Species identification of European forest pathogens of the genus Milesina (Pucciniales) using urediniospore morphology and molecular barcoding including M. woodwardiana sp. nov.

Ben Bubner¹,*; Ramona Buchheit²,*; Frank Friedrich³; Volker Kummer⁴; Markus Scholler²,*

¹ Thünen Institute of Forest Genetics, Eberswalder Chaussee 3a, 15377 Waldsieversdorf, Germany
² State Museum of Natural History Karlsruhe, Erbprinzenstraße 13, 76133 Karlsruhe, Germany
³ Karlsruhe Institute of Technology, Competence Center for Material Moisture, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen
⁴ University of Potsdam, Biodiversity Research/Plant Systematics, Maulbeerallee 1, 14469 Potsdam, Germany

Corresponding author: Markus Scholler (markus.scholler@smnk.de)

Academic editor: Marco Thines | Received 5 October 2018 | Accepted 11 February 2019 | Published 5 March 2019


Abstract
Species of rust fungi of the genus Milesina (Pucciniastreaceae, Pucciniales) are distributed mainly in northern temperate regions. They host-alternate between needles of fir (Abies spp.) and fronds of ferns (species of Polypodiales). Milesina species are distinguished based on host taxonomy and urediniospore morphology. In this study, 12 species of Milesina from Europe were revised. Specimens were examined by light and scanning electron microscopy for urediniospore morphology with a focus on visualising germ pores (number, size and position) and echinulation. In addition, barcode loci (ITS, nad6, 28S) were used for species delimitation and for molecular phylogenetic analyses. Barcodes of 72 Milesina specimens were provided, including 11 of the 12 species.

Whereas urediniospore morphology features were sufficient to distinguish all 12 Milesina species except for 2 (M. blechni and M. kriegeriana), ITS sequences separated only 4 of 11 species. Sequencing with 28S and nad6 did not improve species resolution. Phylogenetic analysis, however, revealed four phylogenetic groups within Milesina that also correlate with specific urediniospore characters (germ pore number and position and echinulation). These groups are proposed as new sections within Milesina (sections Milesina, Vogesiaceae M. Scholler & Bubner, sect. nov., Scolopendriorum M. Scholler & Bubner, sect. nov.

* Authors contributed most and equally to this publication.

Copyright Ben Bubner et al. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
and Carpaticae M. Scholler & Bubner, sect. nov.). In addition, Milesina woodwardiana Buchheit & M. Scholler, sp. nov. on Woodwardia radicans, a member of the type section Milesina, is newly described. An identification key for European Milesina species, based on urediniospore features, is provided.

Keywords
Abies alba, Polypodiales, GBOL, germ pores, host alternation, Uredinopsis, Europe

Introduction

Several genera of rust fungi (Pucciniaceae) in Europe alternate their hosts between Abies spp. (aecial host with spore states 0 and I) and ferns of the order Polypodiales (telial host with spore states II, III and IV or III and IV). These are species of the genera Calypsospora J.G. Kühn (Thekopsora Magnus p.p.), Hyalopsora Magnus, Milesina Magnus (= Milesia F.B. White; see Aime et al. 2018b) and possibly Uredinopsis Magnus (Gäumann 1959; Klenke and Scholler 2015). All are assigned to the Pucciniastraceae.

The genus Milesina Magnus was monographed by Faull (1932). Today, 36 species are known worldwide (Kirk et al. 2001) and 11 species in Europe (Gäumann 1959; Klenke and Scholler 2015). Most species occur in parts of the northern hemisphere with temperate climates and Abies populations (Faull 1932). A few species are also found in the southern hemisphere outside the natural area of Abies species (Liu 1971), for example, South and Central America, South Africa and New Zealand (Berndt 2008). According to Sinclair and Lyon (2005), species of the genus Milesina and Uredinopsis (both called fir-fern rust) may cause needle browning and/or defoliation and occasionally cause economic damage in Christmas tree plantations in Canada. Aeciospores from needles infect ferns. Symptoms on fern fronds are pale green to yellow spots, which later become necrotic and are typically confined by veins (Sinclair and Lyon 2005).

Fern rust species on the telial hosts are characterised and distinguished mainly by host taxonomy (telial host genus), size, shape and ornamentation of urediniospores (e.g. Moss 1926, Faull 1932, Berndt 2008) and the results of inoculation experiments (Hunter 1935, 1936, Kamei 1940, Klebahn 1916; Mayor 1944). In contrast to urediniospores, teliospores are either not formed regularly or at all and teliospore features are obscure. So far, it is not possible to morphologically distinguish species on their aecial hosts Abies spp. (spore and sori features). Urediniospore features alone were also hardly sufficient to distinguish species (e.g. Faull 1932, Gäumann 1959, Majewski 1977). Thus, fern host identification is often the only criterion to link records to a certain species. This is problematic because some fern species may be infected by two (or possibly even more) Milesina spp. (e.g. Dryopteris spp. and Polystichum spp. in Europe; Gäumann 1959, Klenke and Scholler 2015).

In the present study, the urediniospore morphology of European Milesina species was investigated by light and scanning electron microscopical techniques. The morphological approach is supplemented by a molecular phylogenetic approach based on the ITS (Internal Transcribed Spacer) region of the rDNA, which has been shown to
be the best marker for barcode species within fungi (Schoch et al. 2012). As secondary barcodes, nad6 (subunit 6 of NADH dehydrogenase) and 28S rDNA have been used. The molecular data were generated within the German Barcode of Life Project GBOL (Geiger et al. 2016). The present study has three objectives, to:

i) provide a detailed morphological description of urediniospores of all European *Milesina* spp., including the development of a method to visualise their germ pores. Germ pores are known to be a valuable taxonomic feature, for example, in grass rust fungi (Cummins 1971). So far, germ pores have not been visualised in the major studies on *Milesina* spp. (Berndt 2008; Faull 1932).

ii) provide molecular barcodes (ITS, nad6, 28S) for Central European species of *Milesina* spp. within the German Barcode of Life project (Geiger et al. 2016).

iii) assess the assignment of morphological species by comparison with the molecular data.

**Methods**

**Herbaria**

Dried herbarium specimens from the following public herbaria were used: B, FH, G, GLM, GZU, HBG, KR, M, PUR, S and W (acronyms according to Index Herbariorum, Holmgren et al. 1990).

**Light microscopy (LM)**

Urediniospores and cross sections of sori (uredinia) from dried *Milesina* specimens were mounted in a mixture of lactic acid and glycerol (Kirk et al. 2001) and examined with a light microscope (Zeiss Axioskop 2 plus) at a magnification of 400× or 1000×. If a sufficient amount of spore material were available, 30 spores per specimen were arbitrarily selected and measured. The number of examined specimens was between two (*M. magnusiana*) and 23 (*M. kriegeriana*). The number of spores examined depended on sample size and varied for each measurement and also between specimens. The length and width of 30 spores (2–4 specimens per species) and sori (only specimens with *Woodwardia* host), the length of 15 spines, the cell wall thickness of 10 spores and the distance between 20 spines were measured for each specimen (2–16 specimens per species). For spine base diameters, see next chapter.

Germ pore number and their position in the wall of urediniospores were evaluated by an adapted technique originally developed for the genus *Tranzschelia* (Scholler et al. 2014). Spores were mounted in Hoyer’s medium (Cunningham 1972) on a slide, then cover slips were pressed until the spores were disrupted and released the plasma. Then the slides were placed on a drier at 40 °C. After two to five days, the numbers of germ pores were counted in phase contrast illumination at 400× magni-
fication for 120 spores of each species. Only the specimens with the best observable germ pores were used for the analysis. In addition, the diameter of pores was measured at 400× magnification.

Specimens were photographed with a Jenoptik ProgRes CT3 digital camera attached to a Zeiss Axioskop 2 plus light microscope (Oberkochen), using differential interference contrast (DIC) and phase contrast as illumination techniques. Images were captured with PROGRES CAPTUREPRO version 2.10.0.1 software. The pictures of the uredinia of Milesina sp. were taken with a ProgRes CT3 digital camera (Jena) attached to a Zeiss Stemi 508 (Zeiss, Oberkochen). All values determined in this study were rounded to one decimal place and outliers were not included in the species description.

Scanning Electron Microscopy (SEM)

Uredinia and urediniospores of dried specimens of Milesina spp. were placed on a holder with conductive double-sided tape (Leit-Tabs, Plano GmbH). Scanning electron microscope images were obtained on a Philips XL 30 FEG environmental scanning electron microscope operated at acceleration voltages of 12 kV at a chamber pressure of 133 Pa (1 Torr). In order to achieve a better contrast and less charge effects, the samples were coated first with a mixture of gold (80%) and palladium (20%) (MED 020, BAL-TEC).

SEM studies were carried out to study surface structures which are not visible by light microscopy. Spine base diameters (30 per species) were also measured with SEM and the software IMAGEJ 1.5.

Statistical Analysis

The statistical analyses for germ pore numbers and boxplots were carried out with the programme R 3.4.3 (R Core Team 2017).

DNA extraction, PCR and sequencing

Samples were prepared from herbarium specimens by excising single rust pustules including the plant material. They were placed into micro tubes with 8–12 ceramic beads, 1.4 mm diameter (Bio-Budget technologies, Krefeld, Germany), frozen at -20 °C overnight and homogenised on a Bead Ruptor (biolabproducts, Bebensee, Germany) at a speed of 7.45 m/s for 25 s. After freezing the samples again for 10 min at -20 °C, homogenisation was repeated. DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. Selected samples were homogenised with glass mini mortars and pestles (Roth, Karlsruhe, Germany) in 400 µl of the homogenisation buffer included in the extraction kit.
Species identification of European forest pathogens of the genus Milesina...

**Table 1.** Primers and PCR conditions.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
<th>Annealing temperature</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>ITS1F</td>
<td>CTTGGTCATTAGAGGAAAGTAA</td>
<td>(Gardes and Bruns 1993)</td>
<td>60–50 °C</td>
<td>10 cycles with -1 °C per cycle (60–50 °C), then 30 cycles (50 °C)</td>
</tr>
<tr>
<td></td>
<td>ITS4rust</td>
<td>CAGATTACAAATTGGGCT</td>
<td>(Beenken et al. 2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ITS5u</td>
<td>CAAGGTTTCTGTAGGTTG</td>
<td>(Pfunder et al. 2001)</td>
<td>60–50 °C</td>
<td>10 cycles with -1 °C per cycle (60–50 °C), then 30 cycles (50 °C)</td>
</tr>
<tr>
<td></td>
<td>ITS4</td>
<td>TCTTCGCTTTATGATGTC</td>
<td>(O’Donnell 1993)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28S</td>
<td>ITS4BRF</td>
<td>GGACCATGTACAAGTGTTGA</td>
<td>(Vialle et al. 2009)</td>
<td>50 °C</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>LR5</td>
<td>ATCCTGAGGGAAACTTC</td>
<td>(Vilgalys and Hester 2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nad6</td>
<td>Nad6PucciF1</td>
<td>TTCGATAATAAGTTGGCTCATAATG</td>
<td>(Vialle et al. 2013)</td>
<td>47 °C</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Nad6PucciR1</td>
<td>AAATACAATAGGCCAACAT</td>
<td>(Vialle et al. 2013)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*voucher KR-M-0035533, KR-M-0048135

Molecular barcodes were generated for three loci: ITS (Internal Transcribed Spacer of the ribosomal DNA in the nucleus), 28S (coding for the large subunit of the ribosomal RNA gene located on the ribosomal DNA in the nucleus), nad6 (coding for subunit 6 of NADH dehydrogenase, mitochondrial DNA). Primer sequences are listed in Table 1.

PCR was performed with the Accuprime Taq Polymerase System (Life Technologies, Karlsruhe, Germany) using the supplied buffer II and the following final concentrations: 2 mM MgCl₂, 0.2 mM of each dNTP and 500 nM of each primer. The PCR programme was as follows: 3 min denaturation at 94 °C, 40 amplification cycles (94 °C for 30 s, 50 °C for 30 s and 68 °C for 60 s) and 7 min strand completion at 68 °C. PCR products were visualised in 1.6% agarose gel. Deviations from the 50 °C annealing temperature are listed in Table 1.

After purification of the PCR product with QIAquick-PCR Purification Kit (Qiagen, Hilden, Germany), it was sent to GATC Biotech AG (Konstanz, Germany) for sequencing. Sequencing was performed with the same primers used for the PCR. Forward and reverse sequences were edited and assembled with the software package GENEIOUS 10.0 (Biomatters, Auckland, New Zealand).

**Phylogenetic analysis**

Several comparison sequences were selected in order to compare the branch length between Milesina species with branch lengths between related genera. Criteria of selection were availability within the GBOL project and membership in the Pucciniales suborder Melampsorineae sensu Aime (2006) and Aime et al. (2018b). The genera
included *Puccinia* (GenBank) *Pucciniastrum*, *Uredinopsis*, *Cronartium* (GenBank) and *Melampsoridium* as outgroup. GenBank accessions of *Cronartium ribicola* ITS sequences are DQ445908 (Hietala et al. 2008), GU727730 (Mulvey and Hansen 2011) and KX574673 (Vogler et al. 2017). GenBank accessions for *Puccinia graminis* are AY874141, AY874143 and AY874146 (Abbasi et al. 2005).

Sequences were aligned with the ClustalW algorithm implemented in the programme BioEdit, version 7.1.3.0 (Hall 1999) using the standard parameters offered by the programme. Alignments were used for phylogenetic reconstruction by three different methods:

i) Neighbour-Joining (NJ) analysis was performed with the programme PAUP* 4.0b10 (Sinauer, Sunderland, MA, USA) using the Kimura-2-parameter substitution model. Node support values for NJ were calculated from 1000 bootstrap replicates.

ii) Maximum-Likelihood (ML) analysis: The original NEXUS alignment was reformatted to the extended PHYLIP format using the programme Mesquite 2.75 (http://mesquiteproject.org/mesquite/mesquite.html). The PHYLIP alignment was analysed under the ML criterion on the web-based RAxML black box (Stamatakis et al. 2008 https://www.genome.jp/tools/raxml/. Both formats were accessed on 01.09.2018). The used substitution model was GTR without GAMMA correction for amongst-site heterogeneity. Node support values were calculated from 100 bootstrap replicates.

c) Bayesian Inference (BI) analysis: The DNA-Substitution model GTR+I+G was used for performing Bayesian analysis with the programme MrBayes 3.2 (Ronquist et al. 2012). Two independent MCMC runs were performed, each with four chains over 1 000 000 generations. Every 100th tree was sampled. Initial burn-in was 25% and summarisations were calculated after the standard deviation of split frequencies reached below 0.01. The resulting tree file contained posterior probability values for node support.

Tree files resulting from the three methods were visualised using the programme TreeGraph 2 (Stöver and Müller 2010). Alignments are provided as NEXUS files in the Online Supplemental Material (Suppl. materials 1–3).

**Results**

**Barcoding success for ITS, nad6 and 28S**

ITS sequences were generated for 72 specimens of 11 *Milesina* species (Table 2). These include 10 of 11 *Milesina* species known to be present in Europe. Only for *M. magnusiana* no material was available. In addition, we sequenced an unknown *Milesina* species
Table 2. Milesina specimens: herbarium, lab and GenBank accession numbers.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host plant species</th>
<th>Voucher (all herbarium KR)</th>
<th>Lab no.</th>
<th>ITS</th>
<th>28S</th>
<th>nad6</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. blechni</em></td>
<td>Struthiopteris spicant</td>
<td>KR-M-0038517</td>
<td>B1426</td>
<td>MH08410</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Struthiopteris spicant</td>
<td>KR-M-0038523</td>
<td>B1427</td>
<td>MH08411</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Struthiopteris spicant</td>
<td>KR-M-0038519</td>
<td>B1428</td>
<td>MH08412</td>
<td>MK302189</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Struthiopteris spicant</td>
<td>KR-M-0038516</td>
<td>B1442</td>
<td>MH08421</td>
<td>MK302193</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Struthiopteris spicant</td>
<td>KR-M-0049039</td>
<td>B1893</td>
<td>MH08463</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. carpatica</em></td>
<td>Dryopteris filix-mas</td>
<td>KR-M-0048589</td>
<td>B1662</td>
<td>MH08451</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris filix-mas</td>
<td>KR-M-0043192</td>
<td>B1780</td>
<td>MH08454</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. exigua</em></td>
<td>Polystichum brunnii</td>
<td>KR-M-0050247</td>
<td>B2206</td>
<td>MH08478</td>
<td>MK302211</td>
<td>MK302182</td>
</tr>
<tr>
<td><em>M. feurichii</em></td>
<td>Asplenium septentrionale</td>
<td>KR-M-0043159</td>
<td>B1964</td>
<td>MH08476</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris carthusiana</td>
<td>KR-M-0043170</td>
<td>B1435</td>
<td>MH08417</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0043182</td>
<td>B1438</td>
<td>MH08418</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0043165</td>
<td>B1440</td>
<td>MH08419</td>
<td>MK302191</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0039321</td>
<td>B1441</td>
<td>MH08420</td>
<td>MK302192</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris carthusiana</td>
<td>KR-M-0048087</td>
<td>B1469</td>
<td>MH08441</td>
<td>MK302203</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris carthusiana</td>
<td>KR-M-0048085</td>
<td>B1470</td>
<td>MH08442</td>
<td>MK302204</td>
<td>MK302166</td>
</tr>
<tr>
<td></td>
<td>Dryopteris carthusiana</td>
<td>KR-M-0048086</td>
<td>B1471</td>
<td>MH08443</td>
<td>MK302205</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0043162</td>
<td>B1472</td>
<td>MH08444</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0048088</td>
<td>B1473</td>
<td>MH08445</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0043151</td>
<td>B1474</td>
<td>MH08446</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0043184</td>
<td>B1475</td>
<td>MH08447</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris filix-mas</td>
<td>KR-M-0043178</td>
<td>B1476</td>
<td>MH08448</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0048357</td>
<td>B1494</td>
<td>MH08449</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0048477</td>
<td>B1602</td>
<td>MH08450</td>
<td>MK302206</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dryopteris dilatata</td>
<td>KR-M-0048480</td>
<td>B1685</td>
<td>MH08452</td>
<td>MK302207</td>
<td></td>
</tr>
<tr>
<td><em>M. kriegeriana</em></td>
<td>Dryopteris carthusiana</td>
<td>KR-M-0048133</td>
<td>B1443</td>
<td>MH08422</td>
<td>MK302194</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0048134</td>
<td>B1444</td>
<td>MH08423</td>
<td>MK302195</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0048132</td>
<td>B1445</td>
<td>MH08424</td>
<td>MK302196</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0035461</td>
<td>B1446</td>
<td>MH08425</td>
<td>MK302197</td>
<td>MK302150</td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0036224</td>
<td>B1447</td>
<td>MH08426</td>
<td>MK302151</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0036225</td>
<td>B1448</td>
<td>MH08427</td>
<td>MK302152</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0025768</td>
<td>B1449</td>
<td>MH08428</td>
<td>MK302153</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0025185</td>
<td>B1450</td>
<td>MH08429</td>
<td>MK302154</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0025184</td>
<td>B1451</td>
<td>MH08430</td>
<td>MK302155</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0025191</td>
<td>B1452</td>
<td>MH08431</td>
<td>MK302156</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0043149</td>
<td>B1852</td>
<td>MH08459</td>
<td>MK302168</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium ruta-muraria</td>
<td>KR-M-0043154</td>
<td>B1853</td>
<td>MH08460</td>
<td>MK302169</td>
<td></td>
</tr>
<tr>
<td><em>M. murariae</em></td>
<td>Polypodium vulgare</td>
<td>KR-M-0043177</td>
<td>B1429</td>
<td>MH08413</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium interjectum</td>
<td>KR-M-0043189</td>
<td>B1431</td>
<td>MH08414</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium vulgare</td>
<td>KR-M-0043190</td>
<td>B1432</td>
<td>MH08415</td>
<td>MK302190</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium vulgare</td>
<td>KR-M-0043161</td>
<td>B1433</td>
<td>MH08416</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium vulgare</td>
<td>KR-M-0043152</td>
<td>B1466</td>
<td>MH08439</td>
<td>MK302164</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium vulgare</td>
<td>KR-M-0048818</td>
<td>B1846</td>
<td>MH08455</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium vulgare</td>
<td>KR-M-0043157</td>
<td>B1847</td>
<td>MH08456</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium vulgare</td>
<td>KR-M-0043146</td>
<td>B1848</td>
<td>MH08457</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium vulgare</td>
<td>KR-M-0043173</td>
<td>B1849</td>
<td>MH08458</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polypodium vulgare</td>
<td>KR-M-0048694</td>
<td>B1778</td>
<td>MH08453</td>
<td>MK302167</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Host plant species</td>
<td>Voucher (all herbarium KR)</td>
<td>Lab no.</td>
<td>ITS</td>
<td>28S</td>
<td>nad6</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------</td>
<td>----------------------------</td>
<td>----------</td>
<td>----------------</td>
<td>----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>M. scolopendrii</td>
<td>Asplenium scolopendrium</td>
<td>KR-M-0043186</td>
<td>B1455</td>
<td>MH908434</td>
<td>MK302198</td>
<td>MK302159</td>
</tr>
<tr>
<td></td>
<td>Asplenium scolopendrium</td>
<td>KR-M-0043153</td>
<td>B1456</td>
<td>MH908435</td>
<td>MK302160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium scolopendrium</td>
<td>KR-M-0025400</td>
<td>B1457</td>
<td>MH908436</td>
<td>MK302199</td>
<td>MK302161</td>
</tr>
<tr>
<td></td>
<td>Asplenium scolopendrium</td>
<td>KR-M-0049066</td>
<td>B1896</td>
<td>MH908464</td>
<td>MK302170</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium scolopendrium</td>
<td>KR-M-0049049</td>
<td>B1897</td>
<td>MH908465</td>
<td>MK302171</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asplenium scolopendrium</td>
<td>KR-M-0049050</td>
<td>B1898</td>
<td>MH908466</td>
<td>MK302208</td>
<td>MK302172</td>
</tr>
<tr>
<td></td>
<td>Asplenium scolopendrium</td>
<td>KR-M-0049051</td>
<td>B1899</td>
<td>MH908467</td>
<td>MK302209</td>
<td>MK302173</td>
</tr>
<tr>
<td>M. sp.</td>
<td>Abies alba</td>
<td>KR-M-0043687</td>
<td>B1458</td>
<td>MH908437</td>
<td>MK302200</td>
<td>MK302162</td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0042052</td>
<td>B1459</td>
<td>MH908438</td>
<td>MK302201</td>
<td>MK302163</td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0018587</td>
<td>B1860</td>
<td>MH908461</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0018624</td>
<td>B1861</td>
<td>MH908462</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0049062</td>
<td>B1902</td>
<td>MH908468</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0049038</td>
<td>B1903</td>
<td>MH908469</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0049065</td>
<td>B1905</td>
<td>MH908470</td>
<td>MK302174</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0049068</td>
<td>B1906</td>
<td>MH908471</td>
<td>MK302175</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0049063</td>
<td>B1907</td>
<td>MH908472</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0048773</td>
<td>B1911</td>
<td>MH908473</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abies alba</td>
<td>KR-M-0050303</td>
<td>B2209</td>
<td>MH908480</td>
<td>MK302215</td>
<td>MK302184</td>
</tr>
<tr>
<td>M. vogesiana</td>
<td>Polystichum aculeatum</td>
<td>KR-M-0003937</td>
<td>GBOL_1_f10</td>
<td>MH908490</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polystichum aculeatum</td>
<td>KR-M-0043175</td>
<td>B1453</td>
<td>MH908432</td>
<td>MK302157</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polystichum aculeatum</td>
<td>KR-M-0043160</td>
<td>B1454</td>
<td>MH908433</td>
<td>MK302158</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polystichum aculeatum</td>
<td>KR-M-0043187</td>
<td>B1467</td>
<td>MH908440</td>
<td>MK302202</td>
<td>MK302165</td>
</tr>
<tr>
<td>M. whitei</td>
<td>Polystichum aculeatum</td>
<td>KR-M-0049177</td>
<td>B1965</td>
<td>MH908477</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polystichum aculeatum</td>
<td>KR-M-0050248</td>
<td>B2207</td>
<td>MH908479</td>
<td>MK302212</td>
<td></td>
</tr>
<tr>
<td>M. woodwardiana</td>
<td>Woodwardia radicans</td>
<td>KR-M-0049033</td>
<td>B1912</td>
<td>MH908474</td>
<td>MK302176</td>
<td></td>
</tr>
<tr>
<td>sp. nov.</td>
<td>Woodwardia radicans</td>
<td>KR-M-0048787</td>
<td>B1914</td>
<td>MH908475</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

from the Canary Islands which politically belongs to Europe but geographically may belong to Africa. All specimens are from the fungus collection of the State Museum of Natural History Museum Karlsruhe, Germany (Acronym KR) as listed in the Methods section. An additional 43 specimens (data not shown) yielded no sequences. Thus, the success rate for sequencing was 63%. Amongst the unsuccessful specimens, 17 were collected before 2010 and 12 failed specimens were collected in 2017. The oldest successfully sequenced specimens were from 1999 (M. murariae, KR-M-0025191; M. scolopendrii KR-M-0025400). No attempts have been made to sequence M. magnusianna because only two old species from 1933 and 1964 (M-0290299, M-0205474) were available. Nine ITS sequences were generated for the genera Chrysomyxa, Melampsoridium and Uredinopsis (Table 3).

All 72 specimens with ITS sequences were sequenced for the loci nad6 and 28S. Twenty nine specimens yielded barcode sequences at the locus nad6 (sequencing success 40%), while 24 specimens were successfully sequenced at the locus 28S (sequencing success 33%, Table 2). Since no nad6 or 28S sequences could be generated for
Species identification of European forest pathogens of the genus Milesina...

Phylogenetic analysis of the ITS barcode

Phylogenetic analysis of the ITS barcode revealed four clades for clades within Milesina species. The nodes for the first, second and fourth clade have maximum support values of 100/1/100 for the three phylogenetic reconstruction methods ML, BI and NJ (Figure 1). In clade 1, the ITS sequences of the pairs M. whitei kriegeriana and M. blechni woodwardiana sp. nov. are almost identical within the pairs but differ between the pairs by one nucleotide (Figure 3). At position 381, the nucleotide T (M. whitei kriegeriana) is replaced by C (M. blechni/M. woodwardiana sp. nov.). Position 1 is the first nucleotide after the signature TCATTA for the 3’ end of the 18S rDNA. This difference is also reflected in the ITS phylogram by a node with weak support of 67/0.69/54 (Figure 1).

In clade 2, M. scolopendrii, M. polypodii and M. murariae cannot be distinguished by ITS sequences. Apart from single nucleotides at unspecified positions, the ITS sequences are identical. The sequence of M. feurichii differs from the other three species by two nucleotides with specific positions (Figure 4). This difference, however, is not reflected by bootstrap or posterior probability support (Figure 1). Clade 3 with M. vogesiaca and M. exigua has only weak support in the BI and NJ analysis (0.73 and 63) while the

### Table 3. ITS barcodes specimens of Pucciniastraceae other than Milesina: herbarium, lab and accession numbers.

<table>
<thead>
<tr>
<th>Species</th>
<th>host plant species</th>
<th>voucher (all herbarium KR)</th>
<th>lab no.</th>
<th>ITS</th>
<th>28S</th>
<th>nad6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysomyxa empetri</td>
<td>Empetrum hermaphroditum</td>
<td>KR-M-0040758 KR-M-0040758</td>
<td>B1252</td>
<td>MH908481</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melamporidium carpini</td>
<td>Carpinus betulus</td>
<td>KR-M-0048587 B1774</td>
<td>MH908486</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melamporidium hirsutukanum</td>
<td>Alnus incana Alnus glutinosa</td>
<td>KR-M-0049100 KR-M-0048149</td>
<td>B2033</td>
<td>MK302178</td>
<td>MK302188</td>
<td></td>
</tr>
<tr>
<td>Pucciniastrum circaeae</td>
<td>Circae intermedia</td>
<td>KR-M-0039060 B2038</td>
<td>MK302179</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uredinopsis filicina</td>
<td>Phegopteris connectilis</td>
<td>KR-M-0050249 B2208</td>
<td>MH908488</td>
<td>MK302213</td>
<td>MK302183</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phegopteris connectilis</td>
<td>KR-M-0012195 B2011</td>
<td>MK302177</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phegopteris connectilis</td>
<td>KR-M-0050313 B2212</td>
<td>MH908489</td>
<td>MK302215</td>
<td>MK302185</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. ITS Phylogram of 11 Milesina species (excluding M. magnusiana). The phylogram is based on a 733-bp alignment. A Maximum Likelihood (ML) tree is shown with support values for ML, Bayesian Inference (BI) and Neighbour Joining (NJ), in the order ML/BI/NJ. Support values are presented when they are above 50 (ML, NJ) or 0.5 (BI). The host is indicated in brackets. Milesina specimens without species designation (host Abies alba) are not colour-coded. For comparison, several sequences were included from closely related genera. They were all newly generated within the GBOL project, except the GenBank sequences for Cronartium spp. The drawings on the right side present the typical arrangement of spines and germ pores (grey dots) on the Milesina urediniospores.
Species identification of European forest pathogens of the genus *Milesina...*

Bootstrap support was below 50 for the ML analysis (Figure 1). In a version of the ITS phylogram with *Puccinia graminis* as outgroup (Online Suppl. material 1: Figure S1), the support values for clade 3, including *M. exigua* are 100/1/100. The only member of the fourth clade is *M. carpathica* with two identical sequences. The ITS sequence is clearly different from all other *Milesina* species investigated. In summary, amongst the 11 *Milesina* species, only four species (*M. feurichii*, *M. vogesiaca*, *M. exigua* and *M. carpatica*) can be unambiguously assigned by their ITS sequences.

Amongst the specimens on the aecial host *Abies alba*, nine grouped into clade 1 and two into clade 2 (Figure 1). Following the distinction at position 381, six *Abies*-dwelling specimens of clade 1 belong to the pair *M. whitei/*kriegeriana and three to the pair *M. blechnil/woodwardiana* sp. nov.

The ITS phylogeny (Figure 1) does not confirm monophyly of *Milesina* species. High support values are available only for a clade containing all *Milesina* species and *Uredinopsis filicina* (node support (100/1/99, Figure 1). This indicates that the genus *Milesina* may be paraphyletic. The specimens of the three genera *Melampsoridium*, *Cronartium* and *Chrysomyxa* form a clade with a node support of 81/-/0.9, indicating that probably none of them is a sister group of *Milesina*. When the branch lengths from the clade defining node to the next deeper node are compared, it is apparent that the branch lengths for the *Milesina* clades are as long or even longer when compared to the branch lengths between the different genera *Melampsoridium*, *Cronartium* and *Chrysomyxa*. This indicates a relatively large genetic distance between the clades within the genus *Milesina*.

**Phylogenetic analysis of nad6 and 28S barcodes**

Due to the low sequencing success of these two markers, only seven (nad6) and eight (28S) *Milesina* species could be included in the analysis. Although no sequences are available for clade 4 (*M. carpathica*), the general pattern of the clades is the same as for the ITS phylogeny. Clade 1 and clade 2 consist of the same species (Figure 2) as in the ITS analysis and cannot be distinguished. In addition and in contrast to the ITS data, the distinction between the species pairs *M. whitei/*kriegeriana and *M. blechnil/woodwardiana* sp. nov. is not possible. In confirmation of the ITS data, the support for a clade that contains all *Milesina* species and *Uredinopsis filicina* is high (99/1/100 for nad6, 96/0.96/64 for 28S, Figure 2). This again indicates that the genus *Milesina* is not monophyletic. The branch lengths from the clade defining node to the next deeper node are shorter between the *Milesina* clades as compared to the branch length between related species. This is in contrast to ITS data.

The ambiguity in ITS data to determine a clade 3, consisting of both *M. vogesiaca* and *M. exigua*, is also found in the nad6 and 28S data. In the nad6 phylogram, *M. exigua* has an unsupported position next to *Uredinopsis filicina*. In the 28S phylogram, *M. exigua* is only in the same clade with *M. vogesiaca* if *Uredinopsis filicina* is included. Even then, the support values of 70/0.8/52 are relatively low.
Figure 2. Phylograms of supplementary barcodes. The nad6 phylogram is based on a 550 bp alignment, the 28S phylogram on a 680 bp alignment. The technical description is the same as for Figure 1. All *Milesina* specimens from Figure 1 were attempted to sequence for the supplementary barcodes. Only the shown specimens resulted in sequences. The non-*Milesina* species were altered depending on availability. No GenBank sequences were included and the genus *Chrysomyxa* was replaced by *Pucciniastrum*.

Morphology of urediniospores

Germ pores
The number and position of the germ pores of all species were visualised. Germ pores provided three important features, namely (i) the number, (ii) the position and,
Species identification of European forest pathogens of the genus *Milesina*...

---

**Figure 3.** Deviations from the consensus ITS sequence of section *Milesina*. The first line indicates the nucleotide positions in base pairs, the second line the consensus sequence. The order of specimens is as shown in Figure 1. “*Milesina sp*” denotes specimens from *Abies alba*. Deviations for single specimens can be found at 5 positions. All specimens of *M. blechni* and *M. woodwardiana* deviate at position 381 from *M. whitei* and *kriegeriana*.

---

**Figure 4.** Deviations from the consensus ITS sequence of section *Scolopendriorum*. Description as for Figure 3. *Milesina feurichii* deviates from the other three species in positions 288 (A) and 521 (G).

---

finally, (iii) the size of pores. The four species with the highest number of germ pores per spore all belong to the section *Milesina* (Figure 5). All other species had similar germ pore numbers.
Figure 5. Boxplot of germ pore numbers of urediniospores of 12 Milesina spp. and four sections. For each species 120 spores from two (M. magnusiana), three (M. feurichii) or four (all other species) specimens were evaluated. Median, whisker, quantile and outliers (dots) are shown.

Specimens examined, urediniospore descriptions and taxonomic novelties

Comparative data of the main morphological spore characters are listed in Table 4.

Milesina blechni (Syd. & P. Syd.) Syd. & P. Syd., Annales Mycologici 8(5): 491 (1910)

Figure 6a, b

Description. Urediniospores hyaline, ellipsoidal to obovoidal, clavate, 27.5–42.5 × 15.0–20.0 µm, mostly 30.0–37.5 × 15.0–19.0 µm; wall 0.5–1.5 µm, mostly 0.8–1.0 µm thick; echinulate without spine-free areas, spines 1.2–2.2 µm, mostly 1.5–2.0 µm long, irregularly distributed, sometimes also in rows, spines typically straight and perpendicular to the wall, distance between spine bases 1.0–5.0 µm, mostly 1.5–4.0 µm, spine base 0.7–1.3 µm, mostly 0.9–1.1 µm diam.; germ pores scattered, 6–13, mostly 10–11, 2.0–3.0 µm diam., Ø 2.4 µm diam.

Comment. Urediniospore features are very similar to those of *M. kriegeriana*. Average urediniospore length measurements are somewhat higher (30.0–37.5 vs. 27.5–35.0 in *M. kriegeriana*).
Table 4. Comparative overview of morphological features of urediniospores and host range in *Milesina*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host plant genus (family)</th>
<th>Frequent spine length [µm]</th>
<th>Smooth spine-free areas</th>
<th>Frequent wall thickness [µm]</th>
<th>Frequent germ pore number</th>
<th>Ø germ pore diam. [µm]</th>
<th>Germ pore distribution</th>
<th>Frequent spore size [µm]</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milesina blechni</td>
<td><em>Struthiopteris spicant</em> (Blechnaceae)</td>
<td>1.5–2.0</td>
<td>no</td>
<td>0.8–1.0</td>
<td>10–11</td>
<td>2.4</td>
<td>scattered</td>
<td>30.0–37.5 × 15.0–19.0</td>
<td>distance between spines mostly 1.5–4.0 µm, spines typically perpendicular to the wall</td>
</tr>
<tr>
<td>Milesina carpatica</td>
<td><em>Dryopteris filix-mas</em> (Dryopteridaceae)</td>
<td>1.0–1.8</td>
<td>no</td>
<td>0.5–1.2</td>
<td>5–7</td>
<td>2.2</td>
<td>scattered</td>
<td>20.0–30.0 × 12.5–19.0</td>
<td>distance between spines mostly 0.5–3.0 µm, spines typically erect</td>
</tr>
<tr>
<td>Milesina exigua</td>
<td><em>Polytrichum aculeatum, P. braunii</em>, (Dryopteridaceae)</td>
<td>no spines</td>
<td>no spines</td>
<td>0.5–0.8</td>
<td>4–6</td>
<td>2.7</td>
<td>bizonate</td>
<td>22.5–30.0 × 12.5–17.5</td>
<td>germ pores concentrated apically or nearly bizonate</td>
</tr>
<tr>
<td>Milesina feurichii</td>
<td><em>Asplenium septentrionale</em> (Aspleniaceae)</td>
<td>±2.0</td>
<td>yes</td>
<td>0.5–1.0</td>
<td>6–7</td>
<td>2.4</td>
<td>scattered</td>
<td>30.0–37.5 × 20.0–22.5</td>
<td>distance between spines mostly 1.0–5.0 µm, spines typically erect</td>
</tr>
<tr>
<td>Milesina kriegeriana</td>
<td><em>Dryopteris borreri, D. carthusiana, D. dilatata, D. filix-mas</em> (Dryopteridaceae)</td>
<td>±2.0</td>
<td>no</td>
<td>0.8–1.0</td>
<td>10–11</td>
<td>2.3</td>
<td>scattered</td>
<td>27.5–37.5 × 15.0–20.0</td>
<td>distance between spines mostly 1.0–4.0 µm, spines typically erect</td>
</tr>
<tr>
<td>Milesina magmasiana</td>
<td><em>Asplenium adiantum-nigrum</em> (Aspleniaceae)</td>
<td>±2.0–2.2</td>
<td>yes</td>
<td>1.0–1.5</td>
<td>5–6</td>
<td>2.9</td>
<td>scattered</td>
<td>30.0–35.0 × 17.5–20.0</td>
<td>distance between spines mostly 3.0–5.5 µm</td>
</tr>
<tr>
<td>Milesina munitiae</td>
<td><em>Asplenium ruta-muraria</em> (Aspleniaceae)</td>
<td>±2.0</td>
<td>yes</td>
<td>2.0</td>
<td>5–6</td>
<td>2.4</td>
<td>scattered</td>
<td>27.5–35.0 × 17.5–22.5</td>
<td>distance between spines 2.0–3.5 µm, spines typically erect, curved near base</td>
</tr>
<tr>
<td>Milesina polypodii</td>
<td><em>Polypodium interjectum, P. x mantoniae, P. vulgaris</em> (Polypodiaceae)</td>
<td>±2.0</td>
<td>yes</td>
<td>0.5–1.0</td>
<td>5–6</td>
<td>2.3</td>
<td>scattered</td>
<td>30.0–40.0 × 17.5–22.5</td>
<td>distance between spines 1.0–4.0 µm, spines typically erect</td>
</tr>
<tr>
<td>Milesina scolopendrii</td>
<td><em>Asplenium scolopendrium</em> (Aspleniaceae)</td>
<td>±2.0</td>
<td>yes</td>
<td>0.5–1.2</td>
<td>6–7</td>
<td>2.4</td>
<td>scattered</td>
<td>27.5–42.5 × 17.5–22.5</td>
<td>distance between spines 2.0–5.0 µm, spines typically erect</td>
</tr>
<tr>
<td>Milesina vogesiaca</td>
<td><em>Polytrichum aculeatum, P. lonchites</em> (Dryopteridaceae)</td>
<td>no spines</td>
<td>no spines</td>
<td>0.5–0.8</td>
<td>5–6</td>
<td>2.8</td>
<td>± bizonate</td>
<td>30.0–40.0 × 17.5–20.0</td>
<td>spores with very inconspicuous flat verrucae (visibly with SEM only)</td>
</tr>
<tr>
<td>Milesina whitei</td>
<td><em>Polystichum aculeatum, P. setiferum</em> (Dryopteridaceae)</td>
<td>1.8–2.5</td>
<td>no</td>
<td>0.8–1.0</td>
<td>9–13</td>
<td>2.3</td>
<td>scattered</td>
<td>27.5–37.5 × 17.5–22.5</td>
<td>distance between spines mostly around 2.0 µm, spines typically perpendicular to the wall</td>
</tr>
<tr>
<td>Milesina woodwardiana sp. nov.</td>
<td><em>Woodwardia radicans</em> (Blechnaceae)</td>
<td>±3.0</td>
<td>no</td>
<td>0.5–1.0</td>
<td>10–14</td>
<td>2.4</td>
<td>scattered</td>
<td>30.0–37.5 × 17.5–22.5</td>
<td>distance between spines mostly 2.0–4.0 µm, spines irregularly directed</td>
</tr>
</tbody>
</table>
Species identification of European forest pathogens of the genus *Milesina...*

*Milesina carpatica* Wróbl., Sprawozdanie Komisji Fizjograficznej 47(II): 166 (1913)
Figure 6c


**Description.** Urediniospores hyaline, ellipsoidal, obovoidal to subglobose, 16.5–32.5 × 10.0–20.0 µm, mostly 20.0–30.0 × 12.5–19.0 µm; wall 0.5–1.8 µm, mostly 0.5–1.0 µm thick; soft (in microscopic mounts they often crack without pressure), very densely echinulate without spine-free areas, spines 1.0–2.0 µm, mostly 1.0–1.8 µm long, irregularly distributed, spines typically straight and perpendicular to the wall, distance between spine bases 0.5–4.0 µm, mostly 0.5–3.0 µm, spine base 0.4–0.7 µm, mostly 0.5–0.6 µm; germ pores scattered, 4–10, mostly 5–7, 1.3–2.5 µm, mostly 1.3–2.5 µm diam., Ø 2.2 µm diam.

**Comment.** Germ pores are more difficult to visualise and need more time to evaluate.

*Milesina exigua* Faull, Contributions from the Arnold Arboretum of Harvard University 12: 218–219 (1931)

*Polystichum aculeatum* (L.) Roth (*P. lobatum* L., *Aspidium lobatum* Sw.). Ukraine, Kolomyja: Knyazhzhvir, Aug 1913, A. Wróblewski (as *M. vogesiaca*; Sydow, Uredineen 2742; GLM, GLM-53030; W, 1916-4289); Knyazhzhvir, Sep 1913, A. Wróblewski (as *M. vogesiaca*; W, 1975-18645).


**Description.** Urediniospores hyaline, ellipsoidal to obovoidal, clavate, 22.5–32.5 × 12.5–17.5 µm, mostly 22.5–30.0 × 12.5–17.5 µm; wall 0.5–0.8 µm; spores
Figure 6. Urediniospores of 11 Milesina species. a Milesina blechni on Struthiopteris spicant (KR-M-0049039, SEM) b Milesina blechni on Struthiopteris spicant, cracked spore with released plasma, germ pores scattered (KR-M-0038523, LM phase contrast) c Milesina carpatica on Dryopteris filix-mas (KR-M-0043192, SEM) d Milesina exigua on Polystichum braunii, smooth surface (M, M-020547, SEM) e Milesina exigua on Polystichum braunii, smooth surface, plasma-free spore, germ pores bipolar (M, M-0205472, LM, phase contrast) f Milesina feurichii on Asplenium septentrionale with smooth areas on surface (KR-M-0043159, SEM) g Milesina feurichii on Asplenium septentrionale, cracked plasma-free spore, germ pores scattered (KR-M-0043159, LM, phase contrast) h Milesina kriegeriana on Dryopteris carthusiana (KR-M-0048085, SEM) i Milesina magnusiana on Asplenium adiantum-nigrum with smooth areas on surface (M, M-0205474, SEM).
Species identification of European forest pathogens of the genus *Milesina*...

Smooth, germ pores low in number, probably around 4–6, 2.0–3.8 µm, mostly 2.0–3.0 µm diam., Ø 2.7 µm diam.; germ pores mostly apically, or both, basally and apically (bizonate).

**Comment.** Klenke and Scholler (2015: 649) list this species (as *M. neoexigua* Berndt) on *Polystichum braunii* for Germany (Baden-Württemberg), based on a specimen from the Black Forest, SW Germany (KR-M-0019138). *P. braunii* is a rare member of the southern Black Forest flora. We revised host and fungus and found that it is *M. vogesiaca* on *P. aculeatum*. Thus, the presence of *M. exigua* in Germany has not been confirmed. Germ pores are more difficult to visualise and need more time to evaluate.


*Figure 6f, g*


**Description.** Urediniospores hyaline, ellipsoidal, obovoidal to subglobose, 27.5–42.5 × 17.5–25.0 µm, mostly 30.0–37.5 × 20.0–22.5 µm; wall 0.5–1.8 µm, mostly 0.5–1.0 µm thick; spores densely echinulate with 1–2, mostly 1 round to ovoidal smooth area, typically located centrally, smooth area 7.5–17.5 × 6.5–10.0 µm, mostly 10.0–15.0 × 7.5–10.0 µm, spines 1.5–2.5 µm, mostly 1.8–2.2 µm long, irregularly distributed, spines typically straight and perpendicular to the wall, distance between spine bases 1.0–9.0 µm, mostly 1.0–5.0 µm, spine base mostly around 1 µm; germ pores scattered, 5–11, mostly 6–7, 1.3–3.0 µm, mostly 2.0–2.5 µm diam., Ø 2.4 µm diam.

**Figure 6.** Continued. **j** *Milesina magnusiana* on *Asplenium adiantum-nigrum*, spore plasma-free, germ pores scattered (M, M-0205474, LM, phase contrast) **k** *Milesina murariae* on *Asplenium ruta-muraria* with smooth areas on surface (KR-M-0035461, SEM) **l** *Milesina murariae* on *Asplenium ruta-muraria*, cracked spore with released plasma, germ pores scattered (KR-M-0043154, LM, phase contrast) **m** *Milesina polypodii* on *Polypodium vulgare* with smooth areas on surface (KR-M-0043173, SEM) **n** *Milesina scolopendrii* on *Asplenium scolopendrium* with smooth areas on surface (KR-M-0049049, SEM) **o** *Milesina vogesiaca* on *Polystichum aculeatum*, surface with very flat warts at the tip of the spore (arrow) (KR-M-0043160, SEM) **p** *Milesina vogesiaca* on *Polystichum aculeatum*, surface smooth (no warts visible at the tip), germ pores bipolar (KR-M-0043175, LM, phase contrast) **q** *Milesina whitei* on *Polystichum sp.* (KR-M-0039378, SEM) **r** *Milesina whitei* on *Polystichum setiferum*, cracked spore with released plasma, germ pores scattered (KR-M-0049177, LM, phase contrast).

*Figure 6h*


**Description.** Urediniospores hyaline, ellipsoidal, obovoidal to oval, clavate, 25.0–47.5 × 12.5–25.0 µm, mostly 27.5–37.5 × 15.0–20.0 µm; wall 0.5–1.2 µm, mostly 0.8–1.0 µm thick; spores echinulate without spine-free areas, spines 1.2–3.0 µm, mostly 1.8–2.2 µm long, irregularly distributed, sometimes in rows, spines typically straight and perpendicular to the wall, distance between spine bases 1.0–6.0 µm, mostly 1.0–
Species identification of European forest pathogens of the genus Milesina...

4.0 µm, mostly around 1 µm; germ pores scattered, 6–14, mostly 10–11, 1.3–3.0 µm, mostly 2.0–2.5 µm, Ø 2.3 µm diam.

Comment. See annotation under M. blechni.

Milesina magnusiana Jaap, Verhandlungen des Botanischen Vereins für die Provinz Brandenburg 57: 16 (1915)
Figure 6i, j

Asplenium adiantum-nigrum L. France, La Corse: Ajaccio, 5 Mar 1933, O. Jaap, II (M, M-0290299, type); Ireland: Kerry, Dingle peninsula, drywall, 30 Aug 1964, Leuze & Doppelbaur (M, M-0205474).

Description. Urediniospores hyaline, ellipsoidal to obovoidal, 21.3–38.8 × 15.0–22.5 µm, mostly 30.0–35.0 × 17.5–20.0 µm; wall 1.0–2.0 µm, mostly 1.0–1.5 µm thick; spores echinulate with 1–2 ovoidal smooth areas, typically located centrally, smooth area 11.5–17.5 × 6.3–10.0 µm, mostly 15.0–17.5 × 7.5–10.0 µm, spines 1.2–2.8 µm, mostly 2.0–2.2 µm long, irregularly distributed, spines often erect, distance between spines 0.5–9.0 µm, mostly 3.0–5.5 µm; germ pores scattered, 4–9, mostly 5–6, 2.0–4.5 µm, mostly 2.5–3.0 µm diam., Ø 2.9 µm diam.

Figure 6k, l


Description. Urediniospores hyaline, ellipsoidal, obovoidal to subglobose, 25.0–42.5 × 15.0–22.5 µm, mostly 27.5–35.0 × 17.5–22.5 µm; wall 1.2–2.2 µm, mostly around 2.0 µm thick; spores echinulate with 1–2, mostly 2 ovoidal smooth areas, typically located centrally, smooth area 11.5–20.0 × 7.5–12.5 µm, mostly 12.5–15.0 × 7.5–10.0 µm, spines 1.5–2.5 µm, mostly 1.8–2.2 µm long, erect, spines curved toward base, denser toward both spore poles, distance between spine bases 0.5–7.0 µm, mostly 2.0–3.5 µm, spine base 0.7–1.4 µm, mostly around 1 µm; germ pores scattered, 3–9, mostly 5–6, 2.0–3.8 µm, mostly 2.0–2.5 µm diam., Ø 2.4 µm diam.


Figure 6m


Species identification of European forest pathogens of the genus Milesina...


Description. Urediniospores hyaline, ellipsoidal, obovoidal to subglobose, 26.5–42.5 x 15.0–25.0 µm, mostly 30.0–40.0 x 17.5–22.5 µm; wall 0.5–2.5 µm, mostly 0.5–1.0 µm thick; spores echinulate with 1–2, mostly 1 ovoidal smooth area, typically located centrally, smooth area 15.0–22.5 x 6.3–11.3 µm, mostly 15.0–17.5 x 7.5–10.0 µm, spines 1.8–2.8 µm, mostly 1.8–2.2 µm long, irregularly distributed, erect, spines denser toward spore base, distances 0.5–7.0 µm, mostly 1.0–4.0 µm, spine base 0.7–1.6 µm, mostly 0.9–1.2 µm diam.; germ pores scattered, 4–10, mostly 5–6, 1.3–3.8 µm, mostly 2.0–2.5 µm diam., Ø 2.3 µm diam.

Milesina scolopendrii (Fuckel) Jaap, Fungi selecti exsiccati no. 571 (1912)

Figure 6n

Description. Urediniospores hyaline, ellipsoidal to obovoidal, clavate, 27.5–49.0 × 17.5–25.0 µm, mostly 27.5–42.5 × 17.5–22.5 µm; wall 0.5–1.8 µm, mostly 0.5–1.2 µm thick; spores echinulate with 1 mostly ovoidal smooth area, located centrally to apically, smooth area 12.5–20.0 × 7.5–11.3 µm, mostly 15.0–17.5 × 7.5/10.0 µm, spines 1.5–2.8 long, irregularly distributed, erect, distances between spine bases 1.0–9.0 µm, mostly 2.0–5.0 µm, sometimes denser toward spore base, spine base 0.8–1.6 µm, mostly 0.9–1.2 µm diam.; germ pores scattered, 4–9, mostly 6–7, 1.25–3.0 µm, mostly 2.0–3.0 µm diam., Ø 2.4 µm diam.


Figure 6o, p


Description. Urediniospores hyaline, ellipsoidal to obovoidal, clavate, 27.5–45.0 × 15.0–25.0 µm, mostly 30.0–40.0 × 17.5–20.0 µm; wall 0.5–1.0 µm, mostly 0.5–0.8 µm thick; spores with flat verrucae verrucae 0.3–0.6 µm, mostly 0.4–0.5 µm in diam., mainly at the upper part of the spore (visible with SEM only); germ pores often bi-
Species identification of European forest pathogens of the genus *Milesina*...  

Milesina whitei (Faull) Hirats., Memoirs of the Tottori Agricultural College 4: 123 (1936)  
Figure 6q, r

*Milesina whitei* (Faull) Hirats., Memoirs of the Tottori Agricultural College 4: 123 (1936)  
Figure 6q, r

**Milesina whitei** (Faull) Hirats., Memoirs of the Tottori Agricultural College 4: 123 (1936)  
Figure 6q, r


**Description.** Urediniospores hyaline, ellipsoidal, obovoidal to oval, 27.5–40.0 × 16.5–25.0 µm, mostly 27.5–37.5 × 17.5–22.5 µm; wall 0.5–1.0 µm, mostly 0.8–1.0 µm thick; echinulate without spine-free areas, spines 1.8–2.8 µm, mostly around 1.8–2.5 µm long, irregularly distributed, straight and perpendicular to the wall, distance between spine bases 1.0–8.0 µm, mostly 1.5–5.0 µm, spine base 0.5–1.2 µm, mostly 0.8–1.1 µm diam.; germ pores scattered, 8–15 (17), mostly 9–13, 1.3–3.0 µm, mostly 2.0–2.5 µm diam., Ø2.3 µm diam.

**Comment.** The North American *Milesina polystichi* (Wineland) Grove (= *Milesia polystichi* Wineland) on *Polystichum munitum* (Kaufl.) Presl. is considered conspecific with *M. whitei* by several authors (e.g. Gäumann 1959). We were able to study isotype material (USA, Oregon, Granite Pass, 5 Sep 1916, leg. R.J. Weir, PUR 004047) and found urediniospores with mostly 5 to 6 germ pores, i.e. many fewer than in *M. whitei*. Due to this striking difference, they are possibly different species.

**Milesina woodwardiana** Buchheit & M. Scholler, sp. nov.  
Mycobank no. MB829596  
Figure 7a–f

**Holotype.** *Woodwardia radicans* (L.) Sm., Spain, Islas Canarias, La Palma, Cubo de la Galga, ca. 2.5 km SW parking place at coastal highway W San Bartolomé, wayside in Laurosilva, 11 Aug 2017, V. Kummer (KR-M-0049033).

Further specimens examined (paratypes)Spain, Islas Canarias: La Palma, Cubo de la Galga, ca. 1.2 km SW of parking lot at coastal highway W San Bartolomé, wayside in Lau-rosilva, 16 Aug 2015, V. Kummer, II (KR, KR-M-0048787); La Palma, Cubo de la Galga,
Description. Spermogonia (0), aecia (I), telia (III) and basidia (IV) unknown. Uredinia hypophyllous, subepidermal, statistically distributed; sori round, wart-like elevations, 0.1–0.3 mm in diam., covered by brownish or yellow-brownish epidermis, on dark necrotic plant tissue margined by nerves, never on nerves directly, sori opening pore-like; peridium hemispheric, peridal cells colourless, about 7.5–25.0 × 7.5–10 µm, upper peridal cells more or less isodiametrical and lateral peridal cells elongated; urediniospores hyaline, ellipsoidal to obovoidal, sometimes subglobose to irregular, 25.5–46.5 × 15.0–25.0 µm, mostly 30.0–37.5 × 17.5–22.5 µm; cell wall thin, 0.5–1.2 µm, mostly 0.5–1.0 µm thick, densely echinulate without spine-free areas, densest at spore base, spines 2.0–3.2 µm long, mostly 3.0 µm long, slightly irregularly distributed, spines orientated in different directions, dense basal spines typically directed toward spore pedicel, distance between spines bases 0.5–5.0 µm, mostly 2.0–4.0 µm, spine base 0.6–1.3 µm, mostly around 1 µm; spore pedicel often laterally or semilaterally inserted, short and wide, 5.5–14 × 12.5–15.5 µm; germ pores scattered, 8–19 (21), mostly 10–14, 1.3–3.0 µm, mostly 2.0–3.0 µm diam., Ø 2.4 µm diam.; germ tubes septate, may develop simultaneously in one spore.

Distribution. The species is only known north-eastern La Palma, Islas Canarias, Spain.

Etymology. Referring to the English botanist Thomas Jenkinson Woodward (1745 – 1820) and the host plant *Woodwardia radicans* named after him.

Comment. This species differs from *M. blechni* by the telial host plant genus (*Woodwardia*), by a higher number of germ pores/spore, longer spines and irregular spine orientation. *Milesina woodwardiana* is the first *Milesina* species known on *Woodwardia* (Berndt 2008; Faull 1932). The absence of potential aecial hosts (*Abies* spp.) in La Palma and all other Canary Islands (Hohenester and Welss 1993, Ginovés et al. 2009) and the non-formation of telia indicate that the species is not host-alternating in La Palma. The *Woodwardia radicans* area (Hohenester and Welss 1993), however, overlaps with those of *Abies × borisii-regis*, *A. cephalonica* and *A. pinsapo* in south-western Europe (Liu 1971). If the rust is present in this area, it may be possible to observe the spore stages 0 and I on *Abies* spp. *Woodwardia radicans* is the only species of *Woodwardia* in Europe. There are numerous other species in SE Asia and N America (Li et al. 2016). These areas may also coincide with the distribution area of *M. woodwardiana*.

*Milesina* spp.

Species identification of European forest pathogens of the genus Milesina...

Figure 7. Spore morphology and symptoms on fern fronds of Milesina woodwardiana sp. nov. a Fronds of the host Woodwardia radicans at the collection site in La Palma. Dark spots indicate areas where sori are formed on the underside (La Palma, Cubo de la Galga, ca. 1.2 km SW of parking lot W San Bartolomé, 11 Aug 2017) b Host leaf with uredinia. Sori (arrows) are restricted to areas between leaf veins (KR-M-0048787, dissecting microscope) c Transverse section of uredinium E=epidermis, P=peridial cells, U=urediniospore, M=mesophyll of host plant (KR-M-0048787, LM, interference contrast) d Urediniospores with long echinulae (KR-M-0049036, paratype, SEM) e Urediniospores, cracked, without plasma, germ pores scattered (KR-M-0049033, paratype; LM, phase contrast) f Germinating urediniospores, arrows point to germ tubes (KR-M-0049033, paratype, LM, phase contrast).

Comment. In this study, Milesina spp. on Abies alba were only sequenced but not morphologically analysed.

Subgeneric classification

Four morphological groups can be distinguished within Milesina with respect to germ pore number, germ pore size, germ pore position and distribution of spines on the spore surface (Figures 6a–r, 7d–f, Table. 4). The morphological differentiation corresponds with the differentiation in four clades found by molecular data (Figure. 1, right panel).

Milesina sect. Milesina

Type species. M. kriegeriana (Magnus) Magnus 1909.

This type section is characterised by urediniospores having numerous scattered germ pores and an echinulate wall without smooth areas. Milesina blechni, M. whitei and M. woodwardiana are additional members of this section.

Milesina sect. Vogesiaceae M. Scholler & Bubner, sect. nov.
MycoBank no. MB829594

Type species. M. vogesiaca Syd. & P. Syd. 1912.

This section is characterised by urediniospores having few bipolarly distributed germ pores and a smooth or almost smooth wall. Milesina exigua is included in this section. Urediniospore features of European Uredinopsis spp. resemble those of Vogesiaceae species. However, Uredinopsis spores have a terminal mucro.

Milesina sect. Scolopendriorum M. Scholler & Bubner, sect. nov.
MycoBank no. MB829597

Type species. M. scolopendrii (Fuckel) Jaap 1912.
This section is characterised by urediniospores having few scattered germ pores and an echinulate wall with smooth areas. *Milesina feurichii, M. polypodi, M. magnusiana* and *M. murariae* are in this section. *M. magnusiana* agrees well with the other species with respect to morphology. Therefore, we placed it in section *Scolopendriorum*, although no ITS data are available.

**Milesina sect. Carpaticae M. Scholler & Bubner, sect. nov.**
MycoBank no. MB829595

**Type species.** *M. carpatica* Wróbl. 1913
This section is characterised by urediniospores having few scattered germ pores and an echinulate wall without smooth areas. It is similar to section *Milesina* in having an echinulate cell wall, but the number of germ pores is lower (only 5–7). The ITS sequences of the two sections are separated by a large genetic distance. So far, this section is represented only by the type species. Possibly, the North American *M. polystichi* belongs to this section as well (see commentary to *M. whitei*).

**Key to European Milesina species**

The following key to European *Milesina* sections and species is based on urediniospore (abbreviated Us) features listed in Table 4. It requires light-microscopical equipment and methods described in the Methods section. The lengths of the urediniospores refer to the main values.

1. Us with terminal mucro ................................................................. *Uredinopsis*
   – Us without terminal mucro (*Milesina*) ............................................. 2
2. Surface of Us smooth or almost smooth, germ pores often formed apically (sect. *Vogesiacae*) .......................................................................................... 3
   – Surface of Us echinulate, sometimes with particularly smooth areas, germ pores scattered ................................................................. 4
3. Us mostly 30.0–40.0 × 17.5–20.0 µm, germ pores up to 4.5 µm diam. (*Polystichum aculeatum*) .................................................................  *M. vogesiaca*
   – Us smaller, mostly 22.5–30.0 × 12.5–17.5 µm, germ pores smaller, up to 3.8 µm diam. (germ pores are often not visible, check numerous Us) (*Polystichum braunii, P. aculeatum*) ................................................................. *M. exigua*
4. Surface of Us with smooth spine-free areas, germ pores ± 6 (Sect. *Scolopendriorum*) ................................................................................................. 5
   – Surface of Us without smooth spine-free areas, germ pores either ± 6 (*M. carpatica, sect. Carpaticae*) or ± 11 (species of sect. *Milesina*) ............  9
5. Us mostly 27.5–35.0 µm long, wall mostly 2.0 µm thick (*Asplenium rutan-muraria*) ................................................................................................. *M. murariae*
Us mostly more than 30.0 µm long, wall mostly thinner (< 2 µm).........6
Us mostly 30.0–40.0 µm long (Polypodium spp.) .................. M. polypodii
Us shorter, mostly 30.0–37.5 µm ........................................ 7
Spine distance mostly 1.0–4.0 µm (Asplenium septentrionale) .... M. feurichii
Spine distance 2.0–5.5 µm ................................................. 7
Us mostly 30.0–40.0 µm (Polypodium spp.) .................... M. polypodii
Us shorter, mostly 30.0–37.5 µm ................................................................ 7
Spine distance mostly 2.0–5.5 µm, Us 30.0–35.0 × 17.5–20.0 µm, germ pore 2.9 µm diam. (Asplenium adiantum-nigrum) .................. M. magnusiana
Spine distance 2.0–5.0 µm, Us 27.5–42.5 × 17.5–22.5 µm, germ pore < 2.5 diam. (Asplenium scolopendrium) ........................................ M. scolopendrii
Us mostly 20.0–30.0 × 12.5–19.0 µm, wall 0.5–1.0 µm thick, spines mostly 1.0–1.8 µm long, germ pores usually 6, mostly 1.3–2.0 µm diam., pores hardly visible (check numerous Us) (Dryopteris filix-mas) (sect. Carpathica) ..........
................................................................................................ 8
Us larger, mostly 27.0–37.5 × 17.5–22.5 µm, germ pores ± 11 ............. 9
Spines ± 3.0 µm long, orientated in different directions, Us mostly 30.0–37.5 × 17.5–22.5 µm (Woodwardia radicans) .................. M. woodwardiana
Spines shorter, < 3.0 µm long, typically perpendicular to the wall ............ 11
Us mostly ≥ 17.5 µm wide, 27.5–40.0 × 16.5–25.0 µm, spines erect (Polystichum aculeatum, P. setiferum) ........................................................ 10
Us ± 17.5 µm wide, sometimes spines arranged in rows, typically erect (the following two species are morphologically barely distinguishable) ........ 12
Us mostly 27.5–37.5 µm long (Dryopteris borreri, D. carthusiana, D. dilatata, D. filix-mas ................................................................. M. kriegeriana
Very similar, Us somewhat longer on average, mostly 30.0–37.5 µm (Struthiopteris spicant) ......................................................... M. blechni

Discussion
Species resolution by urediniospore features

In previous studies of the genus Milesina (e.g. Wróblewski 1913; Faull 1932; Kuprevič and Tranzschel 1957; Berndt 2008), size and shape of urediniospores, wall thickness, spine length and density were the main features used to characterise their morphology. In this study, additional morphological features and criteria are provided to distinguish species (Figure 5, Table 4).

The number, position and size of germ pores have not been documented even in more recent studies of Milesina (Berndt 2008). Additionally, in Chrysomyxa, another genus of Pucciniarstaceae (Cao et al. 2017), no germ pores were shown. Germ pores in Milesina are documented in Cummins and Hiratsuka (2003). The authors report “bizonate, obscure” germ pores for species of the genus. We found this character only in the two species of the section Vogesiacae. A further observation of germ pores is reported for two North American species Milesina polypodophila (Bell) Faull and Milesina marginalis...
Species identification of European forest pathogens of the genus Milesina...

Faull & Watson (Moss 1926) where germ pores showed a scattered distribution. With our light microscopic method, detection of germ pores was easy and could be realised within short time. In two species, *M. carpatica* and *M. exigua*, germ pores were more difficult to visualise and need more time to evaluate. In general, however, this method is suitable to document an important morphological and taxonomically relevant feature. It may also help to characterise other genera in the Pucciniastraceae. Another feature, smooth areas on the surface of urediniospores has not been documented so far. It is a special character of species of section Scolopendriorum. All of these features in combination allow identification of *Milesina* species in Europe by urediniospore features alone, using a light microscope even without knowledge of the host plant species. Only one pair of species, the common *M. blechni* and *M. kriegeriana*, is difficult to distinguish morphologically. We only found differences in spore length measurements.

In general, identification using only the host is unreliable, since the range of telial hosts in *Milesina* has been only scarcely studied. This holds true even for common species like *M. kriegeriana*, a species which has obviously a much wider host range with species in different host families (Berndt 2008) than listed in European compilatory literature (e.g. Gäumann 1959; Majewski 1977; Klenke and Scholler 2015). *Polystichum aculeatum* is known for hosting *M. carpatica* and *M. vogesiaca* (e.g. Gäumann 1959; Majewski 1977; Klenke and Scholler 2015). In the present study, *M. exigua* was also found on *P. aculeatum*, demonstrating again that the host range may be wider for several *Milesina* species and that host identification is not sufficient to identify *Milesina* spp.

Species resolution by barcoding

We were able to classify four sections by phylogenetic analysis of ITS sequences (Fig. 1) but we were only able to differentiate 4 of 11 species. This low differentiation is in contrast to another genus in the suborder Melampsorineae sensu Aime (2006). In the genus *Chrysomyxa*, almost all species could be resolved on the basis of ITS barcodes (Cao et al. 2017). Still, amongst the three tested barcodes ITS, nad6 and 28S in the present study, ITS showed the highest species resolution because it could resolve the pairs *M. whitei*, *kriegeriana* and *M. blechni*, *woodwardiana*.

The alternative barcode nad6 has been tested on different rust species (Vialle et al. 2009, Feau et al. 2011, Vialle et al. 2013). It was chosen for the present study because, in a study on *Melampsora* spp., it was the only barcode amongst six tested barcodes (ITS, 28S; CO1, nad6, MS277, MS208) that could distinguish the species *Melampsora laricis-tremulae* and *Melampsora pinitorqua* (Vialle et al. 2013) on the basis of a Single Nucleotide Polymorphism. As seen from the branch lengths in Figure 1 and Figure 2 (compare also the scale bars), nad6 sequences show the lowest variation between the sections *Milesina*, Scolopendriorum, and Vogesiacae as compared to the other two markers. Within the sections *Milesina* and Scolopendriorum, the sequences were completely identical amongst the species. This low variation makes this marker more suitable for studies on infrafamiliar level than for species distinction on the infrageneric level.
The fungal barcode 28S rDNA is the second most widely used, following ITS (Schoch et al. 2012). It is used for phylogenetic analysis of rusts on the infralaminar or higher taxonomic level (Maier et al. 2003, Aime et al. 2006, Yun et al. 2011), but also the identification of closely related species (V, Beenken 2014, Maier et al. 2016, Beenken et al. 2017, Demers et al. 2017, Zhao et al. 2017). In the studies that directly compare ITS with 28S data, species resolution of ITS and 28S are comparable (Ali et al. 2016, Beenken et al. 2012, Beenken et al. 2017, Feau et al. 2011) or ITS (namely the sub region ITS2) shows a slightly better resolution (Kenaley et al. 2018, McTaggart and Aime 2018). However, when species could not be distinguished with ITS data, distinction was also not possible with 28S data. For instance, European specimens of *Coleosporium* species *C. euphrasiae* (Schumach.) G. Winter, *C. campanulae* (Pers.) Lév. and *C. senecionis* (Pers.) Fr., each occurring on telial hosts in different plant families, could neither be distinguished by ITS nor by 28S sequences. This is also clearly the case in the present study for the *Milesina* species in the sections *Milesina* and *Scolopendriorum* that colonise different fern species, but have almost identical ITS and 28S sequences.

One possible solution to lacking species resolution is to declare all specimens with the same ITS sequence data as one species, which was the original concept of ITS barcoding (Schoch et al. 2012). However, it is not only the telial host that differs between the *Milesina* species in the section *Scolopendriorum*, but also the features of the urediniospores (see identification key and Table 4). In the case of contradicting results, it is not advisable to weight the ITS data as more reliable than the morphological/host data. In the ascomycete genus *Fusarium*, several species complexes could not be resolved by ITS data, but with newly developed barcodes TOPI (Topoisomerase I) and PGK (phosphoglycerate kinase, Al-Hatmi et al. 2016). It is also possible that, for the rust fungi, new barcodes can be developed on the basis of genomic data (Aime et al. 2018b) that finally allow morphologically determined species to be resolved.

**Success of sequencing**

Not all specimens studied morphologically were used for sequencing (i.e. old specimens, type specimens, specimens with little spore material) and not all of those specimens, where DNA was extracted, were successfully sequenced. The rate of successful ITS sequencing (63%) is relatively low. In a previous study on *Melampsora* rust fungi on *Salix*, the rate of ITS sequencing success (93%) was much higher (Bubner et al. 2014). The *Melampsora* study comprised exclusively freshly collected specimens not older than half a year, whereas we also analysed herbarium material that was several years old. However, freshly collected specimens (from 2017) also failed, while the two oldest successful specimens are from 1999. Therefore, it is not only the age of the samples that explains the low success of sequencing. It is possible that the small and fragile sori of *Milesina* species (in comparison, for instance, to *Melampsora* sori on *Salix*) are more prone to DNA decay on the herbarium specimens.
Even more surprising is the low success rate for the 28S sequencing. The 28S sequencing was performed only on samples with successful ITS sequencing. Template DNA should be present because both loci belong to the same multicopy rDNA region on the nuclear DNA. Despite this linkage, 28S is reported to have a PCR success rate of only 80% as compared to ITS in a large scale study on Basidiomycota including Pucciniomycotina (Schoch et al. 2012). Nevertheless, in recent phylogenetic studies on rusts, often concatenated alignments of ITS and 28S are used (e.g. Beenken 2017, Demers et al. 2017, McTaggart and Aime 2018, Vialle et al. 2013) which requires that both ITS and 28S can be sequenced. Although phylogenetic studies rarely report a success rate, it can be assumed that routine sequencing of both loci is possible. The low sequencing success in Milesina could be a genus-specific problem. We used 28S primers (ITS4BRF, LR5) which Vialle et al. (2013) successfully used for sequencing Melampsora spp. on poplars for Milesina spp., however, with much less success. Possibly 28S rDNA of Milesina is more difficult to sequence than in other rust genera. Other primer combinations for sequencing 28S rDNA are available and could be tested. The requirements of further testing demonstrate that, in the genus Milesina, species identification by barcode sequencing is still far from being routine.

Section Vogesiacae

The support values for section Vogesiacae are smaller than for the other three Milesina sections when M. exigua is included. Interestingly, the support values are 100/1/100 for an ITS tree with Puccinia graminis as outgroup. The decision to place both M. vogesiaca and M. exigua into one section is more strongly supported by morphological than by molecular data. Milesina vogesiaca and M. exigua are the only two species that have urediniospores without ornamentation and a bizonate position of germ pores. Further support for a molecularly and morphologically defined clade is given through Uredinopsis filicina. In the ITS phylogram, it groups behind a node with high support values that includes both M. vogesiaca and M. exigua. The inclusion of U. filicina within a clade, that comprises all Milesina species (also M. carpatica), indicates that the genus Milesina is paraphyletic. The paraphyly is also indicated in the nad6 and 28S phylograms. Furthermore, M. vogesiaca, M. exigua and U. filicina form a group with high support values in 28S phylogram.

By morphology of urediniospores, U. filicina (the type species of Uredinopsis) is similar to the two Milesina species in the section Vogesiacae because it also has smooth urediniospores (Gäumann 1959). Germ pores have not been analysed so far. Germ pores are reported for two North American species, U. osmundae Magnus and U. atkinsonii Magnus (Moss 1926). Urediniospores are smooth and show the same bizonate position of germ pores as documented for M. vogesiaca and M. exigua. A recent study on molecular age estimates in Pucciniales presents 28S data of Melampsorineae that also comprises a limited selection of Uredinopsis and Milesina specimens (Figure 3 in Aime et al. 2018a). The species U. osmundae, U. filicina and M. vogesiaca group to-
Together, while *M. scolopendrii* and an undetermined *Milesina* species form a sister clade. This confirms the topology of the trees in the present study. The molecular and the morphological data indicate that at least *U. filicina* is actually a *Milesina* species in the clade *Vogesiaceae*. To place *U. filicina* (and possible other species of *Uredinopsis*) in the genus *Milesina*, however, requires a more comprehensive sampling of *Uredinopsis* species and sequencing of both ITS and 28S rDNA (study in preparation).

**Host alternation in section *Milesina***

Amongst the 11 sequences of specimens found on the aecial host *Abies alba*, nine could be assigned to the section *Milesina*. Although this section contains four species, the question which species is able to form aeciospores on *Abies alba* can be narrowed down to three species. Only telial hosts of *M. blechni*, *M. kriegeriana* and *M. whitei* grow in the distribution area of *Abies alba* in Europe. Therefore, *M. woodwardiana* can be excluded, because the host *Woodwardia radicans* is restricted to Macaronesia and the Mediterranean (Hohenester and Welss 1993, Li et al. 2016) from where no *Milesina* sequences from the aecial host (*Abies* spp.) are available. In addition, *M. woodwardiana* obviously does not form telia and, consequently, no basidia and basidiospores. Basidiospores, however, are necessary to infect *Abies*.

The answer to the question which of the two species *M. whitei* / *M. kriegeriana* has an alternate host needs further field observation and experimental studies (inoculation experiments). Our specimens most probably belong to *M. kriegeriana*, because they were all collected in the Black Forest area (SW Germany) where we found *M. kriegeriana* many times on the telial host but not *M. carpatica*. Inoculation experiments should not only include the hosts of *M. whitei* (*Polystichum* spp.) and *M. kriegeriana* (*Dryopteris* spp.), but also *Struthiopteris spicant*, the host of *M. blechni*. This would further help to answer the question whether *M. blechni* and *M. kriegeriana* are distinct species or not. Despite the SNP at position 381, both species are very similar in urediniospore morphology. Inoculation experiments would provide further arguments to clarify the status of the two species. Another approach to analyse both host alternation and species distinction in the section *Milesina* would be to measure gene flow between the aecial (*Abies*) and the different telial hosts (ferns) by population genetics. Gene flow measurements in rust fungi have been applied to *Melampsora larici-populina* (Barres et al. 2012; Elefsen et al. 2014).

**Conclusion**

Both morphological features of the urediniospores and ITS sequences provide data to distinguish subgeneric groups (sections) in the genus *Milesina*. Apart from the two re-
lated species, *M. blechni* and *M. kriegeriana* in the section *Milesina*, morphological characteristics of urediniospores are sufficient to distinguish all European species in the genus *Milesina*. In contrast, ITS, nad6 and 28S barcodes worked only for the sections *Carpatiaceae* and *Vogesiacae* and failed to resolve species in the sections *Milesina* and *Scolopendriorum*. Therefore, morphology of urediniospores, in conjunction with host determination, is still a more secure and faster tool to identify species in *Milesina* on the telial host. Other markers have to be developed for quicker and more secure identification with barcodes.

**Acknowledgements**

Curators of the herbaria B, FH, G, GLM, HBG, KR, M, PUR, S and W provided loans for *Milesina* specimens. Numerous collectors provided additional specimens: Ludwig Beenken, Thomas Brodtbeck, Horst Jage, René Jarling, Christian Scheuer and Hjalmar Thiel. Uwe Braun delivered taxonomic expertise, Matthias Lutz and Larry Dunkle reviewed earlier drafts of the manuscript and Wolfgang Mayer provided literature on *Milesina*. Special thanks are due to Sascha Hentschel and Petra Knauer for technical assistance and Max Wiensers, student assistant, who managed the database input and part of the herbarium preparation work. Barcoding and field studies were supported by the German Federal Ministry of Education and Research (BMBF FKZ 01LI15011) (grant to B. Bubner, M. Scholler). Field studies in the Black Forest National Park were supported by institutions (Regierungspräsidium Karlsruhe, Black Forest National Park) of the state of Baden-Württemberg, Germany (project “Pilzflora Wilder See”, grant to M. Scholler).

**References**


Elefsen SE, Frey P, Sverrisson H, Hallsson JH (2014) Microsatellite analysis of Icelandic populations of the poplar fungal pathogen Melampsora larici-populina shows evidence...


Kamei S (1940) Studies on the cultural experiments of the fern rusts of *Abies* in Japan. II. Journal of the Faculty of Agriculture, Hokkaido Imperial University 47: 93–191.


Species identification of European forest pathogens of the genus Milesina...


Supplementary material 1

*Milesina ITS*
Authors: Ben Bubner, Ramona Buchheit, Frank Friedrich, Volker Kummer, Markus Scholler
Data type: multimedia
Explanation note: Fig. S1.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/mycokeys.48.30350.suppl1

Supplementary material 2

*Milesina Nad6*
Authors: Ben Bubner, Ramona Buchheit, Frank Friedrich, Volker Kummer, Markus Scholler
Data type: multimedia
Explanation note: Fig. S2.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/mycokeys.48.30350.suppl2

Supplementary material 3

*Milesina 28S*
Authors: Ben Bubner, Ramona Buchheit, Frank Friedrich, Volker Kummer, Markus Scholler
Data type: multimedia
Explanation note: Fig. S2.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/mycokeys.48.30350.suppl3
Two new species of *Verruconis* from Hainan, China

Min Qiao¹, Weiguang Tian¹², Rafael F. Castañeda-Ruiz², Jianping Xu¹⁴, Zefen Yu²

¹ Laboratory for Conservation and Utilization of Bio-resources, Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming, Yunnan 650091, China  ² School of Life Science, Yunnan University, Kunming, China  ³ Instituto de Investigaciones Fundamentales en Agricultura Tropical “Alejandro de Humboldt”, Calle 1 Esq. 2, Santiago, de Las Vegas, Cuba  ⁴ Department of Biology, McMaster University, Hamilton, Ontario, L8S 4K1, Canada

Corresponding author: Zefen Yu (zfyuqm@hotmail.com)

Academic editor: Cecile Gueidan | Received 4 December 2018 | Accepted 30 January 2019 | Published 5 March 2019


Abstract

Two new species of the genus *Verruconis*, *V. hainanensis* and *V. pseudotricladiata*, were described using combined morphological and DNA sequence data. The DNA sequences of respective strains including nuclear ribosomal DNA genes (nuSSU, ITS, nuLSU) and fragments of three protein-coding genes (ACT1, BT2, TEF1) were sequenced and compared with those from closely-related species to genera *Ochroconis* and *Verruconis* (Family Sympoventuriaceae, Order Venturiales). Morphologically, both species showed typical ampulliform conidiophores and conidiogenous cells, features not seen in other species of *Verruconis*. The conidia of *V. hainanensis* are fusiform and those of *V. pseudotricladiata* are Y or T shaped, similar to old members of a closely-related genus *Scolecobasidium*. The addition of these two new species provides a new perspective on the heterogeneity of *Scolecobasidium*.

Keywords

Aquatic hyphomycetes, dematiaceous fungi, phylogenetic placement, new taxon
Introduction

The genus *Verruconis* Samerp. et al. was proposed for the neurotropic opportunist *Ochroconis gallopava* (W.B. Cooke) de Hoog (Samerpitak et al. 2014). The thermophilic characteristic of this genus is remarkable because all three proposed species of *Verruconis* can grow at 35–42 °C. In addition to the difference in growth temperature, *Verruconis* and *Ochroconis* de Hoog & Arx also differed in conidia colour (Samerpitak et al. 2014). However, a recent molecular phylogenetic analysis placed the mesophilic *V. panacis* T. Zhang & Y. Zhang into *Verruconis*, a result suggesting that both genera are more heterogeneous in their morphological and growth requirements than previously thought (Zhang et al. 2018).

Besides *V. panacis*, other three *Verruconis* species were transferred from other genera. The type species, *V. gallopava* (W.B. Cooke) Samerp. & de Hoogs [≡ *Dactyliaria gallopava* (W.B. Cooke) G.C. Bhatt & W.B. Kendr., ≡ *Ochroconis gallopava* (W.B. Cooke) de Hoog] was transferred from *Diplorhinastrichum* Höhn.; *V. verruculosa* (R.Y. Roy et al.) Samerp. & de Hoog (≡ *Scolecobasidium verruculosum* R.Y. Roy et al.) was transferred from *Scolecobasidium* and *V. calidifluminalis* (Yarita et al.) Samerp. & de Hoog (≡ *Ochroconis calidifluminalis* Yarita et al.) was transferred from *Ochroconis*. These reclassifications suggested that genera *Ochroconis*, *Verruconis* and *Scolecobasidium* E.V. Abbott are closely related and that both morphological and molecular data are needed in order to derive robust classifications. *Ochroconis*, typified by *O. constricta* (E.V. Abbott) de Hoog & Arx, transferred from *Scolecobasidium*, was set up to comprise species with unbranched, subspherical to cylindrical or clavate conidia. Based on these criteria, many *Scolecobasidium* species were transferred to *Ochroconis*, while species in the genus *Scolecobasidium* were restricted to those with T- or Y-shaped or bi-lobed, two- to many-celled conidia and ampulliform conidiogenous cells, possessing one to three conidium-bearing denticles at the apex of the conidiogenous cells (de Hoog and von Arx 1973). However, there is a significant disagreement amongst mycologists about whether the genus *Ochroconis* should be established and some researchers still placed species with unbranched conidia under *Scolecobasidium* (Ellis 1976; Matsushima 1980, 1985, 1987, 1993, 1996; Punithalingam and Spooner 2011; Lu et al. 2013; Ren et al. 2013; Xu et al. 2014).

Samerpitak et al. (2014) revised the genera *Ochroconis* and *Scolecobasidium* using DNA sequences of the nuclear ribosomal RNA gene clusters and three protein-coding genes (actin: ACT1, β-tubulin: BT2, translation elongation factor 1-α: TEF1). They found that the type species of *Scolecobasidium*, *S. terreum* E.V. Abbott, ex-type strain CBS 203.27, originally described as having the T-shaped conidia, had lost the ability to produce conidia. Interestingly, this strain was phylogenetically distant from other strains with Y-shaped conidia as described for *S. terreum* in all analyses. Consequently, type strain *S. terreum* CBS 203.27 is now regarded as a non-representative strain of the species and, indeed, the validity of this species has been questioned and *Scolecobasidium* is considered to be of doubtful identity.
However, Gams thought that an ex-type culture was not so important to decide if a genus is retained, because there are other cultures of \textit{S. terreum} available all over the world, which clearly define the identity of this characteristic fungus. He even thought that CBS 510.71, the ex-type of \textit{Humicola minima} Fassat., a species with characteristic Y-shaped conidia, may replace \textit{S. terreum} (Gams 2015). However, in Samerpitak’s analysis, many \textit{Scolecobasidium} species were scattered outside the Family Sympoventuriaceae. Consequently, the genus \textit{Scolecobasidium} has been questioned (Samerpitak et al. 2014). Since then, several new \textit{Ochroconis} species have been described under \textit{Ochroconis} (Giraldo et al. 2014; Samerpitak et al. 2015a; 2015b; 2017; Crous et al. 2016; 2017), while the number of \textit{Scolecobasidium} species has not increased since 2014 (Index Fungorum 2018). Species with forked conidia, similar to \textit{S. terreum}, were also added to \textit{Ochroconis} based on phylogenetic relationships amongst members of Sympoventuriaceae (Giraldo et al. 2014). The strict morphological characters to demarcate \textit{Scolecobasidium} were abandoned in favour of the molecular phylogenetic approach. Subsequent analyses based on combined molecular sequence information, ecological and physiological traits and morphological differences resulted in the establishment of the genus \textit{Verruconis}.

Hainan Province, China is a centre of biodiversity for aquatic hyphomycetes. Since 2015, we have reported several new aquatic hyphomycetes from this area (Guo et al. 2015; Qiao et al. 2017, 2018). During further studies of aquatic hyphomycetes on submerged decaying leaves collected from a stream in Hainan Province, we encountered two fungi which resembled species of \textit{Scolecobasidium}. Based on phylogenetic analyses, we identified that the fungi belonged to \textit{Verruconis}. In this paper, we describe the two fungi as new species and determined their phylogenetic placement based on the combined sequences of SSU, ITS, LSU, BT2, TEF1 and ACT1.

\textbf{Materials and methods}

\textbf{Collection of samples, isolation and characterisation}

Submerged dicotyledonous leaves were collected from a stream in Hainan. Samples were collected in zip-lock plastic bags and labelled and then transported to the laboratory. The rotten leaves were cut into several 2–4 × 2–4 cm sized fragments in the laboratory and then spread on to the surface of CMA (20 g cornmeal, 18 g agar, 40 mg streptomycin, 30 mg ampicillin, 1000 ml distilled water) medium for 10 days; a single conidium was isolated and cultivated on CMA in Petri plates using sterilised needles while viewing with a BX51 microscope. Morphological observations were then made from CMA after incubation at 28 °C for one week. Measurement data were based on 30 random conidia and 10 conidiophores. Pure cultures were deposited in the Herbarium of the Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University, Kunming, Yunnan, P.R. China (YMF, formerly Key Laboratory of Industrial Microbiology and Fermentation Technology of Yunnan) and at the China General Microbiological Culture Collection Center (CGMCC).
**Table 1.** Species, strains and their corresponding GenBank accession numbers of sequences used for phylogenetic analyses.

<table>
<thead>
<tr>
<th>Taxon strain</th>
<th>ACT</th>
<th>BT2</th>
<th>ITS</th>
<th>LSU</th>
<th>SSU</th>
<th>TEF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ochroconis anellii (Graniti) de Hoog &amp; Arx CBS 284.64*</td>
<td>KF155912</td>
<td>KF156184</td>
<td>FR832477</td>
<td>KF156138</td>
<td>KF156070</td>
<td>KF155995</td>
</tr>
<tr>
<td>O. anomalã A. Nováková &amp; Mart.-Sánch. CBS 131816*</td>
<td>KF155935</td>
<td>KF156194</td>
<td>H575201</td>
<td>KF156137</td>
<td>KF156065</td>
<td>KF155986</td>
</tr>
<tr>
<td>O. constricta (E.V. Abbott) de Hoog &amp; Arx CBS 202.27</td>
<td>KF155942</td>
<td>KF156161</td>
<td>AR161063</td>
<td>KF156147</td>
<td>KF156072</td>
<td>KF156003</td>
</tr>
<tr>
<td>CBS 211.53*</td>
<td>KF155939</td>
<td>KF156163</td>
<td>KF156024</td>
<td>KF156149</td>
<td>KF156074</td>
<td>KF156004</td>
</tr>
<tr>
<td>CBS 269.61</td>
<td>H575201</td>
<td>KF156197</td>
<td>KF156022</td>
<td>KF156058</td>
<td>KF155981</td>
<td></td>
</tr>
<tr>
<td>O. cordanae Samerp., Crous &amp; de Hoog CBS 172.74</td>
<td>KF155906</td>
<td>KF156198</td>
<td>KF156023</td>
<td>KF156151</td>
<td>KF156091</td>
<td>–</td>
</tr>
<tr>
<td>CBS 780.83</td>
<td>KF155905</td>
<td>KF156199</td>
<td>HQ667539</td>
<td>KF156059</td>
<td>KF155979</td>
<td></td>
</tr>
<tr>
<td>O. gamsii de Hoog CBS 239.78*</td>
<td>KF155936</td>
<td>KF156190</td>
<td>KF156019</td>
<td>KF156150</td>
<td>KF156088</td>
<td>KF155982</td>
</tr>
<tr>
<td>CBS 101179</td>
<td>KF155906</td>
<td>KF156192</td>
<td>KF156020</td>
<td>KF156151</td>
<td>KF156091</td>
<td>–</td>
</tr>
<tr>
<td>O. globalis Samerp., A.P.M. Duarte, Angeli &amp; de Hoog CBS 131956</td>
<td>KF956094</td>
<td>KF961067</td>
<td>KF961088</td>
<td>KF961100</td>
<td>KF961117</td>
<td>KF961081</td>
</tr>
<tr>
<td>CBS 780.83</td>
<td>KF956086</td>
<td>KF961072</td>
<td>KF961094</td>
<td>KF961106</td>
<td>KF961116</td>
<td>KF961082</td>
</tr>
<tr>
<td>O. humicola (G.L. Barron &amp; L.V. Busch) de Hoog &amp; Arx CBS 116655*</td>
<td>KF155904</td>
<td>KF156195</td>
<td>HQ667521</td>
<td>KF156085</td>
<td>KF156068</td>
<td>KF155984</td>
</tr>
<tr>
<td>O. icarus Samerp., A. Giraldo, Guarro &amp; de Hoog CBS 475.80*</td>
<td>HQ916976</td>
<td>KF156197</td>
<td>KF156022</td>
<td>KF156058</td>
<td>KF155981</td>
<td></td>
</tr>
<tr>
<td>CBS 172.74</td>
<td>KF155906</td>
<td>KF156198</td>
<td>KF156023</td>
<td>KF156151</td>
<td>KF156091</td>
<td>–</td>
</tr>
<tr>
<td>CBS 780.83</td>
<td>KF155905</td>
<td>KF156199</td>
<td>HQ667539</td>
<td>KF156059</td>
<td>KF155979</td>
<td></td>
</tr>
<tr>
<td>O. macrozamiae Crous &amp; R.G. Shivas CBS 102491</td>
<td>KF155938</td>
<td>KF156191</td>
<td>KF156019</td>
<td>KF156152</td>
<td>KF156092</td>
<td>KF155978</td>
</tr>
<tr>
<td>O. minima (Fassat.) Samerp. &amp; de Hoog CBS 423.64</td>
<td>KF155943</td>
<td>KF156173</td>
<td>HQ667523</td>
<td>KF156085</td>
<td>KF156068</td>
<td>KF155984</td>
</tr>
<tr>
<td>CBS 131815*</td>
<td>KF155916</td>
<td>KF156178</td>
<td>HQ667566</td>
<td>KF156127</td>
<td>KF156061</td>
<td>KF155977</td>
</tr>
<tr>
<td>O. ramosa A. Giraldo, Gené, Deanna A. Sutton &amp; Guarro UTHSC 04-2729</td>
<td>LM644599</td>
<td>LM644604</td>
<td>HQ667525</td>
<td>LM644565</td>
<td>KF156083</td>
<td>–</td>
</tr>
<tr>
<td>CBS 118.91</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>KF156047</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CBS 863.95</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>KF156114</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>O. sexualis Samerp., Van der Linde &amp; de Hoog dH 19815</td>
<td>KF155903</td>
<td>KF156188</td>
<td>KF156017</td>
<td>KF156019</td>
<td>KF156090</td>
<td>KF155977</td>
</tr>
<tr>
<td>CBS 135766</td>
<td>KF155944</td>
<td>KF156174</td>
<td>HQ667524</td>
<td>KF156132</td>
<td>KF156064</td>
<td>KF155980</td>
</tr>
<tr>
<td>O. verrucosa (Zachariah, Sankaran &amp; Leelav.) Samerp. &amp; de Hoog CBS 437.64*</td>
<td>KF155908</td>
<td>KF156182</td>
<td>KF156038</td>
<td>KF156135</td>
<td>KF156060</td>
<td>KF155978</td>
</tr>
<tr>
<td>CBS 138185*</td>
<td>KF155911</td>
<td>KF156183</td>
<td>FR832474</td>
<td>KF156136</td>
<td>KF156069</td>
<td>KF155994</td>
</tr>
<tr>
<td>O. tshawytschae (Doty &amp; D.W. Slater) Kiril. &amp; Al-Achmed CBS 225.77</td>
<td>KF155909</td>
<td>KF156164</td>
<td>HQ667536</td>
<td>KF156085</td>
<td>KF156068</td>
<td>KF155984</td>
</tr>
<tr>
<td>CBS 130.65</td>
<td>KF155916</td>
<td>KF156178</td>
<td>HQ667566</td>
<td>KF156127</td>
<td>KF156061</td>
<td>KF155989</td>
</tr>
<tr>
<td>CBS 228.66</td>
<td>KF155915</td>
<td>KF156179</td>
<td>KF156106</td>
<td>KF156128</td>
<td>KF156064</td>
<td>KF155992</td>
</tr>
<tr>
<td>CBS 100438*</td>
<td>KF155918</td>
<td>KF156180</td>
<td>HQ667562</td>
<td>KF156126</td>
<td>KF156062</td>
<td>KF155990</td>
</tr>
<tr>
<td>O. verruculosa (R.Y. Roy, R.S. Dwivedi &amp; R.R. Mishra) Samerp. &amp; de Hoog CBS 469.95*</td>
<td>KF155934</td>
<td>KF156196</td>
<td>HQ667543</td>
<td>KF156105</td>
<td>KF156096</td>
<td>KF155975</td>
</tr>
<tr>
<td>Verruconis calidifluminalis (Yarita, A. Sano, de Hoog &amp; Nishim.) Samerp. &amp; de Hoog CBS 125818*</td>
<td>KF155901</td>
<td>KF156202</td>
<td>AB836098</td>
<td>KF156108</td>
<td>KF156046</td>
<td>KF155959</td>
</tr>
<tr>
<td>V. gallopava (W.B. Cooke) Samerp. &amp; de Hoog CBS 437.64*</td>
<td>HQ916989</td>
<td>KF156203</td>
<td>HQ667553</td>
<td>KF156112</td>
<td>KF156053</td>
<td>KF155968</td>
</tr>
<tr>
<td>CBS 118.91</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>KF156110</td>
<td>KF156047</td>
<td>–</td>
</tr>
<tr>
<td>CBS 863.95</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>KF156114</td>
<td>KF156052</td>
<td>–</td>
</tr>
<tr>
<td>Verruconis verruculosa (R.Y. Roy, R.S. Dwivedi &amp; R.R. Mishra) Samerp. &amp; de Hoog CBS 119775*</td>
<td>KF155919</td>
<td>KF156193</td>
<td>KF156014</td>
<td>KF156016</td>
<td>KF156055</td>
<td>KF155974</td>
</tr>
<tr>
<td>YMF1.04165*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>KF156066</td>
<td>KF156047</td>
<td>–</td>
</tr>
<tr>
<td>Verruconis hainanensis Z.F. Yu &amp; M. Qiao YMF1.04915*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>KF156066</td>
<td>KF156047</td>
<td>–</td>
</tr>
</tbody>
</table>

Note: Numbers in bold are those generated in this study. Marked with * are type strains.
DNA extraction, PCR and sequencing

Total DNA was extracted from fresh mycelia as described by Turner et al. (1997). Six markers, nuSSU, D1/D2 region of nuLSU, ITS and part of ACT1, BT2 and TEF1 were amplified by PCR using primers as reported earlier (Feng et al. 2013). PCR amplifications were performed using the methods described previously (Wang et al. 2014). The PCR products were then sent to the Beijing Tsingke Biotechnology Co. of China Ltd and sequenced on both strands with the same primers that were used for amplification.

Sequence alignment and phylogenetic analysis

Preliminary BLAST searches with nuSSU and nuLSU gene sequences of the new isolates indicated that they had a close phylogenetic relationship with sequences from the genus *Verruconis*, *Ochroconis* and *Scolecobasidium*. Based on this, we downloaded sequences at the six marker loci from strains belonging to genera *Ochroconis* and *Verruconis*, including 42 strains representing 21 species of *Ochroconis* and four species of *Verruconis*. The sequences of these representative strains were combined with those from our own cultures (see Table 1 for all GenBank accession numbers). *Scolecobasidium excentricum* R.F. Castañeda, W. Gams & Saikawa was specified as an outgroup.

Six alignment files were generated, one for each gene and converted to NEXUS files with ClustalX 1.83 (Thompson et al. 1997) to identify the phylogenetic positions of two species. The six alignments were then combined with BioEdit 7.1.9.0 (Hall 1999). All characters were weighted equally and gaps were treated as missing characters. Maximum likelihood (ML) analysis was computed by RAxML (Stamatakis 2006) with the PHY files generated with ClustalX 1.83 (Thompson et al. 1997), using the GTR-GAMMA model. Maximum likelihood bootstrap proportions (MLBP) were computed with 1000 replicates. Bayesian inference (BI) analysis was conducted with MrBayes v3.2.2 (Ronquist et al. 2012). The Akaike information criterion (AIC) implemented in jModelTest 2.0 (Posada 2008) was used to select the best fit models after likelihood score calculations were done. The base tree for likelihood calculations was ML-optimised. HKY+I+G was estimated as the best-fit model under the output strategy of AIC, Metropolis-coupled Markov chain Monte Carlo (MCMCMC) searches were run for 2000000 generations, sampling every 1000th generation. Two independent analyses with four chains each (one cold and three heated) were run until the average standard deviation of the split frequencies dropped below 0.01. The initial 25% of the generations of MCMC sampling were discarded as burn-in. The refinement of the phylogenetic tree was used for estimating Bayesian inference posterior probability (BIPP) values. The Tree was viewed in FigTree v1.4. The values of Maximum likelihood bootstrap proportions (MLBP) greater than 70% and Bayesian inference posterior probabilities (BIPP) greater than 0.95 at the nodes are shown along branches.
Results

Phylogenetic analysis

The phylogenetic relationships amongst the known representative taxa are completely congruent with the previous studies (Samerpitak et al. 2014; Giraldo et al. 2014). *Ochroconis* and *Verruconis* formed two distinct clades. Within the *Ochroconis* clade, three species, *O. minima* (Fassat.) Samerp. & de Hoog, *O. ramose* A. Giraldo et al., *O. icarus* Samerp.et al. with T-shaped conidia fell into a highly-supported sub-clade. Both *V. bainanensis* and *V. pseudotricladiata* were nested in a well-supported subclade, with *V. panacis* as the closest sister species. The sub-clade comprising the two new species and *V. panacis* is closer to the clade composed of *V. calidifluminalis* and *V. gallopava* than to *V. verruculosa* (Figure 1).

![Figure 1. Phylogenetic tree based on Bayesian analysis of the combined sequences of SSU, ITS, LSU BT2, TEF1 and ACT1. *Scolecosbasidium excentricum* is used as the outgroup. Bayesian posterior probabilities, greater than 0.95, are given above the nodes. Maximum likelihood bootstrap values, greater than 75%, are given below the nodes. The scale bar shows the expected changes per site.](image-url)
Taxonomy

_Verruconis hainanensis_ Z.F. Yu & M. Qiao, sp. nov.
MycoBank MB828550

Figure 2

**Etymology.** Latin, _hainanensis_, refers to the collection locality.

**Description.** Colonies on CMA medium compact, restricted, brown to fuliginous, 13 mm at 20 °C after 20 days, 16 mm at 25 °C, 11 mm at 30 °C, no growth at 35 °C. Aerial hyphae subhyaline to brown, smooth- or somewhat rough-walled. _Conidiophores_ semi-macronematous, mononematous, sometimes slightly moniliform, unbranched or branched at the apex with 2–4 divergent conidiogenous cells, brown basal cell, pale brown branches, smooth, up to 25 µm long. _Conidiogenous_ cells mostly monoblastic, discrete, scattered, brown to fuliginous or pale brown, lageniform to ampulliform, pale brown, 3.4–6.0 × 2.2–3.6 µm, with a fimbriate denticle-like at the conidiogenous locus after rhexolytic conidial secession. _Conidia_ solitary, acrogenous, fusiform, rostrate at the apical cell, 3-septate, dark at the septa, coarsely verrucose, more or less equilateral, slightly constricted at the median septum, bicoloured, with brown middle cells and subhyaline end cells, 23–30.2 × 3.6–5.7 µm, with an inconspicuous basal frill.

**Type.** CHINA. From leaves of an unidentified dicotyledonous plant submerged in a stream, Qixianling, Hainan Province, 18°68’N, 109°69’E, 902 m alt., 16 June 2016,

![Figure 2. Culture and anamorph of _Verruconis hainanensis_ (YMF 1.04165). a Culture on CMA at 25 °C after 20 days b conidiophores and monoblastic conidiogenous cells c Conidia; Scale bars: 2 cm (a); 10 µm (b, c).](image)
Z.F. Yu (dried slide YMFT 1.04165, holotype; live culture YMF 1.04165 – ex-type culture; CGMCC–3.18974–isotype).

**Notes.** *Verruconis hainanensis* shares the fusiform conidial shape with some described *Scolecobasidium* species, such as: *S. cateniphorum* Matsush., *S. caffrum* Matsush., *S. houhense* D.W. Li & Jing Y. Chen and *S. tropicum* Matsush., but all these taxa are readily distinguishable from the new Chinese species. Specifically, *S. cateniphorum* is distinguished by its 1-septate, smooth or inconspicuous echinulate, 10–24 × 2–3.5 µm conidia (Matsushima 1975). *S. caffrum* and *S. tropicum* both have 2-septate conidia, but *S. caffrum* has conidia mostly smooth or inconspicuously rough, 20–35 × 4–7.5 µm, with pale brown central and subhyaline end cells (Matsushima 1996) and *S. tropicum* has conidia with smooth or inconspicuous verruculose, smaller, 14–20 × 4.5–6 µm, with pale brown central and subhyaline end cells (Matsushima 1983). *S. houhense* with 3-septate conidia is superficially similar to *V. hainanensis*, but *S. houhense* is characterised by minutely verruculose conidia, 26–31 × 4.5–5.5 µm, brown, with central cells darker than end cells and slightly protuberate and with a dark basal scar and its conidiogenous cells and conidiophores are different from those of *V. hainanensis* (Li et al. 2010). The distinct dark scar, described from *S. houhense*, has been reported by Matsushima (1975) in *Nakataea fusispora* (Matsush) Matsush., but it is absent in *V. hainanensis*.

**Verruconis pseudotricleadiata** Z.F. Yu & M. Qiao, sp. nov.
MycoBank MB828551
Figure 3

**Etymology.** Latin, *pseudotricleadiata* refers to similar conidia shape to *Scolecobasidium tricleadiatum*.

**Description.** Colonies on CMA medium compact, restricted, brown to fuliginous, surface velvety or floccose, 12 mm at 20 °C after 20 days, 14 mm at 25 °C, 10 mm at 30 °C, no growth at 35 °C. Mycelium subhyaline to pale brown and smooth or somewhat rough-walled. *Conidiophores* semi-macronematous, mononematous, straight or flexuous, 1–4 septa, sometimes moniliform (composed of 2–5 globose serial cells), pale brown, smooth, 6.5–27.2 × 2.1–3.5 µm, sometimes reduced to conidiogenous cells that arise from assimilative hyphae. *Conidiogenous* cells monoblastic, rarely polyblastic after sympodial elongation, globose, ampulliform, lageniform to clavate, 3.0–5.3 × 2.3–3.8 µm, integrated or discrete, mostly determinate, with an inconspicuous or distinct fimbriate denticle-like at the conidiogenous locus after rheolytic conidial secession. *Conidia* mostly acrogenous, subhyaline to pale brown, smooth to verruculose, staurosporic, unbranched or branched: i) unbranched conidia (main axis) cylindrical-clavate, 2–4 septate, slightly constricted at the septa, mostly smooth, rarely verruculose, 16–20 × 3.3–4.7 µm, with an inconspicuous basal frill and often with a globose or ellipsoidal, 0–1 septate, 5.6–12.3 × 2.8–4.5 µm primary branch at the apex; ii) branched conidia staurosporic, Y-, or T-shaped, composed of the main axis and two branches (primary and secondary); iia) main axis cylindrical-clavate to
Two new species of *Verruconis* from Hainan, China

Figure 3. Cultures and anamorph of *Verruconis pseudotricladiata* (YMF 1.04915). a Cultures on CMA at 25 °C after 20 days b branched Y-shaped conidia c unbranched conidia d T-shaped conidia h Conidiophores and conidiogenous cells. Conidiogenous cells on hyphae (black arrow). Scale bars: 2 cm (a); 10 µm (b–h).
clavate, 1–3-septate, mostly 2-septate, smooth or rarely verruculose, very pale brown, 15.6–20.6 × 3.8–5.7 μm; ii) primary branches obclavate, 1–2 septate, verruculate toward the apex, smooth at the basal cell, 17.9–18.2 × 2.9–4.7 μm, at an angle of 45° arising from the apex of main axis; iic) secondary branches ovoid to obclavate, smooth or verruculose towards the apex, 0–2-septate, (−5.6)12.3–17.9 × 2.8–4.5 μm, arising eccentrically from the basal cell of the primary branches.

**Type.** CHINA. From leaves of an unidentified broad-leaf species submerged in a stream, Diaoluo Mountain, Hainan Province, 18°41'N, 109°41'E, 254 m alt., 16 June 2016, Z.F. Yu (dried slide YMFT 1.04915, holotype; live culture YMF 1.04915 ex-type; CGMCC–3.18939–isotype).

**Notes.** *Verruconis pseudotricladiata* is similar to *S. tricladiatum* Matsush. on the general conidial morphology, but in *S. tricladiatum*, the conidiophores are mostly moniliform, irregularly branched forming profuse fascicles and, on pure culture, lack staurosporic conidia or rarely formed on the conidiogenous cells, the conidia are mostly unbranched, ellipsoidal to fusiform, (1–) 3–4 (−5)-septate, (9.5–)14–22 (−28) × 4–5 (−6) μm, pale olivaceous or pale brown, verruculose conidia (Matsushima 1971).

**Discussion**

The Index Fungorum currently lists 66 names in *Scolecobasidium*. However, 22 of these 66 names have been transferred into genera *Dactylaria* Sacc., *Paradendryphiella* Woudemb. & Crous, *Ochroconis*, *Trichoconis* Clem., *Neta* Shearer & J.L. Crane and *Verruconis* (Index Fungorum 2018). Of the remaining 42 species, the majority lacks authentic culture materials and DNA sequence data, making the revision of *Scolecobasidium* very difficult. However, since 2014, the number of *Scolecobasidium* species has not increased, while many new species have been reported under *Ochroconis*, including species with forked conidia (Giraldo et al. 2014). Although *Scolecobasidium* is still listed as an accepted genus of Ascomycota (Wijayawardene et al. 2017), this genus will likely be phased out. Thus, we have placed our strains into *Verruconis* based on phylogenetic analysis.

Morphologically, the two new species resemble some members of the genus *Scolecobasidium*. Conidiophores composed of 2–5 globose serial cells are very typical in old members of *Scolecobasidium*, such as *S. alabamense* Matsush., *S. amazonense* Matsush., *S. cateniphorum* Matsush. and *S. lanceolatum* Matsush. However, amongst these species, only the LSU sequence of *S. cateniphorum* was available. Further, Y-branched conidia of *V. pseudotricladiata* was previously only described in *S. tricladiatum*, while T-shaped branched conidia appeared in four species, including the type species *S. terreum*, *O. minima* (Fassat.) Samerp. & de Hoog, *O. ramosa* Samerp. et al. and *O. icarus* Samerp. et al. In the molecular phylogenetic tree, inferred from the combined sequences of six marker loci, except for the type species, three species with T-shaped branched conidia form a single clade with high support within *Ochroconis*. In the combined analysis of SSU and LSU, *S. tricladiatum* strain P051 is closely related...
Two new species of *Verruconis* from Hainan, China

Two new species of *Verruconis* from Hainan, China

To *V. pseudotricladiata* and *S. terreum* 043 fell into *Ochroconis*, nested with other species with T-shaped branched conidia (data not shown). The phylogenetic analysis is partly consistent with the morphological comparison. The article, comprising sequences of *S. tricladiatum* strain P051 and *S. terreum* 043, has not been published and we do not know if two species have been identified correctly. Anyhow, molecular data for our strains will help improve the taxonomy and revision of *Scolecobasidium*.

When the genus *Verruconis* was established, the thermophilic character was one of the main characteristics distinguishing this genus from *Ochroconis*. The first three species included in this genus all have a high optimal growing temperature of 35–42 °C and maximum growing temperature of 47–50 °C (Samerpitak et al. 2014). However, both our species and their close relative *V. panacus* are mesophilic, which blurred a major distinguishing feature between *Verruconis* and *Ochroconis*. Morphologically, *Verruconis* is characterised by poorly differentiated, flexible, mostly cylindrical to acicular, with 0(−1) thin septa conidiophores, sometimes without conidiophores (Samerpitak et al. 2014). However, conidiophores of two new species are distinct, only occasionally reducing to conidiogenous cells. Ampulliform conidiogenous cells also appeared in *O. minima* and *O. icarus*, but no species in *Verruconis* and *Ochroconis* have similar conidiophores to those of the two new species, which were composed of 2–5 globose serial cells. Based on the phylogenetic relationships amongst the species, the distributions of morphological features indicate that conidiophores and conidiogenous cells are important features for defining these two related genera. Our results suggest that the analyses of more sequences and more cultures in this group of fungi are needed to provide a robust revision of the three genera *Verruconis*, *Ochroconis* and *Scolecobasidium*.

**Acknowledgements**

This work was financed by the National Natural Science Foundation of PR China (31770026, 31760012). We are grateful to two reviewers for critically reviewing the manuscript and for providing helpful suggestions to improve this paper.

**References**


Two new species of *Verruconis* from Hainan, China


Biatora alnetorum (Ramalinaceae, Lecanorales), a new lichen species from western North America

Stefan Ekman¹, Tor Tønsberg²

¹ Museum of Evolution, Uppsala University, Norbyvägen 16, SE-752 36 Uppsala, Sweden ² Department of Natural History, University Museum, University of Bergen, Allégaten 41, P.O. Box 7800, NO-5020 Bergen, Norway

Corresponding author: Stefan Ekman (stefan.ekman@em.uu.se)

Academic editor: T. Lumbsch | Received 10 January 2019 | Accepted 21 February 2019 | Published 5 March 2019


Abstract
Biatora alnetorum S. Ekman & Tønsberg, a lichenised ascomycete in the family Ramalinaceae (Lecanorales, Lecanoromycetes), is described as new to science. It is distinct from other species of Biatora in the combination of mainly three-septate ascospores, a crustose thallus forming distinctly delimited soralia that develop by disintegration of convex pustules and the production of atranorin in the thallus and apothecia. The species is known from the Pacific Northwest of North America, where it inhabits the smooth bark of Alnus alnobetula subsp. sinuata and A. rubra. Biatora alnetorum is also a new host for the lichenicolous ascomycete Sclerococcum toensbergii Diederich.

Keywords
Biatora flavopunctata, Biatora pallens, Lecania, BAli-Phy

Introduction
During field work in the Pacific Northwest of the United States and Canada in 1995–2018, the second author came across a distinct crustose and sorediate lichen on the smooth bark of alders. Ascospores produced in the scattered pale-coloured apothecia turned out to be mostly three-septate, which prompted a search amongst the many names once described in or combined into the genus Bacidia De Not. As we were unable to find any previous description of a species fitting this morphology, it is described here as new to science. Morphological characteristics, primarily the combination of the structure of the proper exciple, sorediate thallus and ascospore shape, led us to suspect the new species to be a member of the genus Biatora Fr. (Printzen 1995, 2014).
Materials and methods

Microscopic quantitative characters were investigated either in a 10% aqueous solution of potassium hydroxide (KOH) (ascospores, paraphyses) or in pure water (all other microscopic characters). Pigments were investigated and characterised using a 10% aqueous solution of KOH and a 50% v/v aqueous solution of commercial-grade nitric acid (70% HNO₃). Measurements of quantitative characters are given either as ‘minimum value – maximum value’ or ‘minimum value – arithmetic mean value – maximum value (s = sample standard deviation, n = sample size)’. Lichen substances were screened with Thin Layer Chromatography (TLC) in solvent systems A, B’ and C following Culberson and Kristinsdottir (1970), Culberson (1972) and Menlove (1974). Aluminium plates were used in systems A and B’ and glass plates in system C, the latter to allow the detection of fatty acids.

In order to obtain some indication of relationships from other than morphological data, we obtained a complete sequence from the internal transcribed spacer (ITS) region of the ribosomal DNA using the laboratory approach described by Ekman and Blaalid (2011). Subsequently, we downloaded the data (S15023) of Printzen (2014) from TreeBase (https://treebase.org) and excised the ITS region. For reasons of computational tractability, we removed Cladostomum griffithii (Sm.) Coppins (shown by Kistenich et al. 2018 to be more closely related to Ramalina Ach.), Mycobilimbia pilularis (Hepp ex Körb.) Hafellner & Türk (the genus already being well represented by two other species), sequences not definitively referred to any taxon (marked “cf.”) and all but one sequence from taxa represented by multiple accessions. Question-mark symbols were either removed (when they were terminal) or replaced by “N” (when they were internal). Finally, all gaps were stripped. To this data, we added our own ITS sequence of Biatora alnetorum (MH818375), generated from Tønsberg 27500 (BG), resulting in a dataset with 45 sequences. We carried out a joint estimation of alignment and phylogeny using BAli-Phy version 3.1.4 (Suchard and Redelings 2006). We set the substitution model to a single GTR+I+Γ (the gamma distribution divided into four categories) and the gap model to a single RS07 model (Redelings and Suchard 2007) without partitioning the data. Priors were kept at their default values. The analysis consisted of 10 parallel runs and included a pre-burn-in of 10 iterations followed by 75000 cycles of Markov chain Monte Carlo (MCMC), sampling states every 50 cycles. The first 25000 cycles of each run were removed as burn-in. A more precise estimate of the time to convergence was obtained with the statreport tool of BAli-Phy.

Results

All numerical parameters of the BAli-Phy analysis had converged after 16650 cycles, but we anyway excluded the first 25000 cycles (resulting in a posterior sample of size 10×(75000-25000)/50 = 10000). In the posterior sample, the average standard deviation of split frequencies at or above 0.1 was 0.015. A majority-rule consensus tree with all compatible groups provided 0.97 posterior probability for the genus Biatora, including B. alnetorum (Fig. 1). In our consensus phylogeny, B. alnetorum appears
**Figure 1.** Majority-rule consensus tree of a Bayesian posterior sample obtained by joint estimation of alignment and phylogeny from ITS sequence data with BAli-Phy. The ingroup consists of the genus *Biatora* and the outgroup of members of *Lecania s. lat.*, *Mycobilimbia*, *Bilimbia* and ‘*Lecidea*’ *albohyalina*. Branch lengths are represented by their average across the posterior sample.

in an unsupported clade (posterior probability 0.64) together with *B. chrysanthoides* Printzen & Tønsberg, *B. sphaeroidiza* Printzen & Holien, *B. pallens* (Kullh.) Printzen and an unnamed *Biatora* species from Norway.
Taxonomy

**Biatora alnetorum** S. Ekman & Tønsberg, sp. nov.

MycoBank No: MB 829438

Figs 2–4

**Diagnosis.** Similar to *Biatora pallens* (Kullh.) Printzen in having 3-septate ascospores and crystals in the exciple, but differs from that species primarily by the sorediate thallus, the production of atranorin in the thallus and proper exciple and in sometimes producing up to 7-septate ascospores.


**Etymology.** The epithet, *alnetorum*, means ‘of the alder stands’ and is a reference to the fact that *Biatora alnetorum* prefers thickets dominated by *Alnus alnobetula* subsp. *sinuata*.

**Description.** Thallus crustose, thin, continuous, finely cracked, whitish, forming ± convex pustules from which soralia develop. Pustules greenish (with no hint of yellow; pale grey in the herbarium), glossy, mostly discrete, firm, rounded or rarely ± ellipsoidal in outline, 0.08–0.32 mm diam. when rounded and 0.12–0.60 × 0.10–0.36 mm diam. when ellipsoid. Soralia forming by disintegration of pustules into convex aggregations of yellowish grass-green (pale straw in the herbarium) and loosely arranged soredia, finally eroding into ± empty and shallow pits. Soredia mostly ellipsoid, rarely globose, strikingly similar in shape and size, firm (not easily disintegrating in squash preparations), 22–47–79 µm long (s = 14, n = 30) and 19–32–46 µm wide (s = 8, n = 30). Prothallus lacking. Photobiont unicellular, chlorococcoid, globose to (irregularly) ellipsoid, 7.5–12.5 µm long.

Apothecia absent or sparse, sometimes abundant, biatorine, 0.3–0.5–0.9 mm diam. (s = 0.1, n = 50), at first flat, later moderately convex, epruinose or thinly pruinose on edge. Disc pale pink (or pale yellowish with age in the herbarium). Margin pale pink to almost white, concolorous with disc or slightly paler, thick, distinct, raised above disc in young apothecia, soon level with the disc, persistent.

Proper exciple laterally 54–68 µm thick, with abundant minute crystals (< 1 µm diam.) that are soluble in KOH, colourless or diffusely pale orange-yellow, prosoplechtenchymatous, composed of radiating hyphae that branch in the inner but not outer part of the exciple, with gelatinised cell walls; cell lumina narrowly cylindrical, 0.7–0.8 µm wide (swelling in KOH); terminal cells not swollen or moderately swollen to 2 µm. Hypothecium colourless to pale orange-yellow, without crystals. Hymenium 41–56–63 µm thick (s = 6, n = 25), colourless or diffusely pale orange-yellow, usually without crystals, rarely with a thin and uneven layer of crystals at the surface. Paraphyses 1.6–2.1–2.8 µm wide in mid-hymenium (s = 0.3, n = 25), unbranched or moderately branched in upper part, sometimes sparingly anastomosed in lower part;
Biatora alnetorum, a new lichen species from western North America

Figure 2. Morphology of Biatora alnetorum. A Habit of lichen thallus with apothecia and soralia in herbarium specimen from 1999 (Tønsberg 27500, BG) B, C section through apothecium (Tønsberg 24077, BG), B showing pigmentation in bright-field illumination and C showing crystals in the proper exciple in cross-polarised light D thallus with soralia in herbarium specimen from 2000 (Tønsberg 28771a, BG) E soralium with soredia in cross-polarised light (Tønsberg 28771a, BG). Scale bars: 0.5 mm (A), 100 µm (B, C), 0.5 mm (D), 50 µm (E).
apices not swollen to ± clavate, 1.6–2.8–4.7 μm wide (s = 0.6, n = 50), without internal pigment. Asci clavate, 8-spored; young spore mass forming a wide and bluntly conical ocular chamber, apex above young spore mass staining blue in IKI except for a pale blue and narrowly conical axial body surrounded by a dark blue zone (i.e. approximately of Biaxtora-type sensu Hafellner (1984)). Ascospores colourless, without perispore or ornamentation, bacilliform to short-acicular, straight or slightly curved to shallowly sigmoid, sometimes coiled in ascus, 17–30–53 μm long (s = 6, n = 50), 1.8–2.4–3.6 μm wide (s = 0.3, n = 50), 7.0–12.9–22.0 times as long as wide (s = 2.9, n = 50), mostly with 3 but sometimes with up to 7 septa.

Pycnidia not seen.

Chemistry: Large amounts of atranorin in thallus and apothecia. Thallus, soralia and proper exciple K+ yellow.

Pigments: No pigments or small amounts of Rubella-orange (Meyer and Printzen 2000) in proper exciple, hypothecium and/or hymenium.

Distribution and ecology. Biaxtora alnetorum is known from the Pacific Northwest of North America in Washington and Alaska (U.S.A.) and British Columbia (Canada). Its vertical distribution ranges from 620 to 1450 m a.s.l. It occurs in openings in humid old-growth coniferous forest and Alnus woodlands and in the alpine scrub zone. B. alnetorum inhabits smooth bark of trunks or, occasionally, branches. The phorophyte is almost exclusively Alnus alnobetula subsp. sinuata (also known as Alnus viridis subsp. sinuata), except for a single record on Alnus rubra. Other lichens occurring together with B. alnetorum include (on Alnus alnobetula) Caloplaca sorocarpa (Vain.) Zahlbr., Biaxtora flavopunctata (Tønsberg) Hinter. & Printzen, B. toensbergii Holien & Printzen, B. vacciniicola (Tønsberg) Printzen and Pertusaria carneopallida (Nyl.) Anzi ex Nyl. and (on Alnus rubra) Parmeliopsis hyperopta (Ach.) Arnold, Japewia subaurifera Muhr & Tønsberg, and Phlyctis speirea G. Merr.

Remarks. Part of the type collection contains the lichenicolous fungus Sclerococcus toensbergii Diederich (Diederich and van den Boom 2017). This fungus was, according to Diederich and van den Boom (2017) and Diederich et al. (2018), previously known to occur on Megalaria pulvorea (Borrer) Hafellner & E. Schreiner (Ramatinaeae) and Pertusaria carneopallida (Nyl.) Anzi ex Nyl. (Pertusariaceae). Biaxtora alnetorum is reported here as a new host for this fungus.

Additional specimens examined. Canada. British Columbia: N of Vancouver, Garibaldi Park, N of Wedgemount Creek, along Wedgemount Trail to Wedgemount Lake, 50°10.1’N, 122°50.2’W, elev. 1450 m, corticolous on horizontal trunks of Alnus alnobetula subsp. sinuata over creek in old-growth coniferous forest, 25 Sept 2000, T. Tønsberg 28708 (BG, CANL, UBC). – U.S.A. Alaska: City and Borough of Juneau, along trail from Juneau to Mt. Robert, 58°17.8’N, 134°22.8’W, elev. 620–630 m, corticolous on ± horizontal trunks of Alnus alnobetula subsp. sinuata, 7 Sept 1999, T. Tønsberg 27490 (BG); 58°17.7’N, 134°22.8’W, elev. 700 m, corticolous on ± horizontal trunks of Alnus alnobetula subsp. sinuata, 7 Sept 1999, T. Tønsberg 27495 (BG); 58°17.7’N, 134°22.5’W, elev. 740–750 m, corticolous on ± horizontal, dead trunks of Alnus alnobetula subsp. sinuata, 7 Sept 1999, T. Tønsberg 27497, 27499, 27500 (BG);
**Figure 3.** Morphology of *Biatora alnetorum*. 

A Thallus with soralia in freshly collected specimen (Tønsberg 48200, UPS) 

B thalli with soralia in freshly collected specimens: *Biatora alnetorum* to the right and the similar *B. flavopunctata* to the left (Tønsberg 48202, BG), separated more or less by the approximately vertical, shallow crack at the centre of the image. Scale bars: 0.5 mm (A, B).
Figure 4. Known world distribution of *Biatora alnetorum*, which includes the western United States and Canada.

Discussion

Morphologically, the new species stands out on account of its combination of pale pinkish apothecia with a proper exciple inspersed with small crystals, mostly 3-septate ascospores and green soralia formed from distinct pustules on the thallus and containing soredia that are remarkably even in size and shape. It agrees with other species of *Biatora* with regard to the radiating and (in lateral view) almost parallel excipular hyphae, the generally strongly gelatinised apothecial tissues, as well as the *Biatora*-type ascus (Hafellner 1984, Printzen 2014). Recent molecular phylogenies (e.g. Printzen 2014, Kistenich et al. 2018) have resulted in an increasingly inclusive delimitation of the genus compared to the first modern circumscription proposed by Printzen (1995). Concomitantly, the morphological amplitude accepted in the genus (tissue structures, chemistry, pigmentation etc.) has expanded. We adhere here to the most recent and quite broad circumscription of *Biatora* advocated by Printzen (2014) and confirmed by Kistenich et al. (2018) as monophyletic, the latter assuming that the genus *Myrionora* R. C. Harris is also included.

Our phylogeny (Fig. 1), which is based only on the internal transcribed spacer region, confirms that *B. alnetorum* is a member of the genus *Biatora* but does not allow any definitive conclusions beyond that. In the consensus tree, the new species appears as sister species to *B. sphaeroidiza* in a poorly supported group also including *B. chrysanthooides*, *B. pallens* and an undescribed species. Taking into account differences in taxon sampling, there are, however, different levels of support but no supported contradictions between our ITS phylogeny and the phylogeny of Printzen (2014), the latter based on more extensive DNA sequence data.

Amongst the previously known species of the genus, *Biatora alnetorum* (Figs 2, 3) is morphologically most likely to be confused with the esorediate *B. pallens* and the sorediate *B. flavopunctata*. *B. pallens* and *B. alnetorum* share the 3-septate ascospores and the presence of crystals in the exciple. *B. alnetorum* is, however, different from that species in forming large, sorediate thalli, the occurrence of atranorin in the thallus and apothecia, larger apothecia that do not become as markedly convex and longer ascospores, with sometimes up to five or seven septa. In *B. pallens*, thalli are esorediate and often small, apothecia become convex with an excluded margin early during development and ascospores are consistently three-septate and short-bacilliform. Small amounts of usnic acid and zeorin occur in the thallus, whereas large amounts of usnic acid form crystals in the proper exciple and upper part of the hymenium (Ekman 1997). In the field, however, *Biatora alnetorum* is most likely to be confused with *B. flavopunctata* (Fig. 3B), which is widespread on alpine shrubs in the Pacific Northwest. Both species are sorediate and often co-occur, forming mosaics on *Alnus* branches. In such situations, *B. alnetorum* is set apart by the presence of discrete and conspicuously yellowish grass-green soralia formed from convex pustules, whereas *B. flavopunctata* possesses pale (yellowish) green soralia not formed from convex pustules. Under the microscope, the soredia of *B. flavopunctata* are mostly globose and fragile, easily disintegrating in squash preparations. Ascospores are non-septate in *B. flavopunctata*
(Tønsberg 1992; Printzen 1995) and chemical constituents include usnic, isousnic and often also stictic and cryptostictic acids in addition to atranorin (Tønsberg 1992, Printzen 1995). The anatomical and chemical differences between B. flavopunctata and B. alnetorum reflect the fact that the two species are unlikely to be closely related within the genus (Fig. 1). In addition, B. alnetorum shares the presence of punctiform soralia and more or less long-bacilliform, mainly three-septate ascospores with B. bacidioides Printzen & Tønsberg (Printzen and Tønsberg 2003). The latter, however, lacks crystals in the exciple, has near-black apothecia and produces argopsin, norargopsin and gyrophoric acid in the soralia.

The distribution of Biatora alnetorum (Fig. 4) coincides with the Vancouverian Subprovince of the Cordilleran-Arctic Province of McLaughlin (2007). To our knowledge, it does not occur in the coastal or inland rain forest zones (Schoonmaker et al. 1997, Goward and Spribille 2005) but seems to prefer somewhat inland conditions. Having said that, B. alnetorum is, although unlikely to be common, probably overlooked and its distribution underestimated.

Acknowledgements

TT is thankful to the University of Bergen for funding fieldwork and to Professor Joe Ammirati, University of Washington, for introducing him to the Goat Marsh area near Mount St. Helens, where many of the finds of the new species were made. He extends his gratitude to the staff at the U.S. National Park Service, Mount Rainier National Park, for the permit to collect lichens in the park in September 2000 and to Susan Bainbridge, Mount Rainier National Park, who accompanied him on a collecting trip in October 2018 in search for fresh material of the new species.

References


Species of *Dendrostoma* (Erythrogloeaceae, Diaporthales) associated with chestnut and oak canker diseases in China

Ning Jiang¹, Xin-Lei Fan¹, Pedro W. Crous², Cheng-Ming Tian¹

¹ The Key Laboratory for Silviculture and Conservation of the Ministry of Education, Beijing Forestry University, Beijing 100083, China ² Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands

Corresponding author: Cheng-Ming Tian (chengmt@bjfu.edu.cn)

Abstract
*Dendrostoma* was recently proposed in Erythrogloeaceae (Diaporthales, Sordariomycetes), with all known members recorded as being plant pathogenic on economically important tree hosts. During our collections of *Dendrostoma* species in China, mild to severe canker symptoms were observed on sweet chestnut (*Castanea mollissima*) and oak (*Quercus* spp.) trees. Dead and dying plant tissues exhibiting *Dendrostoma* canker symptoms were sampled for fungal isolation. A total of 37 *Dendrostoma* isolates were obtained and analysed using morphological characteristics and molecular data (ITS, LSU, RPB2, TEF1-α). Based on these data, 10 novel clades could be distinguished, which also proved to represent morphologically distinct species described here as *Dendrostoma aurorae*, *D. castaneae*, *D. castaneicola*, *D. chinense*, *D. dispersum*, *D. parasiticum*, *D. qinlingense*, *D. quercus*, *D. shaanxiense* and *D. shandongense* spp. nov. A key to species of the genus is also provided.

Keywords

Introduction
The family Erythrogloeaceae was established to accommodate *Chrysocrypta*, *Disculoides*, and *Erythrogloeum*, which exhibit epiphyllous acervuli along with subcylindrical to ampulliform conidiogenous cells and aseptate conidia (Senanayake et al. 2017).
Erythrogloeum (Petrak 1953) is the type genus of Erythrogloeaceae and causes severe anthracnose on Hymenaea courbaril in South America (Ferreira et al. 1992). Chrysocrypta was first proposed in Cryphonectriaceae, being associated with leaf spots on Corymbia spp. in Australia (Crous et al. 2012a), but was subsequently transferred to Erythrogloeaceae, based on DNA sequence data (Senanayake et al. 2017). Disculoides was introduced with two initial species, D. eucalypti and D. eucalyptorum, discovered on diseased Eucalyptus leaves in Australia (Crous et al. 2012b). Two additional Disculoides species, D. calophyllae and D. corymbiae, were subsequently reported as foliar pathogens of Corymbia calophylla (Crous et al. 2016, 2017).

Dendrostoma (Erythrogloeaceae, Diaporthales) was recently introduced as a phytopathogenic fungal genus causing canker diseases on several economic hardwoods such as Malus spectabilis, Osmanthus fragrans and Quercus acutissima (Fan et al. 2018). Subsequently, Dendrostoma leiphaemia on Quercus trees was transferred from Amphiporthe based on ITS and LSU sequences analysis (Senanayake et al. 2018). Dendrostoma represents one of four genera in the family, but is the only one known to have a sexual morph. Hence, Erythrogloeaceae can be distinguished from the other diaporthalean families by multiguttulate and bicellular ascospores that are constricted at the septum and acervular conidiomata, with subcylindrical to ampulliform conidiogenous cells and hyaline to olivaceous, aseptate conidia (Rossman et al. 2007, Voglmayr and Jaklitsch 2014, Senanayake et al. 2017, Voglmayr et al. 2017, Fan et al. 2018).

The Erythrogloeaceae, including Chrysocrypta, Dendrostoma, Disculoides and Erythrogloeum, represent a family of fungal pathogens occurring on several commercially important tree genera such as Corymbia, Eucalyptus, Hymenaea, Malus, Osmanthus and Quercus in Australia, Brazil, China and Costa Rica (Petrak 1953, Ferreira et al. 1992, Crous et al. 2012a, b, 2016, 2017, Fan et al. 2018). Considering the importance of these tree diseases and the lack of taxonomic information on Dendrostoma, we conducted several surveys for members of the genus in China.

The aims of present study were (i) to describe the important Dendrostoma spp. associated with canker diseases on chestnut and oak trees in China and (ii) to provide a multi-gene phylogeny for the genus Dendrostoma based on a large set of freshly collected specimens in China. In agreement with previous taxonomic studies in Erythrogloeaceae, where different Disculoides spp. were discovered on Myrtaceae (Crous et al. 2012a, b, 2016, 2017), several Dendrostoma spp. were found on Fagaceae (Castanea and Quercus), being associated with mild to severe canker diseases. The Dendrostoma species were subsequently classified based on morphological characteristics and phylogenetic data.

**Materials and methods**

**Sample collections and fungal isolates**

Surveys for Dendrostoma species were conducted in plantations, nurseries, parks, gardens, on mountains and natural reserves in Beijing, Hebei, Shaanxi, Shandong, Tian-
Species of Dendrostoma associated with chestnut and... 69

jin and Zhejiang Provinces in China from 2017 to 2018. Typical canker symptoms were observed on stems, branches and twigs of different hosts, including Castanea mollissima, Quercus aliena, Q. aliena var. acuteserrata, Q. wutaishanica and other Quercus species (Fig. 1). Diseased samples were collected and placed in paper bags, then transferred to the laboratory for further study.

A total of 37 Dendrostoma isolates were established by removing a mucoid spore mass from sporulating ascomata and conidiomata produced on diseased bark, spreading the suspension on the surface of potato dextrose agar (PDA) plates and incubating

Figure 1. Chestnut plantations and Dendrostoma canker symptoms. A A chestnut plantation on the mountain B A chestnut plantation on the plain C Collection of the dead trees killed by Dendrostoma pathogens D–H Dendrostoma canker symptoms on host branches.
the plates at 25 °C in the dark for up to 24 h. Single germinating spores were then transferred to clean plates under a dissecting microscope with a sterile needle. Specimens and isolates were deposited in the Museum of Beijing Forestry University (BJFC). Axenic cultures are maintained in the China Forestry Culture Collection Centre (CFCC).

**Morphological analysis**

The identification of *Dendrostoma* spp. was based on morphological features observed on the natural substrates. Cross-sections for ascomata and conidiomata from tree barks were prepared by hand using a double-edged blade under a dissecting microscope. At least 10 conidiomata/ascomata, 10 asci and 50 conidia/ascospores were measured to calculate the mean size and standard deviation. Measurements are reported as maxima and minima in parentheses and the range representing the mean plus and minus the standard deviation of the number of measurements is given in parentheses (Voglmayr et al. 2017). Microscopy photographs were captured with a Nikon Eclipse 80i compound microscope equipped with a Nikon digital sight DS-Ri2 high definition colour camera, using differential interference contrast illumination. Nomenclatural novelties and descriptions were deposited in MycoBank (Crous et al. 2004). Cultural characteristics were recorded for isolates incubated on PDA in the dark at 25 °C.

**DNA extraction, PCR amplification and sequencing**

Genomic DNA was extracted from fungal colonies growing on PDA using a modified cetyl trimethyl ammonium bromide method (CTAB; Doyle and Doyle 1990, Zhang et al. 2010). The ITS region was amplified using the primers ITS1 and ITS4 (White et al. 1990), the LSU region with the primers LR0R and LR5 (Vilgalys and Hester 1990), the *RPB2* region with primers fRPB2-5F and fRPB2-7cR (Liu et al. 1999) and the partial *TEF1-α* gene with the primers EF1-728F and EF1-986R (Carbone and Kohn 1999). The PCR mixture for all regions consisted of 1 µl genomic DNA, 3 mM MgCl$_2$, 20 µM of each dNTP, 0.2 µM of each primer and 0.25 U rTAQ DNA polymerase (TaKaRa, Shiga). Amplification of LSU and ITS were accomplished by an initial step of 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 51 °C and 40 s at 72 °C, with a final extension of 10 min at 72 °C. For *TEF1-α* amplification, the 35 cycles consisted of initiation at 95 °C for 8 min, denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, elongation at 72 °C for 1 min and a final extension of 5 min at 72 °C. For *RPB2*, amplification of 35 cycles consisted of initiation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 52 °C for 1 min, elongation at 72 °C for 1 min and a final extension of 10 min at 72 °C. The DNA sequencing was performed using an ABI PRISM 3730XL DNA Analyzer with BigDye Terminater Kit v. 3.1 (Invitrogen, Carlsbad) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing).
Species of Dendrostoma associated with chestnut and...

Phylogenetic analyses

Sequences generated from the above primers of the different genomic regions (ITS, LSU, TEF1-α and RPB2) were analysed in comparison with those of Dendrostoma mali (CFCC 52102), D. leiphaemia (CBS 187.37), D. osmanthi (CFCC 52106, CFCC 52107, CFCC 52108 and CFCC 52109) and D. quercinum (CFCC 52103, CFCC 52104 and CFCC 52105) from Fan et al. (2018) and Senanayake et al. (2018). Corymbia corymbiae (CBS 132528), Disculoides eucalypti (CBS 132183) and D. eucalyptorum (CBS 132184) were selected as the outgroup taxa (Crous et al. 2012a, b). All sequences were aligned using MAFFT v. 6 (Katoh and Toh 2010) and edited manually using MEGA v. 6 (Tamura et al. 2013). Phylogenetic analyses were performed using PAUP v. 4.0b10 for maximum parsimony (MP) analysis (Swofford 2003) and PhyML v. 3.0 for Maximum Likelihood (ML) analysis (Guindon et al. 2010). The first analyses were performed on the combined multi-gene dataset (ITS, LSU, TEF1-α and RPB2) to compare isolates of Erythrogloeaceae species to ex-type sequence data from recent studies (Table 1).

A partition homogeneity test with heuristic search and 1000 replicates was performed using PAUP v. 4.0b10 to assess the discrepancy amongst the ITS, LSU, TEF1-α and RPB2 sequence datasets in reconstructing phylogenetic trees. MP analysis was run using a heuristic search option of 1000 search replicates with random-additions of sequences with a tree bisection and reconnection algorithm. Maxtrees were set to 5000, branches of zero length were collapsed and all equally parsimonious trees were saved. Other calculated parsimony scores were tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC). ML analysis was performed using a GTR site substitution model including a gamma-distributed rate heterogeneity and a proportion of invariant sites (Guindon et al. 2010). The branch support was evaluated using a bootstrapping method of 1000 replicates (Hillis and Bull 1993). Phylograms were shown using FigTree v. 1.3.1 (Rambaut and Drummond 2010). Novel sequences generated in the current study were deposited in GenBank (Table 1) and the aligned matrices used for phylogenetic analyses in TreeBASE (accession number: S22929).

Results

Phylogenetic analyses

The alignment based on the combined sequence dataset (ITS, LSU, TEF1-α and RPB2) included 46 ingroup taxa and three outgroup taxa, comprising 3536 characters in the aligned matrix. Of these, 2612 characters were constant, 175 variable characters were parsimony-uninformative and 749 characters were parsimony informative (101 from ITS, 21 from LSU, 389 from TEF1-α and 238 from RPB2). The MP analysis resulted in 108 equally most parsimonious trees (TL = 1590, CI = 0.744, RI = 0.897, RC = 0.668); the first tree is shown in Fig. 2. The phylogram based on the four gene sequences indicated 10 new species in Dendrostoma (Fig. 2), as described below.
Table 1. Isolates and GenBank accession numbers used in the phylogenetic analyses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Culture</th>
<th>Location</th>
<th>Host</th>
<th>Host family</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrycorypta corymbiae</em></td>
<td>CBS 133258*</td>
<td>Australia</td>
<td>Corymbia sp.</td>
<td>Myrtaeae</td>
<td>ITTS LSU TEF1−a RPB2</td>
</tr>
<tr>
<td><em>Dendrostoma aurumae</em></td>
<td>CFCC 52755*</td>
<td>China</td>
<td>Castanea mollissima</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma castaneae</em></td>
<td>CFCC 52745*</td>
<td>China</td>
<td>Castanea mollissima</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma chinense</em></td>
<td>CFCC 52755*</td>
<td>China</td>
<td>Castanea mollissima</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma dispersum</em></td>
<td>CFCC 52750*</td>
<td>China</td>
<td>Quercus</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma castaneicola</em></td>
<td>CFCC 52743*</td>
<td>China</td>
<td>Castanea mollissima</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma cinnamome</em></td>
<td>CFCC 52744*</td>
<td>China</td>
<td>Castanea mollissima</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma eumelia</em></td>
<td>CFCC 52755*</td>
<td>China</td>
<td>Castanea mollissima</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma japonicum</em></td>
<td>CFCC 52763*</td>
<td>China</td>
<td>Castanea mollissima</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma pyriforme</em></td>
<td>CFCC 52760*</td>
<td>China</td>
<td>Castanea mollissima</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma quercus</em></td>
<td>CFCC 52734*</td>
<td>China</td>
<td>Quercus</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma quercus acutissima</em></td>
<td>CFCC 52735*</td>
<td>China</td>
<td>Quercus</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Dendrostoma quercus</em></td>
<td>CFCC 52737*</td>
<td>China</td>
<td>Quercus</td>
<td>Fagaceae</td>
<td></td>
</tr>
<tr>
<td><em>Disculoides eucalypti</em></td>
<td>CBS 132183*</td>
<td>Australia</td>
<td>Eucalyptus sp.</td>
<td>Myrtaeae</td>
<td></td>
</tr>
<tr>
<td><em>Disculoides eucalyptorum</em></td>
<td>CBS 132184*</td>
<td>Australia</td>
<td>Eucalyptus sp.</td>
<td>Myrtaeae</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Phylogenetic tree based on an MP analysis of a combined DNA dataset of ITS, LSU, TEF1-α and RPB2 gene sequences for the species of Dendrostoma. Bootstrap values ≥ 50% for MP and ML analyses are presented at the branches. Isolates representing ex-type material are marked with *.
Taxonomy


**Type species.** *Dendrostoma mali* X.L. Fan & C.M. Tian.

**Description.** Sexual morph: *Pseudostromata* small to large, distinct, circular, erumpent, consisting of an inconspicuous ectostromatic disc, semi-immersed to superficial, causing a pustulate bark surface. *Ectostromatic disc* flat or concave, orange, surrounded by bark flaps. *Central column* beneath the disc more or less conical. *Stromatic zones* lacking. *Ascomata* perithecial, conspicuous, umber to fuscous black, embedded in orange to umber pseudostromatic tissue, regularly scattered, surrounding the ectostromatic disc, with small to long ostioles that emerge within the ectostromatic disc. *Ostioles* flat in the disc or sometimes slightly projecting, cylindrical, sometimes obscuring the disc, covered by an orange, umber to fuscous black crust. *Paraphyses* deliquescent. *Asci* fusoid, 8-spored, 2–3-seriate, with an apical ring, becoming detached from the perithecial wall. *Ascospores* hyaline, fusoid to cylindrical, symmetrical to asymmetrical, straight to curved, b icellular, with a median septum, constricted at the septum, smooth, multiguttulate. Asexual morph: *Conidiomata* pycnidial, spherical to conical to pulvinate, occurring separately, immersed to semi-immersed in bark; wall of several layers of yellow *textura angularis*. *Central column* beneath the disc conical or not. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* lining the inner walls of cavity, hyaline, smooth, subcylindrical to ampulliform. *Conidia* hyaline, aseptate, smooth, multiguttulate or not, thin-walled, ellipsoid to fusoid, straight to curved.

*Dendrostoma aurorae* C.M. Tian & N. Jiang, sp. nov.

MycoBank: MB826795

Figure 3

**Diagnosis.** *Dendrostoma aurorae* differs from *D. chinensis* and *D. shandongense* by the existence of obvious central column.

**Holotype.** CHINA. Shaanxi Province: Lan’gao County, chestnut plantation, 32°13′43′′N, 109°00′44′′E, 1820 m a.s.l., on branches of *Castanea mollissima*, 3 Jul. 2017, N. Jiang (holotype: BJFC-S1561; ex-type culture: CFCC 52753).

**Etymology.** *Aurorae*, referring to the orange conidiomata with exuding conidial tendrils.

**Description.** Sexual morph not observed. Asexual morph: *Conidiomata* pycnidial, conical to pulvinate, occurring separately, bright yellow to orange, semi-immersed in bark, 300–500 μm high, 800–1400 μm diam.; wall of several layers of bright yellow *textura angularis*; conidiomata exuding slimy orange masses of conidia; *central column* beneath the disc more or less conical, pale yellow. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* lining the inner walls of the cavity, hyaline, smooth,
Species of *Dendrostoma* associated with chestnut and...

Subcylindrical to ampulliform, 4–15 × 2.5–4 µm. *Conidia* hyaline, aseptate, smooth, multiguttulate, thin-walled, ellipsoid to fusoid, straight to curved, (7.2–)8.1–9.8(–10.3) × (2.1–)2.3–2.6(–2.8) µm, l/w = (2.7–)3.2–4.1(–4.2) (n = 50).

**Culture characters.** On PDA, cultures are initially white, becoming isabelline after 2 weeks. The colonies are flat with irregular edge; texture uniform within 1 month at 25 °C in the dark.

**Additional specimen examined.** CHINA. Shaanxi Province: Lan’gao County, chestnut plantation, 32°13’43”N, 109°00’44”E, 1820 m a.s.l., on branches of *Castanea mollissima*, 3 Jul. 2017, N. Jiang, living culture CFCC 52754 (BJFC-S1562).

**Notes.** *Dendrostoma aurorae* was discovered on stems of dying chestnut trees and appears morphologically similar to the chestnut blight pathogen, *Cryphonectria parasitica*. However, these two diaporthalean pathogens can be distinguished by the existence of a central column inside the conidiomata of *Dendrostoma aurorae*. In the genus *Dendrostoma*, *D. aurorae* differs from *D. chinensis* and *D. shandongense* by the existence of an obvious central column.

---

**Figure 3.** Morphology of *Dendrostoma aurorae* from *Castanea mollissima* (BJFC-S1561). **A–C** Habit of conidiomata on branches **D** Transverse section of conidioma **E** Longitudinal section through conidioma **F, H** Conidia **G** Conidiogenous cells. Scale bars: 1 mm (**A**); 0.5 mm (**B, C, E**); 0.2 mm (**D**); 5 µm (**F, H**); 10 µm (**G**).
*Dendrostoma castaneae* C.M. Tian & N. Jiang, sp. nov.
MycoBank: MB826796

Figure 4

**Diagnosis.** *Dendrostoma castaneae* is distinguished from the phylogenetically closely related species *D. castaneicola* by its narrower conidia.


**Etymology.** Castaneae, referring to the host genus, Castanea.

**Description.** Sexual morph not observed. Asexual morph: Conidiomata pycnidial, pulvinate, occurring separately, bright yellow to orange, immersed in bark, 400–600 µm high, 900–2200 µm diam.; wall of several layers of brown textura angularis; central column beneath the disc irregular, pale yellow. Conidiophores reduced to conidiogenous cells. Conidiogenous cells lining the inner walls of the cavity, hyaline, smooth, subcylindrical to ampulliform, 3–10 × 2–3.5 µm. Conidia hyaline, aseptate, smooth, multiguttulate, thin-walled, ellipsoid, straight to curved, (9.3–)10.4–12.3(–13.3) × (2.1–)2.2–2.7(–2.9) µm, l/w = (3.4–)4.2–5.2(–5.9) (n = 50).

**Culture characters.** On PDA, cultures are initially white, exhibiting grey after 2 weeks. Colonies are flat with irregular edge; texture initially uniform, producing concentric circles with faint orange conidiomata distributed outside the rim within 1 month at 25 °C in the dark.

**Additional specimens examined.** CHINA. Hebei Province: Chengde City, Xinglong County, chestnut plantation, 40°21'44"N, 117°51'29"E, 256 m a.s.l., on branches of *Castanea mollissima*, 27 Apr. 2018, N. Jiang & C.M. Tian, living culture CFCC 52748 (BJFC-S1556); Hebei Province: Chengde City, Xinglong County, chestnut plantation, 40°21'44"N, 117°51'29"E, 256 m a.s.l., on branches of *Castanea mollissima*, 27 Apr. 2018, N. Jiang & C.M. Tian, living culture CFCC 52751 (BJFC-S1557); Hebei Province: Chengde City, Xinglong County, chestnut plantation, 40°21'44"N, 117°51'29"E, 256 m a.s.l., on branches of *Castanea mollissima*, 27 Apr. 2018, N. Jiang & C.M. Tian, living culture CFCC 52747 (BJFC-S1559); Hebei Province: Chengde City, chestnut plantation, 40°37'39"N, 118°27'22"E, 256 m a.s.l., on branches of *Castanea mollissima*, 28 Apr. 2018, N. Jiang & C.M. Tian, living culture CFCC 52750 (BJFC-S1558); Hebei Province: Chengde City, chestnut plantation, 40°37'39"N, 118°27'22"E, 256 m a.s.l., on branches of *Castanea mollissima*, 28 Apr. 2018, N. Jiang & C.M. Tian, living culture CFCC 52752 (BJFC-S1560); Tianjin City: Jizhou District, chestnut plantation, 40°06'33"N, 117°42'45"E, 185 m a.s.l., on branches of *Castanea mollissima*, 25 Apr. 2018, N. Jiang & C.M. Tian, living culture CFCC 52749 (BJFC-S1554); Tianjin City: Jizhou District, chestnut plantation, 40°06'33"N, 117°42'45"E, 185 m a.s.l., on branches of *Castanea mollissima*, 25 Apr. 2018, N. Jiang & C.M. Tian, living culture CFCC 52746 (BJFC-S1555).

**Notes.** *Dendrostoma castaneae* is the most common species in this genus occurring on the host *Castanea mollissima* in China and is associated with canker symptoms on stems and branches. As shown in Fig 2, *Dendrostoma castaneae* is the closest relative of
Species of *Dendrostoma* associated with chestnut and...

**Figure 4.** Morphology of *Dendrostoma castaneae* from *Castanea mollissima* (BJFC-S1553). **A, B** Habit of conidiomata on branches **C** Transverse section of conidioma **D** Longitudinal section through conidioma **E, G** Conidia **F** Conidiogenous cells. Scale bars: 1 mm (**A–D**); 10 µm (**E–G**).

*D. castaneicola*; however, they can be distinguished by conidial width (2.2–2.7 µm in *D. castaneae* vs. 3.2–3.8 µm in *D. castaneicola*).

*Dendrostoma castaneicola* C.M. Tian & N. Jiang, sp. nov.
MycoBank: MB826797
Figure 5

**Diagnosis.** *Dendrostoma castaneicola* differs from the two phylogenetically closely related species, *D. castaneae* and *D. shaanxiense*, by its white central column.


**Etymology.** *Castaneicola*, referring to the host genus, *Castanea.*
**Description.** Sexual morph not observed. Asexual morph: Conidiomata pycnidial, conical to pulvinate, occurring separately, reddish-orange, semi-immersed in bark, 300–550 μm high, 900–1600 μm diam.; wall of several layers of faint yellow textura angularis; central column beneath the disc more or less conical, white. Conidiophores reduced to conidiogenous cells. Conidiogenous cells lining the inner walls of the cavity, hyaline, smooth, subcylindrical to ampulliform, 5–14 × 2–3.5 μm. Conidia hyaline, aseptate, smooth, multiguttulate, thin-walled, ellipsoidal to fusoid, straight, (9.3–)10.5–12.8(–13.8) × (3.1–)3.2–3.8(–4.1) μm, l/w = (2.3–)3–4(–4.4) (n = 50).

**Culture characters.** On PDA, cultures are initially white, becoming black after 2 weeks. The colonies are flat with irregular edge; texture uniform, producing a circle with faint orange conidiomata distributed along the edge of the circle within 1 month at 25 °C in the dark.
Species of *Dendrostoma* associated with chestnut and...

**Additional specimen examined.** CHINA. Hebei Province: Chengde City, Xinglong County, chestnut plantation, 40°21'44"N, 117°51'29"E, 256 m a.s.l., on branches of *Castanea mollissima*, 27 Apr. 2018, N. Jiang & C.M. Tian, living culture CFCC 52744 (BJFC-S1552).

**Notes.** *Dendrostoma castaneicola*, *D. castaneae* and *D. shaanxiense* comprise three closely related pathogen species causing chestnut canker diseases in China, all three species occurring on *Castanea mollissima*. They differ with regard to conidiomatal characteristics, including conidial dimensions (Table 2) and the central column colour (pale yellow central column in *D. castaneae* vs. white in *D. castaneicola* vs. bright yellow in *D. shaanxiense*). Additionally, *Dendrostoma shaanxiense* was only discovered in the Shaanxi Province, whereas *D. castaneae* and *D. castaneicola* were both distributed in Hebei Province.

*Dendrostoma chinense* C.M. Tian & N. Jiang, sp. nov.
MycoBank: MB826798

**Figure 6**

**Diagnosis.** *Dendrostoma chinense* differs from *D. shandongense* by the appearance of conidiomata and is again similar to *D. shandongense* in its conidial characteristics.

**Holotype.** CHINA. Shandong Province: Rizhao City, Donggang District, chestnut plantation, 35°42'28"N, 119°46'23"E, 452 m a.s.l., on branches of *Castanea mollissima*, 14 Apr. 2017, N. Jiang (holotype: BJFC-S1563; ex-type culture: CFCC 52755).

**Etymology.** Chinense, referring to the country, China.

**Description.** Sexual morph not observed. Asexual morph: Conidiomata pycnidial, spherical, occurring separately, black, semi-immersed in bark, 250–450 µm high, 600–850 µm diam.; wall of several layers of white textura angularis. Conidiophores reduced to conidiogenous cells. Conidiogenous cells lining the inner walls of the cavity, hyaline, smooth, ampulliform, 7–14 × 1–2.5 µm. Conidia hyaline, aseptate, smooth, multiguttulate or not, thin-walled, fusoid to ellipsoid, apex acutely rounded, base truncate, (6.9–)7.7–9.1(−9.7) × (3.3–)3.4–3.7(−3.9) µm, l/w = (1.9–)2.2–2.6(−2.7) (n = 50).

**Culture characters.** On PDA, cultures are initially white, becoming olive green in the outer zone after 2 weeks. Colonies are flat with a regular edge; texture uniform within 1 month at 25 °C in the dark.


**Notes.** *Dendrostoma chinense* and *D. shandongense* have been occasionally discovered on the same branches and share similar conidial shape and dimensions. However,
Figure 6. Morphology of *Dendrostoma chinense* from *Castanea mollissima* (BJFC-S1563). A, B Habit of conidiomata on branches. C Transverse section of conidioma. D Longitudinal section through conidioma. E, G Conidia. F Conidiogenous cells. Scale bars: 1 mm (A); 0.5 mm (B–D); 10 µm (E–G).

the conidiomatal appearance of these two species is quite different (black conidiomata in *Dendrostoma chinense* vs. orange conidiomata in *D. shandongense*).

*Dendrostoma dispersum* C.M. Tian & N. Jiang, sp. nov.
MycoBank: MB826799
Figure 7

**Diagnosis.** *Dendrostoma dispersum* can be distinguished from the phylogenetically closely related *D. mali* and *D. quercinum* based on its conidial dimensions.
Species of *Dendrostoma* associated with chestnut and...


Etymology. *Dispersum*, referring to the conidiomata scattered on the bark surface.

Description. Sexual morph not observed. Asexual morph: Conidiomata pycnidial, conical to spherical, occurring separately, bright yellow, semi-immersed in bark, 500–800 µm high, 900–1500 µm diam.; wall of several layers of bright yellow *textura angularis*; central column beneath the disc conical, bright yellow. Conidiophores reduced to conidiogenous cells. Conidiogenous cells lining the inner walls of the cavity, hyaline, smooth, subcylindrical to ampulliform, 6–15 × 2.5–5 µm. Conidia hyaline, aseptate, smooth, multiguttulate, thin-walled, ellipsoid to fusoid, straight to curved, (10.9–11.1–12.2(–12.8) × (1.9–)2–2.3(–2.4) µm, l/w = (4.8–)4.9–5.9(–6.3) (n = 50).

Culture characters. On PDA, cultures are initially white, becoming faint yellow after 2 weeks. The colonies are flat with regular edge; texture uniform, producing concentric circles within 1 month at 25 °C in the dark.

![Figure 7. Morphology of *Dendrostoma dispersum* from *Quercus* sp. (BJFC-S1537). A, B Habit of conidiomata on branches C Transverse section of conidioma D Longitudinal section through conidioma E, G Conidiogenous cells F Conidia. Scale bars: 1 mm (A); 0.5 mm (B–D); 10 µm (E, F), 5 µm (G).](image-url)

Notes. Dendrostoma dispersum is phylogenetically close to D. mali and D. quercinum (Fig. 2). Conidial dimensions of Dendrostoma mali and D. quercinum were described from PDA plates (Fan et al. 2018) and D. dispersum can be differentiated from D. mali by having much longer conidia (11.1–12.2 µm in D. dispersum vs. 3–4.5 µm in D. mali) and from D. quercinum by narrower conidia (2–2.3 µm in D. dispersum vs. 2.5–3 µm in D. quercinum).

Dendrostoma parasiticum C.M. Tian & N. Jiang, sp. nov.
MycoBank: MB826822
Figure 8

Diagnosis. Dendrostoma parasiticum is distinguished from D. quercus by its shorter and narrower conidia.

Holotype. CHINA. Shaanxi Province: Shangluo City, Zhashui County, Longtougou Village, 33°39’27”N, 109°07’15”E, 2504 m a.s.l., on branches of Quercus wutaisinanica, 8 Jul. 2017, N. Jiang (holotype: BJFC-S1570; ex-type culture: CFCC 52762).

Etymology. Parasiticum, referring to the fungus causing canker diseases on different hosts.

Description. Sexual morph not observed. Asexual morph: Conidiomata pycnidial, conical to spherical, occurring separately, yellow, semi-immersed in bark, 350–600 µm high, 1000–1800 µm diam.; wall of several layers of bright yellow textura angularis; central column beneath the disc conical, bright yellow. Conidiophores reduced to conidiogenous cells. Conidiogenous cells lining the inner walls of the cavity, hyaline, smooth, subcylindrical to ampulliform, 7–12 × 2–3.5 µm. Conidia hyaline, aseptate, smooth, multiguttulate, thin-walled, fusoid, straight, (9.2–)9.3–11.7(–13.6) × (2.7–)2.8–3.3(–3.6) µm, l/w = (2.7–)3–3.9(–4.2) (n = 50).

Culture characters. On PDA, cultures are initially white, becoming dark orange after 2 weeks. The colonies are flat with irregular edge; texture uniform, producing concentric circles within 1 month at 25 °C in the dark.

Additional specimens examined. CHINA. Shaanxi Province: Shangluo City, Zhashui County, chestnut plantation, 33°39’27”N, 109°07’15”E, 2504 m a.s.l., on branches of Castanea mollissima, 8 Jul. 2017, N. Jiang, living culture CFCC 52762 (BJFC-S1569); Shaanxi Province: Ankang City, Xiangxidong Park, 32°40’32”N, 109°18’57”E, 2504 m a.s.l., on branches of Castanea mollissima, 29 Jun. 2017, N. Jiang, living culture CFCC 52763 (BJFC-S1571); Beijing City: Mentougou District, Xiaolongmen Forest Park, 39°17’25”N, 115°45’23”E, 452 m a.s.l., on branches of Castanea mollissima, 17 Aug. 2017, N. Jiang & X.L. Fan, living culture CFCC 52764 (BJFC-S1572); Beijing City: Yanqing District, Yudu Mountain, 40°53’48”N,
Species of *Dendrostoma* associated with chestnut and...

![Image](image.jpg)

**Figure 8.** Morphology of *Dendrostoma parasiticum* from *Quercus wutaishanica* (BJFC-S1570). **A, B** Habit of conidiomata on branches **C** Transverse section of conidioma **D** Longitudinal section through conidioma **E, G** Conidia **F** Conidiogenous cells. Scale bars: 2 mm (**A**); 1 mm (**B**); 0.5 mm (**C, D**); 10 µm (**E–G**).


**Notes.** *Dendrostoma parasiticum* constitutes a widely distributed species occurring on several Fagaceae tree species including *Castanea mollissima*, *Quercus aliena*, *Q. aliena* var. *acutiserrata* and *Q. wutaishanica*. *Dendrostoma parasiticum* appears to be associated with tree dieback, canker and even tree death, although its pathogenicity remains unproven. *Dendrostoma parasiticum* is close to *D. quercus* in the phylogram (Fig. 2), but differs from *D. quercus* with shorter (9.3–11.7 µm in *D. parasiticum* vs. 13.3–16.1 µm in *D. quercus*) and narrower (2.8–3.3 µm in *D. parasiticum* vs. 3.5–4.2 µm in *D. quercus*) conidia.
**Dendrostoma qinlingense** C.M. Tian & N. Jiang, sp. nov.
MycoBank: MB826823
Figure 9

**Diagnosis.** *Dendrostoma qinlingense* produces the largest conidia amongst known species of the genus.

**Holotype.** CHINA. Baoji City, Mei County, Taibai Mountain, 34°15′43″N, 107°88′42″E, 2752 m a.s.l., on branches of *Quercus wutaishanica*, 13 Jul. 2017, N. Jiang (holotype: BJFC-S1539; ex-type culture: CFCC 52732).

**Etymology.** *Qinlingense*, referring to the Qinling Mountain.

**Description.** Sexual morph not observed. Asexual morph: Conidiomata pycnidial, conical to pulvinate, occurring separately, dark yellow, semi-immersed in bark, 400–700 µm high, 1100–1600 µm diam.; wall of several layers of bright yellow textura angularis; central column beneath the disc conical, dark orange. Conidiophores reduced to conidiogenous cells. Conidiogenous cells lining the inner walls of the cavity, hyaline,

![Image of Dendrostoma qinlingense](image-url)

**Figure 9.** Morphology of *Dendrostoma qinlingense* from *Quercus wutaishanica* (BJFC-S1539). A, B Habit of conidiomata on branches C Transverse section of conidioma D Longitudinal section through conidioma E, G Conidiogenous cells F Conidia. Scale bars: 1 mm (A); 0.5 mm (B–D); 10 µm (E–G).
Species of *Dendrostoma* associated with chestnut and... 85

smooth, ampulliform, 6–22 × 2–3.5 µm. *Conidia* hyaline, aseptate, smooth, multigut-tulate, thin-walled, fusoid, straight, (15.6–)16–18(–18.6) × (3.1–)3.3–3.7(–3.8) µm, l/w = (4.2–)4.4–5.2(–5.8) (n = 50).

**Culture characters.** On PDA, cultures are initially white, exhibiting light grey after 2 weeks. The colonies are flat with irregular edge; texture uniform, producing concentric circles with sparse conidiomata irregularly distributed on the centre of the plate within 1 month at 25 °C in the dark.

**Additional specimen examined.** CHINA. Shaanxi Province: Baoji City, Mei County, Taibai Mountain, 34°15’43”N, 107°88’42”E, 2752 m a.s.l., on branches of *Quercus aliena* var. *acutiserrata*, 13 Jul. 2017, N. Jiang, living culture CFCC 52733 (BJFC-S1540).

**Notes.** *Dendrostoma qinlingense* was discovered on two *Quercus* species on the Qinling Mountain in northwest China. This species is phylogenetically related to *Dendrostoma osmanthi* on *Osmanthus fragrans*. However, *Dendrostoma qinlingense* differs from *D. osmanthi* by much larger conidia (16–18 × 3.3–3.7 µm in *D. qinlingense* vs. 7.5–10 × 2–2.5 µm in *D. osmanthi*).

**Dendrostoma quercus** C.M. Tian & N. Jiang, sp. nov.  
MycoBank: MB826824  
Figure 10

**Diagnosis.** *Dendrostoma quercus* is recognised by the existence of dimorphic conidia, which is unique in the genus.

**Holotype.** CHINA. Hebei Province: Qinhuangdao City, Zu Mountain, 40°14’13”N, 119°43’28”E, 1125 m a.s.l., on branches of *Quercus* sp., 2 May 2018, N. Jiang & C.M. Tian (holotype: BJFC-S1547; ex-type culture: CFCC 52739).

**Etymology.** *Quercus*, referring to the host genus, *Quercus*.

**Description.** *Sexual morph*: *Pseudostromata* erumpent, consisting of an inconspicuous ectostromatic disc, semi-immersed to superficial, causing a pustulate bark surface, 1000–1500 µm diam. *Ectostromatic disc* flat or concave, pale brown to brown, sometimes concealed by ostioles, surrounded by bark flaps, 400–800 µm diam.; *central column* yellowish to brownish. *Stromatic zones* lacking. *Perithecia* conspicuous, umber to fuscous black, 350–500 µm diam. *Ostioles* 5–8 per disc, flat in the disc or sometimes slightly projecting, cylindrical, covered by an orange, umber to fuscous black crust, 60–80 µm diam. *Paraphyses* slightly deliquescent. *Asci* fusoid to slightly fusiform, 8-spored, ascospores regularly disposed, with an apical ring, 55–65 × 8–11 µm. *Ascospores* hyaline, fusoid to cylindrical, smooth, often containing one guttule per cell to multiguttulate, symmetrical to asymmetrical, straight curved, bicellular, (13.4–)13.8–15.6(–16.6) × (5.1–)5.3–5.8(–5.9) µm, l/w = (2.4–)2.5–2.8(–2.9) (n = 50). *Asexual morph*: *Conidiomata* pycnidial, conical, occurring separately, pale yellow, semi-immersed in bark, 700–1000 µm high, 700–950 µm diam.; wall of several layers of pale yellow *textura angularis*; *central column* beneath the disc conical, yellow. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* lining the inner walls of the cavity, hyaline,
Figure 10. Morphology of Dendrostoma quercus from Quercus sp. (BJFC-S1547). A–C Habit of pseudostromata on branches D Transverse section of pseudostroma E, H Habit of conidiomata on branches F Transverse section of conidioma G Longitudinal section through conidioma I Conidiogenous cells producing dimorphic conidia J Secondary conidia K Asci and ascospores L Ascospores M Primary conidia. Scale bars: 1 mm (A, H); 0.5 mm (B–G); 10 µm (I, K–M); 5 µm (J).

smooth, subcylindrical to ampulliform, 4.5–9 × 2–4 µm. Conidia hyaline, aseptate, smooth, multiguttulate, thin-walled, dimorphic, type one (> 99%) ellipsoid to fusoid, straight to curved, (11–)13.3–16.1(–16.9) × (3.4–)3.5–4.2(–4.5) µm, l/w = (2.6–)3.3–4.4(–4.9) (n = 50); type two (< 1%) fusoid, apex acutely rounded, 13–16 × 4–6 µm.
Species of *Dendrostoma* associated with chestnut and...

**Culture characters.** On PDA, cultures are initially white, becoming dark grey after 2 weeks. The colonies are flat with irregular edge; texture uniform, producing concentric circles with sparse conidiomata irregularly distributed within 1 month at 25 °C in the dark.

**Additional specimens examined.** CHINA. Hebei Province: Qinhuangdao City, Zu Mountain, 40°14′13″N, 119°43′28″E, 1125 m a.s.l., on branches of *Quercus* sp., 2 May 2018, N. Jiang & C.M. Tian, living culture CFCC 52734 (BJFC-S1548); Hebei Province: Qinhuangdao City, Zu Mountain, 40°14′13″N, 119°43′28″E, 1125 m a.s.l., on branches of *Quercus* sp., 2 May 2018, N. Jiang & C.M. Tian, living culture CFCC 52735 (BJFC-S1541); Hebei Province: Qinhuangdao City, Zu Mountain, 40°14′13″N, 119°43′28″E, 1125 m a.s.l., on branches of *Quercus* sp., 2 May 2018, N. Jiang & C.M. Tian, living culture CFCC 52736 (BJFC-S1542); Hebei Province: Qinhuangdao City, Zu Mountain, 40°14′13″N, 119°43′28″E, 1125 m a.s.l., on branches of *Quercus* sp., 2 May 2018, N. Jiang & C.M. Tian, living culture CFCC 52737 (BJFC-S1543); Hebei Province: Qinhuangdao City, Zu Mountain, 40°14′13″N, 119°43′28″E, 1125 m a.s.l., on branches of *Quercus* sp., 2 May 2018, N. Jiang & C.M. Tian, living culture CFCC 52738 (BJFC-S1544); Hebei Province: Qinhuangdao City, Zu Mountain, 40°14′13″N, 119°43′28″E, 1125 m a.s.l., on branches of *Quercus* sp., 2 May 2018, N. Jiang & C.M. Tian, living culture CFCC 52740 (BJFC-S1545).

**Notes.** *Dendrostoma quercus* is associated with oak branch cankers and forms both sexual and asexual fruiting structures beneath cankered bark. Within the genus, *D. quercus* produces the second largest conidia, smaller only than those of *D. qinlingense* (Table 2). The presence of dimorphic conidia in *Dendrostoma*, however, is a feature unique to *D. quercus*.

---

**Dendrostoma shaanxiense** C.M. Tian & N. Jiang, sp. nov.
MycoBank: MB826825
Figure 11

**Diagnosis.** *Dendrostoma shaanxiense* is distinguished from the closely related species *D. castaneae* by smaller l/w ratio and from *D. castaneicola* by its narrower conidia.

**Holotype.** CHINA. Shaanxi Province: Ankang City, Xiangxidong Park, 32°40′32″N, 109°18′57″E, 1079 m a.s.l., on branches of *Castanea mollissima*, 1 Jul. 2017, N. Jiang (holotype: BJFC-S1549; ex-type culture: CFCC 52741).

**Etymology.** *Shaanxiense*, referring to the Shaanxi Province in China.

**Description.** **Sexual morph** not observed. **Asexual morph: Conidiomata** pycnidial, conical to pulvinate, occurring separately, dark orange, semi-immersed in bark, 350–650 µm high, 1050–1400 µm diam.; wall of several layers of bright yellow *textura angularis*; central column beneath the disc conical, bright yellow. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* lining the inner walls of the cavity, hyaline, smooth, subcylindrical to ampulliform, 5–11 × 2.5–3.5 µm. *Conidia* hyaline, aseptate, smooth, multiguttulate, thin-walled, ellipsoid to fusoid, straight to curved, (8.6–)9.5–11.1(–11.7) × (2.3–)2.5–3.1(–3.4) µm, l/w = (2.8–)3.3–4.2(–4.9) (n = 50).
Figure 11. Morphology of *Dendrostoma shaanxiense* from *Castanea mollissima* (BJFC-S1549). **A, B** Habit of conidiomata on branches **C** Transverse section of conidioma **D** Longitudinal section through conidioma **E, G** Conidia **F** Conidiogenous cells. Scale bars: 1 mm (**A**); 0.5 mm (**B–D**); 10 µm (**E–G**).

**Culture characters.** On PDA, cultures are initially white, turning purple after 2 weeks on PDA. The colonies are flat with irregular edge; texture uniform, producing concentric circles within 1 month at 25 °C in the dark.

**Additional specimen examined.** Shaanxi Province: Ankang City, Xiangxidong Park, 32°40'32"N, 109°18'57"E, 1079 m a.s.l., on branches of *Castanea mollissima*, 1 Jul. 2017, N. Jiang, CFCC 52742 (BJFC-S1550).

**Notes.** *Dendrostoma shaanxiense*, *D. castaneae* and *D. castaneicola* are phylogenetically closely related species occurring on the same host, *Castanea mollissima* (Fig. 2). However, *Dendrostoma shaanxiense* has conidia with a smaller l/w ratio than *D. castaneae* (3.3–4.2 in *D. shaanxiense* vs. 4.2–5.2 in *D. castaneae*) and has narrower conidia than *D. castaneicola* (2.5–3.1 µm diam. in *D. shaanxiense* vs. 3.2–3.8 µm diam. in *D. castaneicola*).
**Dendrostoma shandongense** C.M. Tian & N. Jiang, sp. nov.
MycoBank: MB826826

Figure 12

**Diagnosis.** *Dendrostoma shandongense* is distinguished from its closest relative *D. chinensis* by the colour of conidiomata.

**Holotype.** CHINA. Shandong Province: Rizhao City, Donggang District, chestnut plantation, 35°42'28"N, 119°46'23"E, 452 m a.s.l., on branches of *Castanea mollissima*, 14 Apr. 2017, N. Jiang (holotype: BJFC-S1567; ex-type culture: CFCC 52759).

**Etymology.** *Shandongense*, referring to the Shandong Province in China.

**Description.** Sexual morph not observed. Asexual morph: Conidiomata pycnidial, spherical, occurring separately, reddish-orange, semi-immersed in bark, 250–400 μm

---

**Figure 12.** Morphology of *Dendrostoma shandongense* from *Castanea mollissima* (BJFC-S1567). **A–C** Habit of conidiomata on branches **D** Transverse section of conidium **E** Longitudinal section through conidioma **F** Conidiogenous cells **G** Conidia. Scale bars: 1 mm (**A**); 0.3 mm (**B–D**); 5 μm (**F**); 5 μm (**G**).
Table 2. Conidial size of *Dendrostoma* species from natural host barks, species with * were measured from conidia produced in PDA.

<table>
<thead>
<tr>
<th>Species</th>
<th>Conidial length (µm)</th>
<th>Conidial width (µm)</th>
<th>Length/width ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dendrostoma aurorae</em></td>
<td>8.1–9.8</td>
<td>2.3–2.6</td>
<td>3.2–4.1</td>
</tr>
<tr>
<td><em>Dendrostoma castaneae</em></td>
<td>10.4–12.3</td>
<td>2.2–2.7</td>
<td>4.2–5.2</td>
</tr>
<tr>
<td><em>Dendrostoma castaneicola</em></td>
<td>10.5–12.8</td>
<td>3.2–3.8</td>
<td>3–4</td>
</tr>
<tr>
<td><em>Dendrostoma chinense</em></td>
<td>7.7–9.1</td>
<td>3.4–3.7</td>
<td>2.2–2.6</td>
</tr>
<tr>
<td><em>Dendrostoma dispersum</em></td>
<td>11.1–12.2</td>
<td>2–2.3</td>
<td>4.9–5.9</td>
</tr>
<tr>
<td><em>Dendrostoma mali</em></td>
<td>3.5–4.5</td>
<td>2–2.5</td>
<td>NA</td>
</tr>
<tr>
<td><em>Dendrostoma osmanthi</em></td>
<td>7.5–10.5</td>
<td>2–2.5</td>
<td>NA</td>
</tr>
<tr>
<td><em>Dendrostoma parasiticum</em></td>
<td>9.3–11.7</td>
<td>2.8–3.3</td>
<td>3–3.9</td>
</tr>
<tr>
<td><em>Dendrostoma qinlingense</em></td>
<td>16–18</td>
<td>3.3–3.7</td>
<td>4.4–5.2</td>
</tr>
<tr>
<td><em>Dendrostoma quercinum</em></td>
<td>10.5–14</td>
<td>2.5</td>
<td>NA</td>
</tr>
<tr>
<td><em>Dendrostoma quercus</em></td>
<td>13.3–16.1</td>
<td>3.5–4.2</td>
<td>3.3–4.4</td>
</tr>
<tr>
<td><em>Dendrostoma shaanxiense</em></td>
<td>9.5–11.1</td>
<td>2.5–3.1</td>
<td>3.3–4.2</td>
</tr>
<tr>
<td><em>Dendrostoma shandongense</em></td>
<td>8.1–8.8</td>
<td>3.8–4.3</td>
<td>1.9–2.3</td>
</tr>
</tbody>
</table>

high, 450–650 µm diam.; wall of several layers of black *textura angularis*. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* lining the inner walls of cavity, hyaline, smooth, ampulliform, 6.5–13 × 1–2.5 µm. *Conidia* hyaline, aseptate, smooth, multiguttulate, thin-walled, fusoid to ellipsoid, apex acutely rounded, base truncate, (7.8–)8.1–8.8(–9) × (3.7–)3.8–4.3(–4.8) µm, l/w = (1.6–)1.9–2.3(–2.4) (n = 50).

**Culture characters.** On PDA, cultures are white. The colonies are flat with irregular edge; texture uniform, producing sparse conidiomata irregularly distributed near the centre of the plate within 1 month at 25 °C in the dark.

**Additional specimen examined.** Shandong Province: Rizhao City, Donggang District, chestnut plantation, 35°42’28"N, 119°46’23"E, 452 m a.s.l., on branches of *Castanea mollissima*, 14 Apr. 2017, N. Jiang, CFCC 52760 (BJFC-S1568).

**Notes.** *Dendrostoma shandongense* and *D. chinensis* occasionally occur on the same branches. These species are best distinguished by the appearance of their conidiomata, which are black in *Dendrostoma chinense* and orange in *D. shandongense*.

**Discussion**

In this study, we reviewed the taxonomic circumscription of *Dendrostoma* using molecular and morphological data. This is the first study that presents a robust phylogeny using a number of *Dendrostoma* isolates from different geographic origins. The results revealed up to 14 species in *Dendrostoma* based on the observation of type specimens and ex-type cultures (*D. leiphaemia* was not observed), of which 10 species were shown to represent new species, namely *D. aurorae, D. castaneae, D. castaneicola, D. chinense, D. dispersum, D. parasiticum, D. qinlingense, D. quercus, D. shaanxiense* and *D. shandongense*.

The 13 type specimens in *Dendrostoma* (except *D. leiphaemia*) were examined to establish robust morphological characteristics amongst specific ranks. Amongst these, 3 species, *Dendrostoma mali, D. osmanthi* and *D. quercinum*, were discovered.
Species of *Dendrostoma* associated with chestnut and...


...to only have a sexual morph on natural hosts; 9 species, *D. aurorae*, *D. castaneae*, *D. castaneicola*, *D. chinense*, *D. dispersum*, *D. parasiticum*, *D. qinlingense*, *D. shaanxiense* and *D. shandongense*, were observed with only an asexual morph and only one species, *D. quercus*, was represented by both asexual and sexual morphs. Hence,
morphological differences amongst *Dendrostoma* species were mainly established based on conidiomata produced on diseased host tissues, including colours of conidiomata, culture characteristics (Fig. 13), existence or non-existence of a central column, conidial shape and dimensions.

*Dendrostoma shandongense* and *D. chinense* are similar in conidial shape and size, but differ markedly from the other species. Additionally, *Dendrostoma shandongense* and *D. chinense* comprise the only two species in the genus with conidiomata lacking a central column structure, although they differ considerably with regard to in conidiomatal appearance (Figs. 6, 13). The remaining eight species differ by the existence of a central column inside the conidiomata and can be further distinguished by their conidial characteristics, namely length, width and l/w ratio. Additionally, a key to the 14 *Dendrostoma* species is provided below.

### Key to *Dendrostoma* species

<table>
<thead>
<tr>
<th></th>
<th>Asexual morphs with or without sexual morphs known from natural substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Only sexual morph known from natural substrates</td>
</tr>
<tr>
<td>2</td>
<td>Central column absent, length/width ratio of conidia &lt; 3</td>
</tr>
<tr>
<td>3</td>
<td>Central column present, length/width ratio of conidia &gt; 3</td>
</tr>
<tr>
<td>4</td>
<td>Conidiomata orange</td>
</tr>
<tr>
<td>5</td>
<td>Conidiomata black</td>
</tr>
<tr>
<td>6</td>
<td>Conidia dimorphic</td>
</tr>
<tr>
<td>7</td>
<td>Conidia monomorphic</td>
</tr>
<tr>
<td>8</td>
<td>Conidial length &gt; 15 µm</td>
</tr>
<tr>
<td>9</td>
<td>Conidial length &lt; 15 µm</td>
</tr>
<tr>
<td>10</td>
<td>Conidial length/width ratio &gt; 4.2</td>
</tr>
<tr>
<td>11</td>
<td>Conidial length/width ratio &lt; 4.2</td>
</tr>
<tr>
<td>12</td>
<td>Conidial length/width ratio 4.2–5.2, conidial width 2.2–2.7 µm</td>
</tr>
<tr>
<td>13</td>
<td>Conidial length/width ratio 4.9–5.9, conidial width 2–2.3 µm</td>
</tr>
<tr>
<td>14</td>
<td>Conidial width/width ratio &gt; 4.2</td>
</tr>
<tr>
<td>15</td>
<td>Conidial width/width ratio &lt; 4.2</td>
</tr>
<tr>
<td>16</td>
<td>Conidial width 2.8–3.3 µm, length/width ratio 3–3.9</td>
</tr>
<tr>
<td>17</td>
<td>Conidial width 2.5–3.1 µm, length/width ratio 3.3–4.2</td>
</tr>
<tr>
<td>18</td>
<td>Ascospores width &gt; 5 µm</td>
</tr>
<tr>
<td>19</td>
<td>Ascospores width &lt; 5 µm</td>
</tr>
<tr>
<td>20</td>
<td>Ascospores length &gt; 15 µm</td>
</tr>
<tr>
<td>21</td>
<td>Ascospores length &lt; 15 µm</td>
</tr>
<tr>
<td>22</td>
<td>On <em>Osmanthus</em>, Ascospores 11.5–14.5 × 3.5–4 µm</td>
</tr>
<tr>
<td>23</td>
<td>On <em>Malus</em>, Ascospores 12–14 × 3–4 µm</td>
</tr>
</tbody>
</table>
The genus *Dendrostoma* was initially proposed to include three presumed plant pathogens causing canker diseases on hardwood trees, namely *D. mali* on *Malus spectabilis*, *D. osmanthi* on *Osmanthus fragrans* and *D. quercinum* on *Quercus acutissima* (Fan et al. 2018). Consistent with the previous study, the newly described 10 species were all isolated from fruiting structures associated with typical canker symptoms on several hardwood tree species, namely *Castanea mollissima* and *Quercus* spp.

The tree genera *Castanea* and *Quercus* in Fagaceae contain numerous important and common tree species in China, including *C. mollissima*, *C. crenata*, *C. henryi*, *C. seguinii*, *Q. acutissima*, *Q. aliena*, *Q. dentata*, *Q. mongolica* and *Q. wutaishanica* (Flora of China website: http://frps.eflora.cn/). *Castanea mollissima* constitutes one of the most important crop tree species widely cultivated in 26 provinces in China. However, many plantations and nurseries planting Chinese chestnut suffer from fungal diseases that cause high production losses (Jiang et al. 2018). In particular, chestnut blight caused by *Cryphonectria parasitica* represents the most serious fungal disease, reducing host vitality and potentially killing the host (Jiang et al. 2018, Rigling and Prospero 2018).

In the present study, seven *Dendrostoma* species were observed on the host *Castanea mollissima* including *D. aurorae*, *D. castaneae*, *D. castaneicola*, *D. chinense*, *D. parasiticum*, *D. shaanxiense* and *D. shandongense*, causing chestnut canker diseases, termed *Dendrostoma* canker herein. *Dendrostoma* canker constitutes a newly discovered disease that has been observed in chestnut plantations and nurseries. Species of *Dendrostoma* usually infect host branches and stems, with occasional infection of twigs. Maturation of the fruiting structures from June to July resulted in death of the infected branches. Notably, no sexual fruiting structures were discovered during our investigations on chestnut trees.

Accurate recognition and identification of plant diseases are essential as fungal pathogens are constantly evolving and traditional control methods are frequently insufficient for disease control. In comparison, in the present study, *Dendrostoma* canker is considered to be caused by up to eight different species of *Dendrostoma*. Further studies are, however, required to confirm their pathogenicity and fully resolve their ecology.

**Acknowledgements**

This study was financed by the National Natural Science Foundation of China (Project No.: 31670647). We thank Yingmei Liang [Museum of Beijing Forestry University (BJFC), Beijing Forestry University], Chungen Piao and Minwei Guo [China Forestry Culture Collection Center (CFCC), Chinese Academy of Forestry, Beijing] for the preservation of materials studied during this study.

**References**


Species of *Dendrostoma* associated with chestnut and...


Four new corticioid species in Trechisporales (Basidiomycota) from East Asia and notes on phylogeny of the order

Shi-Liang Liu¹, Hai-Xia Ma², Shuang-Hui He¹, Yu-Cheng Dai¹

¹ Institute of Microbiology, Beijing Forestry University, Beijing 100083, China ² Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Hainan Key Laboratory of Tropical Microbe Resources, Haikou 571101, China

Corresponding author: Shuang-Hui He (shuanghuihe@yahoo.com); Yu-Cheng Dai (yuchengd@yahoo.com)

Academic editor: O. Raspé | Received 27 November 2018 | Accepted 21 February 2019 | Published 8 March 2019


Abstract

Four new species in Trechisporales from East Asia, Dextrinocystis calamicola, Subulicystidium acerosum, S. tropicum and Tubulicium bambusicola, are described and illustrated, based on morphological and molecular evidence. The phylogeny of Trechisporales was inferred from a combined dataset of ITS-nrLSU sequences. In the phylogenetic tree, Sistotremastrum formed a family-level clade of its own, sister to the Hydnodontaceae clade formed by all other genera. Dextrinocystis, is for the first time, confirmed as a member of Hydnodontaceae. A key to all the accepted genera in Trechisporales is given.

Keywords

Hydnodontaceae, Sistotremastrum family, phylogeny, taxonomy, wood-inhabiting fungi

Introduction

Trechisporales K.H. Larss. is a rather small but strongly supported order in Agaricomycotina (Hibbett et al. 2007; Larsson 2007). At present, eight to twelve genera, Brevicellicium K.H. Larss. & Hjortstam, Fibriciellum J. Erikss. & Ryvarden, Fibrodon...
Parmasto, **Luellia** K.H. Larss. & Hjortstam, **Porpomyces** Jülich, **Subulicystidium** Parmasto, **Trechispora** P. Karst. (type genus, including **Cristelloporia** I. Johans. & Ryvarden, **Echinotrema** Park-Rhodes, **Hydnodon** Banker and **Scytinopogon** Singer) and **Tubulicium** Oberw., are placed in the family Hydnodontaceae, while **Sistotremastrum** J. Erikss. should be placed in a family of its own (Larsson 2001, 2007; Larsson et al. 2004; Binder et al. 2005; Hibbett et al. 2007, 2014; Birkebak et al. 2013; Telleria et al. 2013a). In addition, four genera, **Dextrinocystis** Gilb. & M. Blackw., **Dextrinodontia** Hjortstam & Ryvarden, **Brevicellopsis** Hjortstam & Ryvarden and **Litschauerella** Oberw. were listed as possible candidates of Hydnodontaceae waiting for molecular confirmation (Larsson 2007; Hibbett et al. 2014). Except for **Scytinopogon** and **Trechispora thelephora** (Lév.) Ryvarden, all the taxa in Trechisporales have resupinate basidiomata and most of them have a non-poroid hymenophore (Fig. 1, Albee-Scott and Kropp 2011; Hibbett et al. 2014). However, the microscopic characters vary significantly amongst different genera and some of them were surprisingly placed in the order solely based on molecular phylogeny (Larsson 2007; Bernicchia and Gorjón 2010).

Except for **Trechispora**, the largest genus in the order, most genera in Trechisporales have mostly few species and some are still monotypic. However, in recent years, many new species have been described, based on both DNA sequence data and morphological characters. Wu et al. (2015) described a cryptic species of **Porpomyces mucidus** (Pers.) Jülich, based mainly on sequence data. Ordynets et al. (2018) studied the short-spored species of **Subulicystidium** and recognised eleven new species. Tens specimens of Trechisporales were collected from East Asia by the senior authors in the past three years. The purposes of the present paper are to study these specimens by using morphological and molecular methods and discuss the phylogeny of the Trechisporales, based on expanded sampling.

**Materials and methods**

**Morphological studies**

Voucher specimens were deposited in the herbaria of Beijing Forestry University, Beijing, China (BJFC) and in the Centre for Forest Mycology Research, U.S. Forest Service, Madison, USA (CFMR). Freehand sections were made from dried basidiomata and mounted in 0.2% cotton blue in lactic acid, 1% phloxine (w/v) or Melzer’s reagent. Microscopic examinations were carried out with a Nikon Eclipse 80i microscope (Nikon Corporation, Japan) at magnifications up to 1000x. Drawings were made with the aid of a drawing tube. All measurements were carried out with sections mounted in Melzer’s reagent. The following abbreviations are used: \( L = \) mean spore length, \( W = \) mean spore width, \( Q = L/W \) ratio, \( n (a/b) = \) number of spores (a) measured from given number of specimens (b). Colour names and codes follow Kornerup and Wanscher (1978).
DNA extraction and sequencing

The CTAB plant genome rapid extraction kit DN14 (Aidlab Biotechnologies Co. Ltd, Beijing) was used for DNA extraction and PCR amplification from dried specimens. The ITS1-5.8S-ITS2 and partial nrLSU markers were amplified with the primer pairs ITS5/ITS4 (White et al. 1990) and LR0R/LR7 (Vilgalys and Hester 1990). The PCR procedures followed Liu et al. (2017). DNA sequencing was performed at Beijing Genomics Institute and the sequences were deposited in GenBank (Benson et al.
The sequence quality control followed Nilsson et al. (2012). BioEdit v.7.0.5.3 (Hall 1999) and Geneious v.11.1.15 (Kearse et al. 2012) were used for chromatogram check and contig assembly.

**Phylogenetic analyses**

The molecular phylogeny was inferred from a combined dataset of ITS1-5.8S-ITS2-nrLSU sequences of Trechisporales sensu Larsson (2007) (Table 1). *Hyphodontia floc-cosa* (Bourdot & Galzin) J. Erikss. and *H. subalutacea* (P. Karst.) J. Erikss. were selected as the outgroup (Wu et al. 2015). The sequences of ITS and nrLSU were aligned separately using MAFFT v.7 (Katoh et al. 2017, http://mafft.cbrc.jp/alignment/server/) with the G-INS-i iterative refinement algorithm. The separate alignments were concatenated using Mesquite v.3.5.1 (Maddison and Maddison 2018). The combined alignments were deposited in TreeBase (http://treebase.org/treebase-web/home.html, submission ID: 23620).

For both Maximum Likelihood (ML) and Bayesian Inference (BI), a partitioned analysis was performed with the following four partitions: ITS1, 5.8S, ITS2 and nrLSU. The ML analysis was performed using RAxML v.8.2.10 (Stamatakis 2014) with the bootstrap values (ML-BS) obtained from 1,000 replicates and the GTR-GAMMA model of nucleotide evolution. The BI was performed using MrBayes 3.2.6 (Ronquist et al. 2012). The best-fit substitution model for each partitioned locus was estimated separately with jModelTest v.2.17 (Darriba et al. 2012) by restricting the search to models that can be implemented in MrBayes. Two runs of four Markov chains were run for 4,000,000 generations until the split deviation frequency value was lower than 0.01. The convergence of the runs was checked using Tracer v.1.7 (Rambaut et al. 2018). Trees and model parameters were sampled every 100th generation. The first quarter of the trees, which represented the burn-in phase of the analyses, was discarded and the remaining trees were used to build a majority rule consensus tree and to calculate Bayesian posterior probabilities (BPP). All trees were visualised in FigTree 1.4.2 (Rambaut 2014).

**Results**

**Phylogenetic inference**

The ITS-nrLSU sequence dataset contained 50 ITS and 51 nrLSU sequences from 58 samples representing 45 ingroup taxa and the outgroup (Table 1). Fourteen ITS and 15 nrLSU sequences were generated for this study. jModelTest suggested GTR+G, SYM+I+G, GTR+I+G and GTR+I+G to be the best-fit models of nucleotide evolution for ITS1, 5.8S, ITS2 and nrLSU markers, respectively, for the Bayesian analysis. BI analysis resulted in an almost identical tree topology compared to the ML analysis.
**Table 1.** Species and sequences used in the phylogenetic analyses.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Voucher</th>
<th>Locality</th>
<th>ITS</th>
<th>nrLSU</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brevicellicium exile</em></td>
<td>MA-Fungi 26554</td>
<td>Spain</td>
<td>HE963777</td>
<td>HE963778</td>
<td>Telleria et al. (2013a)</td>
</tr>
<tr>
<td><em>B. olivascens</em></td>
<td>MA-Fungi 41366</td>
<td>Spain</td>
<td>HE963785</td>
<td>HE963786</td>
<td>Telleria et al. (2013a)</td>
</tr>
<tr>
<td><em>B. sp</em></td>
<td>MPM 2012</td>
<td>Portugal</td>
<td>–</td>
<td>HE963774</td>
<td>Telleria et al. (2013a)</td>
</tr>
<tr>
<td><em>Dextrinocystis calamicola</em></td>
<td>BJFC: He 5693</td>
<td>China</td>
<td>MK204533</td>
<td>MK204546</td>
<td>This study</td>
</tr>
<tr>
<td><em>D. calamicola</em></td>
<td>BJFC: He 5700</td>
<td>China</td>
<td>MK204534</td>
<td>MK204547</td>
<td>This study</td>
</tr>
<tr>
<td><em>Fibroadontia alba</em></td>
<td>TNM: F25503</td>
<td>Taiwan</td>
<td>JQ612713</td>
<td>JQ612714</td>
<td>Yurchenko and Wu (2014)</td>
</tr>
<tr>
<td><em>E. alba</em></td>
<td>BJFC: He 4761</td>
<td>China</td>
<td>MK204529</td>
<td>MK204541</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. brevidens</em></td>
<td>TNM: Wu 9807-16</td>
<td>Taiwan</td>
<td>KC928276</td>
<td>KC928277</td>
<td>Yurchenko and Wu (2014)</td>
</tr>
<tr>
<td><em>E. gesypina</em></td>
<td>BJFC: He 3559</td>
<td>China</td>
<td>MK204528</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td><em>Hyphodontia floccosa</em></td>
<td>AFTOL-ID 599</td>
<td>–</td>
<td>DQ249274</td>
<td>AY646100</td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>H. subalutacea</em></td>
<td>GEL 2196</td>
<td>–</td>
<td>DQ340341</td>
<td>DQ340362</td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>Litschauerella sp.</em></td>
<td>BJFC: He 3171</td>
<td>China</td>
<td>MK204555</td>
<td>MK204566</td>
<td>This study</td>
</tr>
<tr>
<td><em>Porphyrosema mucidus</em></td>
<td>BJFC: Dai 12692</td>
<td>Czech Republic</td>
<td>KT157833</td>
<td>KT157838</td>
<td>Wu et al. (2015)</td>
</tr>
<tr>
<td><em>P. submucicula</em></td>
<td>BJFC: Cui 5183</td>
<td>China</td>
<td>KT152143</td>
<td>KT152145</td>
<td>Wu et al. (2015)</td>
</tr>
<tr>
<td><em>Sulicystidium boidinii</em></td>
<td>KAS: L 1584a</td>
<td>Reunion</td>
<td>MH041527</td>
<td>–</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. acrusum</em></td>
<td>BJFC: He 3804</td>
<td>China</td>
<td>MK204539</td>
<td>MK204543</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. brachyporum</em></td>
<td>O: F: KHL 16100</td>
<td>Brazil</td>
<td>MH000599</td>
<td>MH000599</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. calamicola</em></td>
<td>BJFC: He 2207</td>
<td>USA</td>
<td>MK204532</td>
<td>MK204549</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. fusiform</em></td>
<td>GB: KHL 10360</td>
<td>Puerto Rico</td>
<td>MK041535</td>
<td>MK041567</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. harpagum</em></td>
<td>KAS: L 1726a</td>
<td>Reunion</td>
<td>MH041532</td>
<td>MH041588</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. inornatum</em></td>
<td>GB: KHL 10444</td>
<td>Puerto Rico</td>
<td>MK041558</td>
<td>MK041569</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. longisporum</em></td>
<td>GB: KHL 14229</td>
<td>Sweden</td>
<td>MK000601</td>
<td>MK000601</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. merideae</em></td>
<td>BJFC: He 2981</td>
<td>China</td>
<td>–</td>
<td>MK04550</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. nana</em></td>
<td>GB: Hjm 16400</td>
<td>Brazil</td>
<td>MK041538</td>
<td>MK041604</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. nikau</em></td>
<td>KAS: L 1296</td>
<td>Reunion</td>
<td>MH041513</td>
<td>MH041565</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. obtusisporum</em></td>
<td>Fr: Piepenbrink &amp; Lotz-Winter W213-3-1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. parvisporum</em></td>
<td>KAS: L 0140</td>
<td>Reunion</td>
<td>MH041529</td>
<td>MH041590</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. perlongisporum</em></td>
<td>TU 124388</td>
<td>Italy</td>
<td>UDB028355</td>
<td>UDB028355</td>
<td>Köljalg et al. (2013)</td>
</tr>
<tr>
<td><em>S. niveocremeum</em></td>
<td>GB: KHL 10602</td>
<td>Brazil</td>
<td>MH000600</td>
<td>MH000600</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. nivocrystallinum</em></td>
<td>O: F: 918488</td>
<td>Colombia</td>
<td>MH041512</td>
<td>MH041564</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. tedderoi</em></td>
<td>TU 110894</td>
<td>Vietnam</td>
<td>UDB014161</td>
<td>–</td>
<td>Köljalg et al. (2013)</td>
</tr>
<tr>
<td><em>S. tropicum</em></td>
<td>BJFC: He 3968</td>
<td>China</td>
<td>MK204531</td>
<td>MK204544</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. rarocrystallinum</em></td>
<td>BJFC: He 3583</td>
<td>China</td>
<td>MK204530</td>
<td>MK204542</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. suecicum</em></td>
<td>KAS: L 1584a</td>
<td>Reunion</td>
<td>MH041529</td>
<td>MH041590</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. havencampii</em></td>
<td>TFB 13611</td>
<td>USA</td>
<td>–</td>
<td>Q684661</td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>S. parvisporum</em></td>
<td>KAS: L 0140</td>
<td>Reunion</td>
<td>MH041529</td>
<td>MH041590</td>
<td>Ordynets et al. (2018)</td>
</tr>
<tr>
<td><em>S. pallescens</em></td>
<td>BJFC: He 5192</td>
<td>Vietnam</td>
<td>–</td>
<td>MK204553</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. pallescens</em></td>
<td>MA-Fungi 82105</td>
<td>Portugal</td>
<td>JX10445</td>
<td>–</td>
<td>Telleria et al. (2013b)</td>
</tr>
<tr>
<td><em>S. gattilferum</em></td>
<td>BJFC: He 3338</td>
<td>China</td>
<td>MK204540</td>
<td>MK204552</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. niveocremeum</em></td>
<td>CBS 427.54</td>
<td>France</td>
<td>MH857380</td>
<td>MH868920</td>
<td>Vu et al. (2019)</td>
</tr>
<tr>
<td><em>S. suecicum</em></td>
<td>GB: KHL11849</td>
<td>Sweden</td>
<td>EU118666</td>
<td>EU118667</td>
<td>Larsson (2007)</td>
</tr>
<tr>
<td><em>Trechispora crassicornis</em></td>
<td>AFTOL-ID 665</td>
<td>–</td>
<td>–</td>
<td>AY635768</td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>T. araneosa</em></td>
<td>GB: KHL 8570</td>
<td>Sweden</td>
<td>AF347084</td>
<td>AF347084</td>
<td>Larsson et al. (2004)</td>
</tr>
<tr>
<td><em>T. bispora</em></td>
<td>CBS 142.63</td>
<td>Australia</td>
<td>MH858241</td>
<td>MH869842</td>
<td>Vu et al. (2019)</td>
</tr>
</tbody>
</table>
and no significant conflicts were found between the two analyses. Only the ML tree is shown in Fig. 2 with ML bootstrap values ≥ 50% and Bayesian posterior probabilities ≥ 0.95 labelled along the branches.

In the tree (Fig. 2), two large clades, corresponding to Hydnodontaceae and Sistotremastrum family, were strongly supported. Except for Sistotremastrum, the other eight genera sampled were nested within the Hydnodontaceae clade. The genera Brevicellicium, Fibrodontia, Porpomyces and Subulicystidium were strongly supported as monophyletic lineages. Dextrinocystis calamicola, the first species sequenced in the genus, formed a sister lineage to Tubulicium with relatively strong support (ML-BS = 78%, BPP = 1). The three species of Scytinopogon were nested within the Trechispora lineage. Subulicystidium acerosum and S. tropicum formed distinct lineages in the genus, while Tubulicium bambusicola is closely related to T. raphidisporum.

Taxonomy

Dextrinocystis calamicola S.H. He & S.L. Liu, sp. nov.
Mycobank: MB 828718

Fig. 3


Etymology. “calamicola” refers to growing on Calamus.

Basidiomata. Annual, resupinate, effused, thin, soft, easily separated from the substrate, at first as irregular small patches, later confluent up to 15 cm long, 2 cm wide. Hymenophore surface smooth, orange white (5A2) to greyish-orange [5B(3–5)], finely cracked with age; margin thinning out, fimbriate, slightly paler than hymenophore surface, becoming indistinct with age.

Microscopic structures. Hyphal system monomitic; generative hyphae with clamp connections, hyaline, thin-walled, frequently branched and septate, loosely interwoven, 2–3 µm in diam. Cystidia-like branches present, branched from subicular hyphae, embedded, hyaline, thick-walled, encrusted at apex, 20–30 × 1.5–2 µm. Hy-
menial cystidia abundant, subulate, projecting beyond hymenium, bi- or multi-rooted, hyaline, distinctly thick-walled with a narrow lumen, slightly encrusted at apex, distinctly dextrinoid, 50–110 × 5–6 µm. Basidia suburniform to subclavate, hyaline,
Figure 3. *Dextrinocystis calamicola* (holotype, He 5701). a basidiomata b, f basidiospores c, g basidia d, h cystidia e, i cystidia-like branches in subiculum j subicular hyphae. Scale bars: 1 cm (a), 10 µm (b–j). b, c Taken in phloxine d, e taken in Melzer’s reagent.

thin-walled, with 4 sterigmata and a basal clamp connection, 20–30 × 5–8 µm; sterigmata mostly cylindrical with a blunt tip; basidioles in shape similar to basidia, but slightly smaller. Basidiospores abundant, oblong ellipsoid to short cylindrical, hyaline, thin-walled, smooth, negative in Melzer’s reagent, acyanophilous, (7–)7.5–8.8(–9) × (3.2–)3.3–4 µm, L = 8.1 µm, W = 3.7 µm, Q = 2.1–2.2 (n = 60/2).

Additional specimens examined. CHINA. Fujian Province, Wuyishan County, Wuyishan Nature Reserve, on dead culms of *Calamus*, 3 Oct 2018, He 5693 (BJFC 026755) & He 5700 (BJFC 026762).

Remarks. The thin whitish basidiomata on a palm tree, distinctly thick-walled cystidia with a dextrinoid reaction in Melzer’s reagent, presence of small cystidia-like branches and short cylindrical basidiospores indicate that the new species is a member of *Dextrinocystis*. Two species, *D. capitata* (D.P. Rogers & Boquiren) Gilb. & M. Blackw. and *D. macrospora* (Liberta) Nakasone have been reported in the genus, both
of which differ from *D. calamicola* by having much larger basidiospores (11–14 × 3–4 µm for *D. capitata* in Gilbertson and Blackwell 1988; 12–19 × 4.5–7 µm for *D. macrospora* in Liberta 1960) and a distribution in America. In the phylogenetic tree, *D. calamicola* formed a sister lineage to *Tubulicium* with relatively strong support (Fig. 2).

**Subulicystidium acerosum** S.H. He & S.L. Liu, sp. nov.
MycoBank: MB 828719
Fig. 4

**Typification.** CHINA. Guizhou Province, Libo County, Maolan Nature Reserve, on fallen angiosperm trunk, 16 Jun 2016, He 3804 (holotype, BJFC 022303).

**Etymology.** “*acerosum*” refers to the presence of numerous needle-like crystals.

**Basidiomata.** Annual, resupinate, effused, very thin, easily separated from the substrate, up to 6 cm long, 2 cm wide. Hymenophore surface smooth, more or less arachnoid, white (5A1) to orange grey (5B2); margin undifferentiated.

**Microscopic structures.** Hyphal system monomitic; generative hyphae with clamp connections, hyaline, thin-walled, frequently branched and septate, loosely interwoven, 2–3.5 µm in diam. Cystidia abundant, subulate, projecting beyond hymenium, hyaline, thick-walled and regularly covered with rectangular crystals at basal part, thin-walled and smooth at apex part, 50–100 × 3–5 µm. Crystals numerous, distributed in whole section or more commonly attached on cystidia, acerose, hyaline. Basidia short clavate, hyaline, thin-walled, with 4 sterigmata and a basal clamp connection, 15–20 × 4–5.5 µm; basidioles in shape similar to basidia, but slightly smaller. Basidiospores narrowly fusiform to slightly vermicular, hyaline, thin-walled, smooth, negative in Melzer’s reagent, acyanophilous, (14.5–)15.5–18(–20) × 1.8–2.2 µm, L = 16.6 µm, W = 2 µm, Q = 8.3 (n = 30/1).

**Remarks.** *Subulicystidium acerosum* is characterised by the long and narrow basidiospores and presence of numerous acerose crystals. The species is similar to *S. longisporum* (Pat.) Parmasto, which differs in having slightly shorter and wider basidiospores (12–16 × 2–3 µm, Q < 7, Ordynets et al. 2018). *Subulicystidium cochleum* Punugu is similar to *S. acerosum* by sharing needle-like crystals but differs in having larger basidiospores (20–27 × 2–3 µm, Punugu et al. 1980; Ordynets et al. 2018). Phylogenetically, *S. acerosum* is distinct from all the other sampled species of *Subulicystidium* (Fig. 2).

**Subulicystidium tropicum** S.H. He & S.L. Liu, sp. nov.
MycoBank: MB 828720
Fig. 5

**Typification.** CHINA. Hainan Province, Wuzhishan County, Wuzhishan Nature Reserve, on fallen angiosperm branch, 10 Jun 2016, He 3968 (holotype, BJFC 022470).

**Etymology.** “*tropicum*” refers to the distribution in tropical areas.
Figure 4. Subulicystidium acerosum (holotype, He 3804). a basidiomata b, f basidiospores c acerose crystals d, e, g cystidia h basidia and a basidiole. Scale bars: 1 cm (a), 10 µm (b–h). b–e Taken in phloxine.

**Basidiomata.** Annual, resupinate, effused, very thin, separable from the substrate, up to 10 cm long, 3 cm wide. Hymenophore surface smooth, white (5A1), orange grey (5B2) to greyish-orange [5B(3–4)], not cracked; margin undifferentiated.

**Microscopic structures.** Hyphal system monomitic; generative hyphae with clamp connections, hyaline, slightly thick-walled, frequently branched and septate, loosely interwoven, 2–3.5 µm in diam. Cystidia abundant, subulate, projecting beyond hymenium, hyaline, thick-walled and regularly covered with rectangular crystals except at the apex, 40–70 × 3–5 µm. Basidia subclavate to suburniform, hyaline, thin-walled, with 4 sterigmata and a basal clamp connection, 12–17 × 4–5 µm; basidioles in shape similar to basidia, but slightly smaller. Basidiospores fusiform to slightly vermicular, hyaline, thin-walled, smooth, negative in Melzer’s reagent, acyanophilous, 11–12.5(–13) × 1.8–2.2 µm, L = 11.9 µm, W = 2 µm, Q = 5.95 (n = 30/1).
Four new corticioid species in Trechisporales from East Asia and notes...

**Figure 5.** *Subulicystidium tropicum* (holotype, He 3968). a basidiomata b, d basidiospores c, e cystidia f basidia g subicular hyphae. Scale bars: 1 cm (a), 10 µm (b–g). b, c Taken in phloxine.

**Additional specimens examined.** CHINA. Hainan Province, Baoting County, Qixianling Forest Park, on fallen angiosperm branch, 18 Mar 2016, He 3583 (BJFC 022083).

**Remarks.** *Subulicystidium tropicum* resembles *S. acerosum* and *S. perlongisporum* Boidin & Gilles by sharing narrow basidiospores in the genus, but differs from *S. acerosum* in having shorter basidiospores and lacking the needle-like crystals and from *S. perlongisporum* in having much shorter basidiospores and a tropical distribution (16–25 x 1.5–2.5 µm for *S. perlongisporum* in Ordynets et al. 2018). The new species is also similar to *S. longisporum*, but differs in having slender basidiospores and a tropical distribution. In the phylogenetic tree, *S. tropicum* formed a distinct lineage in *Subulicystidium* (Fig. 2).
**Figure 6.** *Tubulicium bambusicola* (holotype, He 4058). 

- **a** basidiomata
- **b, d** basidiospores
- **c, e** cystidia
- **f** basidia and a basidiole
- **g** subicular hyphae. Scale bars: 1 cm (**a**), 10 µm (**b–g**). **b, c** Taken in phloxine.

---

**Tubulicium bambusicola** S.H. He & S.L. Liu, sp. nov.

MycoBank: MB 828721

Fig. 6

**Typification.** THAILAND. Chiang Rai Province, Doi Mae Salong, on dead culms of bamboo, 22 Jul 2016, He 4058 (holotype, BJFC 023499).

**Etymology.** “*bambusicola*” refers to growing on bamboo.

**Basidiomata.** Annual, resupinate, effused, closely adnate, thin, at first as irregular small patches, later confluent up to 15 cm long, 5 cm wide. Hymenophore surface
Four new corticioid species in Trechisporales from East Asia and notes...

smooth, pilose under lens due to the projecting cystidia, pale orange (5A3) to greyish-orange [5B(3–6)], finely cracked with age; margin undifferentiated.

**Microscopic structures.** Hyphal system monomitic; generative hyphae with clamp connections, hyaline, thin-walled, moderately branched, frequently septate, loosely interwoven, 2–3 µm in diam. Cystidia abundant, subulate, projecting beyond hymenium, multi-rooted, hyaline, distinctly thick-walled, slightly amyloid, covered with dendroid branching hyphae, 70–100 × 10–16 µm. Basidia subclavate, hyaline, thin-walled, with 4 sterigmata and a basal clamp connection, 18–25 × 8–10 µm; basidioles in shape similar to basidia, but slightly smaller. Basidiospores narrowly fusiform to vermicular, bi-apiculate, hyaline, thin-walled, smooth, negative in Melzer’s reagent, acyanophilous, (17–)20–29(–30) × (2–)2.2–3(–3.2) µm, L = 23.9 µm, W = 2.6 µm, Q = 9–9.5 (n = 60/2).

**Additional specimens examined.** CHINA. Guizhou Province, Libo County, Maolan Nature Reserve, on rotten culms of bamboo, 11 Jul 2017, He 4776 (BJFC 024293).

**Remarks.** *Tubulicium bambusicola* is distinguished by its large vermicular basidiospores and growing on bamboo. Three taxa, *T. raphidisporum* (Boidin & Gilles) Oberw., Kism.-Hor. & L.D. Gómez, *T. vermiferum* (Bourdot) Oberw. and *T. vermiferum* var. *hexasterigmatum* J. Kaur & Dhingra are similar to *T. bambusicola* by sharing long vermicular basidiospores but differ in the width of basidiospores (≥ 3.5 µm) and growing on woody plant. *Tubulicium junci-acutus* Boidin & Gaignon on *Juncus acutus* differs from *T. bambusicola* by having shorter and wider basidiospores (15–20 × 3–4.25 µm, Boidin and Gaignon 1992).

**Discussion**

Nine genera in the Trechisporales were included in the present analyses and the results mostly agree with previous studies (Larsson 2007; Birkebak et al. 2013; Telleria et al. 2013a). Most of the sampled genera were retrieved as monophyletic except *Scytinopogon*, which was nested within the *Trechispora* lineage (Fig. 2). A *Dextrinocystis* species was sequenced for the first time and its position in Hydnodontaceae was confirmed. As indicated by the morphology (Burdsall and Nakasone 1983; Gilbertson and Blackwell 1988; Moreno and Esteve-Raventós 2007; Nakasone 2013), the genus is closely related to *Tubulicium*. However, *Tubulicium* is morphologically heterogenous, with different basidiospores (Moreno and Esteve-Raventós 2007; Hjortstam and Ryvarden 2008) and only species with fusiform to vermicular basidiospores were sequenced. Moreover, *Dextrinocystis* is well distinguished from *Tubulicium* by its distinctly dextri-noid cystida and cylindrical basidiospores (Gilbertson and Blackwell 1988; Nakasone 2013). Thus, at present, the authors prefer to retain them as separate genera until more species are sequenced.

*Subulicystidium* is a well-circumscribed genus characterised by the unique cystidia encrusted with rectangular crystals and fusiform to vermicular basidiospores (Bernichia and Gorjón 2010; Ordynets et al. 2018). Although all the sampled species formed a strongly supported lineage in the tree (Fig. 2), the species *S. oberwinkleri* Ordynets,
Riebesehl & K.H.Larss. was not congeneric with other species and excluded from our analyses. Ordynets et al. (2018) showed that S. oberwinkleri formed a distinct basal lineage in the ITS-nrLSU tree. The phylogenetic position of the species in Trechisporales needs to be further studied.

Key to accepted genera in Trechisporales

1. Basidiomata clavarioid ..................................................... *Scytinopogon*
   
2. Basidiomata resupinate or stipitate hydnoid .......................................................... 2
   
2. Hymenophore poroid .................................................................................. 3
   
3. Hymenophore non-poroid .............................................................................. 4
   
3. Basidiospores smooth ................................................................. *Porpomyces*
   
   
5. Basidiomata brown ................................................................. *Luellia*
   
6. Basidiomata light coloured ........................................................................... 5
   
7. Cystidia present, large and distinct.......................................................... 6
   
8. Cystidia absent or indistinct ......................................................................... 8
   
9. Cystidia distinctly dextrinoid in Melzer’s reagent ....................... *Dextrinocystis*
   
10. Cystidia negative or amyloid in Melzer’s reagent ........................................ 7
   
11. Cystidia regularly encrusted with rectangular crystals........... *Subulicystidium*
   
12. Cystidia usually covered with dendroid hyphae ........................................ 9

   
14. Generative hyphae without ampullate septa ........................................... 9

15. Subhymenial hyphae isodiametric ........................................... *Brevicellicium*
   
16. Subhymenial hyphae not isodiametric .................................................... 10
   
17. Hyphal system dimitic; basidia with 4 sterigmata ..................................... *Fibrodontia*
   
18. Hyphal system monomitic; basidia with 4–8 sterigmata .... *Sisotremastrum*

Acknowledgements

The authors thank Dr. Karen Nakasone (Center for Forest Mycology Research, Northern Research Station, U.S. Forest Service, Madison, USA) for literature loan and critical suggestions on the manuscript. This study was supported by the Fundamental Research Funds for the Central Universities (No. 2016ZCQ04) and the National Natural Science Foundation of China (Nos. 31750001 & 31670013).

References

Four new corticioid species in Trechisporales from East Asia and notes...


**Stephanospora mayana** (Stephanosporaceae, Russulales), a new sequestrate fungus from Yucatán Peninsula, Mexico

Javier Isaac de la Fuente¹, Gonzalo Guevara-Guerrero¹, Iván Oros-Ortega², Romeo Sánchez-Zavalegui³, Iván Córdova-Lara⁴, Jesús García-Jiménez¹

1 Tecnológico Nacional de México. Instituto Tecnológico de Ciudad Victoria. Blvd. Emilio Portes Gil #1301Pte. CP87010, Ciudad Victoria, Tamaulipas, Mexico
2 Tecnológico Nacional de México. Instituto Tecnológico de la Zona Maya, Carretera Chetumal-Escárcega, km 21.5, CP 77965, AP 207, Ejido Juan Sarabia, Quintana Roo, Mexico
3 Tecnológico Nacional de México, Instituto Tecnológico de Chetumal. Av. Insurgentes # 330, Col. David G. Gutiérrez, CP 77013, Chetumal, Quintana Roo, Mexico
4 Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, CICY. Calle 43, Col. Chuburná de Hidalgo, CP 97025, Mérida, Yucatán, Mexico

**Corresponding author:** Jesús García-Jiménez (jgarjim@yahoo.com.mx)

**Academic editor:** T. Lumbsch | Received 1 November 2018 | Accepted 8 February 2019 | Published 8 March 2019


**Abstract**

*Stephanospora mayana* is presented as a new species from the Yucatán Peninsula, Mexico. This species is distinguished by the yellowish pileus, basidiospores with a small corona (4–6 × 1–2.5 µm), and variable size (8.0–17.0 × 6.0–11.0), thin pileus (21–40 µm) and the ecological association to lowland forest with *Haematoxylum campechianum*, *Gymnopodium floribundum*, *Coccoloba diversifolia*, *Metopium brownei* and *Pinus caribaea*. It differs from the American species of *Stephanospora*, like *S. michoacanensis* and *S. chilensis*, by its larger basidiospores. Descriptions, photographs and discussions are presented.

**Keywords**

Campeche, Macrofungi, Quintana Roo, tropical truffles, truffle-like fungi

**Introduction**

The species within *Stephanospora* Pat. were previously accommodated in Hymenogastraceae Vittad by Cunningham (1979) as *Octaviania* Vittad. and also in Octavianiaceae Locq. ex Pegler & T.W.K. Young by Pegler and Young (1979), due to the spiny basidiospores. They also included *Hydnangium* Wallr., *Sclerogaster* R. Hesse and *Wakefieldia* Corner &
Hawker. Nevertheless, Overwinkler and Horak (1979) placed these species in the family Stephanosporaceae Overwinkler & Horak along with *Lindtneria* Pilát. due to the spines around the basidiospore base, forming what is called a corona. Actually, Stephanosporaceae includes both sequestrate and resupinate species with or without a corona (Martín et al. 2004; Vidal 2004; Castellano et al. 2007; Lebel et al. 2015). *Stephanospora* is a genus with sequestrate species characterized by the subhypogeous habit, spiny or crested basidiospore ornamentation, and the conspicuous corona at the basidiospore base. Most of the species have a yellowish to orange pileus, pale-orange, olive-grey to pale-brown hymenophore and lack a stipe (Castellano et al. 1986; Pegler et al. 1993; Montecchi and Sarasini 2000; Vidal 2004). According to Lebel et al. (2015), 15 species are recognized worldwide.

Most *Stephanospora* species grow in association with broadleaf trees in Oceania (Cunningham 1979; Bougher and Lebel 2001; Lebel et al. 2015) or temperate forest in Europe (Palacios and Lakisbar 1991; Pegler et al. 1993; Vidal 2004; Fraiture and Novello 2013) and America (Vidal 2004; Guevara-Guerrero et al. 2015). Some additional undescribed species and genetic sequences were mentioned by Lebel et al. (2015) from Belize, Costa Rica and the Caribbean. In the USA, no species have been described from fruiting bodies, but DNA sequences have been included in a couple of analyses (Edwards and Zak 2010; Lebel et al. 2015). Most species can be found growing under mycorrhizal trees species such as *Podocarpus*, *Eucalyptus*, *Quercus* or *Pinus*, but no evidence of ectomycorrhizal associations has been observed (Tedersoo et al. 2010). The genus is represented in Mexico so far by a single species, *S. michoacanensis* Guevara & Castellano from central Mexico (Guevara-Guerrero et al. 2015).

In recent mycological exploration conducted by us on the Yucatán Peninsula in southern Mexico, some interesting sequestrate fungi were found, collected and identified as *Stephanospora*. The specimens were collected under *Haematoxylum campechianum* L., *Gymnopodium floribundum* Rolfe, *Metopium brownei* (Jacq.) Urband, and *Pinus caribaea* Morelet in lowland forest and pine savanna. Due to the basidiospore size, small corona, the association to lowland forest and pine savanna, and a molecular analyses of DNA we conclude that it is a novel species and we propose it as *S. mayana* de la Fuente, García-Jiménez, Guevara-Guerrero & Oros-Ortega.

**Methods**

**Sampling data**

Basidiomata were collected at Calakmul municipality in the state of Campeche and Othón P. Blanco municipality, in the state of Quintana Roo, Mexico. The vegetation is a disturbed lowland forest with *Coccoloba diversifolia* Jacq, *M. brownei*, *H. campechianum*, *G. floribundum*, and *Acoelorraphe wrightii* (Griseb. & H. Wendl.) H. Wendl. ex Becc. (Valdés and Islebe 2011) and pine savanna with *P. caribaea*, *C. diversifolia*, *Curatella americana* L., *Crescentia cujete* L., and *Byrsonima crassifolia* (L.) Kunth (Macario and Sánchez 2011) (Fig. 1). Methods for collecting, sampling and
Stephanospora mayana, a new sequestrate fungus from...

Describing sequestrate fungi were used (Castellano et al. 1986). Hand cuts sections were made from dried specimens mounted in KOH 5% and Meltzer reagent for microscopic description. Colour terminology was according to the Handbook of Colour (Kornerup and Wanscher 1978). All the specimens were curated and deposited at the mycological herbarium José Castillo Tovar of Instituto Tecnológico de Ciudad Victoria (ITCV).

**Figure 1.** Habitat of *Stephanospora mayana*. a Lowland Forest at Blasillo b pine savanna at Xnohá.
Molecular analysis

For DNA extraction from basidiomata tissue we used the protocol reported by Cordova et al. (2014). Briefly, 0.1 g of the tissue was pulverized in liquid nitrogen and 1 ml extraction buffer of CTAB (20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 2% CTAB, 1.4 M NaCl, and 2-mercaptoethanol) was added and incubated for 20–30 min at 65 °C and then vigorously mixed with a solution of phenol-chloroform isoamyl alcohol. After centrifugation, the supernatant was precipitated using cold isopropanol and sodium acetate and then incubated at –20 °C for 1 h. The DNA was pelleted by centrifugation and dried at room temperature. Finally, the DNA was resuspended in 100 µL of nuclease-free ultrapure water. Quantity and quality of the DNA was estimated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The ITS region of the ribosomal DNA was amplified using the primers ITS1F/ITS4B reported by Gardes and Bruns (1993). The final concentration of the PCR reaction was: 1× of MyTaq reaction buffer, 0.4 µM of primer, 40 ng of DNA and 1.5 Unit of MyTaq DNA polymerase (Bioline, USA Inc.). The PCR conditions used for amplification were according to Gardes and Bruns (1993). The PCR products were observed on a 1.5 % agarose gel stained with ethidium bromide and visualized by UV transillumination in a Gel-DOC (Bio-Rad) equipment. Bands amplified were removed and purified with the QIA quick gel extraction kit (QIAGEN). Purified PCR products were sequenced using automated equipment in Davis Inc., CA, USA. Both sides of the cloned inserts were sequenced. Sequences were aligned with MUSCLE (Edgar 2004). Alignments were manually checked and ambiguous regions were excluded. Sequences produced in this study are deposited in GenBank under accession number MK033630. A search of GenBank nucleotide databank (NCBI) for homologous sequences was performed by BLAST analyses.

Phylogenetic analyses was performed from sequences obtained from basidiomata. References sequences (Lebel et al. 2015) and consensus sequence were aligned using BioEdit version 7.0.4.1 (Hall 1999). The tree was built in MEGA X (Kumar et al. 2018) using maximum likelihood analyses and the Kimura 2-parameter model (Kimura 1980) of nucleotide substitution with bootstrap values based on 1000 runs. Pilocerema fallax and Athelia arachnoidea were used as outgroups (Lebel et al. 2015).

Results

Molecular analyses

A total of 48 sequences of Stephanospora species, including the new species, were analyzed (Fig. 2). The sequence consensus from the holotype clustered in the Stephanospora Clade III Subclade A (i) from Lebel et al. (2015). The designation of S. mayana as a new species is supported by ITS rDNA analyses and morphological features.
**Figure 2.** Phylogenetic tree inferred under the maximum-likelihood (ML) criterion from the ITS rDNA alignment corresponding the *Stephanospora* Clade III (i) dataset from Lebel et al. (2015). The tree was rooted using midpoint rooting. Numbers on the branches represent support values from 1,000 ML bootstrap replicates. The branches are scaled in terms of the expected number of substitutions per site. Accession numbers in the sequence labels indicate sequences from GenBank.

**Taxonomy**

*Stephanospora mayana* de la Fuente, García-Jiménez, Guevara-Guerrero & Oros-Ortega, sp. nov.

MycoBank: MB 828118

Figure 3a–f

**Figure 3.** *Stephanospora mayana* (JF-397-ITCV-HOLOTYPE). a Basidiomata showing the pileus and hymenophore b basidiospores c corona d hymenophoral trama e pileus hyphae f hyphae from the locules. Scale bars: 10 mm (a); = 10 µm (b, c, e, f); 40 µm (d).

**Diagnosis.** *Stephanospora mayana* can be distinguished by the yellowish net-like pileus, the variable spore size (8.0–17.0 × 6.0–11.0 µm), thin pileus (21.0–40.0 µm) and the ecological association to lowland forest and pine savanna with *H. campechi-anum, G. floribundum, C.diversifolia, M. brownei, and P. caribaea.*
Stephanospora mayana, a new sequestrate fungus from...

**Etymology.** Named *mayana* in reference to the Mayan zone where this species was found.

**Description.** Basidiomata hypogeous to subhypogeous, scattered, 3.0–15 × 2.0–6.0 mm, globose to subglobose, without rizomorphs or stipe. Pileus yellowish to slightly orange (5A6-30A3-6), bruising pale orange when touched, wet to dry, sometimes net-like, dehiscent, showing locules inside. Hymenophore brittle, grayish (5C4), with empty rounded to angular locules, reaching 0.5 mm long, sometimes with white short and slender hyphae projecting from pileus to locules, trama sometimes orange (5A7-5B7), odour and taste strongly fruity.

Pileus 21.0–40.0 µm thick, composed of loosely interwoven, slender to inflated hyphae, 1.7–4.2 µm in diameter, orange to pale orange-yellow in KOH, thin-walled. Hymenophoral trama irregular, 62.0–100.0 µm wide, composed of irregular, globose, isodiametric and compacted hyphae, 13.5–26.3 µm in diameter, hyaline to slightly yellowish in KOH, thin-walled. Basidia 24.2–30.5 × 9.5–11.1 µm, clavate to subclavate, hyaline in KOH, guttulate, 2-spored, with long sterigmata, reaching 7 µm long, thin-walled, collapsing after basidiospore development. Basidiospores (8.0–) 10.0–16.0 (–17.0) × (6.0–) 8.5–10.5 (–11.0) µm (*L* = 12.10, *W* = 9.31, *Q* = 1.30, *N* = 90) ellipsoid to subglobose, with truncate to acute spines projecting 2.0 µm long, forming ridges reaching 3.5 µm high, sometimes coalescing, with a complete to partial corona 4.0–6.0 × 1.0–2.5 µm long, sometimes with 2–4 projecting spines, 1.5 µm long, with hilar appendage conspicuous, reaching 3 µm long, bright yellowish in KOH, orange in Meltzer reagent, with greenish to yellowish cell wall, 1.5–2.0 µm thick. Hyphae from the locules hyaline, 3.0–5.0 µm diameter, thin-walled. Clamp-connections absent in all tissues.

**Distribution.** Known from the Mexican states of Campeche and Quintana Roo where it is associated to lowland forest and pine savanna under *G. floribundum*, *H. campechianum*, *M. brownei*, and *P. caribaea*.

**Additional material examined.** Mexico, Quintana Roo, Othón Pompeyo Blanco municipality, Santa Elena Town, 18°30′N, 88°23′W, 07 October 2017, de la Fuente and Sánchez-Zavalegui 327 (Paratype); State of Campeche, Calakmul municipality, Xnohá town, 17°53′N, 89°10′W, 30 November 2017, de la Fuente 387 (paratype); Blasillío town, 18°31′N, 88°18′W, growing on abandoned termite mounds, 09 June 2018, de la Fuente 405 (paratype). (All in ITCV.)

**Discussion**

This species belongs to the *Stephanospora* clade III A (i) following Lebel et al. (2015). All the species in this clade are characterized by having basidiospores with ornamentation that does not project more than 2.5 µm, basidia with sterigma up to 7 µm long, and a small corona that never surpasses 7 µm in width (Lebel et al. 2015). However, the Mexican material has larger basidiospores than any other species in this clade (up to 17 µm), unlike *S. poropingao* T. Lebel & Castellano, *S. papua* T. Lebel & Castellano, *S. novae-caledoniae* T. Lebel, Castellano & K. Hosaka, and...
**Table 1.** Comparative morphology of *Stephanospora* species in the clade IIIA (i) according to Lebel et al. (2015).

<table>
<thead>
<tr>
<th>Taxa</th>
<th><em>S. poropingao</em></th>
<th><em>S. novae-caledoniae</em></th>
<th><em>S. cribbae</em></th>
<th><em>S. papua</em></th>
<th><em>S. mayana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Basidiomata size</td>
<td>5–25 mm</td>
<td>5–18 mm</td>
<td>5–25 mm</td>
<td>5–22 mm</td>
<td>3–13 mm</td>
</tr>
<tr>
<td>Pileus surface</td>
<td>Fibrillose</td>
<td>Smooth</td>
<td>Fibrillose</td>
<td>Irregular</td>
<td>Net-like</td>
</tr>
<tr>
<td>Pileus colour</td>
<td>Bright yellow, orangish-yellow to orangish-brown</td>
<td>Pale yellow to bright orange</td>
<td>Yellow to orange-yellow</td>
<td>Pale orange-yellow</td>
<td>Pale yellow</td>
</tr>
<tr>
<td>Hymenophore colour</td>
<td>Greyish-olive, olive-brown to yellow</td>
<td>White to pale yellow</td>
<td>Greyish olive to olive-brown-yellow</td>
<td>Pale yellow</td>
<td>Cream, greyish olive</td>
</tr>
<tr>
<td>Odour</td>
<td>Not recorded</td>
<td>Faintly sweet</td>
<td>Faintly coconut</td>
<td>Not recorded</td>
<td>Fruity</td>
</tr>
<tr>
<td>Basidiospores size</td>
<td>11–14 × 11–13 µm</td>
<td>11–14 × 09–12 µm</td>
<td>11–13,5 × 9,5–12 µm</td>
<td>09–11 × 07–8,5 µm</td>
<td>08–17 × 06–11 µm</td>
</tr>
<tr>
<td>Spines</td>
<td>Robust</td>
<td>Cylindrical, flattened to acute</td>
<td>Fine</td>
<td>Cylindrical or flattened</td>
<td>Truncated to acute</td>
</tr>
<tr>
<td>Corona size</td>
<td>05–09 × 01–03 µm</td>
<td>03–05 × 01–02 µm</td>
<td>03–05 × 01–02 µm</td>
<td>04–05 × 01–02 µm</td>
<td>04–06 × 01–2,5 µm</td>
</tr>
<tr>
<td>Pileus thickness</td>
<td>100–150 µm</td>
<td>40–145 µm</td>
<td>80–130 µm</td>
<td>30–140 µm</td>
<td>21–40 µm</td>
</tr>
<tr>
<td>Distribution</td>
<td>New Zealand, northwestern North Island</td>
<td>New Caledonia</td>
<td>Australia, Victoria, Queensland New South Wales</td>
<td>Papua New Guinea</td>
<td>Southern Yucatán Peninsula, Mexico</td>
</tr>
<tr>
<td>Habitat</td>
<td><em>Agathis</em>-broadleaf, podocarp-broadleaf forest</td>
<td>Mixed forest with <em>Nothofagus</em> spp</td>
<td><em>Eucalyptus</em> and <em>Acacia</em> Woodland</td>
<td>Mixed forest with <em>Eucalyptus</em></td>
<td>Lowland forest and pine savanna</td>
</tr>
</tbody>
</table>

*S. cribbae* T. Lebel & Castellano (up to 14 µm). *Stephanospora kanuka* T. Lebel & Castellano has similar pileus colour, sweet odour, and basidiospore length, but it has fine spines, an orange to yellowish hymenophore, and a fibrillose pileus (Lebel et al. 2015). *Stephanospora cribbae* T. Lebel & Castellano is similar to *S. mayana* in its yellowish pileus, corona size, and greyish hymenophore but differs in the smaller basidiospore size, the fibrillose pileus, and the coconut odour (Lebel et al. 2015). *Stephanospora michoacanensis* differs from *S. mayana* in having smaller basidiospores, the fruit odour absent, a cream colour pileus, and its association with oak-pine forest (Guevara-Guerrero et al. 2015). *Stephanospora chilensis* (E. Horak) J.M. Vidal differs in having an orange pileus and hymenophore, as well as smaller basidiospores (Vidal 2004).

*Stephanospora mayana* is in an unsupported clade with undescribed species from Belize (KM086881) and close to another unsupported clade with undescribed taxa from the USA and Spain (Lebel et al. 2015). Further collections and descriptions of taxa are required for Belizean and US material to better place this new species.

**Acknowledgements**

The first author and Sánchez-Zavalegui thank CONACYT for the financial support, León Ibarra and Miguel Ángel Domínguez-Domínguez for field and technical support, and Luis Alberto Lara-Pérez for support with molecular biology techniques. Guevara-Guerrero and García-Jiménez thank CONACYT, PRODEP, Tecnológico Nacional de México, and Instituto Tecnológico de Ciudad Victoria for financial support.
References


