCR amplification and DNA sequencing

Fresh fungal mycelium grown on MEA for four weeks at 20°C was used for DNA extraction (Jeewon et al. 2002). Genomic DNA extraction and PCR reactions were carried out using ITS4/ITS5 for internal transcribed spacer nrDNA (ITS), LR5/LROR for large subunit nrDNA (LSU), NS1/NS4 for large subunit nrDNA (SSU) and 983F/2218R for translation elongation factor 1 (tef1) genes according to the same protocol of Maharachchikumbura et al. (2012). The PCR products were observed on 1% agarose electrophoresis gel stained with ethidium bromide. Purification and sequencing of PCR products were carried out at the Kunming Institute of Botany, Chinese Academy of Science, Kunming, China. Sequence quality was checked and sequences were condensed with DNASTAR Lasergene v.7.1. Sequences derived in this study were deposited in GenBank (Table 1).

Sequence alignment and phylogenetic analysis

BLASTn searches were made using the newly generated sequences to assist in taxon sampling for phylogenetic analyses. In addition, representatives of the Phaeosphaeriaceae were selected following Tennakoon et al. (2016) and Wanasinghe et al. (2018) (Table 1). Combined multi-locus sequence data of ITS, LSU, SSU and tef1 regions were aligned using default settings of MAFFT v.7 (Katoh et al. 2017) and manually adjusted using BioEdit 7.1.3 (Hall 1999) to allow maximum alignment and minimum gaps. Maximum likelihood analysis was performed by RAxML (Stamatakis and Alachiotis 2010) implemented in raxmlGUIv.1.3 (Silvestro and Michalak 2012). The search strategy was set to rapid bootstrapping and the analysis carried out using the GTRGAMMAI model of nucleotide substitution with 1000 replicates. The model of evolution was estimated by using MrModeltest 2.2 (Nylander 2004).

For the Bayesian inference (BI) analyses of the individual loci and concatenated ITS, LSU, SSU and tef-1 alignment, the above mentioned model test was used to determine the best fitting nucleotide substitution model settings for MrBayes v. 3.0b4. A dirichlet state frequency was predicted for all three data partitions and GTR+I+G as the best model for all single gene and combined datasets. The heating parameter was set to 0.2 and trees were saved every 1000 generations (Ronquist and Huelsenbeck 2003). The Markov Chain Monte Carlo (MCMC) analysis of four chains started in parallel from a random tree topology. The Bayesian analysis lasted 10,000,000 generations (average standard deviation of split frequencies value = 0.0098) and the consensus trees and posterior probabilities were calculated from the 9,998,000 trees sampled

Table 1. Isolates used in this study and their GenBank and culture accession numbers. The strain of *Sulcispora supratumida* sp. nov. is set in bold font and all ex-type strains are annotated with "T".

Taxon	Culture accession no	ITS	LSU	SSU	tef-1
Allophaeosphaeria muriformia	MFLUCC 13-0349^T	KP765680	KP765681	KP765682	–
A. subcylindrospora	MFLUCC 13-0380^T	KT314184	KT314183	KT314185	–
Amarenographium ammophilae	MFLUCC 16-0296^T	KU848196	KU848197	KU848198	MG520894
Ampelomyces quisqualis	CBS 129.79^T	HQ108038	JX681064	EU754029	–
Bhatiellae rosae	MFLUCC 17-0664^T	MG828873	MG828989	MG829101	–
Chaetosphaeronema hispidulum	CBS 216.75	KF251148	KF251652	EU754045	–
Dactylidina dactylidis	MFLUCC 14-0963^T	MG828887	MG829003	MG829114	MG829199
D. shoemakeri	MFLUCC 14-0966^T	MG828886	MG829002	MG829113	MG829200
Dematiopleospora mariae	MFLUCC 13-0612^T	–	KJ749653	KJ749652	KJ749655
Didymella exigua	CBS 183.55^T	GU237794	EU754155	EU754056	–
Didymocyrtis caloplacae	CBS 129338	JQ238641	JQ238643	–	–
D. ficuzzae	CBS 128019	KP170647	JQ238616	–	–
D. cladoniicola	CBS 128026	JQ238626	–	–	–
Embarria clematidis	MFLUCC 14-0976^T	MG828871	MG828987	MG829099	MG829194
Entodesmium rude	CBS 650.86	–	GU301812	–	GU349012
Equiseticola fusispora	MFLUCC 14-0522^T	KU987668	KU987669	KU987670	MG520895
Galliicola pseudophaeosphaeria	MFLUCC 14-0527^T	KT326692	KT326693	–	MG829203
Hawksworthiana clematidicola	MFLUCC 14-0910^T	MG828901	MG829011	MG829120	MG829202
H. lonicerae	MFLUCC 14-0955^T	MG828902	MG829012	MG829121	MG829203
Italica achilleae	MFLUCC 14-0959^T	MG828903	MG829013	MG829122	MG829204
Juncaceicola alpine	CBS 456.84	KF251181	KF251684	–	–
J. luzulae	MFLUCC 16-0780	KX449529	KX449530	KX449531	MG520898
Leptospora rubella	CPC 11006	DQ195780	DQ195792	DQ195803	–
Loratospora aestuarii	JK 5535B	–	GU301838	GU296168	–
L. luzulae	MFLUCC 14-0826	KT328497	KT328495	KT328496	–
Melnikia anthoxanthii	MFLUCC 14-1010^T	KU848205	KU848204	–	–
Muriphaeosphaeria galatellae	MFLUCC 14-0614^T	KT438333	KT438329	KT438331	MG520900
Neosetophoma italica	MFLUCC14-0826^T	KP711356	KP711361	KP711366	–
N. samarorum	CBS 138.96^T	FJ427061	KF251664	GQ387517	–
Neostagonospora caricis	CBS 135092/S616^T	KF251163	KF251667	–	–
N. eligiae	CBS 135101^T	KF251164	KF251668	–	–
Nodulosphaeria hirta	MFLUCC 13-0867	KU708849	KU708845	KU708841	KU708853
N. senecionis	MFLUCC 15-1297	KT290257	KT290258	KT290259	–
Ophiobolus cirsii	MFLUCC 13-0218^T	KM014664	KM014662	KM014663	–
O. disseminans	AS2L14-6	–	–	KP117305	–
Ophiosphaerella agrostidis	MFLUCC 11-0152^T	KM434271	KM434281	KM434290	KM434299
Paraleptosphaeria dryadis	CBS 643.86	J F740213	GU301828	KC584632	GU349009
Paraphoma chrysanthemicola	CBS 522.66	FJ426985	KF251670	GQ387521	–
P. radicina	CBS 111.79^T	KF251172	KF251676	EU754092	–
Parastagonospora nodorum	CBS 110109^T	KF251177	KF251681	EU754076	–
P. poagena	CBS 136776^T	KJ869116	KJ869174	–	–
Phaeosphaeria chiangraina	MFLUCC 13-0231^T	KM434270	KM434280	KM434289	KM434298
P. oryzae	CBS 110110^T	KF251186	KF251689	GQ387530	–
P. papayae	S528	KF251187	KF251690	–	–

Taxon	Culture accession no	ITS	LSU	SSU	tef-1
Phaeosphaeria pleurospora	CBS 460.84	AF439498	–	–	–
Phaeosphaeriopsis glaucopunnctata	MFLUCC 13-0265^T	KJ522473	KJ522477	KJ522481	MG520918
P. triseptata	MFLUCC 13-0271^T	KJ522475	KJ522479	KJ522484	MG520919
Poaceicola arundinis	MFLUCC 15-0702^T	KU058716	KU058726	–	MG520921
P. italica	MFLUCC 13-0267^T	KX926421	KX910094	KX950409	MG520924
Populocrescntia forlicesesensis	MFLU 15-0651^T	KT306948	KT306952	KT306955	MG520925
Premilcurensis senecionis	MFLUCC 13-0575^T	KT728365	KT728366	–	–
Sclerostagonospora sp.	CBS 123538	FJ372393	FJ372410	–	–
Scolicosporium minkeviciusii	MFLUCC 12-0089^T	–	KF366382	KF366383	–
Septoriella leuchtmannii	CBS 459.84^T	KF251188	KF251691	–	–
Setomelanomma holmii	CBS 110217	–	GU301871	GQ387572	GU349028
Setophoma sacchari	CBS 333.39^T	KF251245	KF251748	GQ387525	–
S. terrestris	CBS 335.29^T	KF251246	KF251749	GQ387526	–
Sulcispora supratumida	**MFLUCC 14-0995**	**KP271443**	**KP271444**	**KP271445**	**MH665366**
Tintelnotia destructans	CBS 127737^T	NR_147684	NG_058274	KY090698	–
T. destructans	CBS 137534	–	KY090663	KY090697	–
Vagicola chlamydospora	MFLUCC 15-0177^T	KU163658	KU163654	–	–
V. vagans	CBS 604.86	KF251193	KF251696	–	–
Vrystaatia aloeicola	CBS 135107	KF251278	KF251781	–	–
Wojnowicia dactylidis	MFLUCC 13-0735^T	KP744470	KP684149	KP684150	–
W. lonicerae	MFLUCC 13-0737^T	KP744471	KP684151	KP684152	–
Wojnowiciella eucalypti	CPC 25024^T	KR476741	KR476774	–	LT990617
Xenoseptoria neosaccardoi	CBS 128665^T	KF251281	KF251784	–	–
X. neosaccardoi	CBS 120.43	KF251280	KF251783	–	–
Yunnanensis phragmitis	MFLUCC 17-0315^T	MF684862	MF684863	MF684867	MF683624
Y. phragmitis	MFLUCC 17-1365^T	MF684869	MF684865	MF684864	MF683625

CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; **CPC**: Culture collection of Pedro Crous, housed at CBS-KNAW; **MFLUCC**: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

after discarding the first 20% of generations as burn-in. Trees obtained in this study were deposited in TreeBASE under accession number S22938. The phylogram was visualised in FigTree v. 1.2.2 (Rambaut and Drummond 2008).

Results

Phylogenetic inferences

The combined ITS, LSU, SSU and tef-1 sequence data set comprised 69 strains of Phaeosphaeriaceae with *Didymella exigua* as the outgroup taxon. All individual trees generated under different criteria and from single gene datasets were essentially similar in topology and not significantly different from the tree generated from the concat-

enated dataset. Maximum likelihood analysis with 1000 bootstrap replicates yielded a tree with the likelihood value of ln: -13019.593920 and the following model parameters: alpha: 0.144187; ∏(A): 0.245356, ∏(C): 0.229408, ∏(G): 0.267562 and ∏(T): 0.257674. The best scoring RAxML tree is shown in Figure 1. Maximum likelihood bootstrap values ≥50% and Bayesian inference (BI) ≥0.9 are given at each node.

The phylogenetic trees obtained from maximum likelihood were topologically congruent to previous studies on Phaeosphaeriaceae (Phookamsak et al. 2014; Thambugala et al. 2014; Tennakoon et al. 2016; Karunarathna et al. 2017; Wanasinghe et al. 2018). This phylogenetic analysis showed the placement of 45 genera within Phaeosphaeriaceae. The here-studied strain clustered with CBS 460.84 (one of Leuchtmann's Swiss strains of *S. pleurospora* from *Carex firma*) with 100% bootstrap support value. The ITS sequence of the CBS 460.84 is almost identical to our strain (MFLUCC 14–0995). However no LSU, SSU and tef-1 sequences were obtained from CBS 460.84 in GenBank. The herbarium specimen of CBS 460.84 is in Westerdijk Fungal Biodiversity Institute (CBS) under accession number CBS H-15991 (SWITZERLAND, Kt. Graubünden, Zügenschlucht near Davos, *Carex firma*, A. Leuchtmann). However, CBS has presently stopped sending specimens on loan, hence we could not compare morphological characters of the here studied strain with CBS 460.84. Additionally *Sulcispora* sisterly clustered with the type species of *Loratospora*, *L. aestuarii* with low support and the second species of *Loratospora*, *L. luzulae*. was distantly clustered.

Taxonomy

Sulcispora supratumida **Senan., Camporesi & K.D. Hyde, sp. nov.**
MycoBank No: MB826887
Facesoffungi No: FoF 04782
Figure 2

Etymology. The species epithet is based on the two Latin words "supra" meaning upper and "tumidus" meaning swollen, referring to the position of swollen cells of ascospores.

Type. ITALY. Province of Forli-Cesena, Premilcuore, Passodella Valbura, on dead leaves of *Anthoxanthum odoratum* L. (Poaceae), 25 May 2013, Erio Camporesi, IT 1306 (MFLU 15–0038, holotype; HKAS 83865, paratype): living cultures, MFLUCC 14–0995.

Description. *Saprobic* on leaves of *Anthoxanthum odoratum* L., visible as black spots, occurring on the upper surface of entire leaf. *Sexual morph*. *Ascomata* 110–150 × 90–140 µm ($\bar{x}$ = 140–125 µm, n = 10), scattered, solitary, immersed, uniloculate, globose, black. *Ostiole* 35–40 µm ($\bar{x}$ = 39 µm, n = 10) wide, papillate, central, periphysate. *Periphyses* 15–20 µm long, hyaline. *Peridium* comprising 2–4 layers of brown to dark brown, thick-walled, cells of *textura angularis* to *textura globularis*. *Hamathecium* comprising

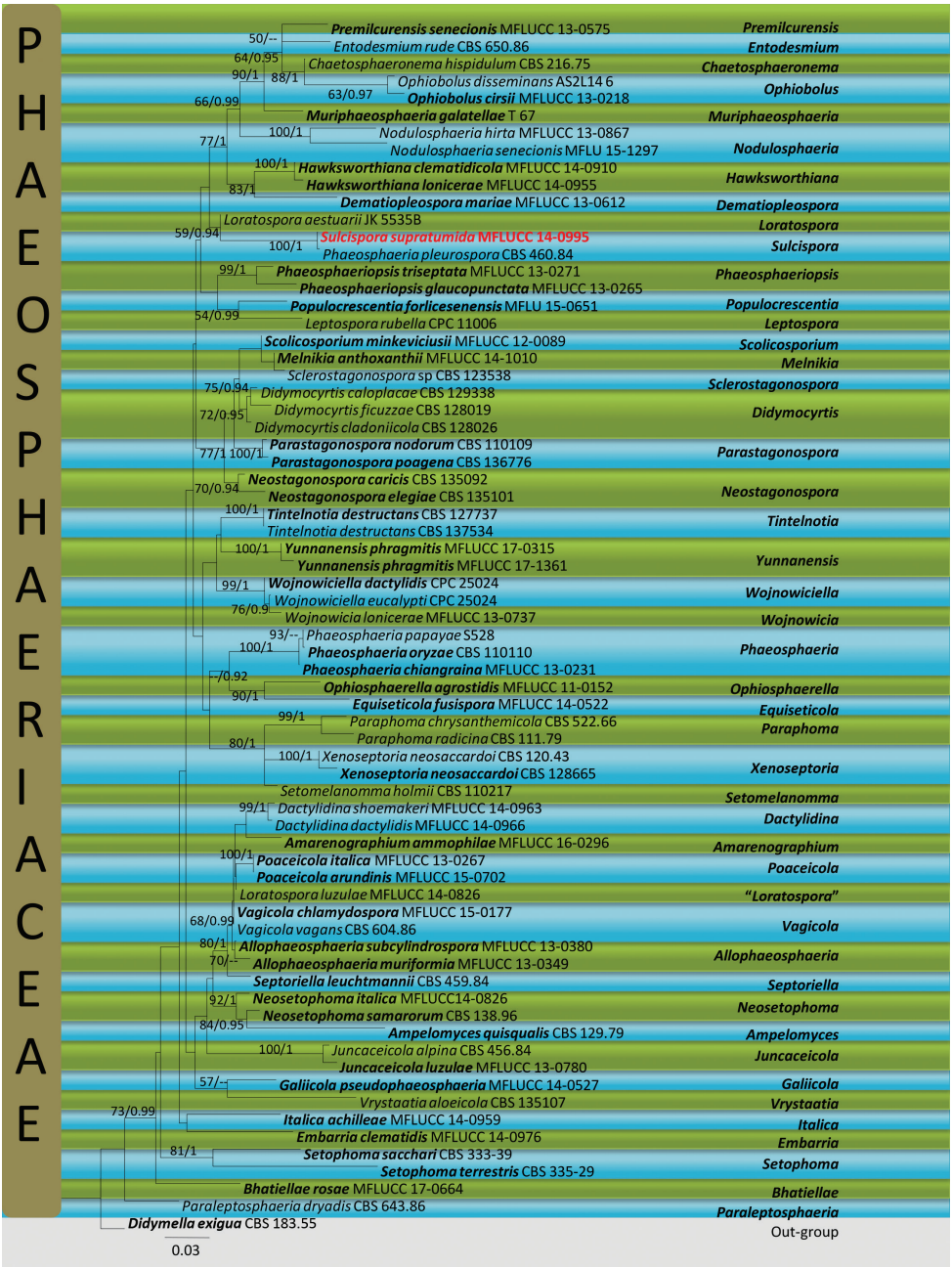

Figure 1. Maximum likelihood majority rule consensus tree based on a combined dataset of ITS, LSU, SSU and tef-1 sequences. Bootstrap support values ≥50% and Bayesian inference (BI) ≥0.9 are given at the nodes. The tree is rooted to *Didymella exigua* (CBS 183.55). The culture accession numbers are given after the species names. All ex-type strains are in bold. The newly introduced species from this study is in bold red.

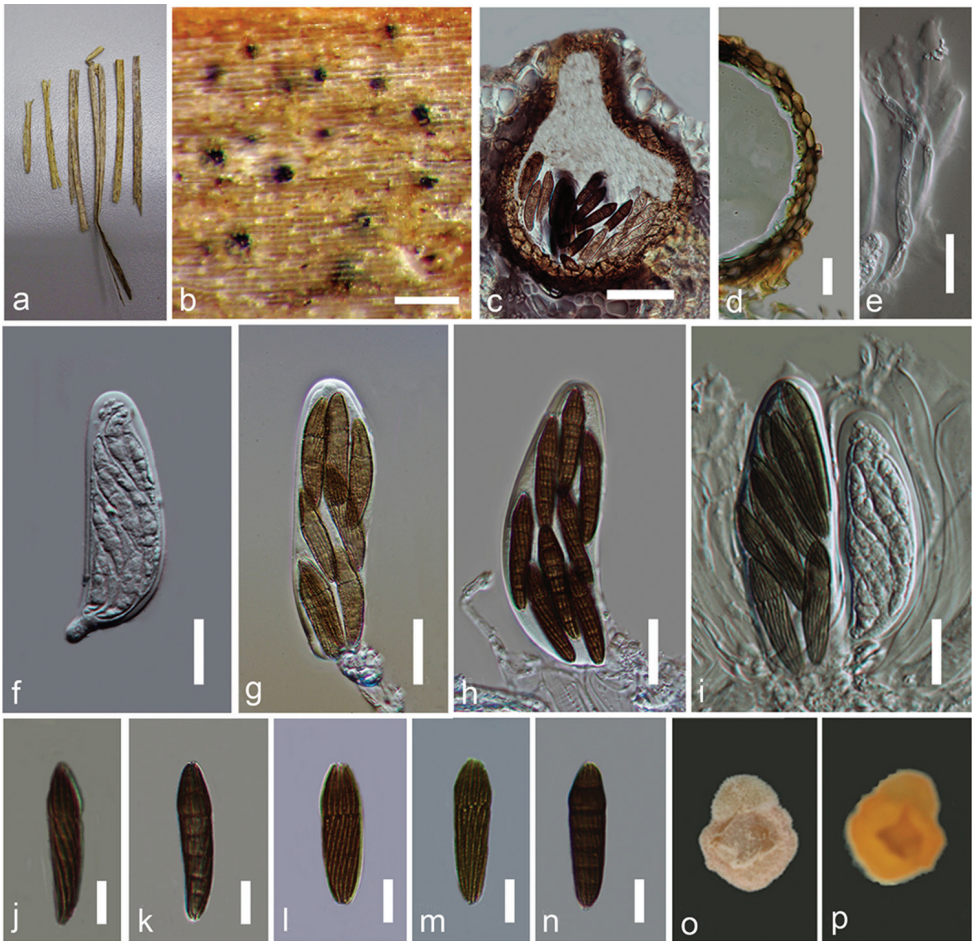

Figure 2. *Sulcispora supratumida* (MFLU 15–0038). **a** Leaves of *Anthoxanthum odoratum* **b** Appearance of ascomata on host surface **c** Cross section of ascoma **d** Peridium **e** Pseudoparaphyses **f–i** Asci **j–n** Ascospores **o** Upper surface of the culture **p** Lower surface of the culture. Scale bars: 200 µm (**b**), 50 µm (**c**), 20 µm (**d–i**), 10 µm (**j–n**).

2–4 µm wide, cellular, hyaline, branched, septate, pseudoparaphyses, constricted at the septa, anastomosing mostly above the asci and embedded in a mucilaginous matrix. *Asci* 85–125 × 20–35 µm ($\overline{x}$ = 100 × 30 µm, n = 20), 8-spored, few, bitunicate, fissitunicate, subglobose to clavate, short pedicellate, apically rounded, with an ocular chamber, arising from the base of the ascoma and attached to parenchymatous cell matrix at base. *Ascospores* 30–35 × 6–9 µm ($\overline{x}$ = 35 × 7 µm, n = 25), bi-seriate to tri-seriate, narrowly fusiform, narrowing towards the end cells, reddish to dark brown, 6-septate, second septum supra-median, slightly constricted, not constricted at other septa, second segment swollen, straight, with 12–16 longitudinal furrows on surface, lacking a mucilaginous sheath. *Asexual morph*. Undetermined.

Table 2. Ascospore morphology comparison of *Sulcispora* species

Species name	Herbarium type data	Host	No of septa	Swollen cell	Reference
Sulcispora pleurospora	FH 196419 (isotype)	*Deschampsia cespitosa* (Poaceae)	5–6	3rd	Shoemaker and Babcock 1989
	F6952, F6949, F6951 (isotype)	*Deschampsia cespitosa* (Poaceae)	6	3rd	In this study
	M (1 collection), ZT (8 collections)	6 monocotyledonous hosts, 1 dicotyledonous host	6–8	3rd or 4th	Leuchtmann 1984
Sulcispora supratumida	ZT (6 collections)	*Seleria caerulea* (Poaceae) *Carex firma* (Cyperaceae)	6	2nd	Leuchtmann 1984
	MFLU 15-0038 (holotype)	*Anthoxanthum odoratum* (Poaceae)	6	2nd	In this study

Culture characteristics. 2 cm diameter after 4 weeks incubated in dark at 25 °C on MEA, pinkish-white, circular, slightly woolly, margin lobate, effuse, lacking aerial mycelium, tightly attached to the media.

Discussion

Shoemaker and Babcock (1989) observed type specimens of *Phaeosphaeria pleurospora* and found that the ascospores of *P. pleurospora* with striated ornamented walls are different to those of other genera in Phaeosphaeriaceae. Hence, they introduced the genus *Sulcispora* to accommodate *P. pleurospora* and placed it in Phaeosphaeriaceae. *Sulcispora pleurospora* has some similarities with *Phaeosphaeria exarata* Shoemaker & C.E. Babc., in having very large cells in the peridium, ascospores with a continuous sheath and ornamented wall of ascospores with coarse, longitudinal ridges (Shoemaker and Babcock 1989).

In this study, a combined gene sequence analysis of taxa amongst the Phaeosphaeriaceae provides substantial evidence to support *Sulcispora* as a distinct genus in Phaeosphaeriaceae. *Sulcispora* differs from other genera in having immersed ascomata with a relatively thin wall, cellular pseudoparaphyses, short pedicellate asci and brown ascospores (Phookamsak et al. 2014).

Leuchtmann (1984) reported variation of ascospore septation amongst several collections of *Phaeosphaeria pleurospora* from different host plants. *Phaeosphaeria pleurospora,* collected from *Sesleria caerulea* (L.) Ard. and *Carex firma* Mygind ex Host, usually formed 6-septate ascospores and the second segment was swollen. Our collection is morphologically identical to Leuchtmann's collection. However, the isotype and some of Leuchtmann's collections from other host plants had 5–8-septate ascospores and the third or fourth segment was swollen (Table 2). Therefore Leuchtmann (1984) characterised *Phaeosphaeria pleurospora* as a species with 5–8 septate ascospores. However, Leuchtmann's collection of *Sulcispora pleurospora* is likely to comprise more than a single species and possibly constitutes a species complex.

Based on the morphology, we identified our collection as different from the isotype of *Sulcispora pleurospora*. Hence, we introduced a new species as *Sulcispora supratumida* sp. nov. However, the ITS sequence of our strain clustered with that of CBS 460.84 (one of Leuchtmann's Swiss strain of *S. pleurospora* from *Carex firma*) with 100% bootstrap support value. There are only two base pair differences between the ITS regions of both strains. Since there are no sequence data of other DNA regions of *Sulcispora pleurospora* deposited in GenBank, we could not confirm whether or not CBS 460.84 is *Sulcispora supratumida*. However, it would eventually be practical to obtain the living strain of CBS 460.84 and generate further sequence data.

Keys for species in *Sulcispora*

1 Ascomata erumpent, long papillate, 5–8-septated, ascospores with 3rd swollen cell ..... ***S. pleurospora***

– Ascomata immersed, short papillate, 6-septated, ascospores with 2nd swollen cell ..... ***S. supratumida***

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Validation of two *Amanita* species from eastern North America: *A. rhacopus* sp. nov. and *A. variicolor* sp. nov.

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Abstract

Members of the mushroom genus *Amanita* usually can easily be identified to the genus in the field, however, species circumscription and identification are often problematic. Several names have been misapplied and cryptic species exist. Here, we formally describe and validate two new species of *Amanita* sect. *Vaginatae* from eastern North America that were recognised under the umbrella European names *A. ceciliae* by past authors: *Amanita rhacopus* **sp. nov.** and *Amanita variicolor* **sp. nov.**

Keywords

Amanitaceae, Agaricales, taxonomy

Introduction

The genus *Amanita* is one of the best-known genera within the Agaricales. The genus contains edible and poisonous mushrooms, is distributed worldwide and is entirely or mostly ectomycorrhizal depending on which side one stands in its currently debated circumscription (Redhead et al. 2016, Tulloss et al. 2016, Hawksworth 2016). Many *Amanita* species are common, quite robust and readily recognisable in the field.

However, cryptic species exist and several European names have been misapplied to taxa from other continents. In eastern North America, more than 75 species are documented and well characterised although several taxa are still not validly published (Lamoureux 2006, Tulloss 2017).

Following an extensive survey conducted by one of us in the province of Québec, Canada, between 1985 and 2005, it was concluded that the region hosts at least 52 taxa of *Amanita* (Lamoureux 2006), which is about twice the 27 species reported by Pomerleau in 1980 for the same region. Amongst these taxa, 11 appear new to science, not corresponding to any known American or European species. Although these species were provisionally named (Lamoureux 2006), the names are not valid since the descriptions of the species lacked a diagnosis and a reference to a holotype.

Two of the new species proposed by Lamoureux (2006) corresponded to what Pomerleau (1980) originally called *A. ceciliae* (Berk. & Broome) Bas or its later synonym *A. inaurata* Secr. ex Gillet. *A. ceciliae* is a European taxon first described in 1854 and this name was later used worldwide to label species with similar appearance. These two new species were described as medium size *Amanita* with cap striated at the margin, a cylindrical stipe without annulus and a friable universal veil, greyish with age with orange tint or not, often leaving some remnants on the cap and at the stipe base. *A. rhacopus* Y. Lamoureux nom. prov. is brown to dark greyish-brown whereas *A. variicolor* Y. Lamoureux nom. prov. has variable intensity of pileus colour but differs mainly by its orange tint especially at the stipe base. Both are in conifer forest mixed with *Betula*. The concept of these two *Amanita* species became well accepted in eastern North America, although *A. ceciliae* ss. auct. amer. is still used by many for both species to avoid the use of *provisorum nomen* (see Tulloss 2017). Recently, DNA sequences of several eastern North America and Québec collections of *A. rhacopus* and *A. variicolor* were released in Genbank (R. Tulloss, unpublished work). The present work aims at validating these two names by designating holotypes and providing diagnoses, detailed descriptions as well as ITS-rDNA barcode sequences (Schoch et al. 2012).

Materials and methods

Specimens examined

Type specimens are deposited in the Cercle des mycologues de Montréal Fungarium, (CMMF). Additional specimens are in the Royal Ontario Museum Fungarium (TRTC), in CMMF or in the private collections of H. Lambert (HL), R. Lebeuf (HRL) or R. Labbé (RLA).

Morphological examination

Collections examined in this study were photographed in the field and macromorphological features were derived from both field notes and pictures. Microscopic studies

were performed in saline solution on fresh material or in 3% ammoniac on exsiccata. Melzer's reagent was used for amyloidity, Cotton Blue for cyanophily and Congo Red for tissue staining. Dimensions of microscopic elements are given as: [a/b/c] (min) D1-D9 (max) where a, b and c represent the number of elements measured, the number of specimens and collections from which the elements were studied, respectively; min and max, the extreme values of the distribution; D1 and D9, the first and ninth decile. Q denotes the length/width ratio of a basidiospore in side view, Qm refers to the arithmetical mean. All microscopic elements were obtained using a Leitz Ortholux II or an Olympus CH-2 microscope equipped with digital camera and were measured from pictures using Piximetre software v.5.6 (Alain Henriot, France).

DNA sequencing and analyses

ITS-rDNA barcode sequences were obtained following Dentinger et al. (2010). DNA was extracted from dried herbarium specimens (Québec collections) or from fresh tissue blotted on FTA cards (Ontario collections). BLAST searches (Altschul et al. 1990) were conducted in Genbank and in UNITE (Kõljalg et al. 2005, Abarenkov et al. 2010) to compare the new sequences with those available in these databases.

Results

DNA sequence analyses

The sequences we obtained from seven *A. rhacopus* collections were identical to each other, whereas those obtained from three specimens of *A. variicolor* differed at two nucleotide positions. The ITS sequences of the holotypes were subjected to BLAST searches against the GenBank and UNITE databases. The holotype sequence of *A. rhacopus* CMMF002171 retrieved 20 sequences with 100% identity deposited as *Amanita* sp. 'rhacopus' by Tulloss et al. of specimens from Connecticut (KY435399, KP224337), Pennsylvania (KX061516, KX270322, KX270321, KX270320, KX270319), Maine (KP224336, KU186825, KP221312, KP662537, KP221311), New York (KP224339), New Jersey (KP224333, KP224331, KP224329), West Virginia (KP224332, KP224330), Texas (KP224334), Tennessee (KP224335) and Québec (KP224338). Then follow several species of *Amanita* sect. *Vaginatae* having sequence similarities of 96% or less, including unnamed species.

The ITS sequence of the holotype of *A. variicolor* CMMM003787 has highest similarities with two sequences deposited in Genbank as *Amanita* sp. '*variicolor*', one by Tulloss et al. (KP711844, specimen CMMF003463 from Québec; unpublished) and one by Bérubé et al. (KJ638268, specimen HL0846, also from Québec; unpublished), showing, respectively, zero to four mismatches out of 509 aligned nucleotides. Other highly similar sequences were from collections labelled *Amanita* sp., one from Arizona (MG518639, T.A. Clements, unpublished, which differs at six sites) and one

from North Carolina (AY456335, Edwards et al. 2004, which differs at nine sites). Then follow several species of sect. *Vaginatae* with 93% similarity or less.

ITS sequences from *A. rhacopus* and *A. variicolor* differ by 8%. They were clearly distinct from a bona fide European sequence of *A. ceciliae* (13% difference from UNITE sequence UDB002316 | RK639).

Taxonomy

***Amanita rhacopus* Y. Lamoureux, sp. nov.**
Mycobank: MB827343
Fig. 1

A. inaurata ss. Pomerl. p. p.; *A. ceciliae* ss. auct. amer. p. p.
non *Amanita inaurata* Secr. ex Gillet, Hyménomycètes (Alençon): 41 (1874) [1878]
non *Agaricus ceciliae* Berk. & Broome, Ann. Mag. nat. Hist., Ser. 2 13: 396 (1854)

Diagnosis. *Amanita rhacopus* differs from other species of *Amanita* section *Vaginatae* by its brown to dark grey-brown pileus, stipe white at times covered with grey chevrons, universal veil grey leaving small to large flakes on pileus and annulus-like remnants at the stipe base, found in stands of conifers (*Abies*, *Picea*, *Pinus*, *Tsuga*) mixed with *Betula*.

Holotype. CANADA, Québec: Mont Orford, in mountain, close to a stand of *Betula papyrifera* in a *Abies balsamea* and *Tsuga canadensis* forest, 45°18'43" North, 72°14'24" West, 11 July 1994, CMMF002171, ITS Genbank accession number MG734660.

Description. Pileus 40-80 mm wide, ovoid to rounded conic slightly umbonate to applanate, smooth, brown to greyish-brown, with time darker in the centre and over inner ends of marginal striations, often with grey velar flakes, margin striated. Lamellae free, crowded, greyish near the pileus margin or completely greyish with age, lamellulae numerous, truncated, of very diverse lengths, unevenly distributed, edges finely powdered, white to whitish. Stipe 70–120 × 7–13 mm, cylindrical (not bulbous), flocculose and white first, then smooth to appressed fibrillose, whitish to greyish, at times with chevron-forming greyish fibrils, without annulus, with grey annulus-like remnants at the base. Universal veil friable, grey, leaving flakes on the pileus and annulus-like remnants at the stipe base. Partial veil absent. Context whitish, unchanging when cut or bruised, odour and taste not distinctive.

Basidiospores [474/11/10] (8.4) 9.5–11.7 (14.5) × (7.9) 9.0–11.1 (13.7) µm, Q= 1.0–1.1(1.2), Qm=1.05, globose to subglobose, smooth, monoguttulate, hyaline, inamyloid and cyanophilous. Basidia (50) 60–75 × 14–16 (18) µm, clavate, usually 4-spored with 4–6 µm long sterigmata, occasionally 2-spored with 5–10 µm long sterigmata, clampless. Subhymenium composed of irregular globose to subglobose 9–18 × 6–9 µm cells. Lamellar trama bilateral consisting of cylindro-clavate, clavate, fusiform to subfusiform, abundantly inflated cells 40–65 × 7–18 µm, mixed with thin-walled,

Figure 1. *Amanita rhacopus*. **a–c** Basidiomes **a** CMMF002171(holotype), photograph by Yves Lamoureux **b** CMMF009640, photograph by Jacqueline Labrecque **c** HL016, photograph by Herman Lambert **d–h** Drawings of typical microscopic structures by Guy Fortin **d** Basidiospores **e** Basidia **f** Acrophysalides **g** Universal veil. h. Caulocystides. Scale bar: 3 cm (**a, b**), 10 µm (**d, e**), 20 µm (**f–h**).

hyaline, 3–6 µm wide filamentous hyphae and of rare 3–6 µm wide, sinuous vascular hyphae. Volva remnants composed of short 3–7 µm wide filamentous ramified hyphae, numerous 25–50 µm wide terminal globose cells (few subglobose), rare to absent vascular hyphae. Pileipellis composed of 4–12 µm wide interwoven gelatinised brown filamentous hyphae mixed with an equal amount of 45–100 × 8–22 µm inflated cylindrical cells, often in chains and some 4–7.5 µm wide vascular hyphae. Pileus context composed of 4–12 µm wide filamentous sometimes partially inflated hyphae and

70–170 × 15–30 µm cylindrical to clavate inflated cells, often in chains with cells of the same diam. and some 4–8.5 µm wide vascular hyphae, ramified and distributed in all parts of the context. Stipitipellis composed of 40–180 (270) × (16) 20–30 (35) µm clavate terminal cells with grey pigment encrusted wall, originating from undifferentiated 4–6 µm wide hyphae. Stipe context composed mainly of 120–360 × 20–50 µm cylindrical cells in chains with ramified 3–5 µm wide filamentous hyphae and 4–7 µm (apex) and 5–23 µm (centre) wide vascular hyphae. Clamps absent.

Ecology and distribution. Solitary or scattered in stands of conifers (*Abies*, *Picea*, *Pinus*) mixed with *Betula*, on mesic to sub-mesic soil, never seen in plantations, from July to September in Québec and, according to sequences in Genbank, in all eastern North America down to Tennessee and Texas.

Etymology. The epithet *rhacopus* refers to the Greek ῥάκος, meaning piece of cloth and πούς, meaning foot.

Specimens examined. Canada, Québec: Québec, Boisé de l'aéroport, R. Labbé (RLA30465), 4 August 2007. Québec, Base de plein air La Découverte, H. Lambert (HL0787), 10 July 2010. Sainte-Catherine-de-la-Jacques-Cartier, Station touristique Duchesnay (sentier 51), H. Lambert (HL002), 7 July 2012. Lac-Beauport, H.Lambert (HL016), 12 July 2008. Québec, Château-Bigot, H. Lambert (HL049), 21 September 2014 (Genbank accession number MG734661). Québec, Base de plein air La Découverte, H. Lambert (HL022), 7 July 2013. Lac-Beauport, Lac Neigette nord, J. Labrecque (CMMF009600), 24 July 2007. Lac-Beauport, Chemin de la Chapelle, J. Labrecque (CMMF008929), 11 August 2006. Québec, Boisé de l'aéroport, R. Labbé (RLA30063), 15 July 2006. Lac-Beauport, Chemin de la Chapelle, J. Labrecque (CMMF009640), 29 July 2007 (Genbank accession number MG734662). Saint-Raymond, lac Sept-Iles, R. Lebeuf (HRL1876), 27 September 2014 (Genbank accession number MG734658). Québec, Château-Bigot, H. Lambert (HL048), 21 September 2014 (Genbank accession number MG734663). Grondines, Highway 40, Renée Lebeuf (HRL0804), 19 August 2011 (Genbank accession number MG734664). Ontario: Algonquin Provincial Park, M. Didukh and B. Dentinger (TRTC156853), 29 September 2007 (Genbank accession number MG734659).

***Amanita variicolor* Y. Lamoureux, sp. nov.**
Mycobank: MB827344
Fig. 2

A. inaurata ss. Pomerl. p. p.; *A. ceciliae* ss. auct. amer. p. p.
non *Amanita inaurata* Secr. ex Gillet, Hyménomycètes (Alençon): 41 (1874) [1878]
non *Agaricus ceciliae* Berk. & Broome, Ann. Mag. nat. Hist., Ser. 2 13: 396 (1854)

Diagnosis. *Amanita variicolor* differs from other species of *Amanita* sec. *Vaginatae* by its versicolour (straw-yellow, orange-brown to blackish brown) pileus, stipe white then covered with brown olive to orange chevron-forming fibrils, stipe base dark orange to

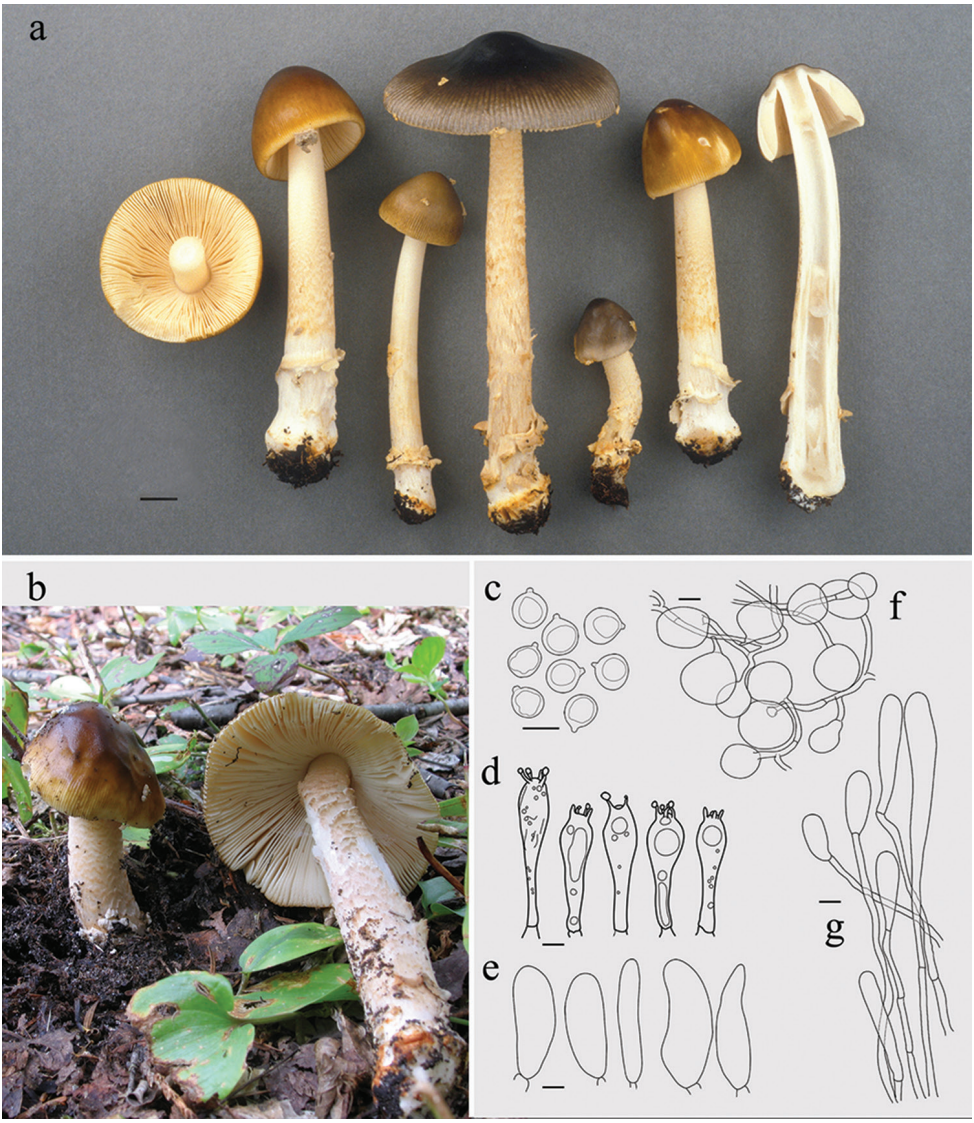

Figure 2. *Amanita variicolor*. **a, b** Basidiomes **a** CMMF003787 (holotype), photograph by Yves Lamoureux **b** HL0257, photograph by Herman Lambert **c–g** Drawings of typical microscopic structures by Guy Fortin **c** Basidiospores **d** Basidia **e** Acrophysalides. f. universal veil **g** Caulocystides. Scale bar: 1 cm (**a**), 10 µm (**c, d**), 20 µm (**e–g**).

rusty, universal veil grey to orange-grey leaving small to large flakes on the pileus and one or two strips at the stipe base, found mainly with *Abies* and *Betula*.

Holotype. CANADA, Québec: Rawdon, in a mixed forest of *Abies balsamea* and *Betula papyrifera*, on moist soil close to a bog, 19 August 2003, CMMF003787, ITS Genbank accession number MG734656.

Etymology. The epithet *variicolor* refers to the very variable colour of the pileus.

Description. Pileus 40–100 mm wide, ovoid to rounded conic at first, then plane with an umbo, smooth, olive yellow, straw yellow, bronze, olive brownish to brown-black and then tinged with olive or orange yellow towards the margin, at times darker in the centre and over inner ends of marginal striations, often with small to large grey or orange grey velar flakes, margin striated. Lamellae free, subcrowded, whitish, greyish to salmon tints. Stipe100–200 × 8–17 mm, cylindrical (not bulbous or barely), flocculose and white at first, typically covered all over by chevron-forming rusty-orange fibrils on a whitish background when mature, without annulus, base always rusty orange with one or two greyish-orange velar strips. Universal veil friable, grey to orange grey, often leaving small to large flakes on the pileus and one or two strips at the stipe base. Partial veil absent. Context white, unchanging when cut or bruised, odour not distinctive, taste not recorded.

Basidiospores: [180/3/3] (8.2) 9.8–11.5 (13.3) × (7.1) 8.8–10.7 (12.2) µm, Q= 1.0–1.2 (1.3), Qm= 1.09, globose to subglobose, smooth, monoguttulate, hyaline, inamyloid and cyanophilous. Basidia 48–65 × 14–19 µm, clavate, 4-spored with sterigmata up to 8.5 µm long. Subhymenium composed of irregular globose to subglobose 11–20 (25) × (6) 10–15 µm cells. Lamellar trama bilateral consisting of cylindro-clavate, clavate, fusiform to subfusiform, abundantly inflated 28–40 (55) × 13–20 µm cells mixed with thin-walled, hyaline, 2–6 µm wide filamentous hyphae and rare vascular hyphae. Volva remnants composed of 4.5–7.5 µm wide filamentous hyphae terminated by 25–60 µm wide subglobose to globose and inflated cells often in chains and rare to absent vascular hyphae. Pileipellis composed of an upper layer of 2.5–6 µm wide radially orientated gelatinised hyphae and a subpellis of mainly filamentous 4–12 µm wide hyphae mixed with cylindrical to fusiform inflated 50–100 × 13–24 µm cells often in chains and some 7–10 µm wide vascular hyphae. Pileus context composed of equal amounts of (4) 5–12 µm wide filamentous hyphae, sometimes inflated, more or less ramified and cylindrical, subfusiform to fusiform 40–110 × 10–33 µm inflated cells often in chains, with some 7–10 (12) µm wide vascular hyphae, sometimes inflated, rarely ramified. Stipitipellis composed of 3–5 (6) µm wide filamentous hyphae terminated by clavate 50–90 (120) × (12) 16–23 µm cells containing reddish-brown pigments. Stipe context composed mainly of cylindrical 150–350 × 20–35 µm cells in chains, 4–13 µm wide filamentous hyphae and some 7–10 µm wide vascular hyphae. Clamps absent.

Ecology and distribution. Solitary, sometimes scattered to gregarious, in stands of conifers (*Abies*, *Picea*, *Pinus, Tsuga)* mixed with *Betula*, on sub-hydric to mesic soil, never seen in plantations, from July to September in Québec and Ontario. Possibly also present further south (see Discussion).

Specimens examined. Canada, Québec: Québec, Base de plein air La Découverte, H. Lambert (HL0846), 15 August 2010. Lac-Beauport, Chemin de la Chapelle, H. Lambert (HL0852), 22 August 2010. Sacré-Sœur-sur-le Fjord, Rivière Sainte-Marguerite, fosses 3, 4, 5, H. Lambert (HL0257), 17 August 2008 (Genbank accession number MG734657). Sacré-Sœur-sur-le Fjord, Parc Saguenay, H. Lambert (HL051), 2015. Ontario : Algonquin Provincial Park, M. Didukh and B. Dentinger (TRTC156902), 1 October 2007 (Genbank accession number MG734655).

Discussion

Amanita rhacopus and *A. variicolor* belong to subgenus *Amanita* section *Vaginatae* (Fr.) Quél. due to the absence of a basal bulb and a partial veil, inamyloid basidiospores and absence of clamp connections at the base of basidia (Bas 1969, Yang 1997). Tulloss (2017) lists 296 names in this section, of which 97 correspond to accepted species, 132 are cryptonyms and 57 are provisory names. Many ITS sequences from members of sect. *Vaginatae* have been deposited in public databases, however, most are unidentified, misidentified or refer to provisory names. In addition, ITS variation within the section is very high and comprises numerous insertion/deletion events that preclude unambiguous sequence alignment in many positions. For these reasons, we have refrained to place *A. rhacopus* and *A. variicolor* in an ITS phylogeny.

In general, *A. variicolor* is larger than *A. rhacopus*. The pileus of *A. rhacopus* is greyish-brown becoming darker with age, never with an orange tint as found on *A. variicolor*. The lamellae of *A. rhacopus* become greyish with time, whereas the lamellae of *A. variicolor* become greyish-salmon. The universal veil texture of *A. rhacopus* is stronger than in *A. variicolor,* leaving a pseudo-annulus at the stipe base, which is more distinct on young basidiomes. In contrast, the universal veil of *A. variicolor* leaves light orange strips on the stipe base. The basal extremity of the stipe is always rusty orange in *A. variicolor*, a characteristic which is never seen in *A. rhacopus*. Both species developed chevron-like motifs on the stipe surface and are growing under conifers and *Betula* mixed forest. *A. variicolor* appears to be less frequent than *A. rhacopus*. They were never observed in pure plantations, only in native forest. *A. variicolor* is growing on rich mossy soil, *A. rhacopus* on dry soil often in spine conifer litter.

Although nucleotide sequence variation alone cannot be used for the circumscription of fungal species, empirical observations as well as a comprehensive study by Nilsson et al. (2008) indicated that ITS intraspecific variation in the Basidiomycetes is typically in the 0-3% range. Here, we found that ITS sequences amongst 27 collections of *A. rhacopus*, from Québec to Texas, were identical and differ from at least 4% from other *Amanita* sequences that have been deposited in public databases so far. This fact, along with its morphological uniqueness, strongly supports the recognition of *A. rhacopus* as a new and widespread species in eastern and southern North America.

While *A. rhacopus* shows remarkable ITS sequence homogeneity from Québec to Texas, *A. variicolor* sequences vary by 0.78% amongst collections from Québec and Ontario and differ by 1.76% from two sequences deposited in Genbank as *Amanita* sp., one from North Carolina (AY456335) and one from Arizona (MG518639). The former sequence comes from a fruiting body collected in a loblolly pine forest, whereas the latter was retrieved from a soil sample under ponderosa pine, douglas fir and gambel oak. Whether or not these two sequences belong to *A. variicolor* needs further investigation. If this is the case, then the distribution range of this species would also encompass areas in the eastern and southern U.S.A.

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RESEARCH ARTICLE

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Additions to tribe Chromosereae (Basidiomycota, Hygrophoraceae) from China, including *Sinohygrocybe* gen. nov. and a first report of *Gloioxanthomyces nitidus*

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Abstract

Sinohygrocybe **gen. nov.**, typified by *S. tomentosipes* **sp. nov.**, is described upon morphological and molecular evidence. The new genus is characterised by its sinuate to subdecurrent or short deccurent, usually furcate and interveined and relatively distant lamellae, dry and whitish tomentose stipe, thin-walled ellipsoid to oviod, non-constricted basidiospores and particularly elongated basidia and a ratio of basidiospore to basidium length of >5 to 8; it is close to genera *Chromosera* and *Gloioxanthomyces* of the tribe Chromosereae, but morphologically differs from *Chromosera* in less umbilicate basidiomata, tomentose stipe and usually longer basidia and differs from *Gloioxanthomyces* in more robust basidioma and less glutinous pileus and/or stipe surface. Phylogenetic analyses, with ITS-LSU-RPB2 data, also indicate that *Sinohygrocybe* forms a very distinct and independent clade at the generic level. In addition, a Chinese new record *G. nitidus* is described here.

Keywords

East Asia, new record species, new taxa, phylogeny overview

Introduction

Hygrophoraceae Lotsy (Hymenomycetes, Basidiomycota) is a large family in Agaricales, including 26 genera and over 600 species (Lodge et al. 2014). In a six-gene phylogenetic tree of Agaricales, Hygrophoraceae, Pterulaceae Corner, Typhulaceae Jülich and some small groups formed a Hygrophoroid clade, which is one of the six largest clades in Agaricales (Matheny et al. 2006); and in a genome based mushroom tree of life, Hygrophoraceae and Clavariaceae Chevall. are representative families of the suborder Hygrophorineae Aime, Dentinger & Gaya, which is one of the seven suborders of the Agaricales (Dentinger et al. 2016). Traditionally, the family Hygrophoraceae referred to a group of agaricoid, waxy-gilled and white-spored mushrooms; and a majority of the members are classified in the type genus *Hygrophorus* Fr. and genus *Hygrocybe* (Fr.) P. Kumm. Morphological characters of the Hygrophoraceae taxa are relatively simple (usually without annulus or volva and a cystidiate) amongst the agaric fungi and their basidioma colours are often very susceptible to the environmental conditions and developmental stages, making their classification and identification difficult, so it is often challenging to make correct identification and taxonomy of them just according to morphological recognition (Young 2005). Modern molecular techniques have been revolutionising the taxonomy and phylogeny of Hygrophoraceae.

Lodge et al. (2014) had conducted the most comprehensive molecular phylogenetic study on the family until now, therefore their systematic viewpoint on Hygrophoraceae is adopted in this paper. According to their study, the family could be divided into four groups at subfamily level, i.e. subfamily Hygrophoroideae E. Larss., Lodge, Vizzini, Norvell & S.A. Redhead, Hygrocyboideae Padamsee & Lodge, Lichenomphalioideae Lücking & Redhead and Cuphophylloid grade. The subfamily Hygrocyboideae could be divided into three tribes, i.e. tribe Chromosereae, Humidicuteae and Hygrocybeae; and the tribe Chromosereae included two sister genera, *Chromosera* Redhead, Ammirati & Norvell and *Gloioxanthomyces* Lodge, Vizzini, Ercole & Boertm.

Chromosera, the type genus of the tribe Chromosereae, was erected to accommodate *Omphalina cyanophylla* (Fr.) Quél. which was originally described from Sweden and combined as *C. cyanophylla* (Fr.) Redhead, Ammirati & Norvell (Redhead et al. 1995, 2012). Now, another four species, formerly placed in *Hygrocybe* or *Hygrophorus*, are also classified into *Chromosera*, i.e. *C. citrinopallida* (A.H. Sm. & Hesler) Vizzini & Ercole originally described from USA, *C. lilacina* (P. Karst.) Vizzini & Ercole originally described from the northern Fennoscandia, *C. viola* (J. Geesink & Bas) Vizzini & Ercole originally described from Belgium and *C. xanthochroa* (P.D. Orton) Vizzini & Ercole originally described from Scotland (Lodge et al. 2014).

Gloioxanthomyces is a small genus with only two known species, the type species *G. vitellinus* (Fr.) Lodge, Vizzini, Ercole & Boertm. originally described from Europe and *G. nitidus* (Berk. & M.A. Curtis) Lodge, Vizzini, Ercole & Boertm. from North America (Crous et al. 2004, Lodge et al. 2014). Before the recognition of *Gloioxanthomyces*, those two species were usually placed in the genus *Hygrocybe* as *H. vitellina* (Fr.) P. Karst and *H. nitida* (Berk. & M.A. Curtis) Murrill, respectively. Morphologi-

cally, the main differences between the two species were in their basidiospore sizes: *G. nitidus* had ellipsoid to oblong basidiospores, measuring 7–10 × 5–6 µm with Q = 1.3–1.8; while *G. vitellinus* had subglobose basidiospores, measuring 6.5–8.5 × 5–7 µm with Q=1.1–1.6 (Boertmann 1990). Since their differences were limited, the two taxa seemed to be conspecific (Boertmann 2011). However, according to the phylogenetic analyses with ITS data by Boertmann (2012), the European collections clearly clustered together as the *G. vitellinus* species clade, while the North American materials independently formed another group as the *G. nitidus* species clade, thus they could actually be sharply defined as two separated sister species.

During the studies on the Chinese Hygrophoraceae in recent years, some collections morphologically corresponding to tribe Chromosereae were collected. Comprehensive observation and analyses revealed some interesting findings, which can contribute to the taxonomic knowledge of the tribe. In this paper, we aim to: 1) formally describe a new genus of tribe Chromosereae from East Asia based upon morphological and molecular analyses and present a Chinese new record of *Gloioxanthomyces nitidus*; 2) reconstruct the phylogeny of the family Hygrophoraceae using 3 gene regions, i.e. the internal transcribed spacer region (ITS), the large subunit nuclear ribosomal RNA region (nrLSU) and the nuclear RPB2 6F to 7.1R region (RPB2). Detailed studies were therefore conducted and the results are presented as follows.

Materials and methods

Morphological studies

Specimens were photographed and annotated in the field and then dried in an electric drier. Macroscopic descriptions were gained from the original field notes and photographs. Colour descriptions followed Kornerup and Wanscher (1978). Tissue sections were immersed in 5% potassium hydroxide (KOH) and/or 1% Congo Red solution for microscopical examinations, but in distilled water for colour descriptions of basidia, pileipellis and stipitipellis. From a mature specimen, over 40 basidiospores and 20 basidia were randomly selected and measured under a light microscope in KOH. The notation (a)b–c(d) was used to describe dimensions where the range b–c representing 90% or more of the measured values and a, d were the extreme values. The length/width ratio of spores was presented as Q and the mean ratio was presented as Q_m. The studied specimens were deposited in the Fungal Herbarium of Guangdong Institute of Microbiology (GDGM), Guangzhou, China.

Molecular studies

Genomic DNA was extracted from the herbarium specimens using the Sangon Fungus Genomic DNA Extraction kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The ITS, LSU and RPB2 gene regions were ampli-

fied by Polymerase Chain Reaction, using universal primers ITS1F/ITS5 and ITS4 (White et al. 1990; Gardes and Bruns 1993), LR0R and LR5 (http://biology.duke.edu/fungi/mycolab/primers.htm) and RPB2-6F and RPB2-7.1R (Matheny 2005), respectively. Amplified products were sequenced by Beijing Genomic Institute (BGI) using the same primers. The abi format sequences were assembled by SeqMan version 7.1.0 (DNAStar, Inc.) and then the assembled sequences were submitted to GenBank.

In this study, two datasets were constructed. The first one is an ITS-LSU-RPB2 matrix of the family Hygrophorceaeae for making a comprehensive phylogenetic tree and analysing the positions of the new taxa; most known species of Hygrophoraceae with available sequences from reliable sources were included in the dataset, each of them having at least an LSU sequence and *Typhula phacorrhiza* (Reichard) Fr. was selected as the outgroup referred from Yang et al. (2013) and Lodge et al. (2014). The second dataset is an ITS matrix of the tribe Chromosereae and *Hygrocybe conica* (Schaeff.) P. Kumm. and *H. conica* var. *conicoides* (P.D. Orton) Boertm. were chosen as outgroups. Each gene was independently aligned on the online MAFFT service (Katoh et al. 2017), then combined by the Geneious software (Biomatters Ltd.) for the first dataset. Maximum likelihood phylogenetic trees were generated by the RAxML software (Stamatakis 2014) on the CIPRES service (Miller et al. 2010) with 1000 bootstrap replications using the default options.

Results

Molecular phylogenetic results

The combined 3-gene dataset composed of 120 samples (Table 1), including 5 newly sequenced samples and 115 published ones. In the final matrix, the ITS, LSU and RPB2 regions comprised positions 1 to 1751, 1752 to 2873, 2874 to 3759, respectively. In the 3-gene Maximum Likelihood tree (Fig. 1), the four Chinese collections (GDGM43351 and GDGM43347 from Sichuan province, GDGM50075 and GDGM50149 from Hunan province) formed a strong monophyletic clade with 100% bootstrap support, which was near the *Chromosera-Gloioxanthomyces* clade composed of members of *Chromosera* and *Gloioxanthomyces* with 76% bootstrap support.

The ITS dataset included 30 samples of all known taxa of tribe Chromosereae and 2 *Hygrocybe* sequences chosen as the outgroups, the matrix length is 679 bp. In the ITS Maximum Likelihood tree (Fig. 2), collections of the species *G. nitidus* and *G. vitellinus* were clustered together with 93% and 100% support values, respectively and the North American and the East Asian *G. nitidus* were clustered as sister groups with 93% support value; all the members of *Chromosera* (except *C. viola*), *Gloioxanthomyces* and *Sinohygrocybe* were clustered together with 95%, 93% and 100% support values, respectively; and the *Chromosera-Gloioxanthomyces* clade was presented as the sister clade of the *Sinohygrocybe* clade with strong support value (100%).

Table 1. Sequences information of samples used for the ITS-LSU-RPB2 combined tree. Newly generated sequences were bold.

Species name	Isolate/voucher ID	ITS	LSU	RPB2
Acantholichen albomarginatus	MDF543	KT429797	KT429809	–
Acantholichen campestris	DIC595b	KT429798	KT429810	KT429818
Acantholichen galapagoensis	MDF057	KT429784	KT429799	KT429811
Acantholichen galapagoensis	MDF058	KT429785	KT429800	KT429812
Acantholichen galapagoensis	MDF089	KT429786	KT429801	–
Acantholichen galapagoensis	MDF090	KT429787	KT429802	KT429813
Acantholichen galapagoensis	MDF093	KT429790	KT429803	KT429814
Acantholichen galapagoensis	MDF094	KT429791	KT429804	KT429815
Acantholichen galapagoensis	MDF100	KT429792	KT429805	KT429816
Acantholichen pannarioides	MDF352	KT429795	KT429807	KT429817
Acantholichen pannarioides	Bungartz 5593	EU825953	EU825953	–
Acantholichen sorediatus	DIC335	KT429794	KT429806	–
Acantholichen variabilis	MDF679	KT429796	KT429808	–
Ampulloclitocybe clavipes	DJL06TN40	–	KF381542	KF407938
Ampulloclitocybe clavipes	AFTOL-ID 542	AY789080	AY639881	AY780937
Arrhenia auriscalpium	Lutzoni Lamoure 910824-3	U66428	U66428	–
Arrhenia lobata	Lutzoni Lamoure 910824-1	U66429	U66429	–
Cantharellula umbonata	RDY-1366 (SFSU)	KF381519	AF261443	–
Cantharocybe brunneovelutina	DJL-BZ-1883 (holotype)	KX452404	HM588721	–
Cantharocybe gruberi	AFTOL-ID 1017	DQ200927	DQ234540	DQ385879
Cantharocybe gruberi	AH24539	JN006422	JN006420	–
Cantharocybe virosa	TENN 63483(holotype)	KX452405	JX101471	–
Chromosera citrinopallida	DUKE8895	U66435	U66435	–
Chromosera citrinopallida	D. Boertmann 2006/2	KF291072	KF291073	–
Chrysomphalina chrysophylla	AFTOL-ID 1523	–	DQ457656	DQ192180
Chrysomphalina chrysophylla	S.A. Redhead 7700	–	U66430	U66430
Chrysomphalina grossula	OSC 113667	–	EU652372	EU644703
Chrysomphalina grossula	OSC 113683	–	EU652373	EU644704
Cora minor	Luecking 15243	EU825968	EU825968	–
Cuphophyllus acutoides var. *pallidus*	CFMR TN-257	–	KF291097	–
Cuphophyllus adonis	MES-152	–	KF291036	KF291037
Cuphophyllus aff. *pratensis*	PBM-752	–	DQ457650	KF442252
Cuphophyllus aurantius	CFMR PR-6601	–	KF291100	KF291102
Cuphophyllus bicolor	DJL-PR-2	–	KF291056	–
Cuphophyllus flavipes	Hattori-JP-6	–	KF291045	KF291047
Cuphophyllus fornicatus	D. Boertmann 2009/94	–	KF291124	–
Cuphophyllus pratensis	DJL-Scot-8	–	KF291058	–
Cuphophyllus sp.	AM01	–	HM026542	–

Species name	Isolate/voucher ID	ITS	LSU	RPB2
Dictyonema glabratum	AFTOL-ID 1995	DQ917656	DQ917661	–
Dictyonema glabratum	Luecking 15581	EU825958	EU825958	–
Dictyonema glabratum	Luecking 16563	EU825956	EU825956	–
Dictyonema glabratum	R06	EU825959	EU825959	–
Dictyonema glabratum	R11	EU825960	EU825960	–
Dictyonema glabratum	R18	EU825961	EU825961	–
Dictyonema glabratum	R20	EU825963	EU825963	–
Gliophorus aff. *psittacinus*	CFMR JP-4	KF291079	KF291080	–
Gliophorus graminicolor	TJB-10048	KF381520	KF381545	KF407936
Gliophorus psittacinus	D. Boertmann 2002/10	KF291075	KF291076	KF291078
Gloioxanthomyces nitidus	GDGM41710	**MG712283-4**	**MG712282**	**MG711911**
Haasiella splendidissima	Herbarium Roux n. 3666	JN944398	JN944399	–
Haasiella splendidissima	Herbarium Roux n. 4044	JN944400	JN944401	–
Haasiella splendidissima	JVG1071013-1	JN944395	JN944396	–
Haasiella venustissima	A. Gminder 971488	KF291092	KF291093	–
Haasiella venustissima	E.C. 08191	JN944393	JN944394	–
Humidicutis sp. 2	CFMR PR4047	–	KF291151	KF291149
Humidicutis sp. 2	DJL-2103 CFMR PR-6524	KF291150	KF291151	
Humidicutis sp. 3	D.J. Lodge DJL-BZ-3	KF291110	KF291111	–
Hygroaster albellus	AFTOL ID 1997	KF381521	EF551314	KF381510
Hygroaster nodulisporus	AFTOL-ID 2020	–	EF561625	KF381511
Hygrocybe acutoconica f. *japonica*	CFMR JP-2	KF291161	KF291162	
Hygrocybe aff. *citrinovirens*	DJL05TN10	KF291090	KF291091	–
Hygrocybe aff. *conica*	PBM 918	AY854074	DQ071739	AY803747
Hygrocybe aff. *prieta*	DJL-BZ-65	KF291168	KF291169	
Hygrocybe caespitosa	DMWV-03-737	KF291104	KF291105	KF291107
Hygrocybe cantharellus	AFTOL-ID 1714	DQ490628	DQ457675	
Hygrocybe ceracea	D. Boertmann 2002/7	KF291108	KF291109	–
Hygrocybe cf. *acutoconica*	DJL04NC2	KF291117	KF291118	KF291120
Hygrocybe chloochlora	DJL-BZ-32	EU435147	EU435147	–
Hygrocybe chlorophana	Boertmann 2002/9	EU435148	EU435148	KF381513
Hygrocybe coccinea	AFTOL-ID 1715	DQ490629	DQ457676	DQ472723
Hygrocybe coccinea	Boertmann02/8	EU435146	EU435146	KF291114
Hygrocybe constrictospora	D. Boertmann 2007/38	KF291115	KF291116	
Hygrocybe glutinipes var. *rubra*	DJL05NC9	EU435149	EU435149	–
Hygrocybe helobia	AK-124	KF291182	KF291183	–
Hygrocybe hypohaemacta	DJL-BZ-105	EU435150	EU435150	KF291165
Hygrocybe konradii var. *konradii*	Boertmann 2004/6	KF306329	KF306330	–
Hygrocybe lepida	Boertmann 2002/2	KF306333	KF306334	–
Hygrocybe melleofusca	DJL-PR-EV	KF291154	KF291155	–
Hygrocybe miniata	AK-110	KF291179	KF291180	

Species name	Isolate/voucher ID	ITS	LSU	RPB2
Hygrocybe miniata f. *longipes*	AFTOL-ID 1891	DQ490630	DQ457677	DQ472724
Hygrocybe noninquinans	DJL-PR-1	KF291127	KF291129	KF291128
Hygrocybe occidentalis var. *occidentalis*	Cancerel PR 02	EU435151	EU435151	–
Hygrocybe punicea	DJL-SCOT-B2	KF291133	KF291134	–
Hygrocybe purpureofolia	DJL04NC1	KF291192	KF291193	
Hygrocybe reidii	DJL-ENG-15-2006	KF291158	KF291159	
Hygrocybe rosea	DJL-PR-4	KF291197	KF291198	–
Hygrophorus agathosmus	EL2-00	–	AY586660	–
Hygrophorus cossus	SJ94064	AY548963	AY548963	
Hygrophorus hyacinthinus	SJ950830	–	HM143012	–
Hygrophorus olivaceoalbus	SJ91060	–	AY586662	–
Hygrophorus russula	JP-3	KF291216	KF291217	KF291219
Hygrophorus sordidus	AFTOL-1338	DQ490632	AF042562	–
Lichenomphalia umbellifera	J. Geml-2	U66445	U66445	KF381515
Neohygrocybe ingrata	GWG H. ingrata 23-10-06 (ABS)	KF291225	KF291226	–
Neohygrocybe ingrata	TN-62 voucher DJL05TN62	KF381525	KF381558	KF381516
Neohygrocybe ingrata	CFMR NY-43	–	KF291223	KF291224
Neohygrocybe ovina	K(M) 187568	KF291228	KF291229	–
Neohygrocybe ovina	GWG H. ovina Rhosisaf (ABS)	KF291233	KF291234	KF291236
Neohygrocybe subovina	WRWV04-752 (DEWV 5366)	–	KF291142	KF291138
Neohygrocybe subovina	CFMR NC-61	KF291136	KF291137	–
Neohygrocybe subovina	DJL04TN16 (GRSM 77065)	KF291140	KF291141	–
Omphalina epichysium	Redhead3140	U66442	U66442	–
Omphalina grossula	Gulden 417/75	–	U66444	U66444
Omphalina hudsoniana	LUTZ-920728.4a	U66446	U66446	–
Omphalina obscurata	Lam L73-101	U66448	U66448	–
Omphalina philonotis	LUTZ930804-5	U66449	U66449	–
Omphalina sphagnicola	LUTZ930810	U66453	U66453	–
Omphalina velutina	LUTZ-930812.1	U66454	U66454	–
Omphalina velutipes Lamoure	L77	U66455	U66455	–
Omphalina wynniae A. H. Smith	82899	–	U66457	U66457
Porpolomopsis aff. *calyptriformis*	DJL05TN80	KF291246	KF291247	KF291249
Porpolomopsis calyptriformis	EB-ENG-3	KF291242	KF291243	KF291245
Porpolomopsis lewelliniae	TJB-10034	KF291238	KF291239	KF291241
Pseudoarmillariella bacillaris	HKAS76377	KC222315	KC222316	–
Pseudoarmillariella ectypoides	AFTOL-ID 1557	DQ192175	DQ154111	DQ474127
Sinohygrocybe tomentosipes	GDGM43351	**MG685872**	**MG696901**	**MG696905**
Sinohygrocybe tomentosipes	GDGM43347	**–**	**MG696900**	**MG696904**
Sinohygrocybe tomentosipes	GDGM50075	**MG685873**	**MG696902**	**MG696906**
Sinohygrocybe tomentosipes	GDGM50149	**MG685874**	**MG696903**	**MG675232**
Typhula phacorrhiza	TP21	AF134710	AF393079	AY218525

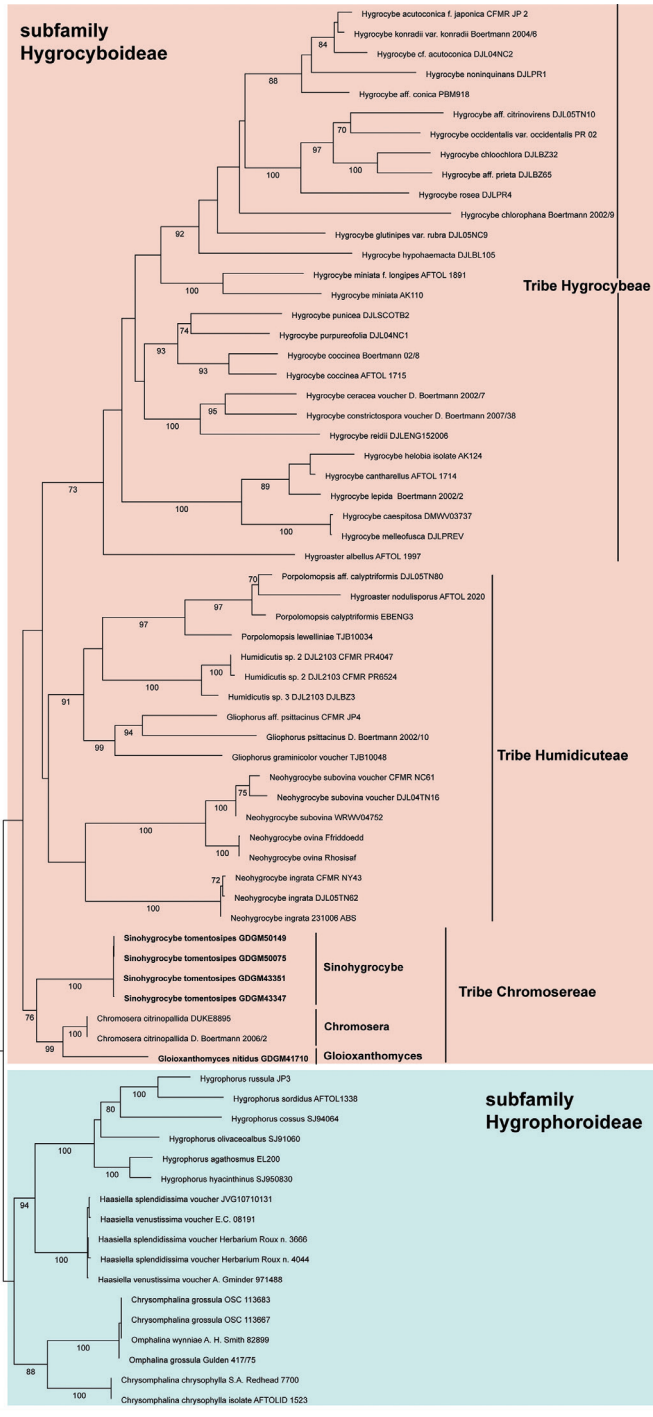

Figure 1. Phylogenetic overview of the family Hygrophoraceae inferred from ITS-LSU-RPB2 data using Maximum Likelihood (ML) method. *Typhula phacorrhiza* was selected as outgroup. Bootstrap values (≥50%) were presented around the branches. The newly generated sequences are shown in bold.

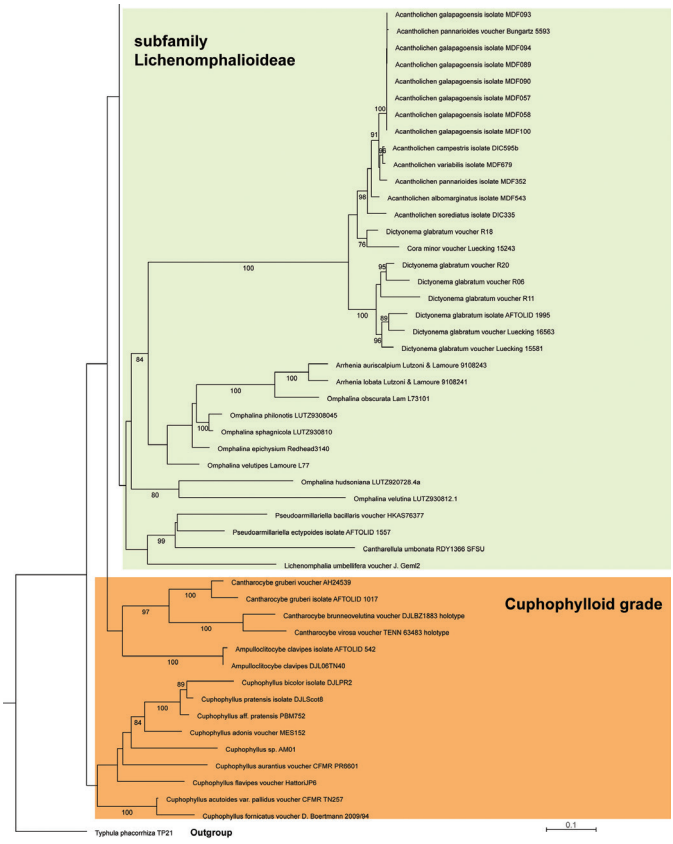

Figure 1. Continued.

Taxonomy

***Sinohygrocybe* C.Q. Wang, Ming Zhang & T.H. Li, gen. nov.**
MycoBank: MB824821

Diagnosis. Differs from *Chromosera* and *Gloioxanthomyces* by its less omphalioid, more robust basidiomata, dry to subviscid pileus, dry and white tomentose stipe, more elongated basidia, higher length ratio (up to 8 times) of basidia to basidospores.

Etymology. *Sino-* refers China, the holotype's location of the genus; *-hygrocybe* indicates that it is a *Hygrocybe*-like genus.

Type species. *Sinohygrocybe tomentosipes* C.Q. Wang, Ming Zhang & T.H. Li

Description. Basidiomata medium-sized, subcaespiotose. Pileus convex to applanate, slightly depressed in the centre, yellow, orangish-yellow to orange, dry to subviscid, slightly when wet, never strongly gelatinised or glutinous. Lamellae adnate to decurrent, concolorous with pileus, with usually furcate and interveined lamellulae. Stipe yellow to whitish or almost concolorous with pileus, yellow or covered by white to yellowish-white tomentum. Basidiospores ellipsoid to oblong, ovoid, Qm = 1.6-1.7,

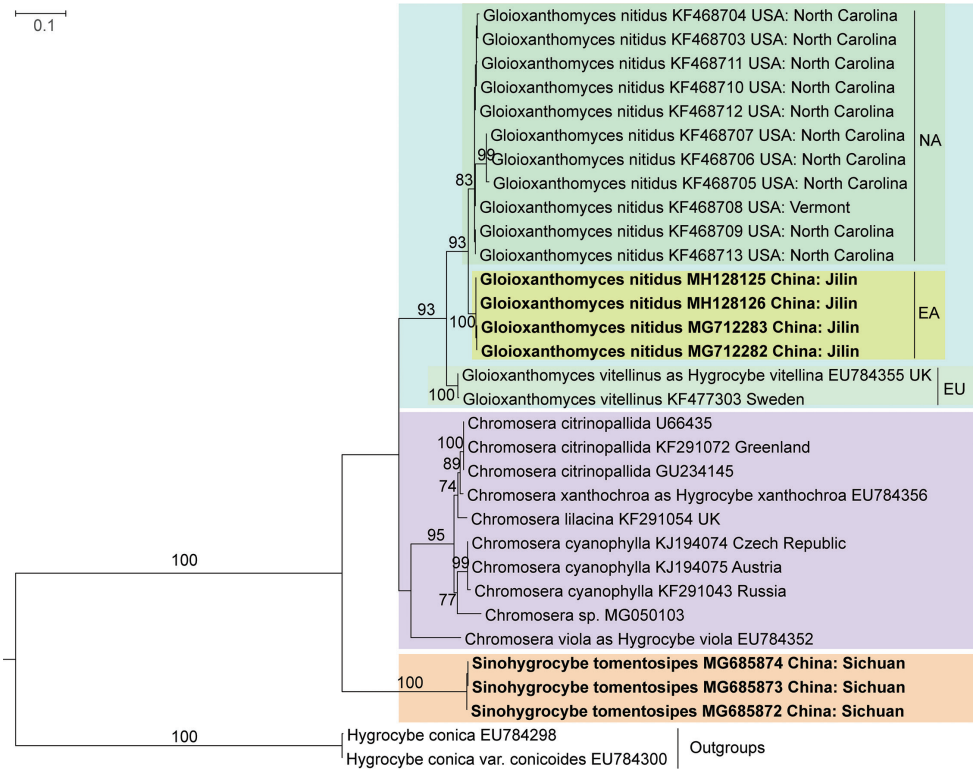

Figure 2. Phylogenetic overview of the tribe Chromosereae inferred from ITS data using ML method. Two *Hygrocybe conica* sequences were rooted as outgroups. Bootstrap values (≥50%) are shown around the branches. GenBank accession numbers of downloaded sequences were added after the species name and the collection locations were added at the ends. NA, EA and EU referred to North America, East Asia and Europe, respectively. The newly generated sequences are shown in bold.

not constricted, thin-walled, inamyloid, hyaline, smooth; basidia usually 4-sterigmate, 41–80 μm long, ratio of basidia to basidiospore length over 5 (up to 8), with basal clamp connection. Pileipellis and stipitipellis a cutis. Lamellar trama subregular. Clamp connections present throughout.

Sinohygrocybe tomentosipes C.Q. Wang, Ming Zhang & T.H. Li, sp. nov.

MycoBank: MB824824
Figs 3, 4

Diagnosis. Differs from the other members of the tribe Chromosereae by its larger and more robust basidiomata, concolorous yellow pileus, lamellae and the subsurface of stipe, usually furcate and interveined lamellae and lamellulae, white fibrillose stipe surface, long basidia (up to 80 μm), ratio of basidia to basidiospore length over 5 and even up to 8.

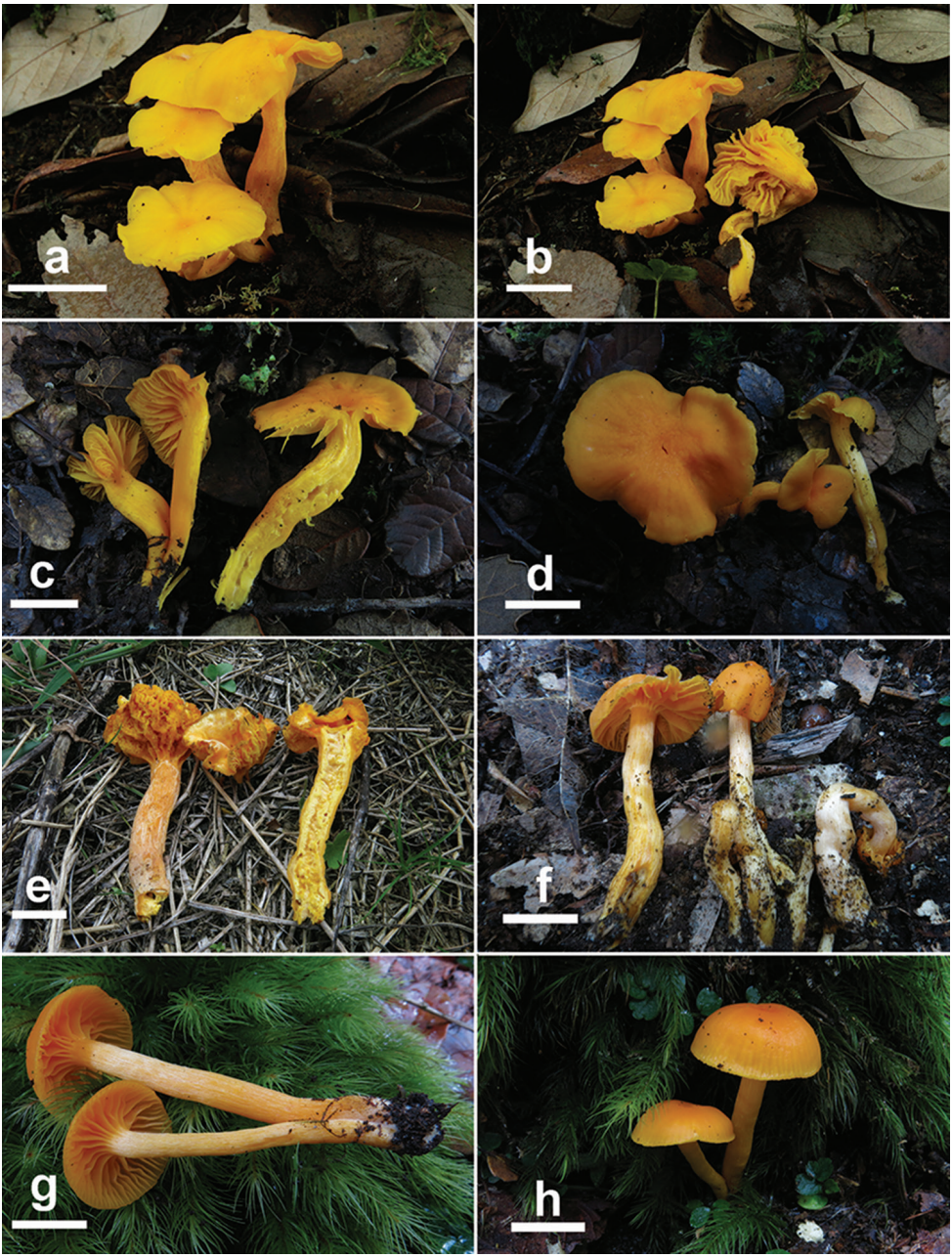

Figure 3. Basidiomata of *Sinohygrocybe tomentosipes* (**a–b** GDGM43351 **c–d** GDGM43352 **e** GDGM43347 **f** GDGM50075 **g–h** GDGM50149). Scale bars: 2 cm.

Etymology. The species epithet *tomentosipes* refers to the tomentose stipe.

Type. China. Sichuan Province, Panzhihua City, Yanbian County, Gesala Ecotourism Area, at 27°16'N, 101°26'E, alt. 3100 m, 24 Aug 2013, Ming Zhang (GDGM43351, holotype).

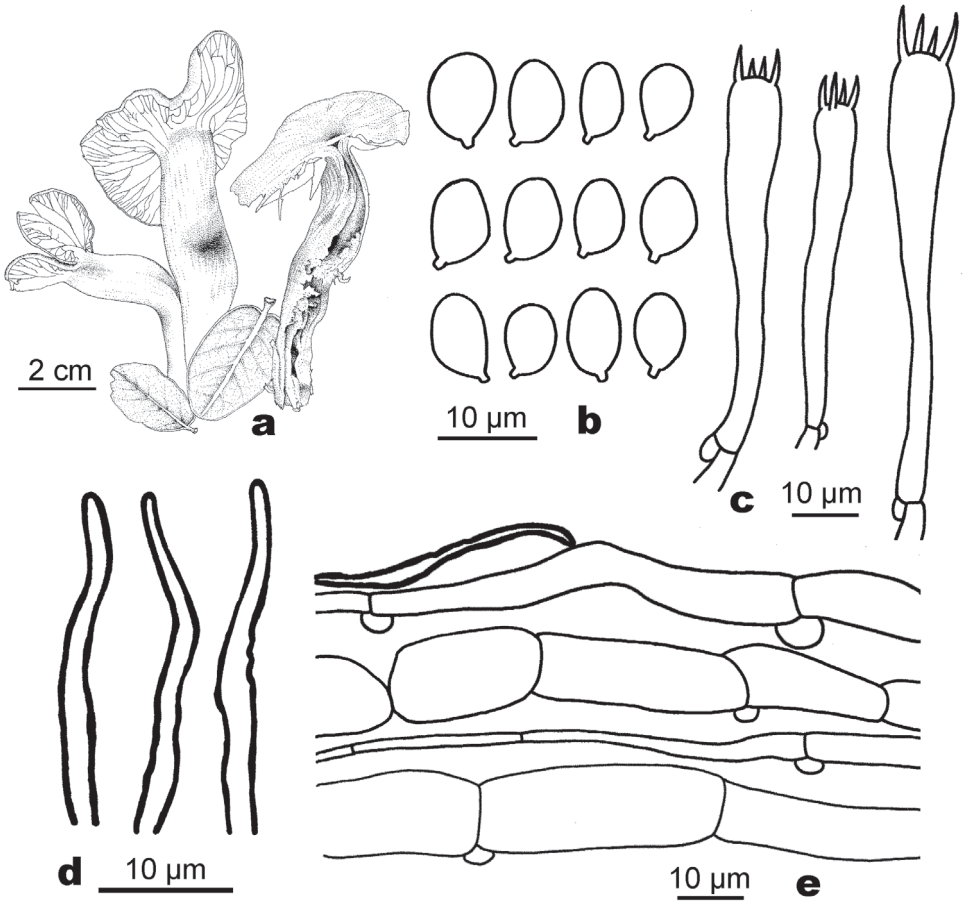

Figure 4. Line drawings of *Sinohygrocybe tomentosipes*. **a** Basidiomata **b** Basidiospores **c** Basidia **d** Elements of pileipellis cells **e** Elements of gill trama.

Description. Basidiomata small to medium-sized. Pileus 2.5–6 cm diam., convex to applanate, usually slightly depressed in the centre, smooth, dry but subviscid when wet, light yellow to vivid yellow (3A5–8) or to deep yellow (4A5–8), or light orange to dark orange (5A5–8), becoming paler when dry; margin even, straight or upturned and occasionally split when mature. Lamellae up to 7 mm wide, adnate to sinuate or decurrent, distant, 17–22 lamellae per pileus, with 1–3 lamellulae between two complete lamellae, usually furcate, often interveined or anastomosing at lamella base, thick, concolorous with the pileus; lamellar base and lamellulae irregular and occasionally the whole hymenophore irregular; lamellar edge even and concolorous. Context concolorous with lamellae and pileus, unchanged when cut. Stipe 4–6.5 × 0.6–1.2 cm, central or occasionally eccentric, subcylindrical, moderately to densely covered with white tiny adpressed fibres. Odour indistinct.

Basidiospores 8–10(–10.5) × (4.5–)5–7(–7.5) μm, Q = (1.3–)1.5–1.8, Q_m = 1.6–1.7, ellipsoid to ellipsoid-oblong, ovoid, not constricted, thin-walled, hyaline, smooth.

Basidia 41–80 × 4–10 μm, strongly elongated, narrow clavate, 4-spored, thin-walled; sterigmata up to 10 μm long; ratio of basidia to basidiospore length over 5 and up to 8. Hymenophoral trama subregular, yellow, made up of thin-walled hyphae 3–15 μm wide and usually less than 100 μm long and some conducting elements. Pileipellis a cutis, made up of repent hyphae 3–9 μm wide with the terminal elements 30–80 μm long. Stipitipellis a cutis, with thin-walled hyphae (5–7 μm wide). Clamp-connections present in all tissues.

Habitat and known distribution. Gregarious, caespitose, or scattered in broad-leaf forest in subtropical temperate transition zone, so far known only from Sichuan and Hunan Provinces in China.

Additional specimens examined. CHINA, Sichuan Province, Panzhihua City, Yanbian County, Gesala Eco-Tourism Area, at 27°16'N, 101°26'E, alt. 3100 m, 24 Aug 2013, Ming Zhang (GDGM43347), Chao-Qun Wang (GDGM43352); Hunan Province, Zhuzhou City, Yanling County, Taoyuandong National Nature Reserve, at 26°19'N, 114°00'E, alt. 1534 m, 23 Nov 2013, Chao-Qun Wang (GDGM50075 and GDGM50149).

Glioxanthomyces nitidus (Berk. & M.A. Curtis) Lodge, Vizzini, Ercole & Boertm., Fungal Diversity 64: 50 (2014)

Figs 5, 6

= *Hygrophorus nitidus* Berk. & M.A. Curtis, Ann. Mag. nat. Hist., Ser. 2 12: 424 (1853).

Description. Pileus 1.5–3.5 cm wide, convex to nearly plane with a slightly depressed disc, strongly glutinous, yellow, light orange yellow to apricot yellow, even whitish-yellow when mature, clearly striate at margin; pileus margin usually slightly undulating, slightly incurved when young, expanded to flat or partially uplifted when mature. Context thin, yellow to nearly concolorous with pileus, hygrophanous and translucent. Lamellae arcuate-decurrent, narrow at both ends, bright yellow or slightly orange yellow, waxy and fragile, subdistant, usually having 1–3 unequal lamellulae between two lamellae; lamellar edge even, usually gelatinised and sometimes translucent. Stipe 2.5–6 × 0.2–0.5 cm, cylindrical, hollow, yellow to slightly greenish-yellow, smooth, sticky or glutinous with a layer of viscid and translucent material when wet, nearly equal mostly but usually tapering at base.

Basidiospores 7–9(11) × 5–6.5(7.5) μm, Q=1.25–1.7, Qm=1.48, ellipsoid, not constricted, smooth, hyaline, thin-walled. Basidia 29–39 × 7.5–10 μm, clavate, 4-spored; sterigmata up to 5 μm. Lamellar trama subregular, with hyphal elements 10–20 μm wide. Pileipellis an ixotrichoderm. Clamp connections present.

Habitat and known distribution. Solitary or scattered, on moist ground in a mixed forest with mosses in North-eastern China, so far known in North America and East Asia.

Figure 5. Basidiomata of *Gloioxanthomyces nitidus* (**a–b** GDGM41710 **c–d** GDGM42150 **e–f** GDGM42151).

Material examined. CHINA. Jilin Province, Antu County, Changbaishan Mountains, 20 August 2012, Ming Zhang, Jiang Xu, Chao-Qun Wang (GDGM41710, GDGM42150 and GDGM42151).

Discussion

Phylogenetically, the distinction of the three subfamilies (Lodge et al. 2014) within Hygrophoraceae has very convincing support in the multi-locus tree of this study

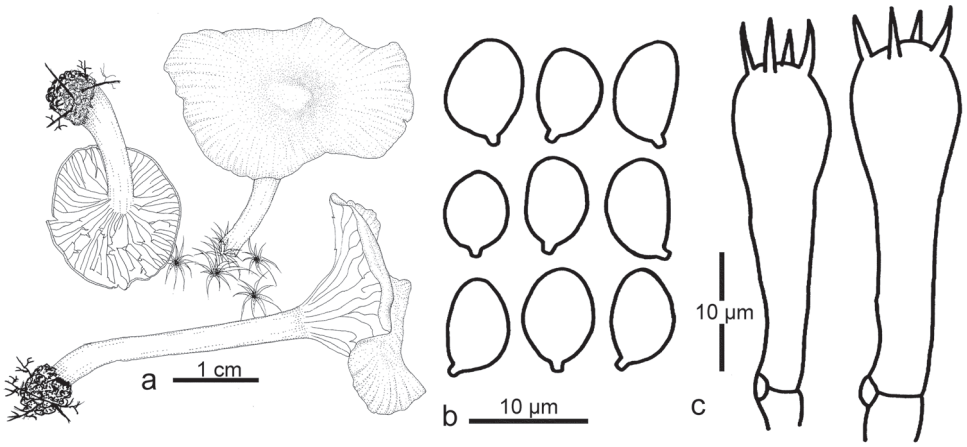

Figure 6. Line drawings of *Gloioxanthomyces nitidus* (GDGM41710). **a** basidiomata **b** basidiospores **c** basidia.

(Fig. 1). In addition, the establishment of the three well-defined monophyletic tribes in subfamily Hygrocyboideae is supported in this phylogenetic frame where the tribe Hygrocybeae with 73% support values and the tribe Humidicuteae with low support value are sister clades, while the tribe Chromosereae with 76% support values is located at their base. However, the cuphophylloid grade appears not to be monophyletic, thus more studies are still needed to understand the phylogenetic positions of *Ampulloclitocybe*, *Cantharocybe* H.E. Bigelow & A.H. Sm. and *Cuphophyllus* (Donk) Bon.

In the multi-gene analyses, *Sinohygrocybe* is placed together with two other genera in Chromoserae. *Chromosera* and *Gloioxanthomyces* are sister genera under the monophyletic tribe Chromosereae, while *Sinohygrocybe* is an independent generic lineage; and the distances between *Sinohygrocybe* and *Chromosera* or *Gloioxanthomyces* are further than the distance between *Chromosera* and *Gloioxanthomyces*. Such results are confirmed in the ITS phylogenetic tree (Fig. 2). According to the Blastn results, the ITS and LSU sequence identities of the new species to the known taxa are not more than 76% and 96%, respectively, with the *Chromosera* and *Gloioxanthomyces* sequences in GenBank. Thus, it is clear the new genus is independent of those two existed genera.

Beside the molecular analyses, morphological data also support its recognition within tribe Chromosereae. *Sinohygrocybe* shares a bright pileus colour and decurrent lamellae with the other genera *Chromosera* and *Gloioxanthomyces* (Table 2). However, the genus *Chromosera*, typified by *C. cyanophylla* (Fr.) Redhead, Ammirati & Norvell, differs from *Sinohygrocybe* in having omphaloid basidiomata, ephemeral dextrinoid reactions in the context, ratio of basidiospore to basidium length <5, ephemeral pigment bodies in the pileipellis and lilac pigments sometimes present (Redhead et al. 1995, Candusso 1997, Lodge et al. 2014); while *Gloioxanthomyces* differs from *Sinohygrocybe* by having weaker/delicate basidiomata, viscid pileus and stipe surface, gelatinised lamellar edge and cheilocystidia, shorter basidia (Boertmann 1990, 2012) with a length ratio of basidium to basidiospore 4–5. *Sinohygrocybe* shares some macroscopic characters with *Hygrocybe*, typified by

Table 2. Type location, basidiospores and basidia dimensions of species of the tribe Chromosereae.

Species name	Type location	Basidiospores (μm)	Basidia (μm)	Reference
Gloioxanthomyces nitidus	USA, South Carolina	6.5–9(11) × 4–6.5(7.5)	29–39 × 7.5–10	Bessette et al. 2010, this study
Gloioxanthomyces vitellinus	Sweden	(6.5)7–9(9) × (5)5.5–7(7.5)	30–45 × 7–10	Boertmann 2010
Chromosera citrinopallida	USA, Washington	7–9(10) × 4.5–5	10–45 × 6–8	Smith and Hesler 1954
Chromosera cyanophylla	Sweden	(6.8)7.2–8.0(8.8) × (3.2)3.6–4.4	24–28 × 5.5–6.5	Holec et al. 2015
Chromosera lilacina	northern Fennoscandia	7–8.5(10) × (4)5–6(6.5)	30–45 × 7–9	Candusso 1997
Chromosera viola	Belgium, Namur Province	6.5–10.5(11) × 5–7(7.5)	36–61 × 8–11	Candusso 1997
Chromosera xanthochroa	Scotland	(5.5)6–8.5(10) × (3.8)4–5.2(5.5)	25–32 × 6.5–7.5(8.5)	Candusso 1997
Sinohygrocybe tomentosipes	China, Sichuan & Hunan Province	8–10(10.5) × (4.5)5–7(7.5)	41–80 × 4–10	This study

H. conica, including bright colour of basidiomata and the distant lamellae, but *Hygrocybe* differs from *Sinohygrocybe* by having more fragile lamellae, more glabrous stipe (at least at the upper portion), often constricted spores and shorter basidia.

Sinohygrocybe samples were collected in both late summer (August) and winter (November), showing that they likely have a quite long fruiting season. It should be noted, however, that they are more abundant at times with lower temperature and higher humidity. Therefore, their fruiting in summer may occur only at higher altitude (with the elevation above 1500 m).

As to the Chinese new *Gloioxanthomyces nitidus* record: 1) phylogenetically, the Chinese samples are nested in the *Gloioxanthomyces* clade as a sister branch to the North American branch (Fig. 2); 2) morphologically, it shares these characters with the North American *G. nitidus*: deep yellow basidiomata fading to whitish with age, viscid, hygrophanous surface, central concave pileus and decurrent lamellae (Bessette et al. 2012); 3) geographically, *G. nitidus* and *G. vitellinus* are distributed in North America and Asia and Europe, respectively, indicating that *Gloioxanthomyces* is a Holarctic genus. It is assumed that both North American and East Asian *G. nitidus* were separated from the same ancestor because of geographical isolation, thus they are very similar at present; however, they may continue to diverge, eventually becoming separate species in the future since they live on detached continents.

Acknowledgments

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A discussion on the genus *Fomitiporella* (Hymenochaetaceae, Hymenochaetales) and first record of *F. americana* from southern South America

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Abstract

Fomitiporella has traditionally been delimited based on the gross morphology of the basidiomes, hyphal structure and basdiospores. Recently, phylogenetic studies supported the incorporation of an extensive number of species within the genus. Although most of its species are nested in the 'Phellinotus clade' (Hymenochaetaceae, Basidiomycota), genera such as *Arambarria*, *Inocutis* and *Phellinotus* were not included in previous analysis. To further our understanding of the genus, new sequences from 28S and ITS nuc rDNA genes were jointly analysed with a large selection of taxa in the 'Phellinotus clade', also with re-examination of morphological and ecological data. Results showed several lineages in what has hitherto been considered to represent *Fomitiporella*, indicating that the genus is paraphyletic as presently circumscribed. There is a well-supported *Fomitiporella* core group that includes the type species and nine other monophyletic lineages with high support, of which those representing *Arambarria*, *Inocutis* and *Phellinotus* are distinct from the *Fomitiporella* core group by macro and micromorphological traits and/or biogeographic distribution. *Fomitiporella americana*, a species described from SE USA, was found in the Patagonian forests of southern Argentina and Chile; it is the taxon responsible for the white heart-rot found on standing *Austrocedrus chilensis* and one of the taxa decaying wooden tiles of historic churches in Chiloé Is., Chile.

Keywords
Hymenochaetaceae; phylogeny; taxonomy; wood-rotting fungi

Introduction

Fomitiporella Murrill [type species *F. umbrinella* (Bres.) Murrill] was originally described to encompass poroid Hymenochaetaceae (Hymenochaetales, Basidiomycota) with resupinate and perennial basidiome that present a thin context, ovoid to globose basidiospores with brown walls and lacking setae of any sort (Murrill 1907). As the species present a dimitic hyphal system, the genus was, for many years, considered a synonym of *Phellinus* Quél. (Ryvarden and Johansen 1980, Larsen and Cobb-Poulle 1990, Ryvarden 1991, Ryvarden and Gilbertson 1994, Dai 1999, Núñez and Ryvarden 2000, amongst others). Nevertheless, the genus received molecular support by Wagner and Fischer (2002) through the comparison and analyses of 28S DNA markers, a fact later on confirmed by Zhou (2014). Ji et al. (2017, 2018) broadened the concept of *Fomitiporella* on the basis of a wide sampling of specimens and species from Central America, USA, Europe, China and Vietnam, studies based on morphological examinations and separated phylogenetic analyses based on nuc rDNA ITS and 28S data sets. Their studies, though, did neither incorporate nor discuss the positions of several genera described previously, namely *Arambarria* Rajchenb. & Pildain, *Inocutis* Fiasson & Niemelä and *Phellinotus* Drechsler-Santos, Robledo & Rajchenb., published in works that showed the complex relations within the members of "Phellinotus clade", where *Fomitiporella* is included (Wagner and Fisher 2002, Rajchenberg et al. 2015, Drechsler-Santos et al. 2016, Pildain et al. 2017).

In Patagonia, Argentina, the native Cordilleran cypress [*Austrocedrus chilensis* (D. Don) Pic. Sern. & Bizzarri, Cupressaceae] has been the subject of continuous research regarding the fungus responsible for the white heart-rot (WHR) present in living trees (Figure 1A). Studies on the wood-rots (Barroetaveña and Rajchenberg 1996) and search of the associated wood-rotting mycobiota (Rajchenberg 2002) were produced but were unsuccessful in identifying the WHR causing agent. Rajchenberg et al. (2015) included strains of this WHR fungus in their phylogenetic study of poroid Hymenochaetaceae from Patagonia. These strains clustered in a group of species that included *Fomitiporella caryophylli* (Racib.) T. Wagner & M. Fischer (a strain from India, Wagner and Fischer 2002) and *Fulvifomes inermis* (Ellis & Everh.) Y.C. Dai (strains from China, Zhou 2014), but the species remained unnamed. In the last years, the search of poroid Hymenochaetaceae (Hymenochaetales, Basidiomycota) from southern Chile allowed us to find specimens that turned out to match the purported species.

The aims of this work were (1) to produce separated and combined phylogenetic analyses based on ITS and 28S markers of *Fomitiporella* in order to discuss its phylogenetic relationships and (2) to record *Fomitiporella americana* from southern South America.

Methods

Study areas. Specimens of poroid Hymenochaetaceae were collected in the Valdivian Rainforest and the Subtropical Xerophytic and Durifoliated Forests of southern Chile (Hueck 1978, Donoso Zegers 1993) and in the Patagonian Andes forests of continental Argentina (Cabrera 1971).

Specimens and cultures. Specimens were dried and preserved in the Phytopathological Herbarium, Centro Forestal CIEFAP at the senior author's address. See Suppl. material 1: Table S1 for specimens' data. Many specimens determined as *Phellinus inermis* (Ellis & Everh.) G. Cunn. (Rajchenberg 2006) [present name *Fomitiporella inermis* (Ellis & Everh.) Murrill] from these areas were incorporated in this study.

Cultures were isolated by placing small portions of contextual tissue of basidiome and/or small portions of the associated wood-rot in the substrate in 2% malt extract agar medium. Morphological features of cultures (Nobles 1965, Stalpers 1978) were used to corroborate their affiliation to Hymenochaetaceae. Strains were deposited at the Culture Collection, Centro Forestal CIEFAP at the senior author's address. Cultures of related specimens were included in the study. Strains isolated from the white heart-rot found in standing *Austrocedrus chilensis* in Patagonia and previously determined as Hymenochaetaceae sp. (Barroetaveña and Rajchenberg 1996, Rajchenberg et al. 2015) were also incorporated, as well as a new strain of *F. inermis* from the USA. See Suppl. material 1: Table S1 for strains' data.

DNA extraction and PCR conditions. DNA was extracted from basidiomes or freshly collected mycelium from pure culture grown in liquid malt peptone broth with 10% (v/v) of malt extract (Merck) and 0.1% (w/v) Bacto peptone (Difco), in 15 ml tubes at 24 °C in the dark. DNA extractions were carried out with the UltraCleanTM Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Solana Beach, California), following the manufacturers' protocols. PCR for the partial 28S (LSU gene that includes the D1/D2 domains) was performed with the primer pairs LROR-LR5 (Vilgalys and Hester 1990) and the full Internal Transcribed Spacer region (i.e. ITS1, ITS2 and the intervening 5.8S RNA gene; further referred as ITS) with ITS5-ITS4 (White et al 1990). The PCR conditions were described in Rajchenberg et al. (2015): 95 °C for 2 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 8 min. The amplified fragments were purified and sequenced at the DNA Synthesis and Sequencing Facility, Macrogen (Seoul, Korea). Sequences generated in this study were submitted to GenBank (cf. Suppl. material 1: Table S1).

Sequence and phylogenetic analyses. Obtained sequences were blasted against the nucleotide database from Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Available ITS and 28S sequences of the genera *Fomitiporella* obtained by Ji et al. (2017) were included. We also included the sequences of *Arambarria*, *Phellinotus*, *Inocutis* and *Phylloporia*. Sequences of *Fomitiporia punctata* MUCL34101 and *Phellinus uncisetus* MUCL46231 were used as outgroups. Suppl. material 2: Table S2 lists the specimens used and their Genbank accession numbers.

Two datasets were analysed for this study: one for the ITS region and one for the 28S gene. Nucleotide sequences for the ITS region and 28S gene were initially edited with BioEdit 7.0.9.0 (Hall 1999), then aligned using L-INS-i strategy as implemented in MAFFT v 7.0 (Katoh and Standley 2013) and manually adjusted using MEGA version 6 (Tamura et al. 2013). Ambiguously aligned regions were eliminated using Gblocks 0.91b (Castresana 2000). The final ITS dataset comprised 53 sequences and 602 characters including gaps and the LSU dataset comprised 47 sequences and 882 characters including gaps. The datasets were combined for concatenated analyses using Mequite 3.40 (Maddison and Maddison 2018). The best-fit models of evolution were determined using the AIC criterion (Akaike 1974), implemented in jModelTest (Posada 2008, http://darwin.uvigo.es) and were HKY+G and TrN+I+G for ITS and 28S respectively. Phylogenetic analysis of the individual and combined dataset was performed using maximum likelihood (ML) and Bayesian Analyses (BA) optimality criteria. The ITS and LSU partitions included 599 and 881 characters, respectively, for a combined data matrix of 1480 characters. The number of included taxa were 44 for both ITS and LSU. Branch support was determined using nonparametric bootstrapping implemented in RAxML 7.2.8 (Stamatakis et al. 2014), using the default parameters, executed on the CIPRES (Cyberinfrastructure for Phylogenetic Research) Science Gateway V. 3.1 (http://www.phylo.org/sub_sections/portal/, Miller et al. 2010) with bootstrap support values calculated with 1000 repetitions. Bayesian phylogenetic analyses were performed using Mr Bayes v. 3.2.2 (Ronquist and Huelsenbeck 2003) for 8,000,000 generations, with four chains and trees sampled every 100 generations. The first 80,000 generations were discarded as the burn-in. Log files for each run were viewed in Tracer v1.6.0 (http://evolve.zoo.ox.ac.uk/software.html/tracer/) to determine convergence. Branch support was assessed using posterior probabilities calculated from the posterior set of trees after stationarity was reached. Trees generated prior to stationarity were discarded and the rest of the trees were summarised in a majority-rule consensus tree from the four independent runs. Alignments have been deposited at TreeBase: http://purl.org/phylo/treebase/phylows/study/22728.

Results

Phylogeny

Two loci analyses of 45 taxa inferred from Bayesian analyses (BA) and Maximum likehood (ML) were performed. The phylogenetic analyses included the simple and combined ITS + 28S concatenated dataset (Figure 2). Combined ITS and 28S analyses confirmed that members of *Fulvifomes*, *Phylloporia*, *Phellinotus*, *Arambarria*, *Inocutis* and *Fomitiporella* are closely related and form a strong monophyletic group (BA 1.0, ML 100) named "Phellinotus clade" by Dreschler et al. (2015). *Fulvifomes* and *Phylloporia* occupy a more basal position and form highly supported subclades. *Phellinotus*,

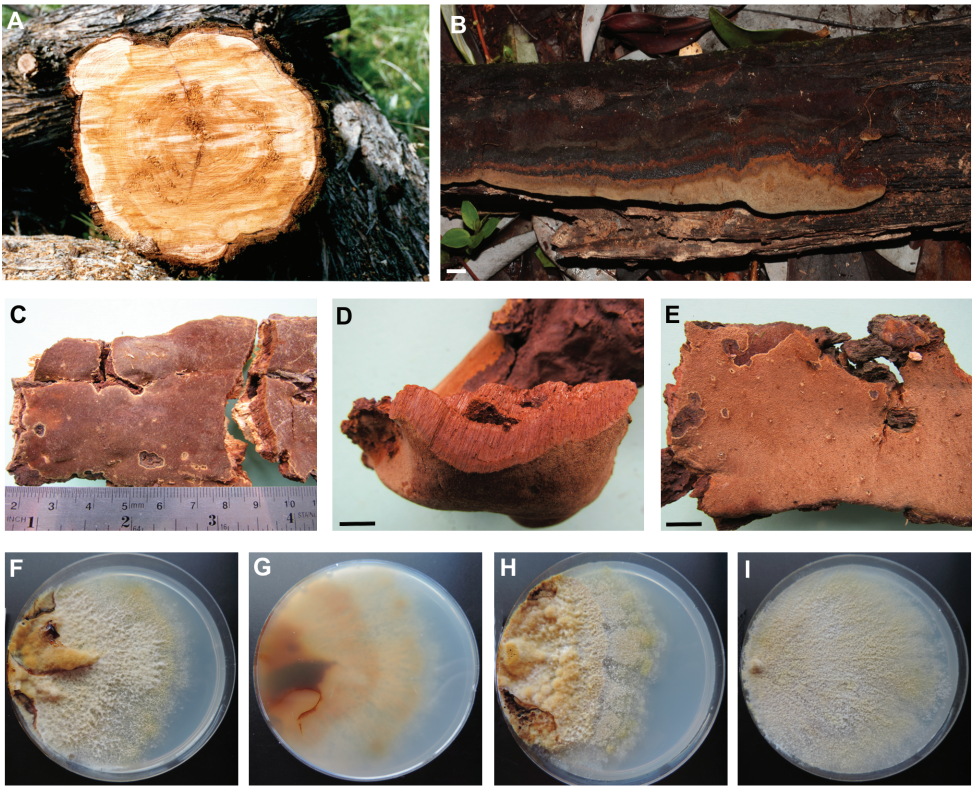

Figure 1. *Fomitiporella americana*, damage and morphology. **A** White heart-rot caused by the fungus in a section of a felled *Austrocedrus chilensis* **B–E** Basidiomes **B** Specimen RDS 1768 (=MR 12602, Chile) **C, E** Specimen MR 10946 (Chile) **D** Specimen MR 12060 (Argentina) **F–I** Macroscopic features of cultures **F** Strain CIEFAPcc 88, frontal view **G** Strain CIEFAPcc 88, reverse view **H** Strain CIEFAPcc 516 **I** Strain CIEFAPcc 595. Scale bar = 10 mm. Petri dishes measure 9 cm in diameter.

Arambarria, *Inocutis* and *Fomitiporella* taxa clustered together as a monophyletic clade. Within this group, five well defined groups were observed in the combined ITS and 28S concatenated analyses (Fig. 1):

(1) *Inocutis*, *Phellinotus* (BA 0.93, ML 50); (2) *Fomitiporella tenuissima* is closely related to *F. mangrovei* but the relationships with the remaining species were not clear (BA 1.0, ML 98); (3) *Arambarria*, *Fomitiporella austroasiana*, *F. cavicola*, *F. caviphila*, *F. resupinata*, *F. umbrinella* (BA 0.9, ML 60); (4) *F. inermis*, *F. subinermis*, *F. chinensis* (BA 1.0, ML 98); and (5) *Fomitiporella micropora*, *F. sinica*, *F. caryophylli*, *F. americana*, *F. vietnamensis*, (BA 1.0, ML 98) . The genus *Fomitiporella,* as currently defined, is paraphyletic, with the type species as part of the clade *Arambarria*, *Fomitiporella austroasiana*, *F. cavicola*, *F. resupinata*, *F. umbrinella* and the additional lineages occurring in the core "Phellinotus clade"; whereas *Fomitiporella sinica*, *F. americana*, *F. caryophylli*, *F. micropora* (BA 1.0, ML 98) may not be closely related to the *Fomitiporella* core group where the type (*F. umbrinella*) is included.

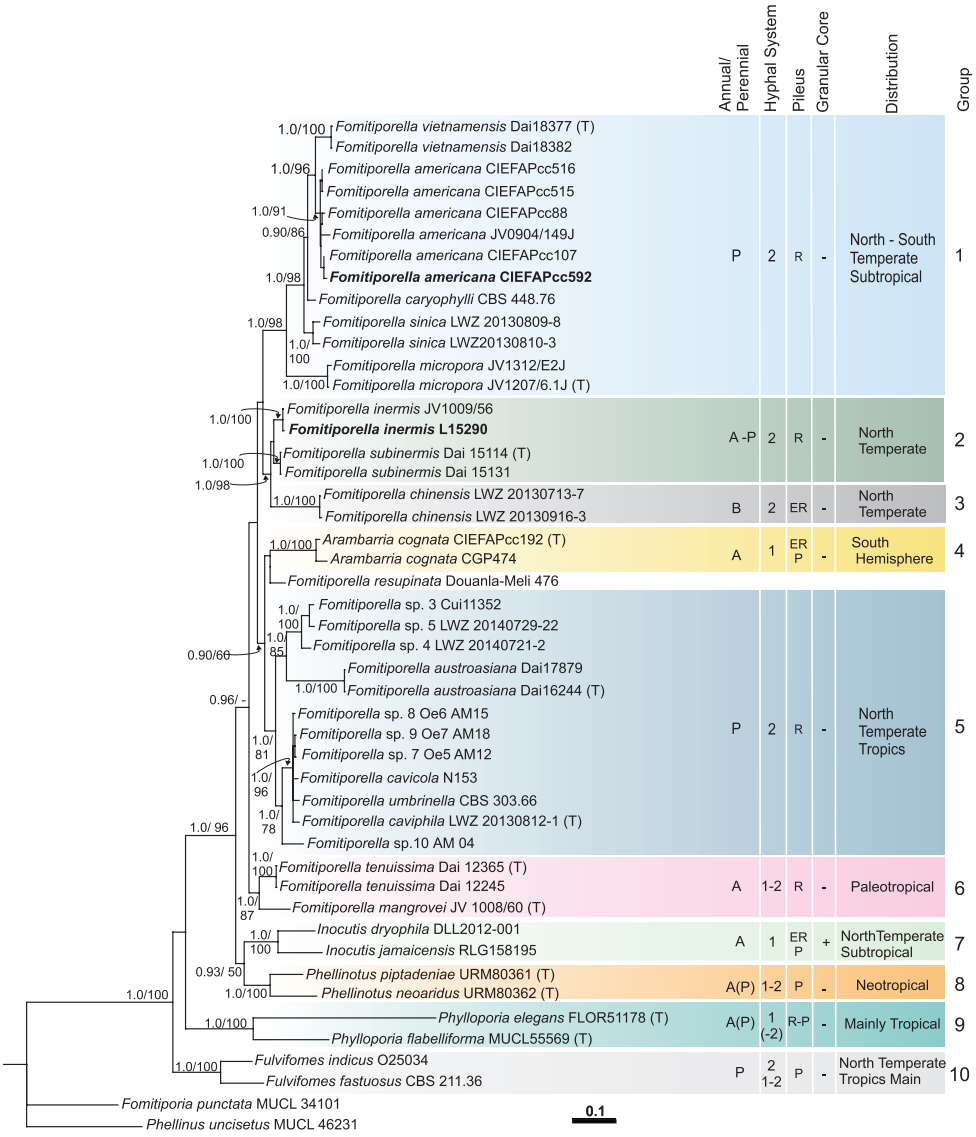

Figure 2. Phylogram generated from nuc rDNA ITS+28S combined sequence data with Bayesian and maximum likelihood analysis. Maximum likelihood (ML) bootstraps from 1000 iterations. Bayesian posterior probabilities (BPP) from 1000 iterations (8 million runs sampling every 100th iteration). Bootstrap values ≥ 50% (ML) followed by the Bayesian posterior probability (≥ 90%) are indicated in the node branches; -: support values lower than 50/90%. Bold type identifies new obtained sequences. T indicates sequences obtained from the genic type species. Horizontal coloured stripes distinguish different clades as treated in the text. Horizontal stripes point out morphological and distributional features of taxa. (A) annual basidiome. (P) perennial basidiome. (1) Monomitic hyphal system. (2) Dimitic hyphal system. (ER) effused reflexed. (P) pileate. (R) resupinate. (-) granular core absent. (+) granular core present.

The internal topology of the "Phellinotus clade" is much better resolved in ITS + 28S than in the single gene datasets, with more than 85% of the nodes receiving strong support (ITS: 75%, Suppl. material 3: Figure S1; 28S: 65%, Suppl. material 4: Figure S2). Within these conflicts that had moderate support (BA <0.90 and/or ML <75%) appears the group formed by *Inocutis* and *Phellinotus* (group not supported in the 28S phylogenetic analysis; cf. Suppl. file S4) and *Arambarria*, *Fomitiporella cavicola*, *F. resupinata*, *F. umbrinella* and *F. tenuissima* with their uncertain position, while *F. americana* appears as a species with north and southern hemisphere members (see below).

Patagonian sequences (CIEFAP515, CIEFAP516, CIEFAP592) of the white heart-rot fungus, responsible for *A. chilensis* decay, fell within *Fomitiporella americana*, a species recently described from SE USA (Ji et al. 2017) (BA 1.0/0.9/0.99, ML 91/70/60 combined ITS + 28S; 28S and ITS, respectively) and differentiated from *Fomitiporella inermis* (formerly *Phellinus inermis*) (Figure 2, Suppl. materials 3, 4: Figures S1, S2). The closest sister group of *F. americana* is *F. vietnamensis* and together with *F. sinica* and *F. caryophylli* formed a strongly supported clade, where *F. micropora* is also included in a basal position (BA 1.0, ML 98 combined ITS + 28S). From the ITS phylogeny, it is noted that another strain that clustered with *F. americana* was the strain 'Achao 50' isolated from roof tiles of an historic wooden church from Chiloé Is. in southern Chile (Ortiz et al. 2014) (Suppl. material 3: Figure S1). Additionally from the ITS phylogeny, it could be observed that strains of *Fomitiporella* sp. (recorded as Hymenochaetales 1, 2 and 4 at GenBank), recorded by Cloete et al. (2015) and isolated from South African grapevines wood-rots associated with esca disease, did not match any known species and represent independent taxa (Suppl. material 3: Figure S1). From 'Achao 50' and Hymenochaetales 1, 2 and 4, there are only ITS sequences available and therefore only included in the ITS analysis.

The newly sequenced strain (L-15290) of *Phellinus inermis* sensu stricto from USA grouped with the other known sequence of *F. inermis* (Ji et al. 2017).

Morphology, ecology and pathogenicity

Specimens previously determined as *Phellinus inermis* (Ellis & Everh.) G. Cunn. (Espinosa 1917, Rajchenberg 1987, 1995, 2006) from southern Argentina and Chile turned out to perfectly match phylogenetically with *Fomitiporella americana* Y.C. Dai, X.H. Ji & Vlasák . They are characterised by resupinate, perennial, flattened to pulvinate basidiomes that may also develop nodulose structures, reaching up to 1.3 mm thick (Figures 1B–E). They present a chestnut, chocolate brown to umbrinous hymenial surface with margins that are lighter in colour and, sometimes, receding. Pores vary from 4.5–7 mm, exceptionally smaller up to 8 mm. Hyphal system is dimitic. Basidiospores are ellipsoid to broadly ellipsoid, always with a straight, ventral, inner side, thick-walled with walls yellowish in water but dark chestnut in 5% KOH, IKI–, 4.5-5.5(6) × 3.5–4.5 µm. Spore size variability was shown by Rajchenberg (1995).

Table 1. Morphological comparison of *Fomitiporella americana* with similar species from different geographic areas.

	Fomitiporella americana **(USA) (Ji et al. 2017)**	***Fomitiporella americana*** **(S Argentina and Chile)**	***Fomitiporella inermis*** **(USA) (Ji et al. 2017)**	***Fomitiporella subinermis*** **(China) (Ji et al. 2017)**	***Fomitiporella sinica*** **(China) (Ji et al. 2017)**
Pores/mm	5–6	4.5–7	5–7	6–7	6–8
Spores length (µm)	(3.5)4–4.5	4.5–5.5(6)	4.5–5(5.5)	(4)4.5–5(5.5)	4–4.5
Spores width (µm)	(2.5)3–3.5(4)	3.5–4.5	3.5–4(4.5)	3.5–4	3–3.5
Spores shape	subglobose*	ellipsoid to broadly ellipsoid	broadly ellipsoid	subglobose*	broadly ellipsoid to globose*
Ecology	fallen trunks (FT)	living trees (L), generally FT	FT	root of trees	L and FT
Hosts	*Quercus* sp. (D) (Fagaceae)	Numerous hosts, see text	*Ilex mucronata* (Aquifoliaceae). Several substrata fide Lowe (1966)	Unknown angiosperm	*Casuarina* sp. (L) (Casuarinaceae) *Melia* sp. (L) (Meliaceae) *Rhododendron* sp. (D) (Ericaceae)

*But no subglobose spore was drawn in Ji et al. (2017). FT= fallen trunk; L= living tree; D= dead; L= living.

Cultures showed macroscopical variation but were otherwise typical of the Hymenochaetaceae (Figures 1F–I) and as previously described by Barroetaveña and Rajchenberg (1996) under Hymenochaetaceae sp. For specimens examined, see Suppl. material 1: Table S1. Table 1 compares specimens from Patagonia with morphologically similar species described from USA and China.

In southern South America, *Fomitiporella americana* has a wide spectrum of hosts that includes living *Austrocedrus chilensis* (Cupressaceae) and dead *Maytenus boaria* (Celastraceae), *Cryptocarya alba* (Lauraceae), *Nothofagus dombeyi* and *N. nitida* (Nothofagaceae), *Diostea juncea* (Verbenaceae), *Escallonia* sp. (Escalloniaceae), *Eucryphia cordifolia* and *Weinmannia trichosperma* (Cunoniaceae), *Peumus boldus* (Monimiaceae), *Luma apiculata* and *Tepualia stipularis* (Myrtaceae). It decays fallen trunks and branches but is also pathogenic to standing *A. chilensis*, being responsible for the WHR that has been recorded many years ago (Barroetaveña and Rajchenberg 1996).

Discussion

This study incorporated for the first time all molecular information available for *Fomitiporella* species and related organisms pertaining to the 'Phellinotus clade' (Drechsler-Santos et al. 2016). Our combined analyses showed that *Fomitiporella* is

paraphyletic as presently circumscribed by Ji et al. (2017, 2018), most notably the unresolved relationships with the well-recognised genera *Inocutis* (Fiasson and Niemelä 1984, Wagner and Fischer 2002), *Phellinotus* and *Arambarria*, all of which present differences in their gross morphology and in the nature of the hyphal structure. Amongst the poroid Hymenochaetaceae, *Inocutis* is distinguished morphologically by a combination of the monomitic hyphal system and the formation of a granular core in the context. It is associated with *Phellinotus* Drechsler-Santos, Robledo & Rajchenb. (Drechsler-Santos et al. 2016), that is distinguished by a monomitic context, dimitic trama of the tubes and by variable presence of a granular core in context (i.e. variably present in *Phellinotus neoaridus* Drechsler-Santos & Robledo). *Arambarria* Rajchenb. & Pildain (Rajchenberg et al. 2015, Pildain et al. 2017) appeared distant from the former and is monomitic throughout the basidiome and lacks a granular core in context. The three genera contrast with the fully dimitic *Fomitiporella* species.

In view of the molecular data presented here, *Fomitiporella* is paraphyletic and the treatment of the genus *Fomitiporella* by Ji et al. (2017, 2018) is artificial. In addition, since the combined phylogenetic analyses retrieved 10 monophyletic lineages with high support within the "Phellinotus clade", it is possible to imagine different taxonomic scenarios:

1) To accept 10 different genera within the group: *Fulvifomes*; *Phylloporia*; *Inocutis*; *Phellinotus*; *Arambarria*; *Fomitiporella* (including *F. umbrinella*, *F. cavicola*, *F. austroasiana* and *Fomitiporella* spp. 3, 4, 5, 7, 8, 9 and 10); and the following taxa as 4 new genera: *Fomitiporella inermis* and *F. subinermis*; *F. chinensis*; *F. tenuissima* and *F. mangrovei*; and subclade *F. sinica*, *F. caryophylli*, *F. americana*, *F. vietnamensis* and *F. micropora*.

The problem with this solution is that a new genus for *F. chinensis* would only include, for the time being, one species.

The case of *F. resupinata* is unclear as it presents an uncertain position close to *Arambarria*. More materials from Africa are needed in order to ascertain its phylogenetic position.

Option '1' would be the easiest solution from an 'operational' point of view, with statistical support comparable to those shown for recent genera that have been treated in the Hymenochaetaceae and accepted phylogenetically: *Onnia* (Ji et al. 2017), *Phellinidium* and *Coniferiporia* (Zhou et al. 2016) and *Neomensularia* (Wu et al. 2016), only to give some examples.

2) To group *Inocutis*, *Phellinotus*, *Arambarria* and *Fomitiporella* s.l. (Ji et al. 2017, 2018) as a unique genus *Fomitiporella*.

This option presents the following problems regarding:

Morphology: the genus would include full monomitic species (those included in *Inocutis* and *Arambarria*), full dimitic species (*Fomitiporella* s.l.) and species with monomitic context and dimitic trama of tubes (*Phellinotus*).

Taxonomy: the genus would encompass three well-established and recognised genera such as *Inocutis*, *Arambarria* and *Phellinotus*.

3) To maintain *Inocutis* and *Phellinotus* as independent genera and to group *Arambarria* under *Fomitiporella* s.l.

This option appears to be convenient but seems not consistent on the basis of the variable phylogenetic supports (Fig. 1, Suppl. files S3, S4) and, morphologically, because of the monomitic hyphal system of *Arambarria*.

Phylogenetic studies showed that specimens, previously recorded as *Phellinus inermis* from southern Argentina and Chile, match *Fomitiporella americana*, a taxon recently described from SE USA (Ji et al. 2017). The species is morphologically similar to *Fomitiporella inermis* and other taxa described from China that were previously recorded as *Fulvifomes inermis* (Ellis & Everh.) Y.C. Dai (Dai 2010). These species are presently accepted as *F. sinica* Y.C. Dai, X.H. Ji & Vlasák and *F. subinermis* Y.C. Dai, X.H. Ji & Vlasák (Ji et al. 2017). The prominent features of these species are shown in Table 1, which underlines that morphological differences between them are subtle, constituting a species complex. From a phylogenetic point of view, *F. americana* comes close to *F. sinica* and *F. caryophylli*, but distant from *F. inermis*, which grouped with *F. subinermis* and *F. chinensis* (Figure 2, ITS+28S). This shows that morphology has been conservative throughout the evolution of this group and is a limited criterion to distinguish taxa.

Fomitiporella americana was originally recorded on *Quercus* sp. but this study shows it is widely distributed on many hosts in southern South America. Our results also show that *F. americana* is one of the wood-rotting agents decaying historic wooden churches in Chiloé Is., southern Chile, recorded as isolate 'Achao50' (Ortiz et al. 2014). During many years, the causing agent of the WHR present in *A. chilensis* standing trees was unknown despite several efforts made to find it (cf. Introduction). Though many basidiomes of *F. americana* (as *Phellinus inermis*) had been found in the past, they never fruited on *A. chilensis*. For this reason, the match between isolates of the WHR fungus and the several materials from S Chile and Argentina came as a surprise, indicating the role of this species in the decay of standing trees. This study shows that *F. americana* appears to have a wide distribution in the Americas, for the moment apparently a species with an amphitropical distribution (i.e. present in temperate to cold temperate areas of the North and South Hemisphere). Whether it is also present in tropical areas needs to be verified; specimens determined as *inermis* have been recorded from Central Argentina (Robledo and Urcelay 2009), but their identity needs to be worked out from a phylogenetic point of view. To date, *F. americana* or any morphological similar taxon has not been recorded from Neotropical areas by Ryvarden (2004). Specimens of *P. inermis* recorded from New Zealand (Cunningham 1965) might represent a different taxon due to its effused-reflexed basidiomes (Parmasto et al. 1980) though they are microscopically akin (Rajchenberg 1987).

Our study incorporated a second strain and sequence of *Fomitiporella inermis* sensu stricto (i.e. L-15290, cf. Figure 2 and Suppl. material 1: Table S1) that perfectly matched that of J. Vlásak 1009/56 gathered on *Ilex mucronata* (Ji et al. 2017). Both showed to be close to *F. chinensis* and *F. subinermis* and far from *F. americana* and *F. sinica*.

As an ending remark, we point out that, before proposing taxonomic inferences coherent with phylogenetic results, it seems cautious to wait till more taxa are sampled and more loci are incorporated into phylogenetic analyses, also including taxa around *F. chinensis* and *F. tenuissima*. Incorporation of more sequences from more taxa may certainly impact the phylogeny, as the resolution of the phylogeny of "Phellinotus clade" is low. Operational units (genera) shown by phylogenetic analyses are certainly correctly defined but, if one admits a large *Fomitiporella* genus, one has to admit that we are unable to understand what biological and morphological features are leading the evolution of this group of Hymenochaetaceae.

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Supplementary material 1

Table S1

Authors: María Belén Pildain, Rodrigo Reinoso Cendoya, Beatriz Ortiz-Santana, José Becerra, Mario Rajchenberg

Explanation note: List of specimens and strains studied. MR= specimens collected by M. Rajchenberg. RDS= R. Drechsler-Santos.

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Supplementary material 2

Table S2

Authors: María Belén Pildain, Rodrigo Reinoso Cendoya, Beatriz Ortiz-Santana, José Becerra, Mario Rajchenberg

Explanation note: List of taxa presented in the phylogenetic analyses with GenBank accession numbers for the ITS and 28S sequences. (T) indicates type specimen. In bold, new generated sequences.

Link: https://doi.org/10.3897/mycokeys.38.27310.suppl2

Supplementary material 3

Figure S1

Authors: María Belén Pildain, Rodrigo Reinoso Cendoya, Beatriz Ortiz-Santana, José Becerra, Mario Rajchenberg

Explanation note: Phylogenetic relationships of 53 Hymenochaetaceae specimens inferred from nuc rDNA ITS sequences. Topology from Bayesian analysis (BA), Maximum likelihood (ML) bootstraps from 1000 iterations. Bayesian posterior probabilities (BPP) from 1000 iterations (8 million runs sampling every 100th iteration). Bootstrap values ≥ 50% (ML) followed by the Bayesian posterior probability (≥ 90%) are indicated in the node branches; -: support values lower than 50/90%. Bold type identifies new obtained sequences. T indicates sequences obtained from the genetic type species.

Link: https://doi.org/10.3897/mycokeys.38.27310.suppl3

Supplementary material 4

Figure S2

Authors: María Belén Pildain, Rodrigo Reinoso Cendoya, Beatriz Ortiz-Santana, José Becerra, Mario Rajchenberg

Explanation note: Phylogenetic relationship between *Fomitiporella* and related genera inferred from the nuc rDNA 28S dataset. Topology from Bayesian analysis (BA), Maximum likelihood (ML) bootstraps from 1000 iterations. Bayesian posterior probabilities (BPP) from 1000 iterations (8 million runs sampling every 100th iteration). Bootstrap values ≥ 50% (ML) followed by the Bayesian posterior probability (≥ 90%) are indicated in the node branches; -: support values lower than 50/90%. Bold type identifies new obtained sequences. T indicates sequences obtained from the genetic type species.

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RESEARCH ARTICLE

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Morphological and phylogenetic characterisation of novel *Cytospora* species associated with mangroves

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Abstract

Mangroves are relatively unexplored habitats and have been shown to harbour a number of novel species of fungi. In this study, samples of microfungi were collected from symptomatic branches, stem and leaves of the mangrove species *Xylocarpus granatum*, *X. moluccensis* and *Lumnitzera racemosa* and examined morphologically. The phylogeny recovered supports our morphological data to introduce three new species, *Cytospora lumnitzericola*, *C. thailandica* and *C. xylocarpi*. In addition, a combined multi-gene DNA sequence dataset (ITS, LSU, ACT and RPB2) was analysed to investigate phylogenetic relationships of isolates and help in a more reliable species identification.

Keywords

3 new species, Cytosporaceae, *Lumnitzera racemosa*, Mangroves, Phylogeny, Taxonomy, *Xylocarpus granatum*, *Xylocarpus moluccensis*

Introduction

Mangroves are forests established in tropical and subtropical backwaters, estuaries, deltas and lagoons. These forests play a major role in the ecology of coastal tropical/subtropical waters, as they serve as hatchery and nursery habitats for marine organisms and protect coastlines from catastrophic events such as storms and tidal surges (Hyde and Jones 1988, Fisher and Spalding 1993, Hyde and Lee 1995, Hyde et al. 1998). The greatest diversity of mangrove species occurs in the mangroves of Indonesia, Malaysia and Thailand (Alias and Jones 2009, Alias et al. 2010).

Reports of fungi associated with mangroves are relatively few and data on diseases of mangroves are uncommon (Cribb and Cribb 1955, Kohlmeyer and Kohlmeyer 1979, Hyde and Jones 1988). So far, a number of fungi collected from mangroves are either saprobes (e.g Swe et al. 2008a, b, Devadatha et al. 2018, Li et al. 2018) or endophytes (e.g Liu et al. 2012, Doilom et al. 2017). One early species documented from mangroves is that of Stevens (1920) who reported a species of *Anthostomella* that was found from a leaf spot in red mangroves (*Rhizophora mangle*) in Puerto Rico. Later, McMillan (1964) reported *Cercospora* which caused leaf spot on red mangroves in Florida and Kohlmeyer (1969) documented an undescribed *Cytospora* species on *R. mangle* in Hawaii. *Cytospora rhizophorae* has also been reported as a marine fungus from *Rhizophora mangle* in southwest Puerto Rico (Wier et al. 2000). Later, Shivas et al. (2009) reported a serious disease, caused by *Pseudocercospora avicenniae*, on leaves of *Avicennia marina* in Cape Tribulation, Queensland.

Cytospora was introduced by Ehrenberg (1818) and belongs to the family Cytosporaceae in *Diaporthales* (Wijayawardene et al. 2018). *Cytospora* species are phytopathogens or saprobes (Wehmeyer 1975, Barr 1978, Eriksson 2001, Castlebury et al. 2002, Wijayawardene et al. 2018). *Cytospora* has a worldwide distribution and is an important pathogenic genus, causing canker and dieback disease on branches of a wide range of plants (Adams et al. 2005, 2006, Hyde et al. 2017, Norphanphoun et al. 2017). Currently, there are 614 epithets for *Cytospora* (Index Fungorum 2018, 14 June 2018) with an estimated 110 species in Kirk et al. (2008). Recently, fourteen new species were introduced to this genus by Norphanphoun et al. (2017). In this study, we report on three novel species of *Cytospora* associated with mangroves in Thailand. Detailed descriptions and illustrations of all the species identified are provided in this paper.

Material and methods

Sample collection and examination of specimens

Samples collected were dead branches of *Xylocarpus granatum* K.D. Koenig, *X. moluccensis* (Lam.) M. Roem. and leaf spots of *Lumnitzera racemosa* Willd. from Phetchaburi and Ranong provinces, Thailand in 2016. Specimens were returned to the laboratory in paper bags, examined and described following Norphanphoun et al.

(2017). Morphological characters of ascomata and conidiomata were examined using a Motic SMZ 168 dissecting microscope. Hand sections were mounted in water and examined for morphological details. Micro-morphology was studied using a Nikon Ni compound microscope and photographed with a Canon EOS 600D digital camera fitted to the microscope. Photo-plates were made using Adobe Photoshop CS6 Extended version 13.0 × 64 (Adobe Systems, USA), while Tarosoft (R) Image Frame Work programme v. 0.9.7 was used for measurements.

Cultures were obtained by single spore isolation method outlined in Chomnunti et al. (2014). Single germinating spores were observed and photographed using a Nikon Ni compound microscope fitted with Canon EOS 600D digital camera. Geminated spores were transferred aseptically to 2% malt extract agar (MEA, malt extract agar powder 32 g in 1000 ml water) and incubated at room temperature (18–25 °C). A tissue isolation method was used for isolation of taxa from leaf spots of *Lumnitzera racemosa*. Leaves with leaf spots were cut into small pieces (0.5 × 0.5 cm^2) using a sterilised blade and surface was sterilised using 70% ethanol for 1 minute, followed by three rinses with sterile distilled water, 1 minute in 3% sodium hypochlorite (NaOCl) and rinsed with sterile water for 1–2 minutes and dried by blotting on sterile filter paper. Four to five segments including the edge of the leaf spot were placed on water agar (WA) plates, supplemented with 100 mg/ml streptomycin. The dishes were incubated at 27 °C ± 2 °C for 7–10 days. Fungal colonies were transferred using single hyphal tips on to potato dextrose agar (PDA) plates throughout a 2-week period. Pure cultures were maintained for further studies on PDA (Bharathidasan and Panneerselvam 2011). The specimens/dried cultures and living cultures are deposited in the Herbarium Mae Fah Luang University (MFLU) and culture collection Mae Fah Luang University (MFLUCC), Chiang Rai, Thailand and duplicated in the International Collection of Micro-organisms from Plants (ICMP). Facesoffungi numbers were registered as in Jayasiri et al. (2015). New taxa are established based on recommendations as outlined by Jeewon and Hyde (2016).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fresh fungal mycelia growing on MEA at room temperature (18–25 °C) for three weeks using a E.Z.N.A.TM Fungal DNA MiniKit (Omega Biotech, CA, USA) following the manufacturer's protocols. Polymerase chain reactions (PCR) were carried out using primer pairs of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') to amplify the ITS region (White et al. 1990), primer pairs of NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') to amplify part of the large subunit rDNA (28S, LSU) (O'Donnell 1993), the partial ACT region was amplified using primers ACT512F (5'-ATGTGCAAGGCCGGTTTCGC-3') and ACT783R (5'-TACGAGTCCTTCTGGCCCAT-3') (Carbone and Kohn 1999) and the partial RPB2 region was amplified using primers bRPB2-6F (5'-TGGGGYATGGTNTGYCCYGC-3') and bRPB2-7.1R (5'-CCCATRGCYTGYTTMCCCATDGC-3') (Matheny 2005).

The amplification reactions were carried out with the following protocol: 50 µl reaction volume containing 2 µl of DNA template, 2 µl of each forward and reverse primers, 25 µl of 2 × Bench Top™Taq Master Mix (mixture of Taq DNA Polymerase (recombinant): 0.05 units/µl, $MgCl_2$: 4 mM and dNTPs (dATP, dCTP, dGTP, dTTP): 0.4 mM) and 19 µl of double-distilled water (ddH_2O) (sterilised water) using the thermal cycle programme in Norphanphoun et al. (2017). Purification and sequencing of PCR products with the same primers mentioned above were carried out at Life Biotechnology Co., Shanghai, China.

Phylogenetic analysis

The sequences were assembled by GENEIOUS Pro v. 11.0.5 (Biomatters) and BLAST searches were made to retrieve the closest matches in GenBank and multiple alignment also included recently published sequences (Norphanphoun et al. 2017, Hyde et al. 2017, 2018). Combined analyses of ITS1, 5.8S, ITS2, LSU, RPB2 and ACT sequence data of 86 taxa were performed under different optimality criteria (MP, ML, BI). *Diaporthe eres* (AFTOL-ID 935) was used as the outgroup taxon. In order to obtain a better picture of the phylogenetic relationships amongst our strains and closely related strains, a separate ITS1+ITS2 phylogeny was inferred, because only ITS sequences were available for many strains in that group and because less ambiguously aligned (and excluded) positions are expected in a dataset with narrower taxonomic coverage. Nineteen strains were selected for this analysis based on preliminary analyses and results from the multigene phylogeny. All sequences were aligned separately using the MAFFT v.7.110 online programme (http://mafft.cbrc.jp/alignment/server/; Katoh and Standley 2013) and Gblocks v. 0.91b was used to exclude ambiguously aligned positions in the ITS and ACT alignments (Castresana 2000, Talavera and Castresana 2007). A partition homogeneity test (PHT) was performed with PAUP 4.0b10* (Swofford 2002) to determine whether the individual datasets were congruent and could be combined. The combined sequence alignments were obtained from MEGA7 version 7.0.14 (Kumar et al. 2015), missing data were coded as question marks (?) and further manual adjustments were made wherever necessary in BioEdit 7.2.3 (Hall 1999). The combined sequence alignment was converted to NEXUS file for maximum parsimony analysis using ClustalX v. 2 (Larkin et al. 2007). The NEXUS file was prepared for MrModeltest v. 2.2 (Nylander 2004) in PAUP v.4.0b10 (Swofford 2002).

Maximum Parsimony (MP) analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10* (Swofford 2002) with 1000 bootstrap replicates using a heuristic search with random stepwise addition and tree-bisection reconnection (TBR), as detailed by Jeewon et al. (2002) and Cai et al. (2005). Maxtrees was set to 1000, branches of zero length were collapsed. The following descriptive tree statistics were calculated: parsimony tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC] and homoplasy index [HI].

Table 1. GenBank accession numbers of the sequences used in phylogenetic analyses.

No	Taxon	Strain[a]	Host	Origin	GenBank accession numbers				References
					ITS	LSU	RPB2	ACT	
1	*Cytospora abyssinica*	CMW 10181[T]	*Eucalyptus globulus*	Wondo Genet, Ethiopia	AY347353	–	–	–	Adams et al. (2005)
2	*C. acaciae*	CBS 468.69	*Ceratonia siliqua*	Spain, Mallorca	DQ243804	–	–	–	Adams et al. (2006)
3	*C. ampulliformis*	MFLUCC 16-0583[T]	*Sorbus intermedia*	Russia	KY417726	KY417760	KY417794	KY417692	Norphanphoun et al. (2017)
4	*C. atrocirrhata*	HMBF156			KF225610	KF225624	–	KF498673	Fan et al. (2015a)
5	*C. austromontana*	CMW 6735[T]	*Eucalyptus pauciflora*	Australia	AY347361	–	–	–	Adams et al. (2005)
6	*C. berberidis*	CFCC 89927[T]	*Berberis dasystachya*	China	KR045620	KR045702	KU710948	KU710990	Liu et al. (2015)
7	*C. berkeleyi*	StanfordT3[T]	*Eucalyptus globulus*	California, USA	AY347350	–	–	–	Adams et al. (2005)
8	*C. brevispora*	CBS 116829	*Eucalyptus grandis*	Venezuela	AF192321	–	–	–	Adams et al. (2005)
9	*C. carbonacea*	CFCC 89947	*Ulmus pumila*	Qinghai, China	KR045622	KP310812	KU710950	KP310842	Yang et al. (2015)
10	*C. centravillosa*	MFLUCC 16-1206[T]	*Sorbus domestica*	Italy	MF190122	MF190068	MF377600	–	Senanayake et al. (2017)
11	*C. ceratosperma*	MFLUCC 16-0625	*Acer platanoides*	Russia	KY563246	KY563248	KY563244	KY563242	Tibpromma et al. (2017)
12	*C. chrysosperma*	HMBF151			KF225605	KF225619	–	KF498668	Fan et al. (2015a)
13	*C. cinereostroma*	CMW 5700[T]	*Eucalyptus globulus*	Chile	AY347377	–	–	–	Adams et al. (2005)
14	*C. cotini*	MFLUCC 14-1050[T]	*Cotinus coggygria*	Russia	KX430142	KX430143	KX430144	–	Norphanphoun et al. (2017)
15	*C. curvata*	MFLUCC 15-0865[T]	*Salix alba*	Russia	KY417728	–	–	KY417694	Norphanphoun et al. (2017)
16	*C. cypri*	CBS 201.42[T]	*Syringa* sp.	Switzerland	DQ243801	–	–	–	Adams et al. (2006)
17	*C. diatrypelloidea*	CMW 8549[T]	*Eucalyptus globulus*	Orbost, Australia	AY347368	–	–	–	Adams et al. (2005)
18	*C. disciformis*	CMW6509			AY347374	–	–	–	Adams et al. (2005)
19	*C. donetzica*	MFLUCC 16-0574[T]	*Rosa* sp.	Russia	KY417731	KY417765	KY417799	KY417697	Norphanphoun et al. (2017)
20	*C. elaeagni*	CFCC 89632	*Elaeagnus angustifolia*	Ningxia, China	KR045626	KR045706	KU710955	KU710995	Fan et al. (2015b)

No	Taxon	Strain[a]	Host	Origin	GenBank accession numbers				References
					ITS	LSU	RPB2	ACT	
21	*C. erumpens*	MFLUCC 16-0580[T]	*Salix* × *fragilis*	Russia	KY417733	KY417767	KY417801	KY417699	Norphanphoun et al. (2017)
22	*C. eriobotryae*	IMI136523[T]	*Eriobotrya japonica*	India	AY347327	–	–	–	Adams et al. (2005)
23	*C. eucalypti*	LSEQ	*Sequoia sempervirens*	California, USA	AY347340	–	–	–	Adams et al. (2005)
24	*C. eucalyptina*	CMW 5882	*Eucalyptus grandis*	Cali, Columbia	AY347375	–	–	–	Adams et al. (2005)
25	*C. fabianae*	Dunnii	*Eucalyptus*		AY347360	–	–	–	Adams et al. (2005)
26	*C. friesii*	CBS 113.81	*Picea abies*	Norway	AY347318	–	–	–	Adams et al. (2005)
27	*C. gelida*	MFLUCC 16-0634[T]	*Cotinus coggygria*	Russia	KY563245	KY563247	KY563243	KY563241	Tibpromma et al. (2017)
28	*C. germanica*	CXY1322	*Elaeagnus oxycarpa*	China	JQ086563	JX524617	–	–	Zhang et al. (2013)
29	*C. gigalocus*	HMBF154			KF225608	KF225622	–	KF498671	Fan et al. (2015a)
30	*C. gigaspora*	CFCC 89634[T]	*Salix psammophila*	China	KF765671	KF765687	KU710960	KU711000	Fan et al. (2015b)
31	*C. hippophaes*	CFCC 89636			KF765678	KF765694	KF765710	–	Fan et al. (2015b)
32	*C. japonica*	CBS375.29	*Prunus persica*	Japan	AF191185	–	–	–	Adams et al. (2002)
33	*C. junipericola*	MFLUCC 17-0882[T]	*Juniperus communis*	Italy	MF190125	MF190072	–	–	Senanayake et al. (2017)
34	*C. kantschavelii*	287-2	*Populus deltoides*	Iran	EF447367	–	–	–	Fotouhifar et al. (2010)
35	*C. kunzei*	CBS 118556	*Pinus radiata*	Eastern Cape, SA	DQ243791	–	–	–	Adams et al. (2006)
36	*C. leucostoma*	CFCC 50015	*Sorbus pohuashanensis*	China	KR045634	KR045714	–	KU711002	Yang et al. (2015)
37	*C. longiostiolata*	MFLUCC 16-0628[T]	*Salix* × *fragilis*	Russia	KY417734	KY417768	KY417802	KY417700	Norphanphoun et al. (2017)
38	*C. lumnitzericola*	MFLUCC 17-0508	*Lumnitzera racernosa*	Phetchaburi, Thailand	MG975778	MH253461	MH253453	MH253457	In this study
39	*C. mali*	CFCC 50044	*Malus baccata*	Haidong, Qinghai	KR045637	KR045717	KU710966	KU711005	Yang et al. (2015)
40	*C. malicola*	167			EF447414	–	–	–	Adams et al. (2002)
41	*C. mali-sylvestris*	MFLUCC 16-0638[T]	*Malus sylvestris*	Russia	KY885017	KY885018	KY885020	KY885019	Hyde et al. (2017)
42	*C. melnikii*	MFLUCC 15-0851[T]	*Malus domestica*	Russia	KY417735	KY417769	KY417803	KY417701	Norphanphoun et al. (2017)

No	Taxon	Strain[a]	Host	Origin	GenBank accession numbers				References
					ITS	LSU	RPB2	ACT	
43	*C. multicollis*	CBS 105.89^{T}	*Quercus ilex* subsp. *rotundifolia*	Spain	DQ243803	–	–	–	Adams et al. (2006)
44	*C. myrtagena*	HiloTib1^{T}	*Tibouchiina urvilleana*	Hilo, Hawaii	AY347363	–	–	–	Adams et al. (2005)
45	*C. nitschkii*	CMW10180^{T}	*Eucalyptus globulus*	Wondo Genet, Ethiopia	AY347356	–	–	–	Adams et al. (2005)
46	*C. nivea*	MFLUCC 15-0860	*Salix acutifolia*	Russia	KY417737	KY417771	KY417805	KY417703	Norphanphoun et al. (2017)
47	*C. palmae*	CXY1280^{T}	*Cotinus coggygria*	Beijing, China	JN411939	–	–	–	Zhang et al. (2014)
48	*C. parakantschavelii*	MFLUCC 15-0857^{T}	*Populus* × *sibirica*	Russia	KY417738	KY417772	KY417806	KY417704	Norphanphoun et al. (2017)
49	*C. parapersoonii*	T28.1^{T}	*Prunus persicae*	Michigan, USA	AF191181	–	–	–	Adams et al. (2002)
50	*C. paratranslucens*	MFLUCC 16-0506^{T}	*Populus alba* var. *bolleana*	Russia	KY417741	KY417775	KY417809	KY417707	Norphanphoun et al. (2017)
51	*C. parasitica*	MFLUCC 16-0507	*Malus domestica*	Russia	KY417740	KY417774	KY417808	KY417706	Norphanphoun et al. (2017)
52	*C. pini*	CBS224.52^{T}	*Pinus strobus*	New York	AY347316	–	–	–	Adams (2005)
53	*C. populina*	CFCC 89644	*Salix psammophila*	Shaanxi, China	KF765686	KF765702	KF765718	–	Fan et al. (2015b)
54	*C. predappioensis*	MFLU 17-0327	*Platanus hybrida*	Italy	MH253451	MH253452	MH253450	MH253449	Hyde et al. (2018)
55	*C. prunicola*	MFLU 17-0995^{T}	*Prunus* sp.	Italy	MG742350	MG742351	MG742352	MG742353	Hyde et al. (2018)
56	*C. pruinopsis*	CFCC 50034^{T}	*Ulmus pumila*	Shaanxi, China	KP281259	KP310806	KU710970	KP310836	Yang et al. (2015)
57	*C. pruinosa*	CFCC 50036	*Syzygium aromaticum*	Qinghai, China	KP310800	KP310802	–	KP310832	Yang et al. (2015)
58	*C. quercicola*	MFLUCC 14-0867^{T}	*Quercus* sp.	Italy	MF190129	MF190073	–	–	Senanayake et al. (2017)
59	*C. rhizophorae*	ATCC38475	*Rhizophora mangle*	LA, USA	DQ996040	–	–	–	He et al. (2003)
60	*C. rhizophorae*	ATCC66924	*Haliclona caerulea*	HI, USA	DQ092502	–	–	–	Unpublished
61	*C. ribis*	CFCC 50026	*Ulmus pumila*	Qinghai, China	KP281267	KP310813	KU710972	KP310843	Yang et al. (2015)
62	*C. rosae*	MFLUCC 14-0845^{T}	*Rosa canina*	Italy	MF190131	MF190075	–	–	Senanayake et al. (2017)
63	*C. rosarum*	218			EF447387	–	–	–	Fotouhifar et al. (2010)

No	Taxon	Strain[a]	Host	Origin	GenBank accession numbers				References
					ITS	LSU	RPB2	ACT	
64	*C. rostrata*	CFCC 89909^{T}	*Salix cupularis*	Gansu, China	KR045643	KR045722	KU710974	KU711009	Unpublished
65	*C. rusanovii*	MFLUCC 15-0854^{T}	*Salix babylonica*	Russia	KY417744	KY417778	KY417812	KY417710	Norphanphoun et al. (2017)
66	*C. sacculus*	HMBF281			KF225615	KF225629	–	KF498678	Fan et al. (2015a)
67	*C. salicacearum*	MFLUCC 16-0509^{T}	*Salix alba*	Russia	KY417746	KY417780	KY417814	KY417712	Norphanphoun et al. (2017)
68	*C. salicicola*	MFLUCC 14-1052^{T}	*Salix alba*	Russia	KU982636	KU982635	–	KU982637	Li et al. (2016)
69	*C. salicina*	MFLUCC 15-0862^{T}	*Salix alba*	Russia	KY417750	KY417784	KY417818	KY417716	Norphanphoun et al. (2017)
70	*C. schulzeri*	CFCC 50040	*Malus domestica*	Ningxia, China	KR045649	KR045728	KU710980	KU711013	Unpublished
71	*C. sibiraeae*	CFCC 50045^{T}	*Sibiraea angustata*	Gansu, China	KR045651	KR045730	KU710982	KU711015	Liu et al. (2015)
72	*C. sorbi*	MFLUCC 16-0631^{T}	*Sorbus aucuparia*	Russia	KY417752	KY417786	KY417820	KY417718	Norphanphoun et al. (2017)
73	*C. sorbicola*	MFLUCC 16-0584^{T}	*Acer pseudoplatanus*	Russia	KY417755	KY417789	KY417823	KY417721	Norphanphoun et al. (2017)
74	*C. sordida*	HMBF159			KF225613	KF225627	–	KF498676	Fan et al. (2015a)
75	*C. sophorae*	CFCC 50047	*Styphnolobium japonicum*	Shanxi, China	KR045653	KR045732	KU710984	KU711017	Fan et al. (2014)
76	*C. sophoricola*	CFCC 89596	*Styphnolobium japonicum*	Gansu, China	KR045656	KR045735	KU710987	KU711020	Unpublished
77	*C. tanaitica*	MFLUCC 14-1057^{T}	*Betula pubescens*	Russia	KT459411	KT459412	–	KT459413	Ariyawansa et al. (2015)
78	*C. thailandica*	MFLUCC 17-0262	*Xylocarpus moluccensis*	Ranong, Thailand	MG975776	MH253463	MH253455	MH253459	In this study
79	*C. thailandica*	MFLUCC 17-0263	*Xylocarpus moluccensis*	Ranong, Thailand	MG975777	MH253464	MH253456	MH253460	In this study
80	*C. tibouchinae*	CPC 26333^{T}	*Tibouchina semidecandra*	La Reunion, France	KX228284	KX228335	–	–	Unpublished
81	*C. translucens*	35			EF447403	–	–	–	Fotouhifar et al. (2010)
82	*C. ulmi*	MFLUCC 15-0863^{T}	*Ulmus minor*	Russia	KY417759	KY417793	KY417827	KY417725	Norphanphoun et al. (2017)

No	Taxon	Strain[a]	Host	Origin	GenBank accession numbers				References
					ITS	LSU	RPB2	ACT	
83	*C. valsoidea*	CMW 4309[T]	*Eucalyptus grandis*	Sibisa, North Sumatra	AF192312	–	–	–	Adams et al. (2005)
84	*C. variostromatica*	CMW 6766[T]	*Eucalyptus globulus*	Australia	AY347366	–	–	–	Adams et al. (2005)
85	*C. vinacea*	CBS 141585[T]	*Vitis* sp.	New Hampshire, USA	KX256256	–	–	–	Lawrence et al. (2017)
86	*C. xylocarpi*	MFLUCC 17-0251	*Xylocarpus granatum*	Ranong, Thailand	MG975775	MH253462	MH253454	MH253458	In this study
87	*Diaporthe eres*	AFTOL-ID 935			DQ491514	–	DQ470919	–	Spatafora et al. (2006)
88	*C.* "rhizophorae"	A761	*Morinda officinalis*	China	KU529867	–	–	–	Unpublished
89	*C.* "rhizophorae"	HAB16R13	*Cinnamomum porrectum*	Malaysia	HQ336045	–	–	–	Harun et al. (2011)
90	*C.* "rhizophorae"	M225	*Rhizophora mucronata*	Philippines	KR056292	–	–	–	Unpublished
91	*C.* "rhizophorae"	MUCC302	*Eucalyptus grandis*	Australia	EU301057	–	–	–	Unpublished

[a] *AFTOL-ID* Assembling the Fungal Tree of Life; *CBS* CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; *CFCC* China Forestry Culture Collection Center; IMI International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, UK; *CPC* Culture collection of Pedro Crous, housed at CBS; *MFLU* Mae Fah Luang University Herbarium Collection; *MFLUCC* Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; [T] Ex-type and ex-epitype cultures.

For both Maximum Likelihood and Bayesian analyses, a partitioned analysis was performed with the following six partitions: ITS1+ITS2, 5.8S, LSU, ACT-exons, ACT-introns and RPB2. Maximum-likelihood (ML) analysis was performed with RAxML (Stamatakis 2006) implemented in the CIPRES Science Gateway web server (RAxML-HPC2 on XSEDE; Miller et al. 2010), 25 categories, 1000 rapid bootstrap replicates were run with the GTRGAMMA model of nucleotide evolution. Maximum likelihood bootstrap values (MLBS) equal or greater than 50% are given above each node.

Bayesian Inference (BI) analysis was performed using the Markov Chain Monte Carlo (MCMC) method with MrBayes 3.2.2 (Ronquist et al. 2012). The best-fit nucleotide substitution model for each dataset was separately determined using MrModeltest version 2.2 (Nylander 2004). GTR+I+G was selected as the best-fit model for the ITS1+ITS2, LSU, ACT (ACT-exons and ACT-introns) and RPB2 datasets and K80 for 5.8S. The MCMC analyses, with four chains starting from random tree topology, were run for 5,000,000 or 10,000,000 generations for the combined dataset or the ITS1+ITS2 dataset. Trees were sampled every 100 generations. Tracer v. 1.5.0 was used to check the effective sampling sizes (ESS) that should be above 200, the stable likelihood plateaus and burn-in value (Rambaut et al. 2013). The first 5000 samples were excluded as burn-in.

The phylogram was visualised in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/; Rambaut 2014) and edited in Adobe Illustrator CC and Adobe Photoshop CS6 Extended version 13.1.2 × 64. Newly generated sequences in this study are deposited in GenBank. The finalised alignment and tree were deposited in TreeBASE, submission ID: 22942 (combined sequence alignment) (Reviewer access URL: http://purl.org/phylo/treebase/phylows/study/TB2:S22942?x-access-code=f9115cf637b0e4171aab1c980eb15830&format=html) and (Reviewer access URL: http://purl.org/phylo/treebase/phylows/study/TB2:S22943?x-access-code=92a782825ac069b3fd761aff21fa2bf4&format=html) 22943 (ITS sequence alignment) (http://www.treebase.org).

Results

Phylogenetic analysis of combined ITS, LSU, ACT and RPB2 sequences

The combined alignment of ITS, LSU, ACT and RPB2 sequences comprised 86 taxa, including our strains, with *Diaporthe eres* (CBS 183.5) as the outgroup taxon. The total length of the dataset was 2037 characters including alignment gaps (1–199, 200–357, 358–518, 519–1056, 1057–1296 and 1297–2037 corresponding to ITS1, 5.8S, ITS2, LSU, ACT and RPB2, respectively). The combined dataset contained 1426 constant, 144 parsimony uninformative and 467 parsimony informative characters. The result from the partition homogeneity test (PHT) was not significant (level 95%), indicating that the individual datasets were congruent and could be combined. The combined dataset was analysed using MP, ML and Bayesian analyses. The trees generated under different optimality criteria were essentially similar in topology and did not differ sig-

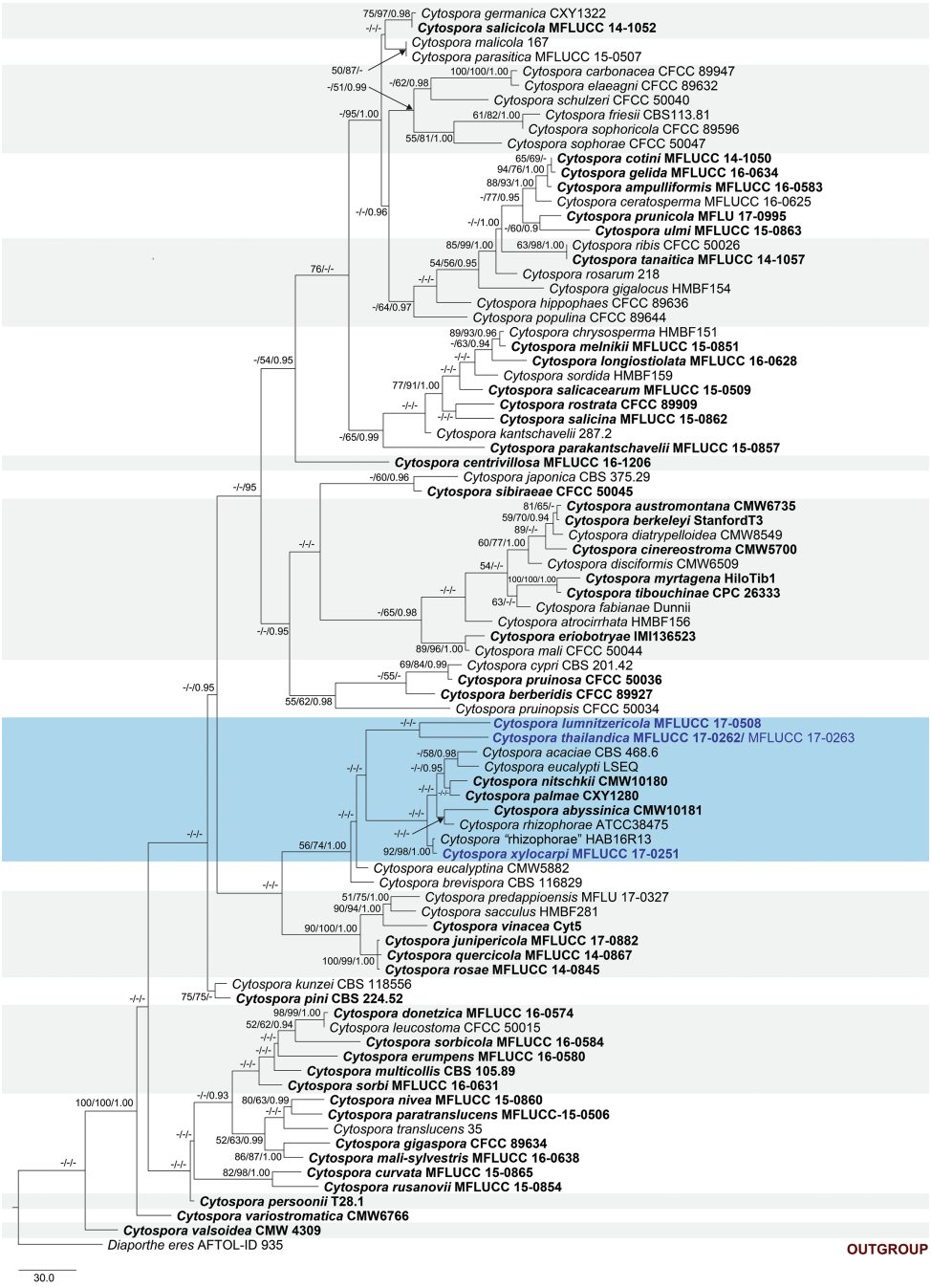

Figure 1. Phylogram generated from maximum parsimony analyses based on analysis of combined ITS, LSU, ACT and RPB2 sequence data. The tree is rooted to *Diaporthe eres* (AFTOL-ID 935). Maximum parsimony and maximum likelihood bootstrap values ≥50%, Bayesian posterior probabilities ≥0.90 (MPBS/MLBS/PP) are given at the nodes. The species obtained in this study are in blue font. Ex-type taxa from other studies are in black bold.

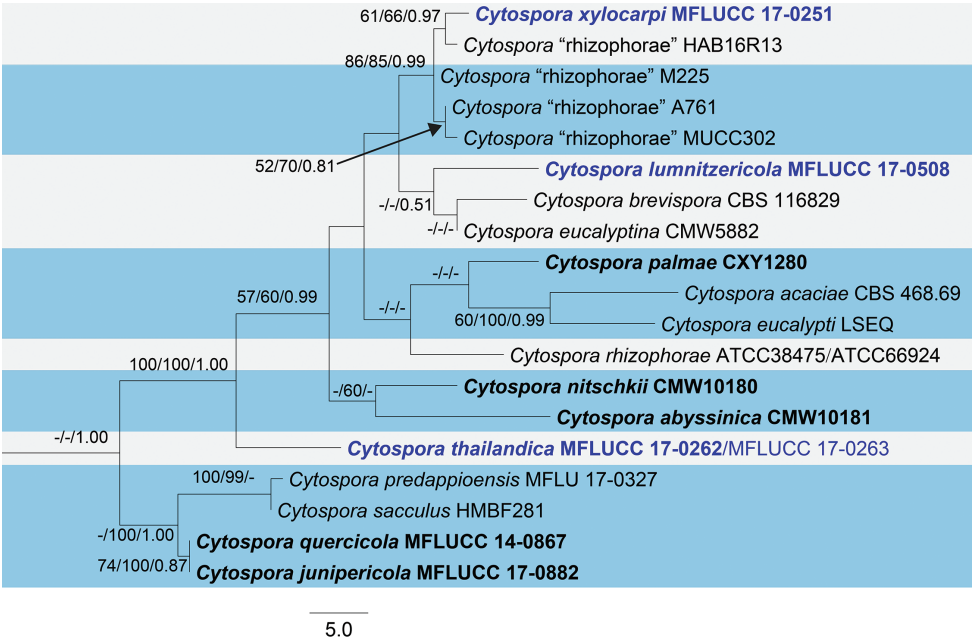

Figure 2. Maximum parsimony phylogenetic tree inferred from ITS1 and ITS2 sequence data. Maximum parsimony and maximum likelihood bootstrap values ≥50%, Bayesian posterior probabilities ≥0.90 (MPBS/MLBS/BIPP) are given at the nodes. The species obtained in this study are in blue font. Ex-type taxa from other studies are in black bold.

nificantly (data not shown). The descriptive statistics of the phylogram generated from MP analysis based on the combined dataset of ITS, LSU, ACT and RPB2 (Fig. 1) were TL = 2418, CI = 0.375, RI = 0.650, RC = 0.244, HI = 0.625. The best scoring likelihood tree selected with a final value for the combined dataset = -14466.797686. The aligned sequence matrix of the ITS1+ITS2 dataset comprising 19 taxa had 279 constant, 23 parsimony uninformative and 57 parsimony informative characters. The descriptive statistics of the most parsimonious tree (Fig. 2) were TL = 2418, CI = 0.375, RI = 0.650, RC = 0.244, HI = 0.625. The best scoring likelihood tree obtained for the ITS1+ITS2 dataset had a log-likelihood of= -1276.782916.

Taxonomy

Cytospora lumnitzericola **Norphanphoun, T.C. Wen & K.D. Hyde, sp. nov.**
Index Fungorum number: IF554778; Facesoffungi number: FoF 04603
Figure 3

Etymology. refers to the host where the fungus was isolated.

Holotype. MFLU 18-1227

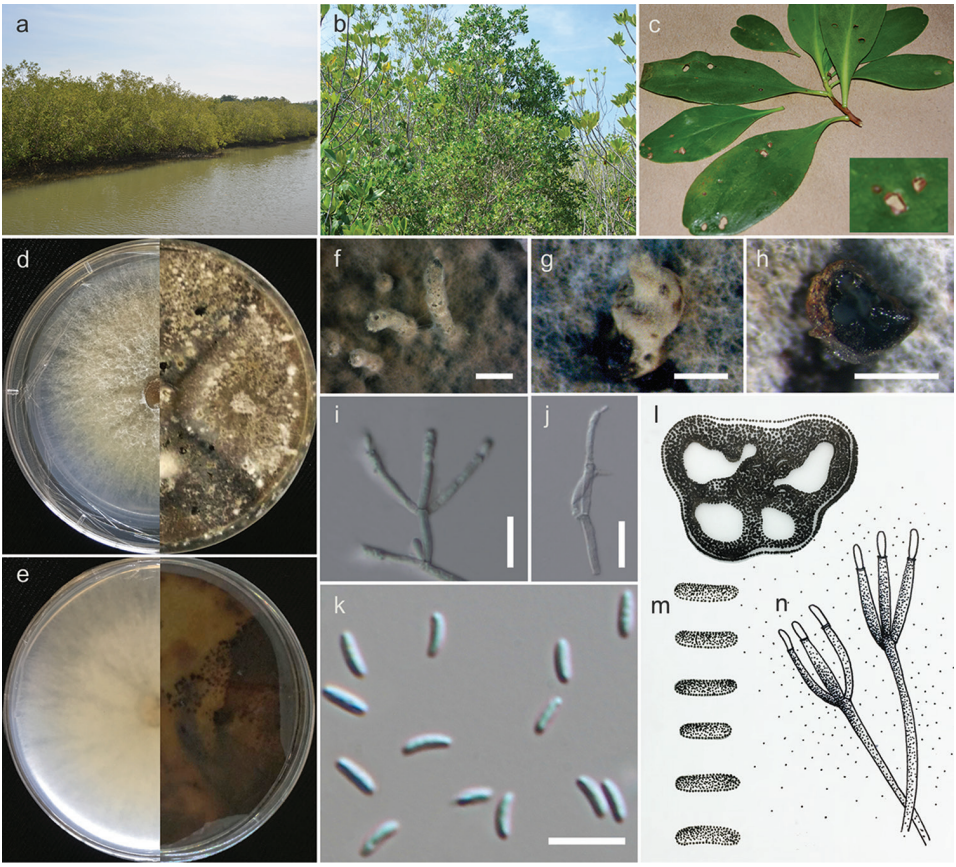

Figure 3. *Cytospora lumnitzericola* (MFLUCC 17-0508, from culture). **a** Mangrove collecting site **b, c** *Lumnitzera racemosa* in mangroves forest **d, e** Colonies on MEA after 6 days (left) and 30 days (right) (d-from above, e-from below) **f, g** Conidiomata produced on MEA **h, l** Transverse sections of conidioma **i, j, n** Conidiogenous cells with attached conidia **k, m** Conidia. Scale bars: **f** = 1000 µm, **g, h** = 500 µm, **i, j** = 10 µm, **k** = 5 µm.

Isolated from leaf spot of *Lumnitzera racemosa*. **Culture characteristic:** Colonies on MEA reaching 5–6 cm diameter after 2 days at room temperature, colonies circular to irregular, medium dense, flat or effuse, slightly raised, with edge fimbriate, fluffy to fairly fluffy, white to grey from above, light yellow to green from below; not producing pigments in agar. **Asexual morph:** *Conidiogenous cells* (8–)8.5–14 × 0.6–1.4(–1.6) µm ($\bar{x}$ = 8.4 × 1.4, n = 15), blastic, enteroblastic, flask-shaped, phialidic, hyaline and smooth-walled. *Conidia* (3.7–)4–4.5 × 1–1.3(–1.5) µm ($\bar{x}$ = 4 × 1.2 µm, n = 30), unicellular, subcylindrical, hyaline, smooth-walled.

Material examined. THAILAND, Phetchaburi Province, the Sirindhorn International Environmental Park, on leaf spot of *Lumnitzera racemosa*, 30 November 2016, Norphanphoun Chada NNS23-2a (MFLU 18-1227 dried culture, **holotype**; PDD, isotype); ex-type-living culture, MFLUCC 17-0508, ICMP.

Notes. Based on the multigene phylogeny, *Cytospora lumnitzericola* is closely related to *Cytospora thailandica* (Fig. 1). Although conidial sizes of both species are similar, they have significant differences in nucleotides: ITS (26 nt), ACT (22 nt), and RPB2 (53 nt) (Table 5). The phylogeny derived from the ITS regions depicts *C. lumnitzericola* as an independent lineage close to *C. brevispora* CBS 116829 and *C. eucalyptina* CMW5882 (Fig. 2). In future, more collections are needed to confirm whether *C. lumnitzericola* can exist as a saprobe or endophyte as well as performing tests to confirm its pathogenicity.

Cytospora thailandica **Norphanphoun, T.C. Wen & K.D. Hyde, sp. nov.**
Index Fungorum number: IF554779; Facesoffungi number: FoF 04605
Figure 4

Etymology. refers to the country where the fungus was collected.

Holotype. MFLU 17-0709

Associated with twigs and branches of *Xylocarpus moluccensis*. **Sexual morph:** Stromata immersed in bark. *Ascostromata* 400–1000 × 70–250 µm diameter, semi-immersed in host tissue, scattered, erumpent, uni- or multi-loculate, with ostiolar neck. *Ostiole* 70–150 µm diameter, numerous, dark brown to black, at the same level as the disc, occasionally area below disc a lighter entostroma. *Peridium* comprising several layers of cell of *textura angularis*, with innermost layer thick, brown, outer layer dark brown. *Hamathecium* comprising long cylindrical, cellular, anastomosed paraphyses. *Asci* (21–)23–25 × 4.1–4.7(–5) µm ($\bar{x}$ = 22 × 4.3 µm, n = 15), 6–8-spored, unitunicate, clavate to elongate obovoid, with a J-, refractive apical ring. *Ascospores* (5.6–)6–6.8 × 1.3–1.5(–2) µm ($\bar{x}$ = 6.6 × 1.5 µm, n = 20), biseriate, elongate-allantoid, unicellular, hyaline, smooth-walled. **Asexual morph:** *Conidiomata* 400–1200 × 180–380 µm diameter, semi-immersed in host tissue, solitary, erumpent, scattered, discoid, circular to ovoid, with multi-loculate, pycnidial, embedded in stromatic tissue, with ostiole. *Ostioles* 230–300 µm long, with an ostiolar neck. *Peridium* comprising few layers of cells of *textura angularis*, with innermost layer thin, pale brown, outer layer brown to dark brown. *Conidiophores* unbranched or occasionally branched at the bases, formed from the innermost layer of pycnidial wall, with conidiogenous cells. *Conidiogenous cells* (3.3–)6–9.1 × 1–1.3(–1.7) µm ($\bar{x}$ = 6 × 1.3 µm, n = 15), blastic, enteroblastic, flask-shaped, phialidic, hyaline and smooth-walled. *Conidia* (3.3–)3.8–4 × 1–1.3(–1.5) µm ($\bar{x}$ = 3.8 × 1.3 µm, n = 30), unicellular, subcylindrical, hyaline, smooth-walled.

Material examined. THAILAND, Ranong Province, Ngao Mangrove Forest, on branches of *Xylocarpus moluccensis*, 6 December 2016, Norphanphoun Chada NG02a (MFLU 17-0709, **holotype**; PDD, isotype); ex-type-living cultures, MFLUCC 17-0262, MFLUCC 17-0263, ICMP.

Notes. *Cytospora thailandica* was collected from branches of *Xylocarpus moluccensis*. The new species resembles some other *Cytospora* species, but is characterised by uni- or multi-loculate ascomata/conidiomata with unicellular, subcylindrical and hyaline spores in both morphs. *Cytospora* species associated with *Xylocarpus granatum* is also

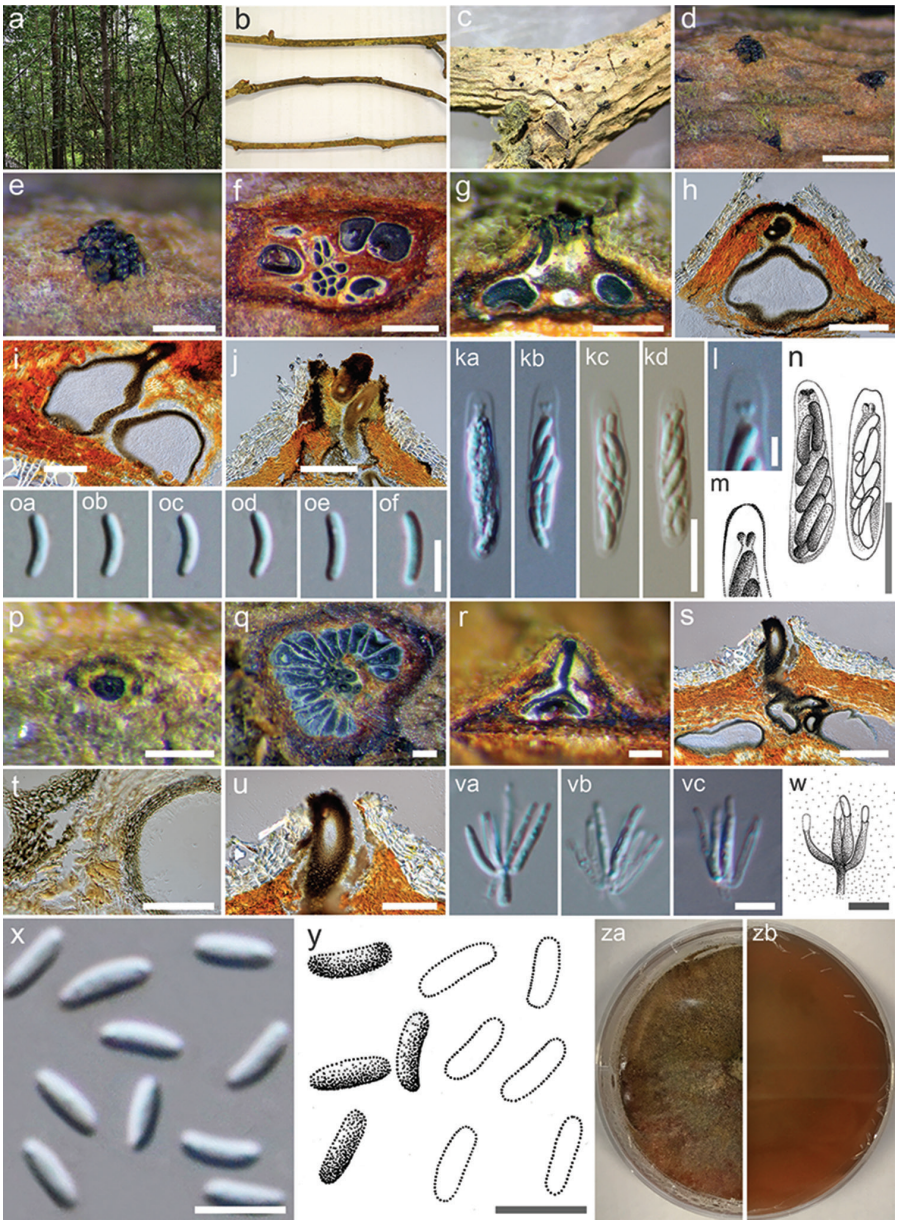

Figure 4. *Cytospora thailandica* (MFLU 17-0709, holotype). **a** *Xylocarpus moluccensis* **b** Branch of *Xylocarpus moluccensis* **c** Ascostromata on host substrate **d, e** Surface of ascomata **f** Transverse sections through ascostroma to show distribution of locules **g–h** Longitudinal sections through ascostroma to show distribution of locules **i** Peridium **j** Ostiolar neck **ka**–**kd, n** Asci **l, m** Apical ring **oa–of** Ascospores **p** Surface of conidioma **q** Transverse sections through conidioma to show distribution of locules **r, s** Longitudinal sections through conidioma to show distribution of locules **t** Peridium **u** Ostiolar neck **va–vc, w** Conidiogenous cells with attached conidia **x, y** Conidia **za, zb** Colonies on MEA (za-from above, zb-from below). Scale bars: **d** = 1000 μm, **e–g** = 400 μm, **h, j, p–s** = 200 μm, **i, u** = 100 μm, **ka–kd, n** = 10 μm, **l, m** = 2 μm, **oa–of, va–vc, w** = 5 μm, **t** = 50 μm, **x, y** = 4 μm.

reported in this study as *C. xylocarpi* (MFLUCC 17-0251, Fig. 5). *Cytospora xylocarpi* is similar to *C. thailandica* in its conidiomata being multi-loculate and in the length of conidia in the asexual morph (*C. xylocarpi*: conidia 3 × 1.1 μm versus 3.8 × 1.3 μm in *C. thailandica*). However, *C. thailandica* differs from *C. xylocarpi* in having shorter ostiolar necks and larger asci and ascospores (Table 2). Phylogenetic analysis of our combined gene also reveals *C. thailandica* is closely related to *C. lumnitzericola* (Fig. 1), but there are nucleotide differences as mentioned in notes of *C. lumnitzericola*. The individual ITS1+ITS2 phylogenetic tree also indicates that *C. thailandica* is distinct with good support (Fig. 2).

Cytospora xylocarpi **Norphanphoun, T.C. Wen & K.D. Hyde, sp. nov.**
Index Fungorum number: IF554810; Facesoffungi number: FoF 04604
Figure 5

Etymology. refers to the host genus that fungus was collected.

Holotype. MFLU 17-0708

Associated with *Xylocarpus granatum* branches. **Sexual morph:** Stromata immersed in bark. *Ascostromata* 230–600 × 90–250 μm diameter, semi-immersed in host tissue, scattered, erumpent, multi-loculate, with ostiolar neck. *Ostiole* 160–200 μm diameter, numerous, dark brown to black, at the same level as the disc, occasionally area surrounded with white hyphae. *Peridium* comprising several layers of cells of *textura angularis*, with innermost layer thick, pale brown, outer layer dark brown to black. *Hamathecium* comprising long cylindrical, cellular, anastomosed paraphyses. *Asci* (22–)24–28.8 × 3.6–4.8(–5.1) μm ($\bar{x}$ = 26 × 4 μm, n = 15), 6–8-spored, unitunicate, clavate to elongate obovoid, with a refractive, J-, apical ring. *Ascospores* (5.5–)6–6.5 × 1.7–1.8(–2) μm ($\bar{x}$ = 5.7 × 1.8 μm, n = 20), biseriate, elongate-allantoid, unicellular hyaline, smooth-walled. **Asexual morph:** *Conidiomata* 700–1200 × 400–480 μm diameter, semi-immersed in host tissue, solitary, erumpent, scattered, multi-loculate, with ostiole. *Ostioles* 200–250 μm long, with 1–2 ostiolar necks. *Peridium* comprising several layers of cells of *textura angularis*, with innermost layer brown, outer layer dark brown to black. *Conidiophores* unbranched or occasionally branched at the bases, formed from the innermost layer of pycnidial wall, with conidiogenous cells. *Conidiogenous cells* (6.3–)7.9–10 × 0.9–1.4(–1.6) μm ($\bar{x}$ = 8.5× 1.4 μm, n = 15), blastic, enteroblastic, flask-shaped, phialidic, hyaline and smooth-walled. *Conidia* (2.4–)3–3.1 × 0.8–1(–1.2) μm ($\bar{x}$ = 3 × 1 μm, n = 30), unicellular, subcylindrical, hyaline, smooth-walled.

Material examined. THAILAND, Ranong Province, Ngao Mangrove Forest, on branches of *Xylocarpus granatum*, 6 December 2016, Norphanphoun Chada NG09b (MFLU 17-0708, **holotype**; PDD); ex-type-living cultures, MFLUCC 17-0251, ICMP.

Notes. The asexual morph of *C. xylocarpi*, studied here, is most similar to *C. rhizophorae* from dead roots of *Rhizophora mangle* L. in Guatemala, in having multi-loculate conidiomata and allantoid, slightly curved, hyaline and 3–6 × 1.1–1.5 μm

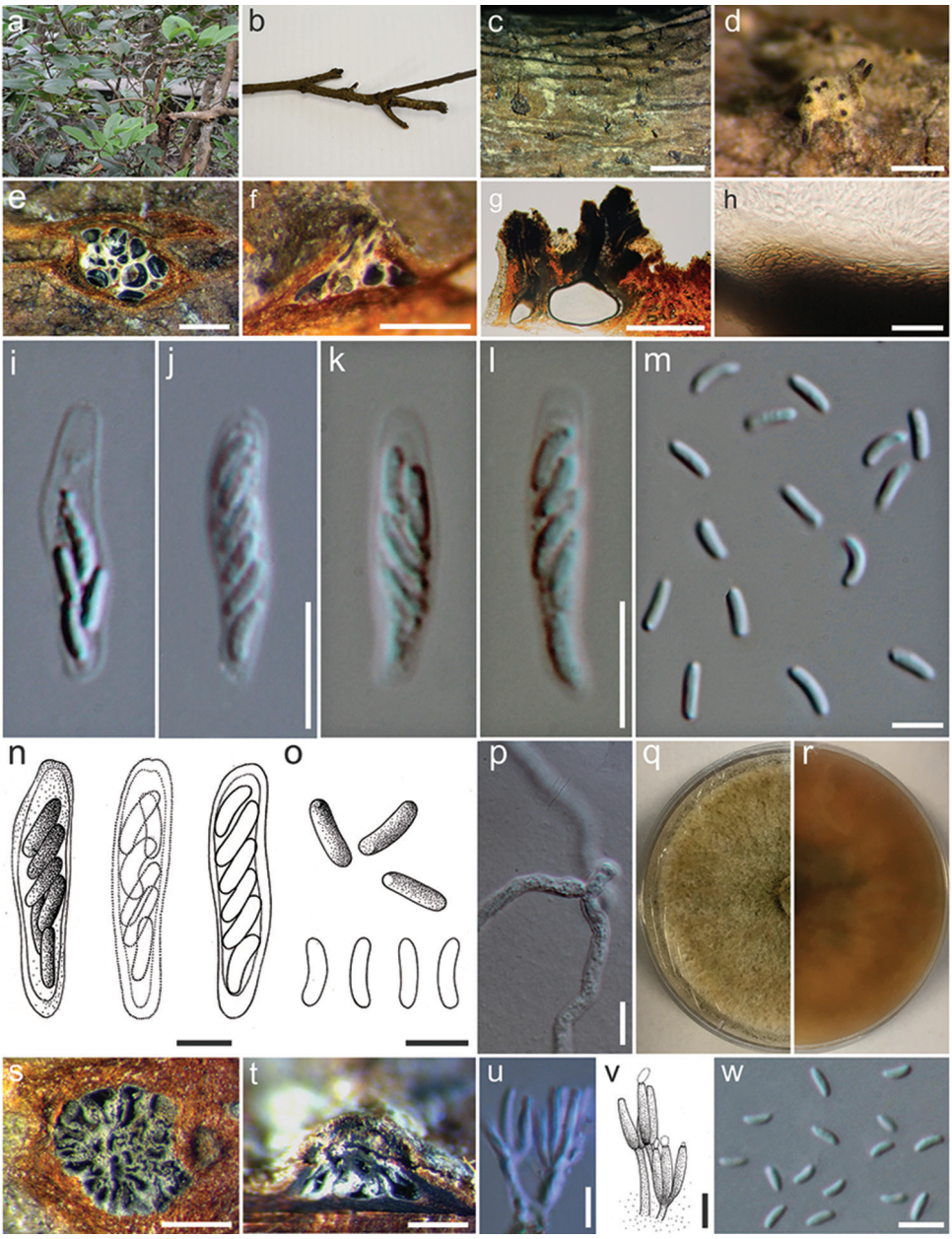

Figure 5. *Cytospora xylocarpi* (MFLU 17-0708, holotype). **a** *Xylocarpus granatum* **b** Branch of *Xylocarpus granatum* **c** Ascostromata on host substrate **d** Surface of ascomata **e** Transverse sections through ascostroma to show distribution of locules **f, g** Longitudinal sections through ascostroma to show distribution of locules **h** Peridium **i–l, n** Asci **m, o** Ascospores **p** Germinating spore **q, r** Colonies on MEA (q-from above, r-below) **s** Transverse sections through conidioma to show distribution of locules **t** Longitudinal sections through conidioma to show distribution of locules **u, v** Conidiogenous cells with attached conidia **w** Mature conidia. Scale bars: **c** = 2000 μm, **d–f** = 500 μm, **g** = 200 μm, **h** = 20 μm, **i, p** = 10 μm, **j–o, u–w** = 5 μm, **s, t** = 400 μm.

Table 2. Synopsis of species of *Cytospora* discussed in the paper.

Taxon	Sexual morph				Asexual morph				References
	Ascostoma	Ostiolar neck	Asci	Ascospores	Conidiomata	Ostiolar neck	Conidiogenous cell	Conidia	
C. lumnitzericola	–	–	–	–	–	–	8.4 × 1.4	4 × 1.2	In this study
C. rhizophorae	–	–	–	–	370–500 × 100–310	30 × 10–25	13–20 × 1–1.8	3–6 × 1.1–1.5	Kohlm. and Kohlm. (1971)
C. thailandica	400–1000 × 70–250	70–150	22 × 4.3	6.6 × 1.5	400–1200 × 180–380	230–300	6 × 1.3	3.8 × 1.3	In this study
C. xylocarpi	230–600 × 90–250	160–200	26 × 4	5.7 × 1.8	700–1200 × 400–480	200–250	8.5× 1.4	3 × 1	In this study

Table 3. GenBank BLAST search from ITS1 and ITS2 of *Cytospora xylocarpi* (MFLUCC 17-0251) with sequence from GenBank identified as *Cytospora rhizophorae*.

Toxon	Strain	Host	Country	Accessions	ITS1	ITS2	ITS1+ITS2	Identities (I), Query cover (QC)	References
C. "rhizophorae"	HAB16R13	*Cinnamomum porrectum*	Malaysia	HQ336045	213/215	167/169	380/384	I=98.9%, QC=99%	Harun et al. (2011)
C. "rhizophorae"	M225	*Rhizophora mucronata*	Philippines	KR056292	213/217	167/169	380/386	I=98.4%, QC=100%	Unpublished
C. "rhizophorae"	A761	*Morinda officinalis*	China	KU529867	213/217	166/169	379/386	I=98.2%, QC=100%	Unpublished
C. "rhizophorae"	MUCC302	*Eucalyptus grandis*	Australia	EU301057	213/217	164/169	377/386	I=97.7%, QC=100%	Unpublished
C. rhizophorae	ATCC38475	*Rhizophora mangle*	LA, USA	DQ996040	187/202	156/166	343/368	I=93.2%, QC=100%	He et al. (2003)

Table 4. Nucleotide differences in the ITS1+ITS2 of *Cytospora xylocarpi* (MFLUCC 17-0251) with sequence from GenBank identified as *Cytospora rhizophorae*.

Taxon	Strain	ITS1																	
		14	**16**	**18**	**30**	**92**	**93**	**96**	**99**	**102**	**103**	**104**	**105**	**113**	**115**	**118**	**119**	**135**	**154**
C. xylocarpi	MFLUCC 17-0251	-	G	A	C	C	C	C	G	G	G	C	G	C	T	T	C	A	G
C. rhizophorae	ATCC38475	G	A	C	T	G	A	T	A	T	T	T	A	T	-	C	T	-	T
C. "rhizophorae"	HAB16R13	?	G	A	C	C	C	C	G	G	G	C	G	C	T	T	C	A	T
C. "rhizophorae"	M225	?	G	A	T	C	C	C	G	G	G	C	G	C	T	T	C	A	T
C. "rhizophorae"	A761	?	G	A	T	C	C	C	G	G	G	C	G	C	T	T	C	A	T
C. "rhizophorae"	MUCC302	?	G	A	T	C	C	C	G	G	G	C	G	C	-	T	C	A	T
Taxon	**Strain**	**ITS2**																	
		13	**24**	**40**	**46**	**47**	**50**	**51**	**75**	**111**	**112**	**115**	**123**						
C. xylocarpi	MFLUCC 17-0251	C	C	A	T	-	T	T	C	A	A	C	T						
C. rhizophorae	ATCC38475	T	T	-	T	-	-	T	C	G	T	A	T						
C. "rhizophorae"	HAB16R13	C	T	A	T	-	T	T	C	A	A	C	C						
C. "rhizophorae"	M225	C	T	A	T	-	T	T	C	A	A	C	T						
C. "rhizophorae"	A761	C	T	A	T	T	-	T	C	A	A	C	T						
C. "rhizophorae"	MUCC302	C	T	A	-	-	-	-	T	A	A	C	T						

Table 5. Nucleotides differences in the ITS, ACT and RPB2 sequences of *Cytospora lumnitzericola*, *C. thailandica* and *C. xylocarpi*.

Taxon	Strain	ITS																	
		29	**88**	**91**	**92**	**93**	**94**	**96**	**97**	**99**	**101**	**102**	**103**	**104**	**105**	**106**	**107**	**108**	**111**
C. lumnitzericola	MFLUCC 17-0508	T	C	T	T	T	T	C	T	C	G	G	A	C	T	A	T	A	G
C. thailandica	MFLUCC 17-0262	T	-	T	-	-	-	T	C	T	C	A	G	-	-	A	C	G	C
C. thailandica	MFLUCC 17-0263	T	-	T	-	-	-	T	C	T	C	A	G	-	-	A	C	G	C
C. xylocarpi	MFLUCC 17-0251	C	C	C	-	-	C	C	C	C	G	G	G	-	-	G	C	G	G
Taxon	**Strain**	**ITS**																	
		119	**120**	**121**	**122**	**123**	**124**	**125**	**134**	**157**	**389**	**396**	**404**	**405**	**412**	**413**	**414**	**415**	**420**
C. lumnitzericola	MFLUCC 17-0508	T	T	C	-	-	-	-	-	T	T	A	A	-	-	-	-	T	G
C. thailandica	MFLUCC 17-0262	C	T	T	C	-	G	G	-	T	T	G	T	T	-	-	-	-	A
C. thailandica	MFLUCC 17-0263	C	T	T	C	-	G	G	-	T	T	G	T	T	-	-	-	-	A
C. xylocarpi	MFLUCC 17-0251	T	C	T	C	C	G	G	A	G	C	A	A	A	C	T	T	T	G
Taxon	**Strain**	**ITS**					**ACT**												
		439	**468**	**485**	**487**	**488**	**74**	**78**	**80**	**92**	**95**	**96**	**97**	**107**	**122**	**125**	**129**	**136**	**137**
C. lumnitzericola	MFLUCC 17-0508	T	T	C	T	A	G	C	A	T	T	-	-	C	T	A	G	A	A
C. thailandica	MFLUCC 17-0262	T	T	T	C	T	T	G	A	A	T	-	-	T	C	T	G	A	G
C. thailandica	MFLUCC 17-0263	T	T	T	C	T	T	G	A	A	T	-	-	T	C	T	G	A	G
C. xylocarpi	MFLUCC 17-0251	C	C	C	T	T	G	C	T	A	C	C	C	T	C	A	A	G	A
Taxon	**Strain**	**ACT**																	
		139	146	147	148	149	150	152	159	165	198	209	210	212	215	216	217	218	223
C. lumnitzericola	MFLUCC 17-0508	A	A	G	C	T	C	C	G	T	C	T	C	G	A	A	A	C	A
C. thailandica	MFLUCC 17-0262	A	G	-	-	T	T	T	T	T	T	T	C	A	A	A	-	C	A
C. thailandica	MFLUCC 17-0263	A	G	-	-	T	T	T	T	T	T	T	C	A	A	A	-	C	A
C. xylocarpi	MFLUCC 17-0251	G	G	-	-	A	A	C	T	C	C	A	T	A	T	G	-	A	-

Taxon	Strain	ACT							RPB2										
		224	225	231	234	242	245	246	4	18	33	42	57	84	85	96	102	108	120
C. lumnitzericola	MFLUCC 17-0508	C	G	C	-	-	A	A	T	T	C	T	C	C	T	T	C	G	A
C. thailandica	MFLUCC 17-0262	T	T	C	T	G	T	G	T	C	A	T	C	T	C	T	C	A	G
C. thailandica	MFLUCC 17-0263	T	T	C	T	G	T	G	T	C	A	T	C	T	C	T	C	A	G
C. xylocarpi	MFLUCC 17-0251	T	T	A	C	G	T	A	C	T	C	C	T	T	C	C	A	A	G
Taxon	**Strain**	**RPB2**																	
		123	**126**	**129**	**144**	**153**	**171**	**174**	**177**	**204**	**210**	**213**	**216**	**222**	**231**	**237**	**243**	**246**	**279**
C. lumnitzericola	MFLUCC 17-0508	C	G	C	G	T	G	C	C	G	C	T	C	T	T	C	T	C	T
C. thailandica	MFLUCC 17-0262	T	A	T	A	C	G	T	C	G	T	C	C	C	T	T	T	T	C
C. thailandica	MFLUCC 17-0263	T	A	T	A	C	G	T	C	G	T	C	C	C	T	T	T	T	C
C. xylocarpi	MFLUCC 17-0251	C	A	C	A	T	A	T	T	C	C	C	T	C	G	C	C	C	T
Taxon	**Strain**	**RPB2**																	
		282	**294**	**306**	**309**	**336**	**339**	**342**	**351**	**352**	**357**	**378**	**390**	**393**	**396**	**402**	**405**	**435**	**441**
C. lumnitzericola	MFLUCC 17-0508	C	A	T	C	T	C	G	T	C	G	A	C	C	G	T	T	C	T
C. thailandica	MFLUCC 17-0262	T	G	C	T	C	A	A	C	T	C	G	C	T	A	T	C	C	T
C. thailandica	MFLUCC 17-0263	T	G	C	T	C	A	A	C	T	C	G	C	T	A	T	C	C	T
C. xylocarpi	MFLUCC 17-0251	T	A	C	C	T	C	G	T	C	C	A	T	T	A	C	T	T	G
Taxon	**Strain**	**RPB2**																	
		456	**465**	**468**	**492**	**498**	**510**	**516**	**517**	**543**	**561**	**570**	**576**	**603**	**612**	**613**	**615**	**627**	**633**
C. lumnitzericola	MFLUCC 17-0508	C	T	C	G	T	T	A	T	T	A	A	G	T	T	C	C	C	G
C. thailandica	MFLUCC 17-0262	C	C	G	C	C	C	A	T	C	A	G	A	C	C	T	G	C	G
C. thailandica	MFLUCC 17-0263	C	C	G	C	C	C	A	T	C	A	G	A	C	C	T	G	C	G
C. xylocarpi	MFLUCC 17-0251	T	T	T	G	T	C	G	C	C	G	G	G	T	C	T	G	G	A
Taxon	**Strain**	**RPB2**																	
		651	**663**	**675**	**678**	**690**	**693**	**699**	**702**	**711**	**732**								
C. lumnitzericola	MFLUCC 17-0508	T	A	C	T	T	G	T	C	C	T								
C. thailandica	MFLUCC 17-0262	C	G	T	C	G	A	C	T	C	C								
C. thailandica	MFLUCC 17-0263	C	G	T	C	G	A	C	T	C	C								
C. xylocarpi	MFLUCC 17-0251	C	A	T	C	T	A	C	C	T	T								

All isolates are new taxa in this study; "-" gap (insertion/deletion); "?" missing data.

conidia (Kohlmeyer and Kohlmeyer 1971). However, the phylogenies, generated herein, show that *C. xylocarpi* is distinct from *C. rhizophorae* (ATCC 38475), a strain from *Rhizophora mangle* that was identified by Kohlmeyer, the author of the species (Fig. 2). The two species also differ by 25 substitutions in ITS1+ITS2 and were collected from different hosts. Therefore, the collection in the present study is designated as a new species.

Our phylogeny also indicates a close relationship to unpublished sequences from GenBank (Figs 1, 2). Given that no morphological descriptions are available for these, the similarity in the ITS1 and ITS2 sequence between our strain and the sequences from GenBank (HAB16R13, M225, A761, MUCC302) are presented in Table 3. Those strains were collected from different hosts (Table 3) and, together with our strain, show substantial variation in ITS1 and ITS2 (Table 4). More collections are needed to further study morphological and genetic variation in this group.

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Multilocus phylogeny reveals taxonomic misidentification of the *Schizopora paradoxa* (KUC8140) representative genome

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Abstract

Schizopora paradoxa, current name *Xylodon paradoxus*, is a white-rot fungus with certain useful biotechnological properties. The representative genome of *Schizopora paradoxa* strain KUC8140 was published in 2015 as part of the 1000 Fungal Genomes Project. Multilocus phylogenetic analyses, based on three nuclear regions (ITS, LSU and *rpb2*), confirmed a misidentification of *S. paradoxa* strain KUC8140 which should be identified as *Xylodon ovisporus*. This wrong identification explains the unexpected geographical distribution of *S. paradoxa*, since this species has a European distribution, whereas the strain KUC8140 was recorded from Korea, Eastern Asia.

Keywords

Hymenochaetales, phylogenetic analyses, taxonomy, white-rot fungi, *Xylodon*

Introduction

The genus *Schizopora* Velen., currently synonymous with *Xylodon* (Pers.) Fr. (Riebesehl and Langer 2017), includes white-rot fungi that play an important role in ecosystem processes as a wood decomposer. The description and identification of *Xylodon* (=*Schizopora*) species, based on morphological characters, has led to inaccuracies due to a lack of clear diagnostic characters and it has been assumed that many *Xylodon* species have a worldwide distribution (Paulus et al. 2000). However, during the last

decade, it has been pointed out that fungal cosmopolitanism could be the result of the application of a morphological species recognition criterion and not the result of an actual biogeographical pattern (Taylor et al. 2006). Moreover, phylogenetic analyses have revealed an undescribed species diversity masked by the morphological species recognition approach (Taylor et al. 2000).

The representative genome of *Schizopora paradoxa* strain KUC8140, current name *Xylodon paradoxus* (Schrad.) Chevall., was sequenced in 2015 as part of the 1000 Fungal Genomes Project (http://jgi.doe.gov/fungi) (Min et al. 2015); this strain was collected from an oak forest in Korea. Usually *X. paradoxus* has been associated with late stages of wood decay, mainly in deciduous forests and shows useful biotechnological properties for bioremediation, such as tolerance to heavy metals or dye decolourising activity (Lee et al. 2014). It has been recorded around the world; however, available genetic data point to a European distribution (Paulus et al. 2000). Within the framework of a broader study of *Xylodon* through molecular approaches, the taxonomic identity of the strain KUC8140 has been assessed.

Materials and methods

In order to infer the taxonomic position of the strain KUC8140, phylogenetic relationships of six *Xylodon* species were addressed. DNA from specimens of *X. paradoxus*, *X. quercinus* (Pers.) Gray, *X. nothofagi* (G. Cunn.) Hjorstam & Ryvarden, *X. raduloides* Riebesehl & E. Langer, *X. flaviporus* (Ber. & M.A. Curtis ex Cooke) Riebesehl & E. Langer and *X. ovisporus* (Corner) Riebesehl & E. Langer was extracted from herbaria specimens and culture collections (Table 1). Three specimens of the sister genus *Lyomyces* P. Karst. were included as outgroup in the phylogenetic analyses (Table 1). DNA isolation was performed using DNeasy™ Plant Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions. Three nuclear regions were amplified and sequenced: nuclear ribosomal internal transcribed spacer (ITS, fungal barcoding; Schoch et al. 2012), nuclear large ribosomal subunit (LSU) and the second largest subunit of RNA polymerase II (*rpb*2). Direct Polymerase chain reactions (PCRs) were performed to obtain sequences from ITS and LSU with the pair of primers ITS5/ITS4 (White et al. 1990) and LR0R/LR5 (Rehner and Samuels 1994), respectively. Nested-PCRs were done to obtain amplifications of *rpb*2 fragments, using RPB2-5F/RPB2-7.1R (Liu et al. 1999, Matheny 2005) for the first amplification followed by RPB2-6F/RPB2-7R2 (Matheny et al. 2007), using 1 µl of the first PCR as target DNA. Amplifications were undertaken using illustra™ PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare, Buckinghamshire, UK) as described in Winka et al. (1998), following thermal cycling conditions in Martín and Winka (2000). Negative controls lacking fungal DNA were run for each experiment to check for contamination. Amplifications were assayed by gel electrophoresis in 2% Pronadisa D-1 Agarose (Lab. Conda, Torrejón de Ardoz, Spain). Amplified DNA fragments were purified from the agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) and sent to Macrogen Korea (Seoul, Korea) for sequencing. Primers,

Table 1. Specimen information, GenBank accession numbers and genome BLAST searches (ID) used in this study. New sequences generated in this study are indicated in bold. n.d.: no data.

Species	Specimen voucher	Country	GenBank accession number		
			ITS	LSU	rpb2
Lyomyces crustosus	HHB 10401	USA	**MH260068**	**MH260061**	**MH259316**
	HHB 13100	USA	**MH260069**	**MH260062**	**MH259317**
	UC 2022841	USA	KP814310	n.d.	n.d.
Xylodon flaviporus	ICMP 13836	Taiwan	AF145585	n.d.	n.d.
	MA-Fungi 79440, 12094IS	Germany	**MH260071**	**MH260066**	**MH259319**
Xylodon nothofagi	ICMP 13839	New Zealand	AF145582	**MH260064**	**MH259322**
	PDD 91630, BCP 3306	New Zealand	GQ411524	n.d.	n.d.
Xylodon ovisporus	ICMP 13835	Taiwan	AF145586	**MH260063**	**MH259320**
	ICMP 13837	Taiwan	AF145587	n.d.	n.d.
Xylodon paradoxus	FCUG 2425	Russia	AF145577	n.d.	n.d.
	MA-Fungi 70444, 11060MD	France	**MH260070**	**MH260065**	n.d.
	MA-Fungi 81294, 13833MD	France	**MH260072**	n.d.	**MH259318**
Xylodon quercinus	H 6013352	Finland	KT361632	n.d.	n.d.
	MA-Fungi 91311, 1JFL	Spain	**MH260073**	**MH260067**	**MH259321**
Xylodon raduloides	ICMP 13833	Australia	AF145580	**KY962853**	n.d.
	MA-Fungi 75310, GP2291	Spain	**KY962825**	**KY962864**	**KY967055**
Schizopora paradoxa	KUC8140	Korea	**ID14957398**	**ID14957349**	**ID1495735**

used for sequencing, were those used for PCR amplifications. Additional searches for the six *Xylodon* species in EMBL/GenBank/DDBJ databases were performed in order to complete the molecular information available for this genus.

Using the BLAST tool from the JGI portal, ITS, LSU and *rpb*2 sequences were extracted from the KUC8140 strain genome (https://genome.jgi.doe.gov/pages/blast-query.jsf?db=Schpa1). The same regions from *X. paradoxus* specimens FCUG-2425, MA-Fungi 70444 and MA-Fungi 81294 were used as reference sequences for BLAST searches, respectively (Table 1). For ITS and LSU, custom search settings were used (blastn; all databases; Expect = $1*10^{-3}$; Word size = 11; Filter low complexity regions; Scoring matrix = PAM30; ITS Job ID = 14957398; LSU Job ID = 14957349). For *rpb*2, default BLAST settings were used (blastn; assembly database; Expect = $1*10^{-5}$; Word size = 11; Filter low complexity regions; Scoring matrix = BLOSUM62; *rpb*2 Job ID = 14957357). The best scoring sequence from the *S. paradoxa* KUC8140 strain genome for each region was extracted and downloaded.

Raw sequence data were processed and assembled with Geneious version 9.0.2. (Kearse et al. 2012). Two individual datasets, ITS-LSU concatenated and *rpb*2, were created to compare the KUC8140 strain with other *Xylodon* species. The combination of novel, GenBank and KUC8140 sequences for each dataset were aligned in Geneious 9.0.2 with the MAFFT nucleotide sequence alignment function (Katoh and Standley 2013). The automatic alignments were reviewed manually through Geneious 9.0.2.

Phylogenetic tree estimation for each alignment was performed using Maximum Likelihood (ML) and Bayesian Inference (BI). ML and bootstrapping analyses were conducted in RAxML (Stamatakis 2006), using default parameters established in the

CIPRES web portal (http://www.phylo.org/portal2/; Miller et al. 2010) and calculating bootstrap statistics from 1000 replicates. Bayesian inference analyses were implemented in BEAST v2.4.3 (Drummond and Rambaut 2007). Site model partition was selected using jModelTest2 (Darriba et al. 2012) and defined using BEAUti v2.4.3 interface. HKY and GTR substitution models were selected for ITS+LSU and *rpb2* alignments, respectively, as the closest available in BEAST from the results obtained in jModelTest2. We used relative timing with an uncorrelated lognormal relaxed clock by calibrating the tree with a value of 1 in the root for the *Xylodon* clade. Birth Death model was used as a tree prior. One MCMC run was specified for 50 million generations, sampling every 5000th generation. Results were visualised in Tracer v.1.6 (Rambaut et al. 2018) to evaluate whether the effective sample size (ESS) values were above 200. The trees obtained were summarised in a maximum clade credibility tree by TreeAnnotator v.1.7. with a burn-in of 5000.

Results and discussion

The ITS+LSU dataset was 1193 characters long (ITS = 594; LSU = 599) and the *rpb2* dataset was 647 characters long. The results of phylogenetic analyses of ITS+LSU and *rpb2* datasets are summarised in Fig. 1, using *phytools* R package (Revell 2012). Each phylogram represents the best tree produced from the RAxML analysis. All effective sample sizes from BEAST analyses were higher than 200 for all parameters. Those clades with Maximum likelihood bootstrap (MLB) percentages ≥ 75% and Bayesian posterior probabilities (BPP) ≥ 0.99 are marked with empty circles in Fig. 1. The remaining support values are represented above branches (MLB/BPP); specimen vouchers and species names are provided on the tip labels.

Our phylogenetic analyses confirmed the misidentification of S. *paradoxa* strain KUC8140, since sequences of this strain grouped in the *X. ovisporus* clade, showing a different evolutionary history from *X. paradoxus*. Therefore, *S. paradoxa* strain KUC8140, from Korea, must be identified as *Xylodon ovisporus,* reported from Asia and West Pacific areas (Wu 2000, Hattori 2003). The new identity of the strain KUC8140 is also supported by geographical data, since *S. paradoxa* has a European distribution. This rectification helps to explain the biogeographical patterns of *Xylodon* and also sustains the idea that "not everything is everywhere" for wood-decay fungi (Lumbsch et al. 2008).

According to our phylogenetic analyses, *Xylodon ovisporus* is the sister species of *X. flaviporus* and morphological characters confirm this relationship. The species can be discriminated by the spore size, shorter in the first one (Hattori 2003). This example accords with studies that warn about misidentifications or mislabelled vouchers in public sequence databases (Bidartondo 2008). It has been estimated that around 20% of DNA fungal sequences in the GenBank repository may have erroneous lineage assignations (Bridge et al. 2003, Nilsson et al. 2006). Assessing accuracy in GenBank and other DNA repositories is a key stage for species identification in current biodiversity analyses based on similarity of DNA sequences (Hibbett et al. 2016). It is especially

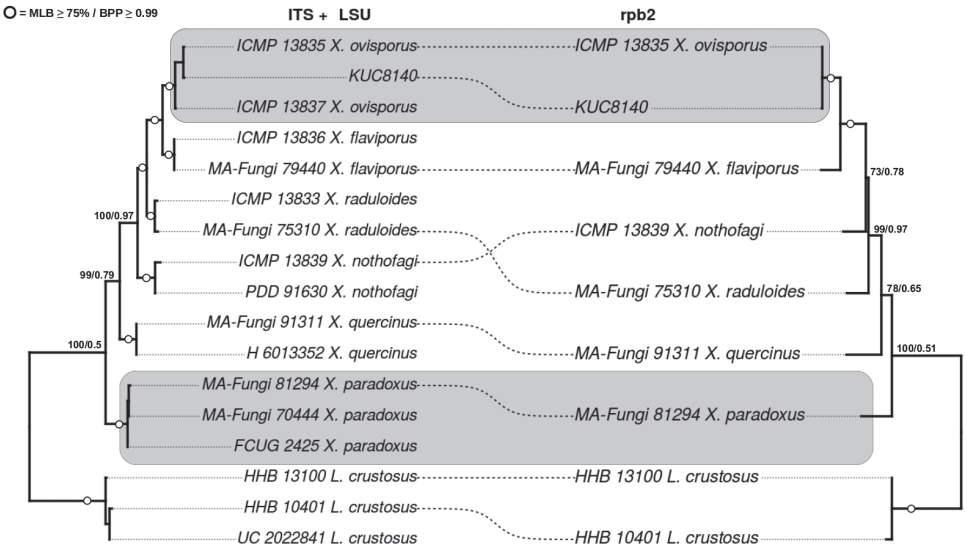

Figure 1. Maximum likelihood trees for ITS+LSU (left) and *rpb2* (right) regions of *Xylodon* species. In order to assess genealogical concordance, dotted lines link the position of the same specimen in both trees. Grey boxes indicate the position of KUC8140 strain with *Xylodon ovisporus* and the position of *X. paradoxus*. Numbers over branches are maximum likelihood bootstrap (MLB) values and posterior probabilities (BPP). Voucher numbers and species names are indicated in Table 1.

important in cases like *Xylodon paradoxus*, with useful biotechnological properties since, according to Bortolus (2008), a wrong taxonomy could lead not only to inaccurate knowledge of nature, but also to important economic losses.

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