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Pyrenochaetopsis kuksensis (*Pyrenochaetopsidaceae*), a new species associated with an ornamental boxwood in the Czech Republic

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Abstract

During the investigation of fungal microbiome associated with boxwood in the Czech Republic, samples from *Buxus sempervirens* L. (*Buxaceae*) plants were collected and used for isolation. Two fungal strains were proposed as a new species *Pyrenochaetopsis kuksensis* based on morphology as well as phylogenetic analyses of ITS, LSU, *rpb2*, and *tub2* sequence data. Detailed descriptions and phylogenetic relationships of the new taxon are provided.

Keywords: *Buxus sempervirens*, Fungi, Phylogeny, *Pyrenochaetopsis*, Taxonomy

Introduction

Buxus sempervirens L. (*Buxaceae*) commonly known as boxwood is an evergreen ornamental shrub grown worldwide. Boxwood is widely used as hedges or topiaries. The history of cultivating the boxwood dates at least to Roman Times, but the fossil records are known in Europe since the Miocene (Lodwick 2017, Ciarallo 2004, Kvacek *et al.* 1982).

De Gruyter *et al.* (2010) established the genus *Pyrenochaetopsis* (*Ascomycota*, *Pezizomycotina*, *Dothideomycetes, Pleosporales, Cucurbitariaceae*) to accommodate several species which were formerly placed in highly polyphyletic genera *Phoma* and *Pyrenochaeta.* The genus is typified by *Pyrenochaetopsis leptospora* (Sacc. & Briard) de Gruyter *et al.* and is characterized by setose pycnidia; acropleurogenous conidiophores and aseptate, cylindrical to allantoid, glutatte conidia (de Gruyter *et al.* 2010). Morphologically, the genus *Pyrenochaetopsis* resembles genera *Pyrenochaeta* and *Paraphoma*, by its setose pycnidia and *phoma*-like conidiogenesis. However, both genera are only distantly related based on molecular phylogeny.

Recently, Valenzuela-Lopez *et al.* (2018) transferred the *Pyrenochaetopsis* genus from the family of *Cucurbitariaceae* to a newly established family of *Pyrenochaetopsidaceae,* based mainly on molecular data of four genomic loci. In order to delineate between the *Pyrenochaetopsis* species and the related genera, a multi locus phylogenetic analysis based on ITS, *LSU*, *rpb2* and *tub2* sequences is necessary (Wang *et al*. 2019, Valenzuela-Lopez *et al.* 2018, Gruyter *et al.* 2010).

Currently, the genus *Pyrenochaetopsis* contains 17 species (Index Fungorum 2021). Species of *Pyrenochaetopsis* have various hosts including human, soil, water or plants and can be plant pathogenic, saprophytic or endophytic on cereals, coffee tree, millet grass or sugarcane (Mapook *et al.* 2020, Wang *et al*. 2019, Valenzuela-Lopez *et al.* 2018, Papizadeh *et al*. 2017, de Gruyter *et al.* 2010, Boerama *et al.* 2004).

During the investigation of fungal microbiome associated with boxwood in the Czech Republic, samples from boxwood plants displaying symptoms of dieback were collected. Several fungal strains were obtained and two strains were proposed as a new species. The taxon is described and illustrated here in terms of morphology and phylogeny based on multi locus ITS, LSU, *rpb2* and *tub2* sequence data. To the best of our knowledge, there are four studies of fungi associated with boxwood plants in the Czech Republic (Crous *et al.* 2020a, Spetik *et al.* 2020, Spetik *et al.* 2019, Safrankova 2012).

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Materials and methods

Collection and isolation

During summer and autumn 2018, branches and twigs of 20 boxwood plants displaying symptoms of dieback (bronze leaves and twigs) were sampled from Nachod region, Czech Republic. Collected samples were placed in sterile plastic zip-bags and stored at 4°C, until fungal isolation. Wooden tissues were debarked, surface sterilized using 1.0 % sodium hypochlorite for 1 minute, then washed three times with a sterile distilled water. Pieces of tissue were aseptically transferred into a plates of Potato dextrose agar (PDA) and incubated at 25°C in the dark for 7 days. The plates were checked every day for fungal growth and transferred onto the new plates, when needed.

Morphology

Cultures were transferred to 2% water agar (WA) with double-autoclaved poplar twigs on the agar surface to enhance sporulation. Plates were incubated at 25°C under near-UV in a 12 h light 12 h dark regime for 2–4 weeks. A single pycnidium from each culture was dissected and the conidiogenous layer and conidia were mounted in 100% lactic acid. Microscope LSM800 (Carl Zeiss, Germany) equipped with Axiocam 305 colour camera was used for bright-field digital images of pycnidia and spores. Microscopic measurements were made with the VHX-6000 (Keyence, Belgium) microscope equipped with VH-ZST optics. The mean, standard deviation and 95% confidence intervals were calculated from measurements of 30 conidia per each isolate. Dimensions are presented as the range of measurements with extreme values in brackets followed by 95% confidence limits and mean ± standard deviation. Cultural characteristics were determined after 10 days on PDA, Malt extract agar (MEA) and Oat meal agar (OA) at 25 °C in the dark. Growth rates and cardinal temperatures for growth were determined on PDA plates incubated at different temperatures (5–35°C at 5°C intervals) in the dark. Colony colours were determined with the reference to the colour chart of Rayner (1970). Reference strains and specimens are maintained at CBS and MEND-F fungal collections.

DNA extraction and amplification

Genomic DNA was extracted from 7 days old mycelium grown on PDA at 25°C in the dark, using a NucleoSpin DNA extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. To confirm the identity of fungal species, a multi-locus DNA analysis was performed. Four genomic loci were used (ITS, LSU, *tub2* and *rpb2*). The internal transcribed spacer region (ITS) was amplified using ITS1/ITS4 primers (White *et al.* 1990). Primer pair L0R0/L5 (Rehner and Samuels 1994; White *et al.* 1990) was used to amplify the large ribosomal subunit (LSU). Primers rpb2-5f2 and rpb2-7cR were used to amplify the RNA polymerase II second largest subunit (*rpb2*) (Liu *et al.* 1999; Sung *et al.* 2007). The partial β-tubulin (*tub2*) region was amplified using the primers T1 (O'Donnell and Cigelnik 1997) and bt2b (Glass and Donaldson 1995). PCR was performed utilizing G2 Flexi DNA polymerase (Promega, Madison, USA) with primers targeting the mentioned gene sequences using respective amplification conditions regarding the authors of primers. Sequencing was done in both directions with the same primer pair used for amplification at the Eurofins Genomics Germany GmbH.

Phylogenetic analysis

Consensus sequences were assembled in BioEdit 7.2.5 and additional reference sequences were obtained from GenBank (**Table 2**). Alignments were performed using MAFFT v. 7 (Katoh *et al.* 2019) with the default settings. Alignments were checked and manual adjustments made when necessary, using BioEdit v. 7.2.5 (Hall *et al.* 1999). Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses were performed using MEGA7 (Kumar *et al.* 2016). The best fitting DNA evolution model was determined also by MEGA7. ML analysis was performed on a Neighbour-Joining starting tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference. MP analysis was done using the Tree-Bisection-Regrafting (TBR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The robustness of the trees (ML and MP) was evaluated by 1000 bootstrap replications. Gaps were treated as missing data. Tree Length [TL], Consistency Index [CI], Retention Index [RI], Relative Consistency Index [RC] and Homoplasy Index [HI] were calculated for the most parsimonious tree.

The alignment and tree files were submitted to TreeBASE (Submission ID: 26867).

TABLE 1. Known distribution and hosts of *Pyrenochaetopsis* species.

Species	Collection No.[1]	Host, substrate	Country	**Refference**
Pyrenochaetopsis americana	FMR 1375	Unknown	USA	Valenzuela-Lopez *et al.* 2018
P. botulispora	UTHSC:DI16-289	Human, respiratory trac	USA	Valenzuela-Lopez *et al.* 2018
P. botulispora	UTHSC:DI16-297	Human, superficial tissue	USA	Valenzuela-Lopez *et al.* 2018
P. botulispora	CBS 142458	Human, bronchial wash sample	USA	Valenzuela-Lopez *et al.* 2018
P. chromolaenae	MFLUCC: 17-1440	*Chromolaena odorata*	Thailand	Mapook *et al*. 2020
P. confluens	CBS 142459	Human, deep tissue	USA	Valenzuela-Lopez *et al.* 2018
P. decipiens	CBS 343.85	*Globodera pallida*	Netherlands	Gruyter *et al*. 2010
P. globosa	CBS 143034	Human, superficial tissue	USA	Valenzuela-Lopez *et al.* 2018
P. indica	CBS 124454	*Saccharum officinarum,* leaf	India	Gruyter *et al*. 2010
P. kuksensis	**CBS 146534**	***Buxus sempervirens*, wood**	**CZE**	**This study**
P. kuksensis	**MEND-F-58**	***Buxus sempervirens*, wood**	**CZE**	**This study**
P. leptospora	CBS 101635	*Secale cereale*	Germany	Gruyter *et al*. 2010
P. leptospora	CBS 122787	Unknown	Netherlands	Valenzuela-Lopez *et al.* 2018
P. microspora	CBS 102876	Human, sinusitis sample	USA	Gruyter *et al*. 2010
P. paucisetosa	CBS 142460	Human, toe nail	USA	Valenzuela-Lopez *et al.* 2018
P. poae	CBS 136769	*Poa* sp.	Netherlands	Crous *et al*. 2014
P. rajhradensis	CBS 146846	*Buxus sempervirens*, wood	Czech Republic	Crous *et al*. 2020b
P. setosissima	CBS 119739	*Coffea arabica*, leaf	Brazil	Valenzuela-Lopez *et al.* 2018
P. sinensis	CGMCC 3.19296	Rhizosphere soil of *Poa pratensis*	China	Hyde *et al.* 2019
P. uberiformis	CBS 142461	Human, superficial tissue	USA	Valenzuela-Lopez *et al.* 2018
P. tabarestanensis	CBS 139506	Soil	Iran	Papizadeh *et al*. 2017
P. terricola	HGUP 1802	Soil	China	Wang *et al*. 2019

Note: [1]BRIP, Queensland Plant Pathology herbarium, Brisbane, Australia; **CBS**, Westerdijk Fungal Biodiversity Institute, Netherlands;; **FMR**, Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain; **CGMCC**, China General Microbiological Culture Collection Center; **HGUP**, Herbarium of Department of Plant Pathology, Guizhou University; **MEND-F**, Fungal Collection of Mendeleum - Institute of Genetics, Mendel University, Czech Republic; **UMP**, University of Melbourne; **UTHSC**, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA

Results

The combined LSU, ITS, *tub2* and *rpb2* sequence data set consisted of 22 *Pyrenochaetopsis* strains with *Xenopyrenochaetopsis pratorum* and *Neopyrenochaetopsis hominis* as the outgroup taxa and consisted of 2205 characters. Of these 1626 were constant, 176 were variable and parsimony-uninformative and 366 were parsimony-informative. A heuristic search of these 366 parsimony-informative characters resulted in 1000 equally parsimonious trees of 1358 steps with CI = 0.59, RI = 0.58 and HI = 0.41. The ML analysis yielded a best scoring tree with the final ML optimization likelihood value of – 9279.66 (ln) and a gamma distribution shape parameter value of $\gamma = 0.2260$. All individual trees obtained from single gene datasets were essentially similar in topology and not substantially different from the tree generated from the concatenated dataset. One of the three ML trees obtained is presented in **Fig. 1** with ML/MP bootstrap support values at the nodes. The four-gene phylogeny (LSU, ITS, *tub2* and *rpb2*) showed the isolates of the present study in a well-supported monophyletic lineage distinct from the previously described *Pyrenochaetopsis* species, which is described here as *Pyrenochaetopsis kuksensis* sp. nov**.** The species described here is highlighted in bold.

TABLE 2. Strains included in the phylogenetic analyses.

Species	Collection number[1]	GenBank accession numbers			
		ITS	LSU	*tub2*	*rpb2*
Pyrenochaetopsis americana	FMR 1375[T]	LT592912	LN907368	LT592981	LT593050
P. botulispora	UTHSC:DI16-289	LT592941	LN907432	LT593010	LT593080
P. botulispora	UTHSC:DI16-297	LT592945	LN907440	LT593014	LT593084
P. botulispora	CBS 142458[T]	LT592946	LN907441	LT593015	LT593085
P. chromolaenae	MFLUCC: 17-1440[T]	MT214378	MT214472	–	MT235827
P. confluens	CBS 142459[T]	LT592950	LN907446	LT593019	LT593089
P. decipiens	CBS 343.85[T]	LT623223	GQ387624	LT623240	LT623280
P. globosa	CBS 143034[T]	LT592934	LN907418	LT593003	LT593072
P. indica	CBS 124454[T]	LT623224	GQ387626	LT623241	LT623281
P. kuksensis	**CBS 146534[T]; MEND-F57**	**MT371092**	**MT371397**	**MT372662**	**MT372656**
P. kuksensis	**MEND-F-58**	**MT371093**	**MT371398**	**MT372663**	**MT372657**
P. leptospora	CBS 101635[T]	JF740262	GQ387627	LT623242	LT623282
P. leptospora	CBS 122787	LT623225	EU754151	LT623243	LT623283
P. microspora	CBS 102876[T]	LT592899	LN907341	LT592968	LT593037
P. paucisetosa	CBS 142460[T]	LT592897	LN907336	LT592966	LT593035
P. poae	CBS 136769[T]	KJ869117	KJ869175	KJ869243	LT623286
P. rajhradensis	CBS 146846[T]	MT853115	MT853182	MT857726	MT857727
P. setosissima	CBS 119739[T]	LT623227	GQ387632	LT623245	LT623285
P. sinensis	CGMCC 3.19296[T]	MK348586	MK348581	MK348221	MK355077
P. uberiformis	CBS 142461[T]; FMR 13769	LT592935	LN907420	LT593004	LT593074
P. tabarestanensis	CBS 139506[T]; IBRC-M 30051	KF730241	KF803343	KX789523	–
P. terricola	HGUP 1802[T]	MH697394	MH697393	MH697392	MH697395
Xenopyrenochaetopsis pratorum	CBS 445.81[T]; FMR 14878[T]	JF740263	GU238136	KT389846	KT389671
Neopyrenochaetopsis hominis	CBS 143033[T]	LN880536	LN880537	LN880539	LT593073

Note: [1]**BRIP**, Queensland Plant Pathology herbarium, Brisbane, Australia; **CBS**, Westerdijk Fungal Biodiversity Institute, Netherlands; **CPC**, Culture collection of Pedro Crouse, Housed at CBS; **FMR**, Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain; **CGMCC**, China General Microbiological Culture Collection Center; **HGUP**, Herbarium of Department of Plant Pathology, Guizhou University; **MEND-F**, Fungal Collection of Mendeleum - Institute of Genetics, Mendel University, Czech Republic; **UMP**, University of Melbourne; **UTHSC**, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA. [T]ex-type.

TABLE 3. Number of a single nucleotide polymorphisms of the most related species to *P. kuksensis* (CBS 146534).

Species	Collection number	Number of nucleotide polymorphisms			
		ITS	LSU	*tub2*	*rpb2*
Pyr. leptospora	CBS 122787	4	0	27	79
Pyr. poae	CBS 136769	5	0	35	78
Pyr. rajhradensis	CBS 146846	4	1	25	82

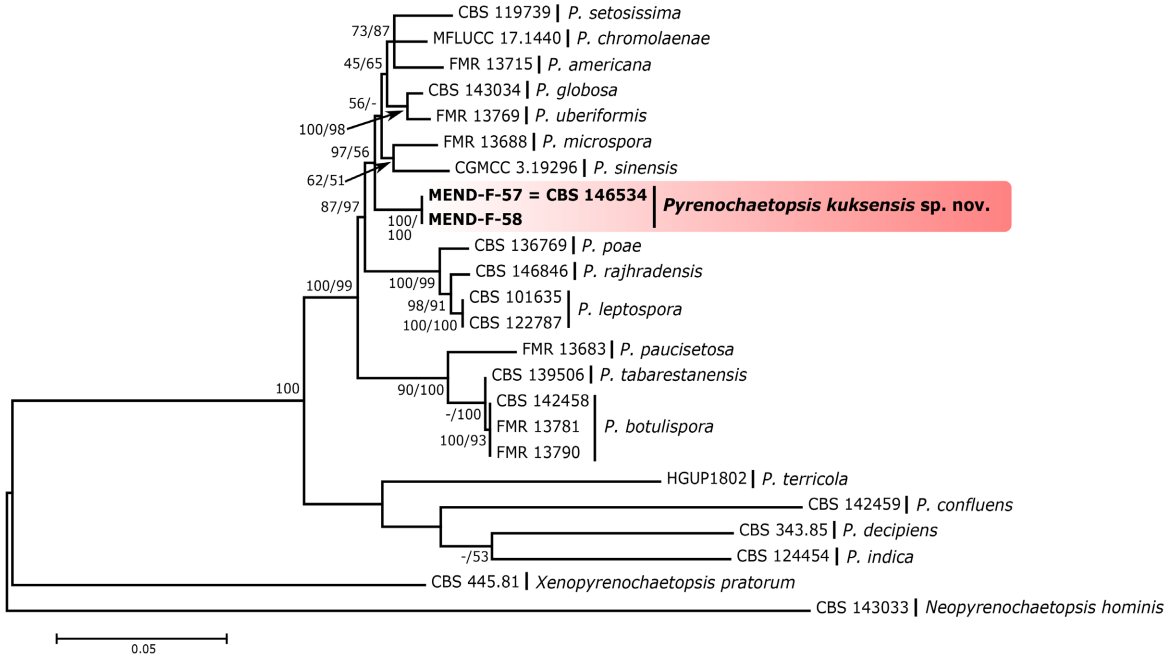

FIGURE 1. Maximum likelihood tree generated from the combined analysis of ITS, LSU, *tub2* and *rpb2* sequence data. ML/MP bootstrap values are given at the nodes. Bootstrap values less than 50 % are not shown. The tree was rooted to *Xenopyrenochaetopsis pratorum* and *Neopyrenochaetopsis hominis*

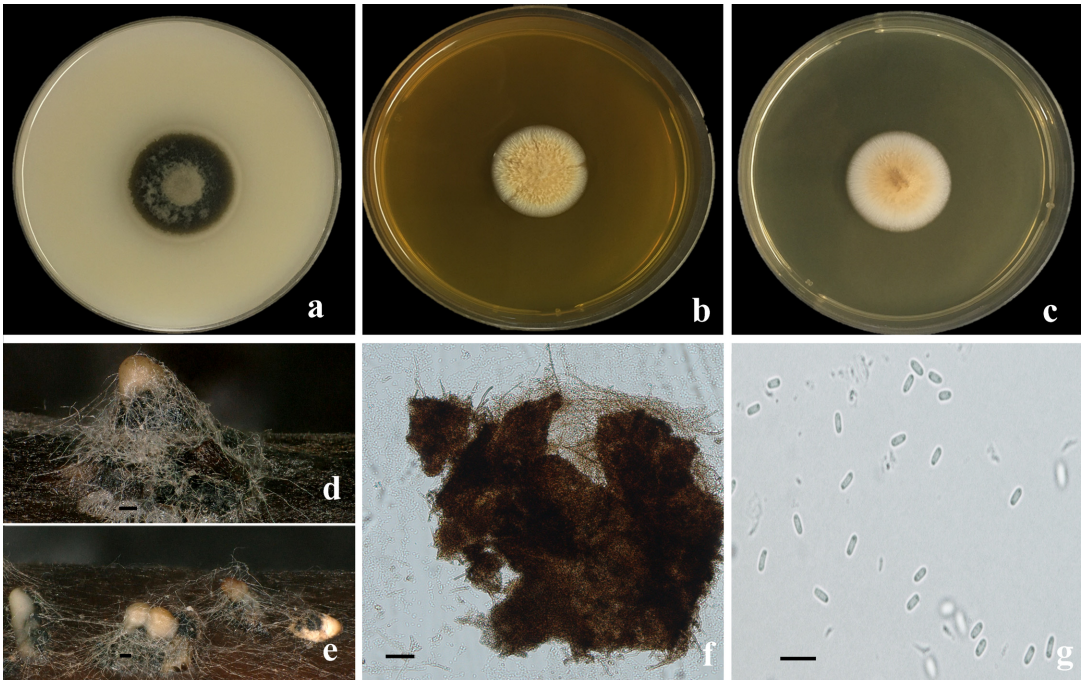

FIGURE 2. *Pyrenochaetopsis kuksensis*. Colony on OA (a). Colony on MEA (b). Colony on PDA (c). Pycnidia forming on poplar twigs (d,e). Cut through pycnidia (f). Conidia (g). Scale bars: d, e = 100 µm. f= 50 µm. g = 10 µm.

Taxonomy

Pyrenochaetopsis kuksensis Spetik, Eichmeier & Berraf-Tebbal, *sp. nov*. (**Fig.2**)
Mycobank number: MB835803

Type:—CZECH REPUBLIC, Nachod: Kuks, castle garden (50°23'49.0"N 15°53'21.7" E), isolated from the wood of *Buxus sempervirens*, September 2018, M. Spetik, Holotype: BRNU 673828, isotype BRNU 673829, ex-type living culture CBS 146534 = MEND-F-57

Saprobic on dead wood of *Buxus sempervirens*. **Asexual morph:** *Conidiomata* pycnidial, brown, solitary or aggregated, semi-immersed, globose to ovoid, setose, ostiolate, unilocula.

Conidiogenous cells phialidic, hyaline, discrete and integrated in septate. *Conidia* hyaline, aseptate, cylindrical to allantoid, slightly guttulate, (2.5–)2.9–4(–3) × (1–)1.4–2(–1.5) µm mean 3 ± 0.4 × 1.4 ± 0.2 µm.
Colonies on PDA reaching 32 mm diam at 25 °C after 10 d, margin regular, floccose, white in outer ring, changing to salmon towards the centre of the colony; reverse white. On MEA reaching 36.5 mm diam after 10 d, margin regular, floccose, erumpent, white in outer ring, changing to salmon towards the centre of the colony; reverse white. On OA reaching 44 mm diam after 10 d, margin regular, floccose, white in outer ring, grey-olivaceous, white in center; reverse grey. No growth at 10° and 37°C was observed.

Etymology:—named after Kuks, where the taxon was collected.

Additional specimens examined:—CZECH REPUBLIC, Nachod: Kuks, castle garden, isolated from the wood of *Buxus sempervirens*, September 2018, M. Spetik, living culture MEND-F-58.

Discussion

The genus *Pyrenochaetopsis* was established to accommodate *Pyrenochaetopsis leptospora* (Sacc. & Briard) de Gruyter *et al.* and is characterized by setose pycnidia, phialidic conidiogenous cells and hyaline, aseptate, cylindrical to allantoid conidia (de Gruyter *et al*. 2010). The new species *P. kuksensis* has morphological characters that fit the generic concepts very well. *Pyrenochaetopsis* species have a broad distribution and various hosts - see **Table 1**. Currently, two *Pyrenochaetopsis* species are known from *Buxus sempervirens* L., both from the Czech Republic - *Pyrenochaetopsis rajhradensis* isolated as an endophyte and *P. kuksensis* described here as saprobe. The morphological characters *P. kuksensis* are very similar to those of *P. leptospora*, *P. poae and P. rajhradensis*. However, the conidia of *P. kuksensis* are smaller 2.9 – 4 × 1.4 – 2 µm than conidia of *P. leptospora* 4.5–7 × 1–2 µm (Boerema *et al.* 2004), *P. poae* 4–5 × 1.5(–2) µm (Crous *et al.* 2004) and *P. rajhradensis* 4.1–4.9 × 1.6–2.2 µm (Crous *et al.* 2020b). Nevertheless, based only on morphological characters is difficult to distinguish between species (Valenzuela-Lopez *et al.* 2018, Wang *et al*. 2019). We confirm an importance of at least four genomic loci (ITS, LSU, *tub2*, *rpb2*) to distinguish between *Pyrenochaetopsis* species. Jeewon & Hyde (2016) suggest that a minimum of >1.5% nucleotide polymorphisms in the ITS regions may indicate a new species. Accordingly, among the 17 known *Pyrenochaetopsis* species, 14 had more than 1.5% nucleotide polymorphisms in ITS region compared to *P. kuksensis*. The other three species, namely: *P. leptospora, P. poae* and *P. rajhradensis* showed less nucleotide similarity than <1.5% compared to *P. kuksensis* ITS regions. Nevertheless, the novelty of specimen was strongly supported by nucleotide comparison of additional three loci – LSU, *tub2*, *rpb2*. Number of single nucleotide polymorphisms in each loci is showed in **Table 3**. Briefly, while comparing nucleotide differences in four regions (ITS, LSU, tub2, rpb2) of *P. kuksensis* against *P. leptospora*, *P. poae* and *P. rajhradensis* the LSU region is the least informative with approximately no differences, ITS region carried approximately 4 % differences, *tub2* loci carried 24% differences and rpb2 loci carried 72% differences. The *tub2* and *rpb2* loci are the most important for resolving interspecific relationship within genus *Pyrenochaetopsis* and we recommend to use them in future studies.

Contribution

This study identified the new species *Pyrenochaetopsis kuksensis* from the shrubs of *Buxus sempervirens* in the Czech Republic and provided the morphological and molecular data for the future studies. It might be possible to identify a new distribution spots of *P. kuksensis* from other shrubs in the world.

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Author Contributions

A.E. designed the study. M.S. performed molecular work. A.B.T. performed the morphological study and phylogenetic data analysis. R.P. helped with microscopy. M.S. wrote the manuscript. A.E., A.B.T., R.P. reviewed and edited the manuscript. All the authors reviewed and approved the final manuscript.

Additional information

Competing Interests: The authors declare no competing interests.

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