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# *Phaeobotryon negundinis* sp. nov. (Botryosphaeriales) from Russia

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**Abstract**

A new species of *Phaeobotryon* was collected from *Acer negundo*, *Forsythia* × *intermedia* and *Ligustrum vulgare* from European Russia. Morphological and phylogenetic analyses of combined ITS, β-tubulin and EF1-α sequence data revealed that these collections differ from all other species in the genus. Therefore it is introduced here as *Phaeobotryon negundinis* sp. nov. It is characterized by immersed-erumpent, uniloculate conidiomata, bearing brown, ovoid, aseptate conidia, with broadly rounded apices and truncate bases. Conidia form a single septum at germination. *Phaeobotryon negundinis* is morphologically similar to *P. cupressi*, but has smaller conidia. This is the first time a *Phaeobotryon* species is reported on *Acer negundo*, *Ligustrum vulgare* and *Forsythia* × *intermedia*.

**Key words** – Botryosphaeriaceae – Dothideomycetes – morphology – phylogeny – taxonomy

## Introduction

The family Botryosphaeriaceae is considered to be one of the largest families in the class Dothideomycetes and members of this family comprise a wide range of morphologically diverse taxa that are characterized by uni- to multi-loculate ascostromata, usually 8-spored, bitunicate asci and hyaline to brown, aseptate to 2-septate ascospores (Liu et al. 2012, Phillips et al. 2013). The asexual morphs produce uni to multi-locular pycnidial conidiomata, with hyaline phialidic conidiogenous cells and hyaline or pigmented, septate or aseptate conidia (Phillips et al. 2008, Liu et al. 2012, Hyde et al. 2013, Phillips et al. 2013, Machado et al. 2014, Thambugala et al. 2014, Doilom et al. 2015).

*Phaeobotryon* was established by Theissen & Sydow (1915) as a monotypic genus typified by *P. cercidis* (Cooke) Theiss. & Syd. In their broad view of *Botryosphaeria* based on morphology of the sexual morph, von Arx & Müller (1954, 1975) considered *Phaeobotryon* to be a synonym of

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*Botryosphaeria*. However, recent studies showed that *Phaeobotryon* is morphologically and phylogenetically distinct from all other genera in Botryosphaeriaceae (Phillips et al. 2008, Liu et al. 2012, Hyde et al. 2013, Phillips et al. 2013). The genus is characterized by immersed to erumpent, multi-locular ascostromata, clavate to cylindrical-clavate asci and short-pedicellate, hyaline or brown, 2-septate ascospores and ellipsoidal to oblong or obovoid, hyaline or brown conidia, that are mostly 2-septate at maturity (Liu et al. 2012, Hyde et al. 2013, Phillips et al. 2013, Fan et al. 2014). Eight epithets are currently listed in Index Fungorum (2016).

In this paper we introduce *Phaeobotryon negundinis* as a new species from *Acer negundo*, *Forsythia* x *intermedia* and *Ligustrum vulgare* collected from Russia. The species was compared morphologically with other known *Phaeobotryon* species and its phylogenetic position was determined from an analysis of ITS, β-tubulin and EF1-α sequence data.

## Materials and Methods

*Sampling and morphology*

Fresh specimens were obtained from *Acer negundo* L., *Forsythia* × *intermedia* Zabel and *Ligustrum vulgare* L. collected in Russia. Morphological examination and photomicrography were carried out as described by Thambugala et al. (2015) and Daranagama et al. (2015). Axenic cultures were prepared according to the method of Chomnunti et al. (2014) and maintained on potato-dextrose agar (PDA; Difco Laboratories). Herbarium material was deposited in the herbarium of Mae Fah Luang University, Chiang Rai, Thailand (MFLU) and cultures deposited at Mae Fah Luang University Culture Collection (MFLUCC). Faces of fungi numbers and Index Fungorum numbers were registered as explained in Jayasiri et al. (2015) and Index Fungorum (2016).

*DNA extraction, PCR amplification and sequencing*

DNA was extracted following the method of Thambugala et al. (2015). PCR amplifications were performed in a total volume of 25 μL of PCR mixtures containing 8.5−9.5 μL dd$H_2O$, 12.5 μL 2×PCR Master Mix (TIANGEN Co., China), 1–2 μL of DNA template, 1 μL of each primer. The EF1-α (EF1-728F and EF1-986R), ITS (ITS5/ITS4), LSU (LR0R/LR5) and β-tubulin (Bt2a and Bt2b) gene regions were amplified following the conditions described in Carbone and Kohn (1999), White et al. (1990), Vilgalys and Hester (1990) and Glass and Donaldson (1995). PCR products were visualized under UV light on 1% agarose gels stained with ethidium bromide. PCR products were purified and sequenced by Invitrogen Biotechnology Co., Ltd. Shanghai, China.

*Phylogenetic analyses*

The phylogenetic position of the new strains was determined in two separate analyses. The first analysis was based on combined EF1-α and ITS sequence data of 56 isolates belonging to *Botryosphaeriaceae* (Table 1) to show the placement of the genus *Phaeobotryon* in the family, with *Phyllosticta citricarpa* as the outgroup taxon. The second analysis, based on combined β-tubulin, EF1-α and ITS sequence data, was done only for species in *Phaeobotryon*, which included 14 isolates and *Barriopsis fusca* as the outgroup taxon. All newly generated sequences were deposited in GenBank (Table 1). Isolates and GenBank accession numbers used in the analyses are listed in Table 1.

The sequence data were combined and aligned with Bioedit v. 7.2.5 (Hall 1999) and MEGA 6.0 (Tamura et al. 2013). Maximum likelihood (ML) analyses were performed using MEGA 6.0. The general time-reversible (GTR) model of evolution including estimation of invariable sites (I) and assuming a discrete gamma distribution (G) was used. All gaps were included in the analyses. ML analyses were performed on a neighbor-joining (NJ) starting tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference and 1000 bootstrap replicates were executed. The best scoring trees were selected for each analysis and figures prepared with Adobe Illustrator CS3.

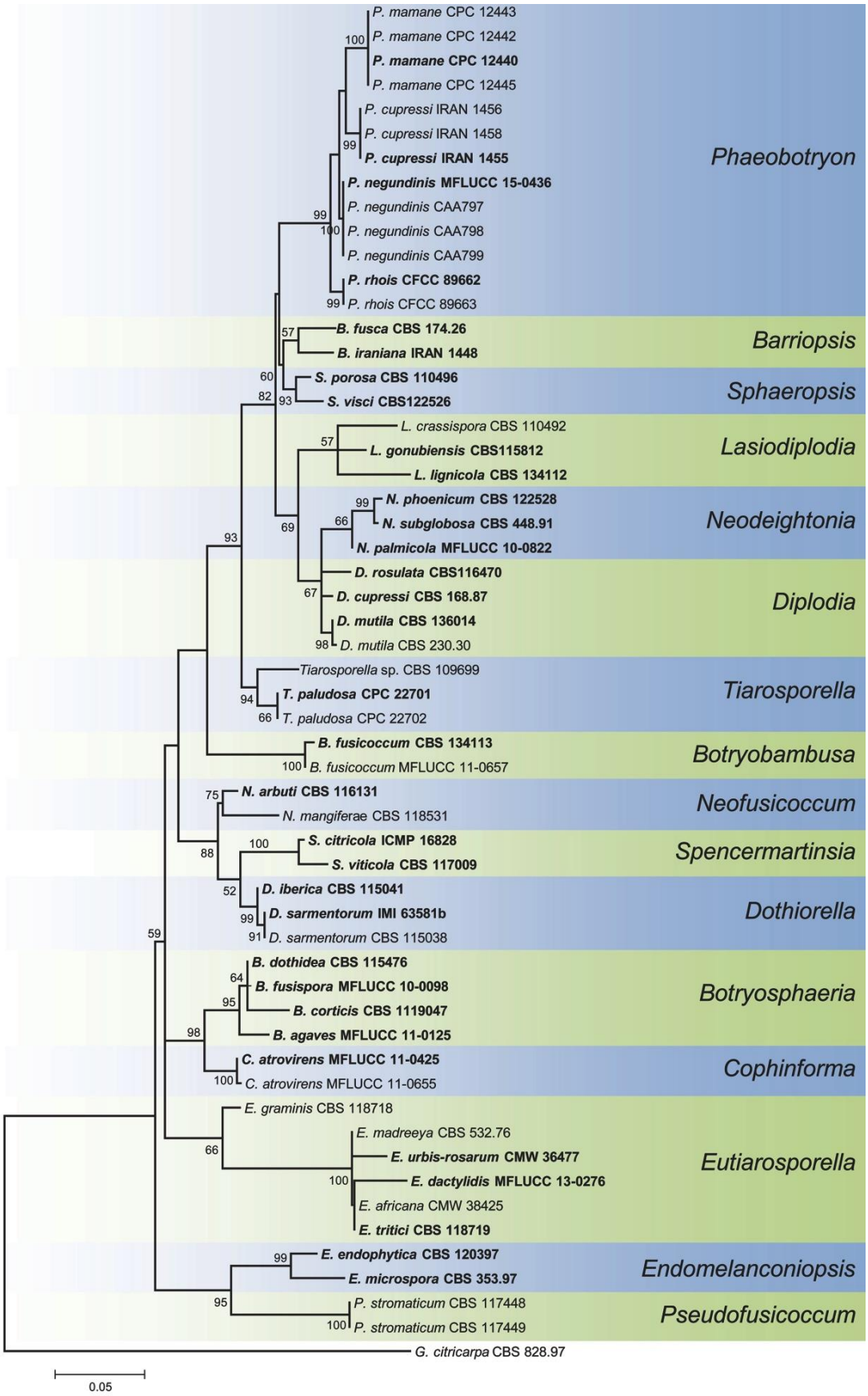


**Fig. 1 –** Phylogram inferred from maximum likelihood analysis of combined ITS and EF-1α sequence data. Strain numbers are given after the taxon names. Holo-, neo- or epitype strains/specimens are in bold face. Bootstrap support values >50% from 1000 replicates are shown above or below the branches.

## Results

Phylogenetic analysis

After alignment, the combined ITS and EF-1α dataset of 55 ingroup taxa had a length of 1060 characters including gaps. Phylogenetic analysis by maximum likelihood resulted in 15 clades corresponding to 15 genera of Botryosphaeriaceae (Fig. 1). The Russian isolates clustered in a well-supported clade within *Phaeobotryon* distinct from *P. rhois* C.M. Tian et al., *P. cupressi* Abdollahz. et al. and *P. mamane* Crous & A.J.L. Phillips. These are the only species for which cultures and sequence data are available. Analysis of the combined ITS, β-tubulin and EF1-α dataset for *Phaeobotryon* resulted in four clades corresponding to four species (Fig. 2). Three of the species are already known, while the Russian isolates formed the fourth clade representing a distinct species that we introduce here as *P. negundinis*.

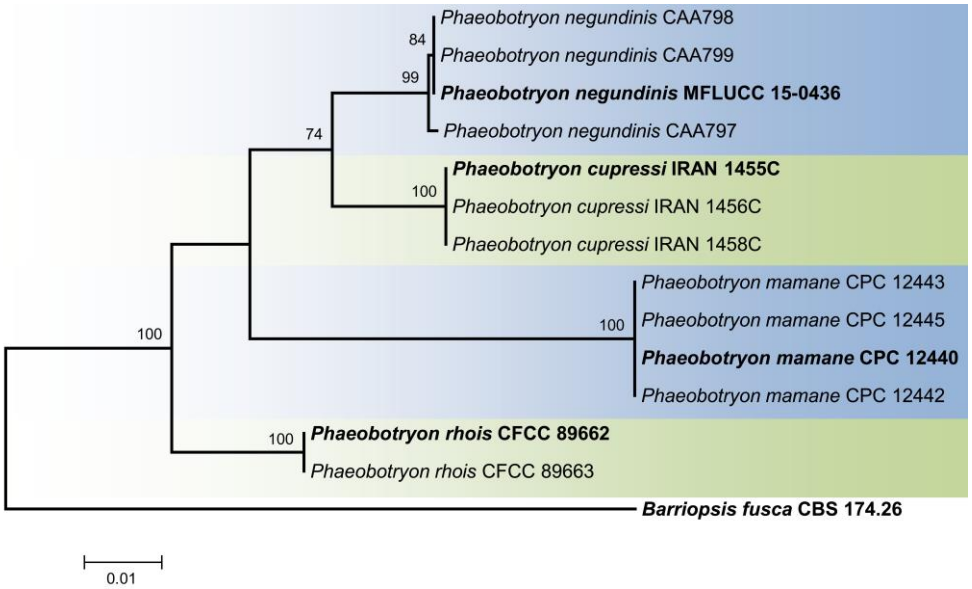


**Fig. 2 –** Phylogram inferred from maximum likelihood analysis of combined ITS, EF-1α and β-tubulin sequence data. The values above/below the branches indicate ML bootstrap support values. Ex-type strains are in boldface.

## Taxonomy

***Phaeobotryon negundinis*** Daranagama, Bulgakov and K.D. Hyde, **sp. nov.**
Facesoffungi number: FoF 01916
Index Fungorum number: IF551954

Etymology – "*negundinis*" refers to the epithet of the host plant *Acer negundo* from which it was first isolated

Holotype – MFLU 16-0475

*Necrotrophic* on dying and recently dead twigs and branches of *Acer negundo*. **Sexual morph**: Not observed. **Asexual morph**: *Conidiomata* 200–350 *μm* high × 210–325 *μm* diam. ($\bar{x}$ = 268 × 253 *μm*, *n* = 6), stromatic, solitary and scattered in small groups, immersed, becoming erumpent through the host tissue, uniloculate, black, globose to subglobose, ostiolate. *Conidiomatal wall* 30–60 *μm* ($\bar{x}$ = 38.5 *μm*, n = 15), comprising several layers of lightly pigmented to dark brown cells of *textura angularis* becoming hyaline towards the towards the conidiogenous region. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 10–28 × (1.8–)2–4 *μm* ($\bar{x}$ = 16.7 × 2.8 *μm*, *n* = 15), lining the conidiomatal cavity, holoblastic, hyaline, subcylindrical. *Conidia* 16–24.5 × 7.9–11.5 *μm* ($\bar{x}$ = 20.1 × 9.5 *μm*, *n* = 30), ovoid with a broadly rounded apex and truncate base, initially hyaline to lightly pigmented, becoming dark brown at maturity, aseptate, forming a single septum at germination, smooth to finely verruculose.

**Table 1** GenBank accession numbers of the strains used in this study.

| Taxon | Culture Accession No. | GenBank Accession No. | | |
|---|---|---|---|---|
| | | β-tubulin | ITS | EF1-α |
| *Barriopsis fusca* | CBS 174.26 | EU673109 | EU673330 | EU673296 |
| *Barriopsis iraniana* | IRAN 1448 | – | FJ919663 | FJ919652 |
| *Botryobambusa fusicoccum* | MFLUCC 11–0657 | – | JX646793 | JX646858 |
| *Botryobambusa fusicoccum* | CBS 134113 | – | JX646792 | JX646857 |
| *Botryosphaeria agaves* | MFLUCC 11–0125 | – | NR111792 | JX646856 |
| *Botryosphaeria corticis* | CBS 119047 | – | DQ299245 | EU017539 |
| *Botryosphaeria dothidea* | CBS 115476 | – | AY236949 | AY236898 |
| *Botryosphaeria fusispora* | MFLUCC 10-0098 | – | JX646789 | JX646854 |
| *Diplodia cupressi* | CBS 168.87 | – | DQ458893 | DQ458878 |
| *Diplodia mutila* | CBS 136014 | – | KJ361837 | KJ361829 |
| *Diplodia mutila* | CBS 230.30 | – | DQ458886 | DQ458869 |
| *Diplodia rosulata* | CBS 116470 | – | EU430265 | EU430267 |
| *Dothiorella iberica* | CBS 115041 | – | AY573202 | AY573222 |
| *Dothiorella sarmentorum* | IMI 63581b | – | AY573212 | AY573235 |
| *Dothiorella sarmentorum* | CBS 115038 | – | AY573206 | AY573223 |
| *Endomelanconiopsis endophytica* | CBS 120397 | – | EU683656 | EU683637 |
| *Endomelanconiopsis microspora* | CBS 353.97 | – | EU683655 | EU683636 |
| *Eutiarosporella africana* | CMW38423 | – | KC769956 | KC769852 |
| *Eutiarosporella africana* | CMW38425 | – | KC769958 | KC769854 |
| *Eutiarosporella dactylidis* | MFLUCC 13–0276 | – | KM978944 | KP031694 |
| *Eutiarosporella madreeya* | CBS 532.76 | – | KC769960 | _ |
| *Eutiarosporella tritici* | CBS 118719 | – | KF531830 | KF531809 |
| *Eutiarosporella urbis-rosarum* | CBS 130405 | – | JQ239407 | JQ239394 |
| *Phyllosticta citricarpa* | CBS 828.97 | – | FJ538318 | FJ538376 |
| *Lasiodiplodia crassispora* | CBS 110492 | – | EF622086 | EF622066 |
| *Lasiodiplodia gonubiensis* | CBS 115812 | – | AY639595 | DQ103566 |
| *Lasiodiplodia lignicola* | CBS 134112 | – | JX646797 | JX646862 |
| *Marasasiomyces karoo* | CBS 118718 | – | KF531828 | KF531807 |
| *Neodeightonia palmicola* | MFLUCC 10-0822 | – | HQ199221 | _ |
| *Neodeightonia phoenicum* | CBS 122528 | – | EU673340 | EU673309 |
| *Neodeightonia subglobosa* | CBS 448.91 | – | EU673337 | EU673306 |
| *Neofusicoccum arbuti* | CBS 116131 | – | AY819720 | KF531792 |
| *Neofusicoccum mangiferae* | CBS 118531 | – | AY615185 | DQ093221 |
| *Phaeobotryon cupressi* | IRAN 1455 | – | FJ919672 | FJ919661 |
| *Phaeobotryon cupressi* | IRAN 1456 | – | FJ919670 | FJ919659 |
| *Phaeobotryon cupressi* | IRAN 1458 | – | FJ919671 | FJ919660 |
| *Phaeobotryon mamane* | CPC 12440 | EU67312 | EU673332 | EU673298 |
| *Phaeobotryon mamane* | CPC 12442 | EU673124 | EU673333 | EU673299 |
| *Phaeobotryon mamane* | CPC 12443 | EU673120 | EU673334 | EU673300 |
| *Phaeobotryon mamane* | CPC 12445 | EU673122 | EU673302 | EU673336 |
| ***Phaeobotryon negundinis*** | **MFLUCC 15-0436** | **KU853996** | **KU820970** | **KU853997** |
| ***Phaeobotryon negundinis*** | **CAA 797** | **KX061510** | **KX061513** | **KX061507** |
| ***Phaeobotryon negundinis*** | **CAA 798** | **KX061511** | **KX061514** | **KX061508** |
| ***Phaeobotryon negundinis*** | **CAA 799** | **KX061512** | **KX061515** | **KX061509** |
| *Phaeobotryon rhois* | CFCC 89662 | – | KM030584 | KM030598 |
| *Phaeobotryon rhois* | CFCC 89663 | – | KM030585 | KM030599 |
| *Pseudofusicoccum stromaticum* | CBS 117448 | – | AY693974 | AY693975 |
| *Pseudofusicoccum stromaticum* | CBS 117449 | – | DQ436935 | DQ436936 |
| *Spencermartinsia citricola* | ICMP 16828 | – | EU673323 | EU673290 |
| *Spencermartinsia viticola* | CBS 117009 | – | AY905554 | AY905559 |
| *Sphaeropsis porosa* | CBS 110496 | – | AY343379 | AY343340 |
| *Sphaeropsis visci* | CBS 186.97 | – | EU673325 | EU673293 |
| *Tiarosporella paludosa* | CPC 22701 | – | KM108378 | – |
| *Tiarosporella paludosa* | CPC 22702 | – | KM108379 | – |
| *Tiarosporella* sp. | CBS 109699 | – | KM108380 | KM108429 |

Abbreviations: CAA: Collection of Artur Alves housed at Department of Biology, University of Aveiro, Portugal; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center; CMW: FABI, University of Pretoria, South Africa; ICMP International Collection of Micro-organisms from

Plants, Landcare Research, New Zealand; CPC Collection of Pedro Crous housed at CBS; IMI International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

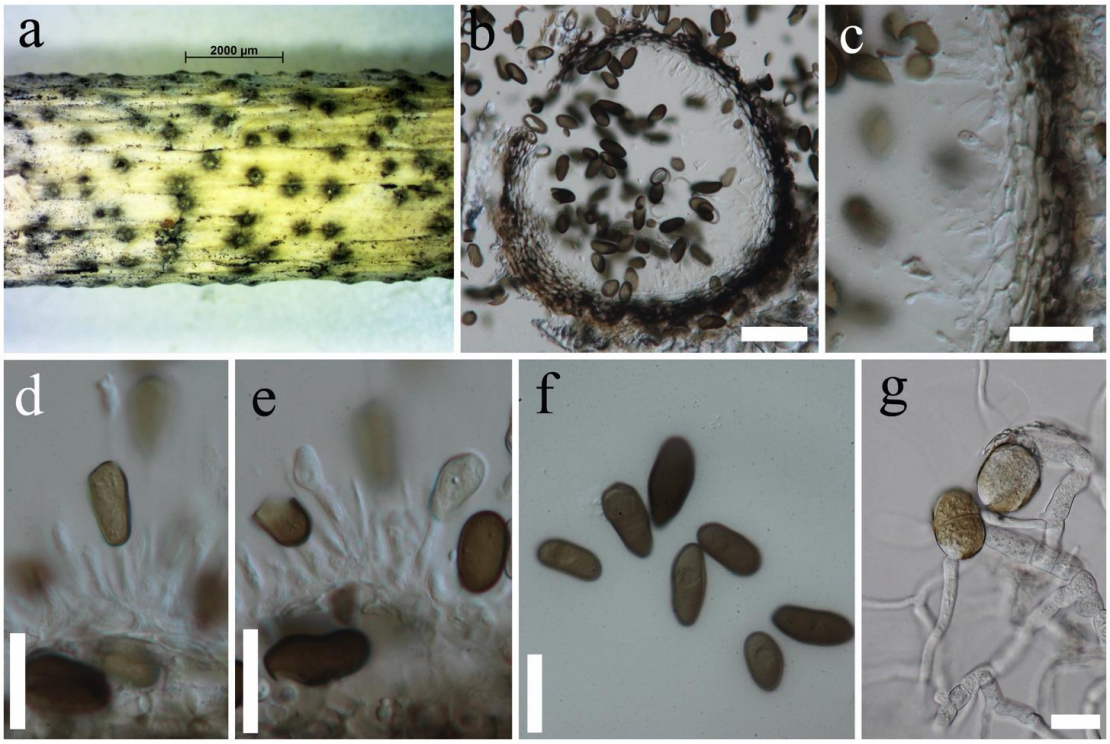


**Fig. 3.** *Phaeobotryon negundinis* (MFLU 16-0475, **holotype**) a. Appearance of conidiomata on host surface. b. Vertical section through conidioma. c. Conidiomatal wall. d, e. Conidia developing on conidiogenous cells f. Conidia. g. Germinating conidia. Scale bars: a, b = 50 µm, c, g = 25 µm, d-f= 20 µm.


**Fig. 4** – *Phaeobotryon negundinis* on *Acer negundo*. Dark pycnidia – a-c. On twigs, d-g. On branches.

Cultural characteristics – Conidia germinating on PDA within 18 h. Colonies fast growing on PDA at 25 °C, covering the medium surface (9 cm Petri-dishes) after 7 days, circular, flat, dense, surface initially white, becoming gray from the centre within 7 days, smooth surface with entire to slightly undulate edge.

Material examined – Russia, Rostov region, Rostov-on-Don city, Botanical Garden of Southern Federal University, Higher Park, on dying and dead twigs and branches of *Acer negundo* L. (Sapindaceae), 05 March 2014, T.S. Bulgakov (MFLU 16-0475, **holotype**), *ibid*. GZAAS 15-0103 isotype; ex-type living culture MFLUCC 15-0436, ICMP. GB Accession numbers: KU853996 (β-tubulin), KU853997 (EF-1α), KU820970 (ITS), KU820971 (LSU); Shakhty city, Central Park, on dying and dead twigs and branches of *Acer negundo* L. (Sapindaceae), 12 March 2014, T.S. Bulgakov, living culture CAA 797; on twigs and branches of *Ligustrum vulgare* L. (Oleaceae), 12 March 2014, T.S. Bulgakov, living culture CAA 798; Krasnosulinsky district, Donskoye forestry, forest nursery, on twigs and branches of *Forsythia* × *intermedia* Zabel (Oleaceae), 21 May 2014, T.S. Bulgakov, living culture CAA 799.

Notes – Phylogenetically *Phaeobotryon negundinis* is most closely related to *P. cupressi*. The two species can be differentiated morphologically on account of the smaller conidia of *P. negundinis*.

## Discussion

In this paper *Phaeobotryon negundinis* is introduced as a new species that is phylogenetically distinct from all other species in the genus. The smaller conidia of *P. negundinis* differentiate it from its nearest relative (*P. cupressi*). The 2-septate, brown ascospores with an apiculus at either end differentiate *Phaeobotryon* from all other genera in *Botryosphaeriaceae*. The sexual morph of *P. negundinis* was not seen on the host and did not form in culture.

Eight epithets are listed in Index of Fungi under *Phaeobotryon*, but only four species are known in culture and with DNA sequence data in GenBank. Unfortunately no DNA sequence data are available for the generic type of *Phaeobotryon*, and no cultures are extant. Therefore all the known species need to be recollected and sequenced to establish a complete phylogeny of the genus.

Although *P. negundinis* was associated with disease symptoms on twigs, leaves and seeds, pathogenicity has not been proved. Considering the number of different fungi that are associated with the same symptoms (Bulgakov, unpublished data) it is not possible to determine if *P. negundinis* is a primary pathogen or a secondary invader of already diseased tissues. Furthermore, there is no information available on the pathogenic status of any of the species in *Phaeobotryon*. This is the first report of a *Phaeobotryon* species on *Acer negundo*, *Forsythia* × *intermedia* and *Ligustrum vulgare*. Considering that *Phaeobotryon* is a relatively small genus with nine species, only five of which are known in culture, it can be expected that further sampling will reveal more species.

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