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Citizen science facilitates phylogenetic placement of old species of non-lichenised Pezizomycotina based on newly collected material

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During the informal Spring Micromyco 2019 meeting, we tested how newly obtained molecular barcodes of common or poorly known saprotrophic microfungi from more or less targeted collections may be useful for identification and taxonomic studies. Our aim was to obtain DNA sequences of fungi enabling their phylogenetic placement and routine identification in the future using molecular barcoding.

As a result, DNA of four species was sequenced for the first time, among them *Leptosphaeria acuta*, for which a new synonym *L. urticae* is proposed. The new combination *Koorchaloma melaloma* is proposed for a species previously known as *Volutella melaloma* and its new synonym is *K. europaea*. This species is accommodated in the *Stachybotryaceae*. A detailed phenotypic description and phylogenetic placement are provided for *Mytilinidion insulare*, a resurrected species hitherto considered a later synonym of *M. gemmigenum*. *Chalara insignis* was placed in the *Helotiales* without any clear relationship to other members of this order. For another two helotialean species, a second referencing sequence was obtained.

Our study showed that for some microfungi, due to the fundamental lack of molecular data, even a single molecular barcode may provide novel and important information on their phylogenetic and taxonomic placement. The results also illustrate that scientific progress can be made through collaboration between academic and amateur mycologists.

Key words: ITS rDNA barcode, phenotype, taxonomy, Dothideomycetes, Leotiomycetes, Sordariomycetes.

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Koukol O., Halasů V., Janošík L., Mlčoch P., Polhorský A., Šandová M., Zíbarová L. (2020): Občanská věda pomáhá díky novým nálezům zařadit staré druhy nelichenizovaných zástupců Pezizomycotina do fylogenetického systému. – Czech Mycol. 72(2): 263–280.

Během neformálního setkání Jarní Micromyco v roce 2019 jsme ověřili, jak mohou být užitečné sekvence nově získané z více či méně cílených sběrů běžných nebo méně známých druhů saprotrofních mikroskopických hub. Naším cílem bylo získat sekvence umožňující jejich zařazení do fylogenetického systému a rutinní určování v budoucnosti založené na sekvenční shodě.

Výsledkem je získání vůbec první sekvence pro čtyři druhy hub. Mezi nimi je i *Leptosphaeria acuta*, pro kterou je navrženo nové synonymum *L. urticae*. Nová kombinace *Koorchaloma melaloma* je navržena pro druh dříve známý jako *Volutella melaloma*, nově je s ním synonymizován *K. europaea* a současně je tento druh zařazen do čeledi *Stachybotryaceae*. Dále jsme provedli detailní popis fenotypu a umístili ve fylogenetickém systému znovu oživený druh *Mytilinidion insulare*, který byl považován za synonymum druhu *M. gemmigenum*. *Chalara insignis* byla zařazena do řádu *Helotiales* bez další příbuznosti k ostatním zástupcům tohoto řádu. Pro další dva druhy z řádu *Helotiales* byla získána druhá referenční sekvence.

Naše studie ukázala, že kvůli obecné absenci molekulárních dat může pro některé mikroskopické houby i jediná sekvence představovat novou a významnou informaci o jejich fylogenezi a taxonomii. Výsledky zároveň dobře ilustrují pokrok, jakého je ve vědě možné dosáhnout spoluprací mezi akademiky a amatérskými mykology.

INTRODUCTION

In recent years, DNA-based approaches in fungal diversity-oriented studies were favoured over phenotype-based approaches, especially in fungal groups with minimum morphological characteristics, such as yeasts (Vu et al. 2019). Unfortunately, less than 35,000 of the currently known 120,000 formally recognised "good" species are represented in the GenBank database by at least one sequence (Hawksworth & Lücking 2017). This is usually the internal transcribed spacer (ITS) of ribosomal DNA, which is largely accepted as the fungal barcode (Kõljalg et al. 2013), but its use as the sole barcode for identification is problematic in particular fungal groups (Raja et al. 2017, Stadler et al. 2020). Other drawbacks of using ITS from GenBank for sequence identification is the insufficient length or poor quality of the deposited sequences (Nilsson et al. 2006). In a recent study, Hofstetter et al. (2019) tested the reliability of identification of macrofungi collected during a large fungal survey project in a beech-dominated old-growth forest in Switzerland. After very detailed verification and in-depth examination of BLAST results, they concluded that around 30% of ITS sequences were associated with incorrect taxon names and also reported of frequent errors in metadata. Therefore, one of the challenges of current mycology is to fill the gaps in public databases with sequences originating from reliably identified vouchered specimens, by which gaps in the knowledge of the phylogenetic position of "old"

fungal species may be bridged. Multiple examples in ascomycetes show that when a polyphasic approach in identification is applied to collections of rare fungi, substantial updates of their taxonomy may be the result (Iturriaga et al. 2017, Agnello et al. 2018, Suija et al. 2020).

Our knowledge of the distribution and occurrence of numerous saprotrophic microfungi is rather scanty. Their fructification may be limited to a short time period with specific microclimatic conditions and thus some species have hitherto been overlooked in systematic surveys in scientific projects. Microfungi recorded and vouchered by amateur mycologists are usually not accompanied by molecular data. To fill these gaps, molecular barcodes were obtained from collections of rare or poorly known microfungi during an informal meeting titled Spring Micromyco 2019 (Koukol 2019b). Professionals, mycology students and keen amateur mycologists contributed with fresh collections which were identified primarily based on phenotypic characteristics. This traditional approach was coupled with isolating them in living cultures. DNA extraction and analysis of molecular data were used to confirm the identification based on the phenotype. The aim of this survey was to assess how useful this combined approach is for the identification of saprotrophic microfungi and to obtain original sequences of species which are not represented in GenBank.

MATERIAL AND METHODS

Fresh material was observed using dissecting and light microscopes. Fungal structures were mounted in water, lactic acid, Melzer's reagent and Lugol's solution. Isolation of selected fungi was performed under a dissecting microscope using sterile 100 units of insulin syringes which make it possible to take minute amounts of fungal tissue (usually hymenium or conidia) and transfer them to Petri dishes with Potato Carrot Agar (PCA) or Water Agar (prepared from 20 g of agar dissolved in 1 litre of distilled water). Petri dishes were kept on the laboratory bench and observed regularly.

To obtain ITS barcode sequences, two approaches were adopted. In one, the process of DNA extraction was omitted and small pieces of fresh fungal tissue (mostly hymenium) were placed directly into the PCR microtube with a sterile needle. In the other, DNA was extracted from fruitbodies or conidiomata (usually 3–5 taken directly from the substrate) or mycelium from culture using the ZR Fungal/Bacterial DNA kit (Zymo Research, Orange, USA). Nuclear rDNA containing the ITS and LSU regions was amplified with primer set ITS1F/NL4 (White et al. 1990, O'Donnell 1993). The PCR products were viewed by electrophoresis on 1% (w/v) TAE agarose gel, stained with ethidium bromide. The PCR products were purified with the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech,

Bade City, Taiwan). Both strands of the PCR fragments were sequenced with the primers used for amplification at the Sequencing Laboratory of the OMICS Core Facility, BIOCEV (Vestec, Czech Republic).

Sequences were checked for ambiguous bases and assembled in Geneious (Biomatters, Auckland, New Zealand). Consensus sequences were used for homology search using the BLAST search (Altschul et al. 1997) in the GenBank database. Phylogenetic analysis based on Bayesian interference, performed using MrBayes v3.2.6 (Ronquist et al. 2012), followed the settings described in Koukol et al. (2018).

RESULTS

In the first step, 11 out of 15 specimens used for PCR without previous extraction of DNA were successfully amplified as viewed on the gel. With the exception of one, they yielded high quality reads, which allowed for their assemblies and further use of consensus sequences. Together with specimens whose DNA was amplified after the routine extraction, at least one sequence was obtained from 14 fungal collections and submitted to GenBank (species marked with an asterisk in the following paragraph and all species listed thereafter). Common and previously thoroughly characterised species, identified already based on phenotype, included *Cistella caricis* (Raitv.) Raitv.* (sequences obtained from two collections), *Cistella typhae* (Svrček) Raitv.*, *Cucurbitaria spartii* (Nees ex Fr.) Ces. & De Not., *Dermea acerina* (Peck) Rehm*, *D. tulasnei* J.W. Groves*, *Hyaloscypha fuckelii* Nannf.*, *Lachnum virgineum* (Batsch) P. Karst., *Lasiobolus intermedius* J.L. Bezerra & Kimbr., *Nematogonum ferrugineum* (Pers.) S. Hughes, and *Sydowiella fenestrans* (Duby) Petr. Two species, *Peziza varia* (Hedw.) Alb. & Schwein.* and *Paramyrothecium roridum* (Tode) L. Lombard & Crous*, were identified based on a combination of phenotypic and molecular data, i.e. on sequences already deposited in GenBank. Collecting data and GenBank accessions are presented in the Electronic supplement to this paper. The following six species are discussed in more detail and also documented with microphotographs.

Brunnipila palearum (Desm.) Baral, in Baral & Krieglsteiner, Beih. Z. Mykol. 6: 51, 1985 Fig. 1a–c

Apothecia of this species were found in large amounts on dead grass, but without mature ascospores. Therefore, culms of grass were placed in a sterile moist chamber and observed again after 12 days. The observed characters including the type of ascus bases and the ascus pore reaction matched the descriptions by

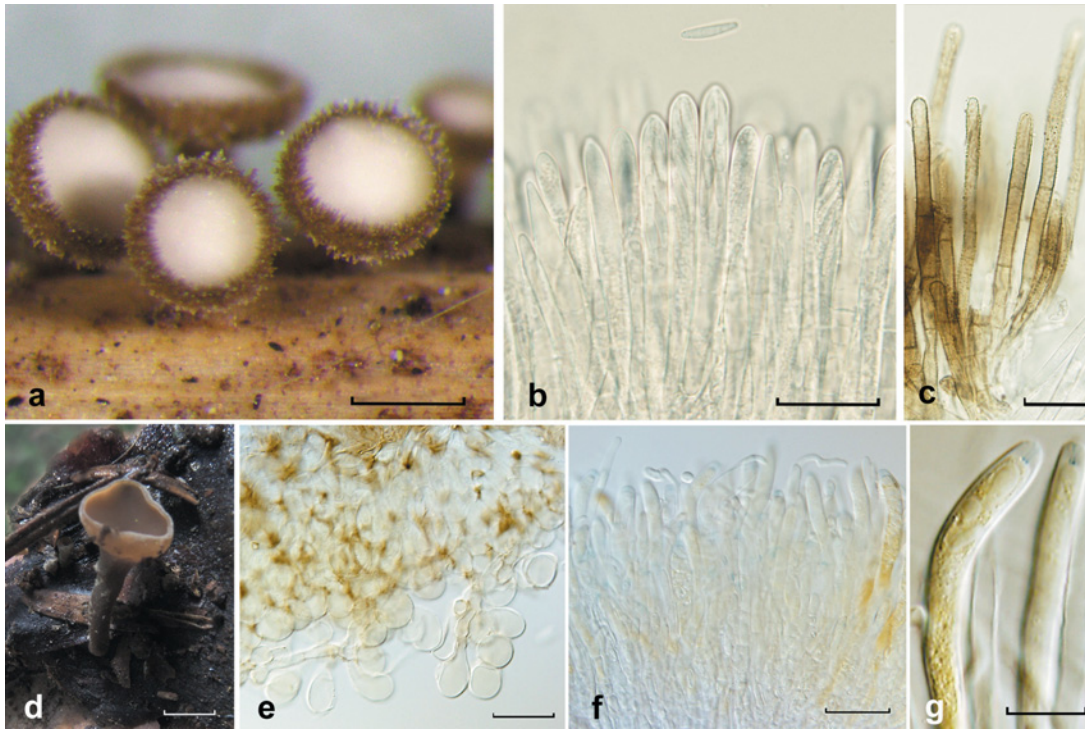

Fig. 1. *Brunnipila palearum* (PRM 953071): **a** – apothecia on dead culms after 12 days in moist chamber; **b** – asci with pointed paraphyses and one released ascospore; **c** – excipular hairs. *Ciboria rufofusca* (PRC 4659): **d** – apothecium growing out of cone scale; **e** – excipular cells; **f** – asci with interspersed paraphyses; **g** – detail of asci with ascus tip stained with Melzer's reagent. Scale bars = 500 µm (a, d), 20 µm (b–c, e–f), 10 µm (g). Photographs O. Koukol (a, b, e–g), M. Šandová (c), M. Piepenbring (d).

Ellis & Ellis (1997) and Šandová et al. (2018) with exception of the hairs, which were 2–4-septate (Fig. 1c). The measurements in living state recorded during the present study were: hairs 70–90 × 4–6 µm, asci 58–70 × 5–5.5 µm, ascospores 10–14 × 2–2.5 µm, paraphyses (5.5)6–7.5(8) µm wide.

The ITS sequence obtained from our collection was identical with sequence LT904856 (specimen voucher PRM 900741) originating from a study by Šandová et al. (2018). The LSU region (containing the variable D1/D2 domain) obtained for the first time revealed 98.2% similarity with *B. fuscescens* (Pers.) Baral (LC424945, specimen voucher TNS F-16635).

Collection studied

Czech Republic. Central Bohemia, Praha-Běchovice, 150 m west of Zelená spring, 50°5'8.6" N, 14°38'2.4" E, alt. 247 m, on dead grass culms, 12 Apr 2019, leg. & det. M. Šandová (PRM 953071).

Ciboria rufofusca (O. Weberb.) Sacc., Syll. fung. (Abellini) 8: 203, 1889 Fig. 1d–g

Multiple apothecia in various stages of maturity were collected on decomposing cone scales of *Abies alba* buried in the litter. This species can be readily recognised based on its characteristic brown stipitate apothecia growing on the cone scales of fir, less frequently also of other conifers (Groves & Elliott 1969, Palmer et al. 1994).

BLAST search showed a 99.8% match with a sequence obtained from an isolate from a pine twig and identified as *Lambertella* sp. (MG098309, strain NW-FVA2685) in a dissertation by Busskamp (2018). Two sequences under the name *C. rufofusca* were already present in GenBank (*C. rufofusca* KF545372 and KF545358, voucher specimens BPI 653662 and BPI 653664, respectively), but these ITS fragments of only 187 bp revealed no similarity with our ITS sequence. Interestingly, these GenBank records were not identical, but differed from each other by 4 bp. BLAST search performed with KF545372 showed an almost identical match with multiple records of *Lambertella corni-maris* Höhn. (with only one out of 187 bp differing) indicating a misnaming of these putative *C. rufofusca* sequences.

Collection studied

Czech Republic. Central Bohemia, between Hvězdonice and Zlenice, 500 m NW of Stará Dubá ruins, 49°52'31.4" N, 14°44'54.9" E, alt. 347 m, on remnants of *Abies alba* cone in the litter, 4 May 2019, leg. & det. L. Janošík (PRC 4659).

Chalara insignis (Sacc., M. Rousseau & E. Bommer) S. Hughes, Can. J. Bot. 31: 622, 1953 Fig. 2a–g

A dense growth of dark phialides with distinct chains of conidia was visible on the substrate already under the dissecting microscope. The morphological characteristics matched those given by Nag Raj & Kendrick (1976). Conidia isolated on PCA germinated and produced a slow growing white mycelium. After 2 months of incubation, aberrant phialides were produced in the central part of the colony. These phialides were mostly shorter, only 23–43 µm long compared to 66–145 µm on the natural substrate (Nag Raj & Kendrick 1976). No released conidia were observed. BLAST search with the ITS sequence indicated placement of *C. insignis* in the *Helotiales* without any clear relationship to other members of this order, as no match above 90% was found. BLAST search performed with the LSU sequence indicated *Chalara breviclavata* Nag Raj & W.B. Kendr. as the most closely related species (474/483 bp similarity).

Collection studied

Czech Republic. Central Bohemia, Nechánice near Kamenice, margin of a mixed forest, 49°55'2.4" N, 14°32'27.8" E, alt. 419 m, on rotten bark of fallen log of *Betula pendula*, 9 Dec 2018, leg. D. Novák, det. O. Koukol (PRC 4658).

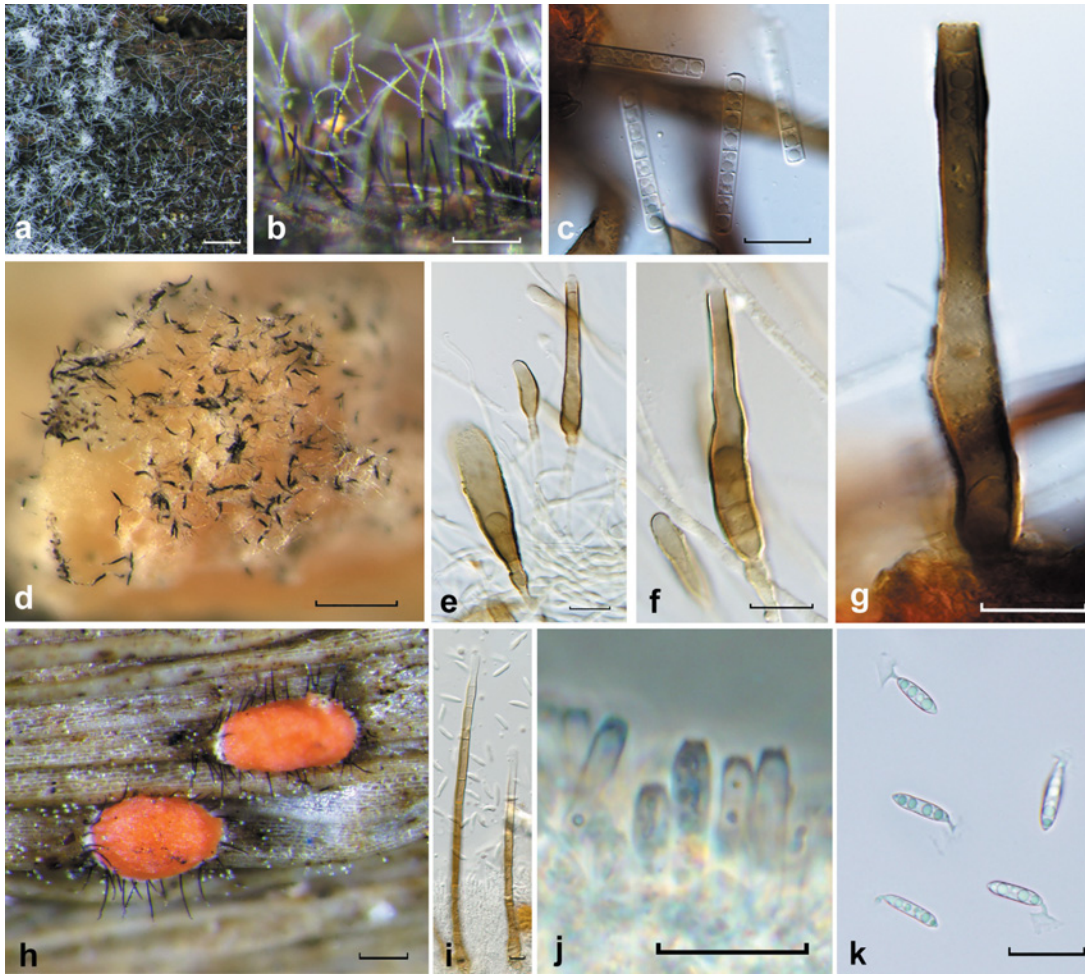

Fig. 2. *Chalara insignis* (PRC 4658): **a–b** – phialides with chains of conidia on rotten bark; **c** – conidia; **d** – phialides in central part of colony on PCA; **e–f** – aberrant phialides in culture; **g** – phialide on substrate. *Koorchaloma melaloma* (PRM 953076): **h** – sporodochia on dead leaves; **i** – setae; **j** – conidiogenous cells; **k** – conidia. Scale bars = 500 µm (a, d), 200 µm (b, h), 20 µm (c, g, k), 10 µm (e–f, i–j). Photographs O. Koukol (a–j) and M. Šandová (k).

Koorchaloma melaloma (Berk. & Broome) Koukol & Šandová, **comb. nov.**

MycoBank MB 836323 Fig. 2h–k

≡ *Volutella melaloma* Berk. & Broome, Ann. Mag. nat. Hist., Ser. 2, 5: 465, 1850

= *Koorchaloma europaea* Treigienė, Mycotaxon 96: 177, 2006

Orange pulvinate sporodochia surrounded by black setae were observed on the substrate. Morphological characters, especially the presence of fusiform conidia

with apical mucoid appendages matched well the short description of *Volutella melaloma* Berk. & Broome given by Ellis & Ellis (1997). However, the morphologically highly similar genus *Koorchaloma* Subram. accommodating mostly tropical species came under our scrutiny. *Koorchaloma* species are characterised by brightly coloured, sporodochial conidiomata, with dark setae and 1-celled, hyaline, fusiform conidia with mucoid appendages at both ends or only at the apex. They occur mostly on dry culms and leaves of monocots (Subramanian 1953, Sarma et al. 2001). Two species of this genus are exceptional in having a mucoid appendage only at the apex, i.e. *Koorchaloma madreeya* Subram., the type of the genus, and *Koorchaloma europaea* Treigienė. Whilst our specimen differed from the description of *K. madreeya* (Nag Raj 1993) by setae having a longer apical cell (20–35.5 µm vs 8–14 µm), narrower conidiogenous cells (2–3 µm vs 3–4 µm) and larger mean conidium length/width ratio (4.5 vs 3.7:1), it was identical to that of *K. europaea* (Treigienė 2006). The protologue of the latter species does not include the size of the conidial appendage, which was 7–8 µm long and 8–9 µm wide in the living state of our fungus, but the illustration clearly shows the same dendriform shape. Similarly, the inflated thin-walled apical cells of the setae were clearly depicted by Treigienė (2006), but not mentioned in the protologue. Interestingly, we observed also setae with a pointed thin-walled apex (probably immature) in our specimen, which agrees with the illustration of *V. melaloma* in Berkeley & Broome (1850) and provides further evidence for conspecificity of these two species.

The species is obviously not host-specific, because it was recorded, as *V. melaloma*, on *Carex* spp. (Berkeley & Broome 1850, Ellis & Ellis 1997), *Iris pseudacorus* (Ale-Agha et al. 2004) and dead stems and leaves of *Heracleum mantegazzianum* (Feige & Ale-Agha 2004). Treigienė (2006) recorded this species, as *K. europaea*, on dead stems and leaves of several plant species in Lithuania, among others also on *Scirpus sylvaticus*, i.e. the same substrate as in our study.

The ITS sequence of this species was obtained for the first time. The closest records in a BLAST search with only 89–90% similarity were multiple species of the genus *Stachybotrys* Corda, indicating its placement in the *Stachybotryaceae* L. Lombard & Crous (*Hypocreales*) or in close relationship to them. *Koorchaloma* did not appear in the closest matches and this genus was not yet included in the most recent phylogenetic study of *Stachybotryaceae* (Lombard et al. 2016). However, our phylogeny based on ITS and combining sequences of the most closely related members of *Stachybotryaceae* with sequences of three *Koorchaloma* species used by Li et al. (2020) confirmed that our fungus is congeneric with *Koorchaloma* (Fig. 3). In view of the other morphological similarities, *V. melaloma* is combined here into the phylogenetically correct genus *Koorchaloma* with *K. europaea* as its later synonym.

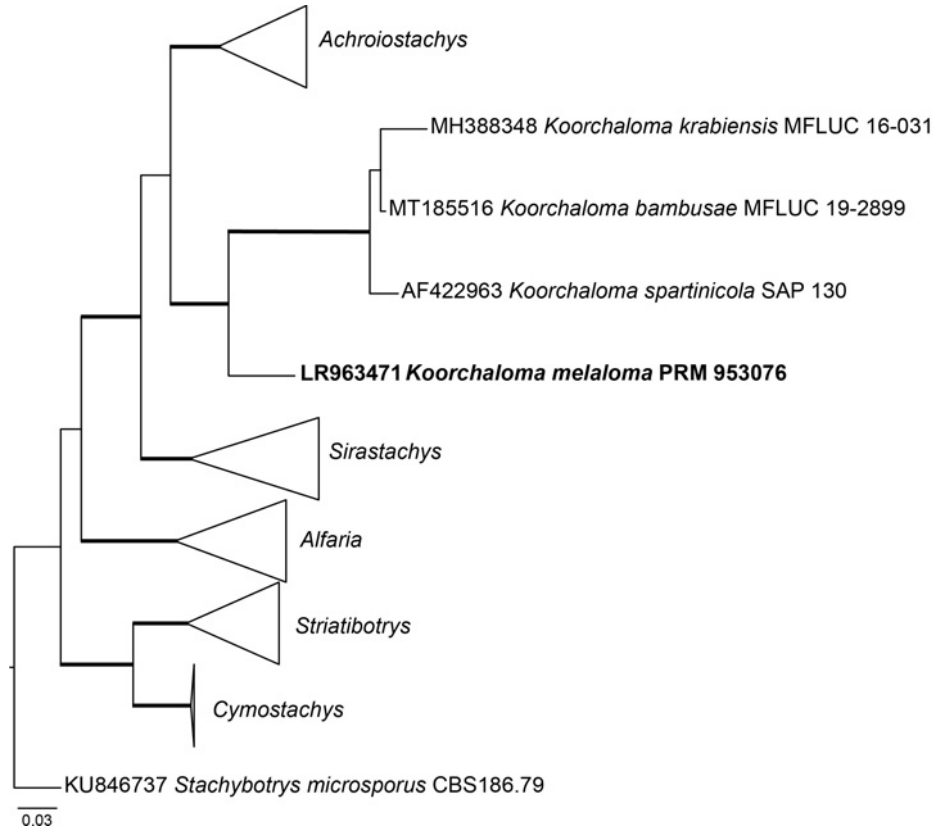

Fig. 3. Phylogenetic tree inferred from Bayesian analysis showing the position of *Koorchaloma melaloma* (in bold) within *Stachybotryaceae* based on ITS. Thick branches represent PP > 0.95. Sequence of *Stachybotrys microsporus* (B.L. Mathur & Sankhla) S.C. Jong & E.E. Davis was used as outgroup. Collapsed branches denote multiple sequences of the given genera. For particular species and sequence codes, refer to Lombard et al. (2016) and Li et al. (2020).

Collection studied

Czech Republic. Central Bohemia, Praha-Běchovice, Klánovický les Nature Reserve, Zelená spring, wet terrain depression, 50°5'9.2" N, 14°38'9.2" E, alt. 247 m, on basal leaves of dead *Scirpus sylvaticus*, 12 Apr 2019, leg. & det. M. Šandová (PRM 953076).

Leptosphaeria acuta (Fuckel) P. Karst., Bidr. Känn. Finl. Nat. Folk 23: 98, 1873
Fig. 4

= *Leptosphaeria urticae* D. Pem, E.B.G. Jones & K.D. Hyde, in Phookamsak et al., Fungal Diversity 95: 21, 2019

Numerous black pseudothecia were collected on the substrate. This species belongs to the hitherto phylogenetically unresolved species group of *Leptosphaeria*

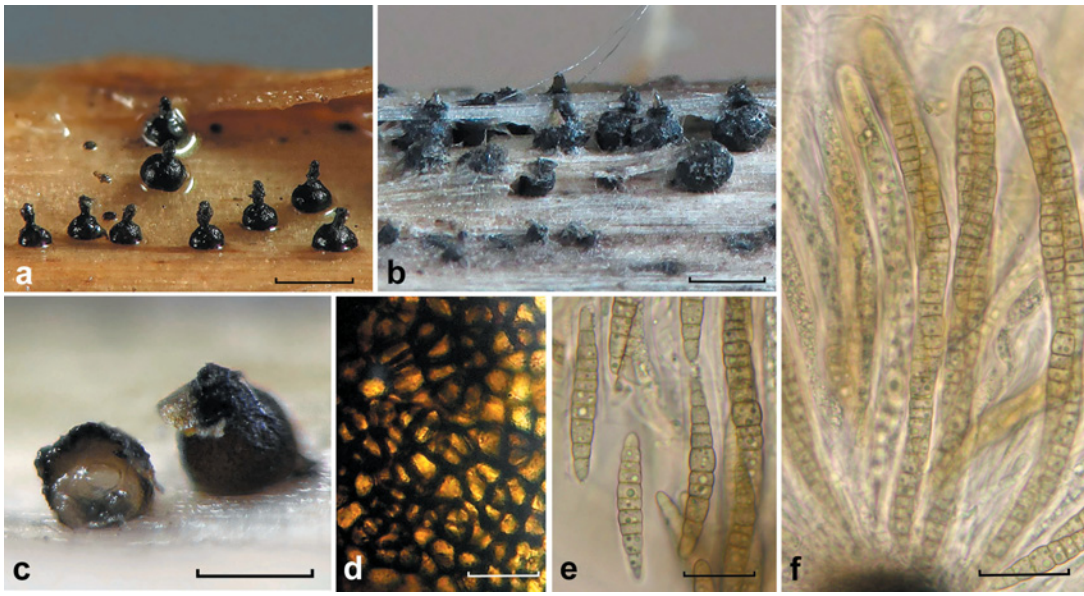

Fig. 4. *Leptosphaeria acuta* (PRM 953072): **a** – pycnidia on dead culm; **b** – pseudothecia partially immersed in dead culm; **c** – cross section through pseudothecium; **d** – ascomatal wall; **e** – ascospores; **f** – asci with ascospores. Scale bars = 500 µm (a–c), 20 µm (d–f). Photographs P. Mlčoch.

multiseptata G. Winter with six and more transversal septa and fusiform ascospores up to 50 µm long without appendages and gelatinous sheath (Müller 1950). Morphologically similar species are species of the genus *Plenodomus* Preuss, which usually have ascospores with a central constriction, the ascomatal wall of a *textura angularis* and strictly parasitic anamorphs. The recently described species *L. urticae* is phylogenetically, ecologically and morphologically identical to *L. acuta* and was erroneously proposed as a new species, obviously due to absence of any sequences of *L. acuta* in GenBank. According to Pem, Jones & Hyde (in Phookamsak et al. 2019), the main diagnostic characteristics are differences in the ITS sequences from the phylogenetically most closely related species *L. italica* Dayar., Camporesi & K.D. Hyde and *L. sclerotioides* (Preuss ex Sacc.) Gruyter, Aveskamp & Verkley. Pem, Jones & Hyde also erroneously interpreted the ascospore septation, which they considered to be different from *L. acuta*. The septation is rather variable in this species even within one collection and thus not a stable diagnostic character.

Collection studied

Czech Republic. Central Bohemia, Praha-Klánovice, Klánovický les Nature Reserve, 50°5'11.4" N, 14°38'45.1" E, alt. 245 m, on dead stem of *Urtica dioica*, 12 Apr 2019, leg. & det. M. Šandová (PRM 953072).

To support future identifications, a key to the phragmosporous species of the *Pleosporales* on *Urtica* (based on both literature records and unpublished data) is provided here.

1 Pseudothecia depressed globose, 250–320(450) µm wide, ascomatal wall of a *textura angularis*, asci 75–100 × 10–12(15) µm, ascospores hyaline to light yellowish, 35–45(50) × 5–8 µm, Q = 3.2, 3(–5)-septate, second cell enlarged ..... *Paraleptosphaeria macrospora* (Thüm.) Gruyter, Aveskamp & Verkley

1' Pseudothecia not depressed, ascomatal wall of a *textura angularis* or *textura prismatica*, ascospores not hyaline or with other characters 2

2 Ascospores constantly 3-septate (rarely 4-septate), up to 30 µm long 3

2' Ascospores with more than 3 septa, longer than 30 µm 6

3 Pseudothecia with mauve tones, globose, 180–250 µm wide, ascomatal wall of a *textura angularis*, asci 70–110 × 7.5–10 µm, ascospores 3-septate (rarely 4-septate), fusiform, second cell enlarged, yellowish to brown, 25–32 × 3–5 µm ..... *Leptosphaeria atropurpurea* Petr.

3' Pseudothecia without mauve tones, dark, globose or conical, ascomatal wall of a *textura angularis* or *textura prismatica*, asci and ascospores with other characters 4

4 Pseudothecia globose to hemiglobose, never concentrically sulcate, less than 250 µm wide, ascomatal wall of a *textura angularis*, ascospores with second cell enlarged 5

4' Pseudothecia conical, concentrically sulcate, more than 250 µm in diam., ascomatal wall of a *textura angularis* or *textura prismatica*, asci (90)100–135 µm long, ascospores yellowish brown to brown, with ascospore cells constricted at the septa 6

5 Asci (50)60–70 × 6–7 µm, ascospores fusiform, hyaline to light brownish, (18.5)20–23(24) × 4.5–6(6.5) µm, Q = 4.2 ..... *Leptosphaeria* aff. *dumetorum* Niessl

5' Asci (55)70–80(95) × (6)8–10(11) µm, ascospores fusiform to narrowly fusiform, hyaline to yellowish brown, (20.5)21.5–30(34) × (4.5)5.5–7(7.5) µm, Q = 5.2 ..... *Leptosphaeria purpurea* Rehm

6 Asci 8–9 µm wide, second cell of ascospores never enlarged, ascospores (20)22–28(31) × (3.5)4–5(5.5) µm, Q = 4.7 ..... *Leptosphaeria doliolum* (Pers.) Ces. & De Not.

6' Asci 7–8 µm wide, second cell of ascospores enlarged, ascospores (22)24–27 × 4–5(6) µm, Q = 5.4 ..... *Leptosphaeria doliolum* var. *dissimilis* Rehm

7 Asci 130–170 µm long, ascospores without central constriction, (6)7–10(11)-septate, yellow-brown to brown, without terminal appendages, 37–50 × 4.5–8 µm ..... *Leptosphaeria acuta* (Fuckel) P. Karst.

7' Asci 80–120 µm long, ascospores constricted at the central septum, 5-septate, hyaline to brownish, often with terminal appendages, (30)35–40 × 4.5–5.5 µm ..... *Leptosphaeria ogilviensis* (Berk. & Broome) Ces. & De Not.

Mytilinidion insulare Sacc. [as '*Mytilidion*'], in Barbey, Fl. Sard. Comp.: 246, 1885 Fig. 5

Black pseudothecia of this species were found on south-facing, recently fallen, but long dead and strongly weathered, decorticated trunk of *Pinus sylvestris*, on bleached wood in upper parts of the tree. Phenotypic characteristics differentiated this fungus from common species of *Mytilinidion* or other members of the *Hysteriales*. A detailed search in the literature revealed the name *M. insulare* with a short, but well-matching description (Barbey 1885). This species, described from the surface of dry wood in Sardinia, was considered a synonym of *M. gemmigenum* Fuckel by Zogg (1962). There are morphological


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i
j

◀ **Fig. 5.** *Mytilinidion insulare* (BRA CR32418): **a** – pseudothecia partially immersed in dead wood; **b** – typical habitat with standing dead pine trees; **c** – asci with ascospores; **d** – cross section through pseudothecium; **e** – pseudoparaphyses; **f** – crozier at ascus base; **g** – ascospores; **h** – germinating ascospores; **i–j** – conidia produced in culture. Scale bars = 500 µm (a), 10 µm (c, e, g–i), 30 µm (d), 5 µm (f), 20 µm (j). Photographs A. Polhorský (a–h) and O. Koukol (i–j).

differences between these two species, such as the production of immersed pseudothecia, smaller number of septa (3–8 vs 3–11) and sizes of the ascospores with more pronounced constrictions at spore septa. Because of the very short diagnosis and hardly any other records in the literature, we provide here a detailed description of *M. insulare* and its phylogenetic placement.

Description on natural substrate (in *living and †dead state). Pseudothecia (0.17)0.36–0.75(0.90) mm long, 0.13–0.27(0.34) mm wide, 0.25–0.34 mm high, first immersed, finally erumpent, parallel to the vascular bundles of the host tissue, navicular, straight or slightly flexuous, gregarious to scattered, not anastomosing, not darkening the substrate, with cristate apex having a longitudinal slit, surface black. Ascomatal wall 23–50 µm thick, basally thickened up to 110 µm, outer layer carbonaceous, inner layer composed of densely packed, thin-walled, hyaline to brownish cells composing a *textura angularis*. Pseudoparaphyses septate, persistent, *1.5–3 µm wide, hyaline, rarely branched, apical cell slightly or not inflated, covered in gelatinous matrix forming an epithecium. Asci †(69)75–95(112) × (12)12.5–13.5(15.5) µm, cylindrical to clavate, bitunicate, 8-spored, biseriate, basally subbiseriate, short-stipitate with croziers. Ascospores */†(18)21–30(38) × (5)5.5–6.5(7) µm, straight to slightly curved phragmospores, indistinctly heteropolar – apex broadly rounded, base attenuated, smooth, without sheath, submature spores hyaline to yellowish, mature ones yellow to brown, (3)4–5(8)-septate, constricted at septa, containing oil guttules.

Description in culture. Brown to grey-brown pulvinate colonies were formed on PCA from germinating ascospores reaching 12–15 mm in diam. in 21 days at 25 °C. Vegetative hyphae with thickened cell wall, brown, 2–3 µm wide. Conidia globose to subglobose, produced from integrated, holoblastic conidiogenous cells in branched, dispersible chains, 3.5–4.5 µm in diam.

A BLAST search with the ITS sequence did not reveal any record with a match higher than 98.5%. Interestingly, the LSU sequence was almost identical (with only one out of 468 bp differing) with sequence MG760876, originating from a fungal strain isolated from an asymptomatic surface-sterilised needle fragment of *Pinus ponderosa* in Arizona (Bowman & Arnold 2018), suggesting a possible conspecific record. Phylogenetic comparison with *M. gemmigenum* is not provided due to absence of its sequences in online databases. However, an unpublished LSU sequence obtained by one of the authors showed a significant difference

from *M. insulare* (840/882 bp match). A detailed study of *M. gemmigenum* will be presented in a future paper.

Collections studied

Slovakia. Žilina Region, Blatnica, Pekárová hill, 48°58'8.4" N, 18°58'18.4" E, alt. 879 m, weathered wood of *Pinus sylvestris*, 5 Apr 2019, leg. & det. A. Polhorský (BRA CR32418); ibid., 21 Sep 2019 (BRA CR32419).

DISCUSSION

Our approach shows how citizen science may contribute to scientific mycology when combined with the technical facilities of a scientific institution. Phenotype-based identification of freshly collected microfungi performed by experienced academic and non-academic mycologists was coupled with molecular data obtained directly from collected specimens or their cultures. Molecular data is essential in confirming species and generic concepts and elucidating the phylogenetic placement of fungi whose DNA has not been sequenced. This was particularly the case with *Mytilinidion insulare*, whose description was rather short and ambiguous (Barbey 1885) and since its synonymisation with *M. gemmigenum* by Zogg (1962) the name has not been in use. We believe that phenotypic differences are sufficient to distinguish these two species, although we could not confirm this based on molecular data. Nevertheless, a type study of *M. insulare* is recommended to confirm its identity with our recent collection (the type specimen was not available during this study due to COVID-19 restrictions). The reason that *M. insulare* has so far been overlooked might have resulted from the extreme niche that it occupies, i.e. tops of standing dead trees.

Our results also point to a general underrepresentation of saprotrophic microfungi, even the common ones, in GenBank. Together with an unfortunate trend in the description of new species from single collections based primarily on sequence similarities without a study of original species descriptions, this may lead to redundant taxonomic novelties (Friggens et al. 2017). This was particularly distinct in the case of *Leptosphaeria acuta*, a very common species which was not represented by any sequence in GenBank prior to our study, so that the morphologically identical *L. urticae* was erroneously introduced as new species (Phookamsak et al. 2019). Also very low sequence similarities obtained for e.g. *Chalara insignis* were rather surprising, which suggests that some well-known fungi represent hitherto unknown, deeply rooting lineages of ascomycetes.

Relying on the BLAST search itself may be misleading for various reasons. One of them may be the preference of BLAST search for listing sequences with higher coverage but lower similarity, as noted also by Hofstetter et al. (2019). To avoid false negative results, i.e. absence of short but highly identical sequences

in the top results, a simple phylogeny may be necessary in elucidating the phylogenetic position and thus the correct name for a given fungus (Koukol 2019a). Although a BLAST search with the ITS sequence obtained from a fungus originally identified as *Volutella melaloma* did not indicate any affinity to species of the genus *Koorchaloma*, whose sequences were nevertheless present in GenBank, Bayesian analysis showed that these species formed a well-supported monophyletic lineage and thus supported the new combination (Fig. 3).

Despite a considerably high success ratio in the amplification of ITS directly from fungal tissues (75% of reactions yielded high quality reads), the use of ITS as the sole barcode may not provide enough information for conclusive identification of particular fungal groups. This was especially the case with three collections of two *Cistella* species, identified based on their phenotype. Two ITS sequences originating from independent collections of *C. caricis* were highly divergent, sharing only 93% similarity, and a sequence obtained from *C. typhae* differed from all other *Cistella* sequences in GenBank (less than 90% similarity). Similarly, our collections of *Dermea acerina* and *D. tulasnei* were identified based on phenotypic characteristics and matched well with previous descriptions (Groves 1946). However, the ITS sequence of *D. acerina* shared only 94% similarity with two sequences of this species present in GenBank. Both of them originate from seemingly reliable sources, but their usability as reference sequences for our collections is questionable. The first one (AF141164) originates from a Canadian collection by J.W. Groves (Abeln et al. 2000), i.e. from a geographically distant area, while the other one (MK894299) from a Ukrainian collection contains multiple ambiguities (Suija et al. 2020). Interestingly, we obtained higher similarity (98.56%) with two sequences originating from the recently introduced genus and species *Neodermea rossica* W.J. Li, D.J. Bhat & K.D. Hyde (Li et al. 2020). This description is based on a single collection from *Acer tataricum* in western Russia and in absence of knowledge of the phylogeographic structure of *D. acerina*, this novelty may merely represent a European population of this primarily North American species. A correct taxonomical combination for *D. acerina* can thus be made only after more collections from the entire geographical distribution range and more genes are included (Suija et al. 2020). These examples indicate that proper barcodes enabling reliable identification should be selected after detailed phylogenetic study of these groups of fungi.

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REFERENCES

ABELN E.C.A., DE PAGTER M.A., VERKLEY G.J.M. (2000): Phylogeny of *Pezicula*, *Dermea* and *Neofabraea* inferred from partial sequences of the nuclear ribosomal RNA gene cluster. – Mycologia 92: 685–693. DOI: https://doi.org/10.1080/00275514.2000.12061209

AGNELLO C., ALVARADO P., LOIZIDES M. (2018): *Sarcopeziza* (*Pezizaceae*, Ascomycota), a new monotypic genus for Inzenga's old taxon *Peziza sicula*. – Ascomycete.org 10: 177–186. DOI: https://doi.org/10.25664/ART-0240

ALE-AGHA N., FEIGE G., CHRISTIAANS B., BRASSMANN M., BALAKIREW S. (2004): New and remarkable microfungi in North Rhine Westphalia, Germany. – Communications in Agricultural and Applied Biological Sciences 69: 489–497.

ALTSCHUL S.F., MADDEN T.L., SCHÄFFER A.A., ZHANG J., ZHANG Z., MILLER W., LIPMAN D.J. (1997): Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. – Nucleic Acids Research 25: 3389–3402. DOI: https://doi.org/10.1093/nar/25.17.3389

BARBEY W. (1885): Florae Sardoae compendium. – Georges Bridel, Lausanne.

BERKELEY M.J., BROOME C.E. (1850): XL.—Notices of British fungi. – Annals and Magazine of Natural History, Series 2, 5: 455–466. DOI: https://doi.org/10.1080/03745486009494947

BOWMAN E.A., ARNOLD A.E. (2018): Distributions of ectomycorrhizal and foliar endophytic fungal communities associated with *Pinus ponderosa* along a spatially constrained elevation gradient. – American Journal of Botany 105: 687–699. DOI: https://doi.org/10.1002/ajb2.1072

BUSSKAMP J. (2018): Schadenserhebung, Kartierung und Charakterisierung des „*Diplodia*-Triebsterbens" der Kiefer, insbesondere des endophytischen Vorkommens in den klimasensiblen Räumen und Identifikation von den in Kiefer (*Pinus sylvestris*) vorkommenden Endophyten. – Ph.D. Dissertation [depon. in Universität Kassel].

ELLIS M., ELLIS J. (1997): Microfungi on land plants. An identification handbook (New enlarged Ed.). – Richmond Publishing Co., Slough.

FEIGE G., ALE-AGHA N. (2004): Mycodiversity on a dead stem of the giant hogweed – *Heracleum mantegazzianum* Sommer et Levier. – Communications in Agricultural and Applied Biological Sciences 69: 479–487.

FRIGGENS N.L., TAYLOR J.E., KOUKOL O. (2017): Diversity and community composition of aquatic ascomycetes varies between freshwater, estuarine and marine habitats in western Scotland. – Mycosphere 8: 1267–1287. DOI: https://doi.org/10.5943/mycosphere/8/9/2

GROVES J.W. (1946): North American species of *Dermea*. – Mycologia 38: 351–431. DOI: https://doi.org/10.1080/00275514.1946.12024065

GROVES J.W., ELLIOTT M.E. (1969): Notes on *Ciboria rufo-fusca* and *Ciboria alni*. – Friesia 9: 29–36.

HAWKSWORTH D.L., LÜCKING R. (2017): Fungal diversity revisited: 2.2 to 3.8 million species. – Microbiology Spectrum 5: FUNK-0052-2016. DOI: https://doi.org/10.1128/microbiolspec.FUNK-0052-2016

Hofstetter V., Buyck B., Eyssartier G., Schnee S., Gindro K. (2019): The unbearable lightness of sequenced-based identification. – Fungal Diversity 96: 243–284. DOI: https://doi.org/10.1007/s13225-019-00428-3

Iturriaga T., LoBuglio K.F., Pfister D.H. (2017): *Bulgariella pulla*, a Leotiomycete of uncertain placement, with an uncommon type of ascus opening. – Mycologia 109: 900–911. DOI: https://doi.org/10.1080/00275514.2017.1418590

Kõljalg U. et al. (2013): Towards a unified paradigm for sequence-based identification of fungi. – Molecular Ecology 22: 5271–5277. DOI: https://doi.org/10.1111/mec.12481

Koukol O. (2019a): Revision of root-associated microfungi of *Pinus wallichiana* in Kashmir Himalaya. – Canadian Journal of Forest Research 49: 326–329. DOI: https://doi.org/10.1139/cjfr-2018-0294

Koukol O. (2019b): Jarní Micromyco 2019 [Spring Micromyco 2019]. – Mykologické Listy 144: 61–62. [in Czech]

Koukol O., Delgado G., Hofmann T.A., Piepenbring M. (2018): Panama, a hot spot for *Hermatomyces* (*Hermatomycetaceae*, *Pleosporales*) with five new species, and a critical synopsis of the genus. – IMA Fungus 9: 107–141. DOI: https://doi.org/10.5598/imafungus.2018.09.01.08

Li W.-J. et al. (2020): Taxonomy and phylogeny of hyaline-spored coelomycetes. – Fungal Diversity 100: 279–801. DOI: https://doi.org/10.1007/s13225-020-00440-y

Lombard L., Houbraken J., Decock C., Samson R.A., Meijer M., Réblová M., Groenewald J.Z., Crous P.W. (2016): Generic hyper-diversity in *Stachybotriaceae*. – Persoonia 36: 156–246. DOI: https://doi.org/10.3767/003158516X691582

Müller E. (1950): Die schweizerischen Arten der Gattung *Leptosphaeria* und ihrer Verwandten. – Ph.D. Dissertation [depon. in ETH Zürich].

Nag Raj T.R. (1993): Coelomycetous anamorphs with appendage-bearing conidia. – Mycologue Publications, Sidney.

Nag Raj T.R., Kendrick W.B. (1976): A monograph of *Chalara* and allied genera. – Wilfred Laurier University Press, Waterloo.

Nilsson R.H., Ryberg M., Kristiansson E., Abarenkov K., Larsson K.-H., Kõljalg U. (2006): Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. – PLoS ONE 1: e59. DOI: https://doi.org/10.1371/journal.pone.0000059

O'Donnell K. (1993): *Fusarium* and its near relatives. – In*:* Reynolds D.R., Taylor A.F., eds., The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics, pp. 225–233. CAB International, Wallingford.

Palmer J.T., Tortić M., Matočec N. (1994): *Sclerotiniaceae* (Discomycetes) collected in the former Federal Republic of Yugoslavia. – Österreichische Zeitschrift für Pilzkunde 3: 41–70.

Phookamsak R. et al. (2019): Fungal diversity notes 929–1035: taxonomic and phylogenetic contributions on genera and species of fungi. – Fungal Diversity 95: 1–273. DOI: https://doi.org/10.1007/s13225-019-00421-w

Raja H.A., Baker T.R., Little J.G., Oberlies N.H. (2017): DNA barcoding for identification of consumer-relevant mushrooms: A partial solution for product certification? – Food Chemistry 214: 383–392. DOI: https://doi.org/10.1016/j.foodchem.2016.07.052

Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Hohna S., Larget B., Liu L., Suchard M.A., Huelsenbeck J.P. (2012): MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. – Systematic Biology 61: 539–542. DOI: https://doi.org/10.1093/sysbio/sys029

Sarma V., Newell S., Hyde K. (2001): *Koorchaloma spartinicola* sp. nov., a new marine sporodochial fungus from *Spartina alterniflora*. – Botanica Marina 44: 321–326. DOI: https://doi.org/10.1515/BOT.2001.041

Stadler M., Lambert C., Wibberg D., Kalinowski J., Cox R.J., Kolařík M., Kuhnert E. (2020): Intragenomic polymorphisms in the ITS region of high-quality genomes of the *Hypoxylaceae* (*Xylariales*, Ascomycota). – Mycological Progress 19: 235–245. DOI: https://doi.org/10.1007/s11557-019-01552-9

SUBRAMANIAN C. (1953): *Koorchaloma*, a new genus of the *Tuberculariaceae*. – Journal of the Indian Botanical Society 32: 123–126.

SUIJA A., HALDEMAN M., ZIMMERMANN E., BRAUN U., DIEDERICH P. (2020): Phylogenetic placement and lectotypification of *Pseudotryblidium neesii* (*Helotiales*, Leotiomycetes). – Fungal Systematics and Evolution 5: 139–150. DOI: https://doi.org/10.3114/fuse.2020.05.09

ŠANDOVÁ M., NILSSON R.H., KOLAŘÍK M. (2018): Relationships within *Capitotricha bicolor* (*Lachnaceae*, Ascomycota) as inferred from ITS rDNA sequences, including some notes on the *Brunnipila* and *Erioscyphella* clades. – Mycological Progress 17: 89–101. DOI: https://doi.org/10.1007/s11557-017-1346-5

TREIGIENĖ A. (2006): New species of *Kananascus* and its anamorph from Lithuania. – Mycotaxon 96: 173–180.

VU D. et al. (2019): Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. – Studies in Mycology 92: 135–154. DOI: https://doi.org/10.1016/j.simyco.2018.05.001

WHITE T.J., BRUNS T.D., LEE S., TAYLOR J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. – In: Innis M.A., Gelfand D.H., Sninsky J.J., White, T.J., eds., PCR protocols: a guide to methods and applications, pp. 315–322. Academic Press, San Diego.

ZOGG H. (1962): Die *Hysteriaceae* s. str. und *Lophiaceae*, unter besonderer Berücksichtigung der mitteleuropäischen Formen. – Beiträge zur Kryptogamenflora der Schweiz 11: 1–190.