Morphologically similar but not closely related: the long-spored species of *Subulicystidium* (Trechisporales, Basidiomycota)

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Received: 26 March 2020 / Revised: 29 April 2020 / Accepted: 1 May 2020
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Abstract
Species boundaries and geographic distribution of corticioid fungi (resupinate Basidiomycota) are often poorly known. Our recent study on *Subulicystidium* showed that species diversity in this genus is at least twice as high as previously recognized. This re-estimation of the species diversity was based on a study of only a part of the genus. The present study sheds light on molecular and morphological diversity of three more species. We generated 27 ITS and 24 28S nuclear ribosomal DNA sequences from 49 specimens labelled as *Subulicystidium cochleum*, *S. longisporum* and *S. perlongisporum* and collected in distant geographic localities. We assessed pairwise dissimilarities and phylogenetic relationships of DNA sequences with Bayesian and maximum likelihood methods. We correlated phylogenetic information with morphological data on spores and cystidia. We found that the three species are not closely related, despite their similarity in spore shape and size. In one of the species, *S. perlongisporum*, we detected the presence of two sympatric lineages. These lineages are not morphologically distinct, except for a small difference in the mean length of cystidia. Our study provides a further example of transoceanic species distribution in Agaricomycetes.

Keywords Cryptic species · Genetic distance · Homoplasic character · Internal transcribed spacer · Large subunit · Traditional morphometrics

Introduction
Species boundaries and geographic distribution of corticioid fungi (resupinate Basidiomycota) are often poorly known. Fungi from the genus *Subulicystidium* Parmasto 1968 (Hydnodontaceae, Trechisporales, Agaricomycetes, Basidiomycota) occur on moderately or strongly decayed wood and are common in many forest ecosystems, especially tropical ones. For many decades, *Subulicystidium* has challenged both morphology- and DNA-based mycology. The presence of numerous transitional forms as to basidiospore size and shape hindered species delimitation (Oberwinkler
1977; Liberta 1980; Duhem and Michel 2001). Recently, Ordynets et al. (2018) described 11 new species based on analyses of ribosomal DNA and morphometry of spores and cystidia. The genus now includes 22 accepted species (Index Fungorum 2019).

In *Subulicystidium*, morphological species identification through a key traditionally begins with defining whether the spores’ length-to-width ratio ($Q$) is lower or higher than four. This criterion is stable and allows one to assign each specimen unambiguously to one of the morphogroup (Boidin and Gilles 1988; Duhem and Michel 2001; Gorjón et al. 2012). For easier unambiguously to one of the morphogroup (Boidin and Gilles (1988) and differs from *S. longisporum* by the presence of needle-like crystals on cystidia and by more strongly curved spores (Punugu et al. 2018), by means of the package “dplyr” (Wickham et al. 2019) in R v. 3.5.3 (R Core Team 2019).

During the measurements of cystidia, the protruding bow-tie crystals were included in the cystidium diameter. In most cases, between 9 and 28 cystidia per specimen were measured (in two cases nine and in one case 38). In total 645 cystidia were measured from 36 sequenced specimens (see Supplementary file 2). These measurements were not checked for outliers and were directly summarized on specimen level with the same method as used for the spores (Ordynets and Denecke 2018). To compare the mean size and shape of spores and cystidia between phylogenetically defined groups, the unpaired two-sample Wilcoxon test was used as implemented in the function “wilcox.test”, with default settings, from the R “stats” package (R Core Team 2019).

**DNA extraction, amplification and sequencing**

Sequences of two nuclear ribosomal DNA regions were considered in our study: internal transcribed spacer (nc ITS rDNA) and ribosomal large subunit-coding DNA (nc 28S rDNA). Total DNA was extracted from dried herbarium specimens. For this, pieces of fungal fruiting bodies totalling about 20 mm$^2$ were placed into 2-ml tubes containing two small and two large sterile metallic beads. The tube content was ground in the mixer mill for 1 min at 30 Hz. Then, tubes were centrifuged at 16,060 g for 30 s. The following methods of total DNA isolation were used:
(1) Most of the specimens were processed with the E.Z.N.A.® Fungal DNA Mini Kit from Omega Bio-tek, Inc. (Norcross, GA, USA). We used the short version of the protocol from the manufacturer’s manual with few modifications. In particular, after adding FG1 buffer, we added to each tube 10 μl of proteinase K (20 mg/ml, Ambion, Thermo Fischer Scientific, Waltham, MA, USA) and used neither RNase A or β-mercaptoethanol. We also used a fixed volume of 300 μl for FG3 buffer and 600 μl for 100% ethanol. We added sterile deionized water instead of elution buffer at the two final elution steps. We performed all the centrifuging steps at 9500g. We used the DNA gained with this protocol in undiluted form for PCR.

(2) For Reunionese specimens collected in 2013 and 2015 (herbaria KAS, FR), the protocol of Izumitsu et al. (2012) was used (see details in Ordynets et al. 2018).

(3) For collections from Russia (herbarium LE), total DNA extraction followed the manufacturer’s protocol of the NucleoSpin Plant II kit (Macherey–Nagel GmbH and Co. KG, Duren, Germany).

(4) In specimens from Sicily and Estonia (herbarium TU), the total DNA was extracted in a lysis buffer (0.8 M Tris-HCl, 0.2 M (NH₄)₂SO₄, 0.2% w/v Tween-20) (Soil BioDyne, Tartu, Estonia) using a proteinase K method (100 μl lysis buffer and 2.5 μl proteinase K; incubation at 56 ºC for 24 h and at 98 ºC for 15 min).

Primer pairs used to amplify the complete ITS region were ITS1f/ITS4, ITS1f/ITS4B and ITS5/ITS4 (White et al. 1990; Gardes and Bruns 1993). If the amplification of the complete ITS region failed, it was performed for shorter ITS portions with primer pairs ITS1f/ITS2 and ITS3f/ITS4. Each of these two fragments represents nearly half of ITS and they were later assembled to a single complete ITS sequence. The D1–D2 domains at the 5′ end of the 28S region were amplified with primer pair LR0R/LR5 (Hopple and Vilgalys 1999) or alternatively with NL1/NL4 (O’Donnell 1992). Finally, for some Sicilian collections, primer pair ITS1f/LB-W was used to recover the full ITS and partial 28S region with a total length of ca. 1000 nucleotides (Tedersoo et al. 2008).

The PCR after extraction methods 1 and 2 were performed on 25 μl solution containing 2.5 μl of extracted DNA and 22.5 μl master mix. One master mix portion contained 15.1 μl of double-distilled H₂O, 5 μl of 5× MangoTaq® Colored Reaction Buffer, 1 μl dNTPs (5 mM), 1 μl MgCl₂ (50 mM), 0.1 μl MangoTaq DNA polymerase 5 units/μl (all components above from Bioline GmbH, Luckenwalde, Germany), 1 μl of bovine serum albumin (20 μg/μl) and 0.4 μl of each forward and reverse primers (25 pmol) from Thermo Fisher Scientific (Waltham, MA, USA). PCR with primer pairs ITS1f/ITS4 and ITS3f/ITS4 was set as initial denaturation at 94 ºC for 3 min followed by 29 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 45 s and extension at 72 ºC for 60 s; final elongation was done at 72 ºC for 7 min. PCR with primer pair LR0R/LR5 differed in having the annealing temperature as 58 ºC and for primer pairs NL1/NL4 and ITS1f/ITS2 at 60 ºC. PCR products were checked on 1% agarose gel stained with GelRed fluorescence dye (Biotium, Hayward, CA, USA). PCR products were cleaned with QiAquick PCR Purification Kit according to the manufacturer’s instructions (QIAGEN GmbH, Hilden, Germany). Sanger sequencing of purified products was performed by the company Eurofins Genomics Germany GmbH (Ebersberg, Germany) and in the facilities of the Senckenberg Research Institute and Natural History Museum (Frankfurt am Main, Germany). The primers used for sequencing were identical to those used for amplification.

The PCR after extraction method 3 was performed on 20 μl solution containing 2 μl of extracted DNA, 7.6 μl of distilled H₂O and 10 μl of iQ Supermix (Bio-Rad Laboratories, USA).

The purification of PCR products was made with the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Lithuania). Sequencing was performed in house on ABI 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

The PCR after extraction method 4 was performed in 25 μl containing 0.5 μl of each primer, 5 μl FirePol Mastermix (Solis BioDyne, Tartu, Estonia), 1 μl of 10 times diluted DNA template and sterilized distilled water. PCR conditions for the amplification of ITS region were set as initial denaturation at 95 ºC for 15 min followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s and extension 72 ºC for 60 s; final elongation was done at 72 ºC for 7 min. PCR products were cleaned using Exo-SAP enzymes (GE Healthcare, Freiburg, Germany) using incubation at 37 ºC for 45 min and at 85 ºC for 15 min. PCR products were sequenced by Macrogen Inc. (Amsterdam, the Netherlands) with the same primers as used for PCR.

DNA sequence—analyses

Raw sequence data were processed with Geneious v. 5.6.7 (http://www.geneious.com, Kearse et al. 2012) and Sequencher v. 5.1 (Gene Codes Corporation, Ann Arbor, USA). For various sequence format conversion and alignment viewing, Mesquite v. 3.6 (build 917) (Maddison and Maddison 2018) and AliView v. 1.19 (Larsson 2014) were used. For phylogenetic data import and processing within R, the following packages were used: “ape” (Paradis et al. 2004), “forcats” (Wickham 2019), “gdata” (Wames et al. 2017), “geiger” (Harmon et al. 2008), “Hmisc” (Harrell Jr et al. 2019), and with contributions from Charles Dupont, many others (2019), “pals” (Wright 2018) and “treeio” (Yu 2019).

In this study, the following numbers of new nc ITS rDNA sequences were generated: 21 sequences for S. perlongisporum, three for S. cochleum and three for...
S. longisporum. For the nc 28S rDNA, the numbers of sequences generated for these same species were 19, 3 and 2, respectively. The newly generated DNA sequences were submitted to GenBank (Benson et al. 2018). Additional six ITS and four 28S sequences of S. perlongisporum and two ITS and three 28S sequences of S. longisporum available in GenBank (e.g. Volobuev 2016) and UNITE database (Nilsson et al. 2018) were used in our analyses after the corresponding herbarium specimens were examined (Table 1).

To relate the DNA sequences of S. perlongisporum, S. cochleum and S. longisporum to the rest of the genus Subulicystidium, we combined our data for these three species with the datasets of 57 ITS and 55 28S DNA sequences of other Subulicystidium species (Table 2). We used ITS and 28S DNA sequences from the holotype of S. oberwinkleri (KAS:L 1860) as an outgroup in all genus-level phylogenetic analyses.

Sequences from each locus, 92 of ITS region and 86 of 28S region, were aligned in MAFFT v. 7 online (Katoh et al. 2017), with L-INS-i algorithm and other settings as default. The small fragments of 18S rDNA and 28S rDNA were automatically trimmed from the target ITS region with the ITSx software (Bengtsson-Palme et al. 2013) implemented in the PlutoF workbench (Abarenkov et al. 2010). The final ITS alignment had 659 nucleotide positions. The 28S alignment was trimmed manually to produce sequences of the same length and with fewer (if any) gaps at both ends, leaving 911 positions in the final version. ITS and 28S alignments were concatenated with the SequenceMatrix v. 1.8 (Vaidya et al. 2011) to produce a matrix with 100 rows and 1570 columns.

We performed phylogenetic reconstruction for Subulicystidium from concatenated ITS+28S alignment using Bayesian and maximum likelihood analyses. For Bayesian inference of phylogeny, GTR+G+I evolutionary model was used in MrBayes 3.2.3 (Ronquist et al. 2012) run on CIPRES Science Gateway v. 3.3 (Miller et al. 2010; http://www.phylo.org). Two independent MCMC processes, each in 4 chains, were run. Ten million trees were generated; the sample size was 4924.1347. The plot of the generation versus alignment was performed with PhyML v. 3.0 (Guindon et al. 2010) after automated model selection with SMS v. 1.8.1, both run online (Lemoine et al. 2019, https://ngphylogeny.fr). Evolutionary model comparison was performed under Bayesian information criterion and resulted in selecting TN93+G+I as the best-fitting model. BioNJ was used as a starting tree. The algorithm to explore the space of tree topologies was subtree pruning and regrafting (SPR). For branch support estimation, an approximate likelihood ratio test was requested that relies on a nonparametric, Shimodaira-Hasegawa-like procedure (SH-like aLRT, Guindon et al. 2010).

To visualize results for the genus-level concatenated ITS+28S dataset, the consensus tree from Bayesian analysis was plotted with both posterior probabilities from Bayesian analysis and SH-like aLRT support values from PhyML. For this, we used R script of Ordynets which is based on the R code from Crane (2013).

In the most abundant species, S. perlongisporum, we analyzed also the intraspecific genetic diversity. We produced a species-level ITS alignment with 27 sequences that after trimming with ITSx was 554 positions long. We calculated pairwise genetic distances between these ITS sequences using “pairDistPlot” function of “adegenet” R package (Jombart 2008) setting the arguments as follows: model = “raw”, pairwise.deletion = TRUE (i.e. with deleting the sites with missing data in a pairwise way). Phylogenetic inference for this dataset was performed in PhyML v. 3.0 online with settings identical to those in the analysis of concatenated genus-level dataset but with HKY85+G as the best model. The result was visualized with the R packages “ggtree” (Yu et al. 2017), “ggstance” (Henry et al. 2019) and “ggplot2” (Wickham 2009).

The multiple sequence alignments, details of phylogenetic analyses and trees were deposited in TreeBASE (Piel et al. 2009) under submission ID S24881. The R code for the DNA-based analyses and visualizations is available on GitHub (https://github.com/ordynets/subLongSpored).

Results

Phylogenetic analyses

The genus-level phylogenetic analyses based on the ITS+28S dataset showed that each of the species Subulicystidium cochleum, S. longisporum and S. perlongisporum is monophyletic (Fig. 1). The three species were not closely related to each other. S. cochleum (branch support PP = 1, aLRT = 0.92) was placed as a sister species to S. acerosum on a highly supported branch (PP = 1, aLRT = 1). S. longisporum (PP = 1, aLRT = 0.95) was nested in the clade dominated by numerous sequences of S. meridense and S. brachysporum (PP = 0.94, aLRT = 0.87). S. perlongisporum (PP = 1, aLRT = 0.98) was
Table 1 DNA sequences of Subulicystidium perlongisporum, S. longisporum and S. cochleum with voucher specimen data and publication source. Abbreviation “na” means our failure to generate DNA sequence of a particular region.

| Species           | Locality                | Collector(s)  | Voucher specimens | GenBank/UNITE accession numbers | Source                          |
|-------------------|-------------------------|---------------|-------------------|---------------------------------|---------------------------------|
| S. cochleum       | Jamaica: Middlesex      | K.-H. Larsson | GB: KHL 10517     | MN207035 MN207023               | This study                      |
| S. cochleum       | Costa Rica: Alajuela    | K.-H. Larsson | GB: KHL 11200     | MN207036 MN207024               | This study                      |
| S. longisporum    | Madagascar: Anony       | K.-H. Larsson | O:F: KHL 14355    | MN207034 MN207026               | This study                      |
| S. longisporum    | Italy: Sicily           | A. Saitta     | O:F: KHL 14355    | MN207034 MN207026               | This study                      |
| S. longisporum    | Sweden: Skåne           | K.-H. Larsson | GB: KHL 14229     | MH000601 MH000601              | Larsson (unpublished)           |
| S. longisporum    | Ukraine: Zakarpatska    | A. Ordynets   | CWU 6737          | MN207038 MN207016               | This study                      |
| S. longisporum    | Taiwan: Nantou          | G. Langer; E. Langer; C.-J. Chen | KAS: GEL 3550 | MN207037 AJ406423 | This study and Langer (2001), respectively |
| S. longisporum    | Germany: Hesse          | A. Ordynets   | KAS: Ordynets 00146 | MN207039 MN207032 | This study                      |
| S. perlongisporum | Réunion: Saint-Benoît   | J. Boidin     | LY 11631          | MN207054 MN207030               | This study                      |
| S. perlongisporum | Brazil: Paraiba         | K.-H. Larsson | O:F: KHL 16062    | MH000600 MH000600              | This study                      |
| S. perlongisporum | Brazil: Saô Paulo, Canaranie | D. Pegler; K. Hjortstam; L. Ryvarden | O:F: LR 24826 | MN207042 MN207029 | This study                      |
| S. perlongisporum | Brazil: Saô Paulo, Saô Paulo | D. Pegler; K. Hjortstam; L. Ryvarden | GB: Hjm 16,340 | na | MN207017 This study |
| S. perlongisporum | Cuba: Villa Clara       | S. Kõljalg; U. Kõljalg | TU 108264 | UDB016775 UDB016775 | Kõljalg (unpublished) |
| S. perlongisporum | Dominican Republic:     | K.-H. Larsson | GB: KHL 9926      | MN207041 MN207027               | This study                      |
| S. perlongisporum | Provincia La Altagracia| K.-H. Larsson | GB: KHL 9943      | MN207051 MN207028               | This study                      |
| S. perlongisporum | Dominican Republic:     | K.-H. Larsson | GB: KHL 10671     | MN207040 na                     | This study                      |
| S. perlongisporum | Madagascar: Ambotanithely | B. Buyck     | PC 0125118; Buyck 001899 | MN207052 MN207015 | This study                      |
| S. perlongisporum | Madagascar: Ilhornbe    | K.-H. Larsson | O:F: KHL 14305    | MN207044 MN207025               | This study                      |
| S. perlongisporum | Italy: Sicily           | A. Saitta     | TU 124387         | UDB028354 UDB028355            | Saitta (unpublished)           |
| S. perlongisporum | Réunion: Saint-Pierre   | E. Langer     | KAS: L 0103       | MN207058 na                     | This study                      |
| S. perlongisporum | Réunion: Saint-Benoît   | E. Langer; E. Hennen | KAS: GEL 5217a | MN207043 AJ406422 | This study and Langer (2001), respectively |
| S. perlongisporum | Réunion: Saint-Paul     | G. Langer; E. Langer; E. Hennen | KAS: GEL 4815 | MN207055 na                     | This study                      |
| S. perlongisporum | Réunion: Saint-Pierre   | M. Striegel   | KAS: L 1726b      | MN207045 MN207022               | This study                      |
| S. perlongisporum | Réunion: Saint-Pierre   | J. Boidin     | LY 12824          | MN207046 MN207031               | This study                      |
| S. perlongisporum | Russia: Kaluga           | S. Volobuev   | LE 302156         | MN207044 MN207025               | This study                      |
| S. perlongisporum | Russia: Briansk         | S. Volobuev   | LE 314099         | MN207044 MN207025               | This study                      |
| S. perlongisporum | Russia: Chuvash Republic| S. Bolshakov  | LE 315315         | MN207043 AJ406422 | This study and Langer (2001), respectively |
| S. perlongisporum | Spain: Gipuzkoa         | J.M. Riezua   | ARAN:Fungi 303195 | MN207057 na                     | This study                      |
| S. perlongisporum | Spain: Gipuzkoa         | J.M. Riezua   | ARAN:Fungi 4160   | MN207057 na                     | This study                      |
| S. perlongisporum | Taiwan: Chiayi          | G. Langer; E. Langer; C.-J. Chen | KAS: GEL 3681 | MN207049 MN207021 | This study                      |
| S. perlongisporum | Taiwan: Miaoli          | G. Langer; E. Langer; C.-J. Chen | KAS: GEL 3388 | MN207047 MN207019 | This study                      |
| S. perlongisporum | Taiwan: Miaoli          | G. Langer; E. Langer; C.-J. Chen | KAS: GEL 3392 | MN207048 MN207020 | This study                      |

most closely related to S. robustius and S. rarocrystallinum (occupying the next more basal node) and to S. boidinii, S. harpagum, S. parvisporum and S. tropicum and to the specimen of S. obtusisporum from Jamaica (occupying the next highest node).

Concatenated ITS+28S DNA sequences from S. perlongisporum were grouped into two clades of unequal size (Fig. 1). The larger clade included the holotype (LY 11631) and sequences of various geographic origin (Paleotropics, Neotropics and temperate Europe) strongly
Table 2  DNA sequences of Subulicystidium species besides *S. perlongisporum*, *S. longisporum* and *S. cochleum* used in this study with information on voucher specimens and publication source. Abbreviation “na” means sequence is not available.

| Species                  | Locality       | Voucher specimen | Collector(s)          | GenBank/UNITE accession numbers | Source                  |
|--------------------------|----------------|------------------|-----------------------|--------------------------------|-------------------------|
| *Subulicystidium acerosum* (holotype) | China: Guizhou | BJFC 022303      | S. H. He              | MK204539 MK204543               | Liu et al. (2019)       |
| *S. boidinii*            | Reunion: Saint-Benoît | KAS: L 1584a    | M. Striegel           | MH041527 na                     | Ordynets et al. (2018) |
| *S. boidinii*            | Costa Rica: Puntarenas | GB: KHL 12830 | K.-H. Larsson         | MH041537 MH041570               | Ordynets et al. (2018) |
| *S. brachysporum*        | Argentina: Misiones | O:F: 506782      | L. Ryvarden           | MH041518 MH041572               | Ordynets et al. (2018) |
| *S. brachysporum*        | Brazil: Paraiba | O:F: KHL 16100   | K.-H. Larsson         | MH000599 MH000599               | Ordynets et al. (2018) |
| *S. brachysporum*        | Brazil: Rondonia | O:F: KHL 15352   | K.-H. Larsson         | MH041553 MH041576               | Ordynets et al. (2018) |
| *S. brachysporum*        | Brazil: Saõ Paulo | GB: Hjm 16,573  | K. Hjortstam          | MH041545 MH041596               | Ordynets et al. (2018) |
| *S. brachysporum*        | Colombia: Magdalena | O:F: 918493 | L. Ryvarden           | MH041522 MH041605               | Ordynets et al. (2018) |
| *S. brachysporum*        | Costa Rica: Alajuela | GB: KHL 11216  | K.-H. Larsson         | MH041517 MH041580               | Ordynets et al. (2018) |
| *S. brachysporum*        | Jamaica: Cornwall | GB: KHL 10763   | K.-H. Larsson         | MH041546 MH041598               | Ordynets et al. (2018) |
| *S. brachysporum*        | Jamaica: Middlesex | GB: KHL 10566   | na                    | MH041599                        | Ordynets et al. (2018) |
| *S. brachysporum*        | Madagascar: Anosy | O:F: KHL 14537   | K.-H. Larsson         | MH041552 MH041573               | Ordynets et al. (2018) |
| *S. brachysporum*        | Puerto Rico: Isabela | GB: KHL 9544   | K.-H. Larsson         | MH041555 MH041560               | Ordynets et al. (2018) |
| *S. brachysporum*        | Puerto Rico: Luquillo | GB: KHL 10406  | K.-H. Larsson         | MH041543 MH041600               | Ordynets et al. (2018) |
| *S. brachysporum*        | Puerto Rico: Luquillo | GB: KHL 10411  | K.-H. Larsson         | MH041549 MH041601               | Ordynets et al. (2018) |
| *S. brachysporum*        | Réunion: Saint-Pierre | KAS: L 0134   | E. Langer             | MH041541 MH041593               | Ordynets et al. (2018) |
| *S. brachysporum*        | Réunion: Saint-Benoît | KAS: L 1584b  | M. Striegel           | MH041544 MH041610               | Ordynets et al. (2018) |
| *S. brachysporum*        | Réunion: Saint-Pierre | KAS: L 1147   | J. Riebesehl; M. Schroth | MH041542 MH041594               | Ordynets et al. (2018) |
| *S. brachysporum*        | Réunion: Saint-Pierre | KAS: L 1498   | M. Striegel           | MH041526 na                     | Ordynets et al. (2018) |
| *S. brachysporum*        | Réunion: Saint-Pierre | KAS: L 1795   | M. Striegel           | MH041525 MH041579               | Ordynets et al. (2018) |
| *S. brachysporum*        | Réunion: Saint-Pierre | LY 12293   | G. Gilles             | MH041550 MH041571               | Ordynets et al. (2018) |
| *S. brachysporum*        | Réunion: Saint-Pierre | LY 12772   | G. Gilles             | na MH041595                    | Ordynets et al. (2018) |
| *S. brachysporum*        | Brazil: Rondonia | O:F: KHL 15318   | K.-H. Larsson         | MH041557 MH041577               | Ordynets et al. (2018) |
| *S. brachysporum*        | Brazil: Saõ Paulo | O:F: LR 24170    | D. Pegler; K. Hjortstam; L. Ryvarden | MH041556 na                     | Ordynets et al. (2018) |
| *S. brachysporum*        | Reunion: Saint-Paul | LY 11378  | J. Koidin             | na MH041574                    | Ordynets et al. (2018) |
| *S. fusisporum* (holotype) | Puerto Rico: Rio Grande | GB: KHL 10360  | K.-H. Larsson         | MH041535 MH041567               | Ordynets et al. (2018) |
| *S. fusisporum*          | Costa Rica: Puntarenas | GB: KHL 12761  | K.-H. Larsson         | MH041536 MH041568               | Ordynets et al. (2018) |
| *S. fusisporum*          | Puerto Rico: Rio Grande | GB: KHL 9093  | K.-H. Larsson         | MH041534 na                     | Ordynets et al. (2018) |
| *S. grandisporum* (holotype) | Costa Rica: Cartago | O:F: 506781 | L. Ryvarden           | MH041547 MH041592               | Ordynets et al. (2018) |
| *S. harpagum* (holotype) | Réunion: Saint-Pierre | KAS: L 1726a  | M. Striegel           | MH041532 MH041588               | Ordynets et al. (2018) |
| *S. harpagum*            | Colombia: Magdalena | O:F: LR 15736  | L. Ryvarden           | MH041531 MH041586               | Ordynets et al. (2018) |
| Species | Locality | Voucher specimen | Collector(s) | GenBank/UNITE accession numbers | Source |
|---------|----------|------------------|--------------|--------------------------------|--------|
|         |          |                  |              | ITS 28S                        |        |
| *S. harpagum* | Jamaica: Cornwall | GB: KHL 10733 | K.-H. Larsson | MH041520 MH041563 | Ordynets et al. (2018) |
| *S. harpagum* | Réunion: Saint-Benoît | KAS: L 0244 | E. Langer | MH041533 MH041609 | Ordynets et al. (2018) |
| *S. inornatum* (holotype) | Puerto Rico: Rio Grande | GB: KHL 10444 | K.-H. Larsson | MH041558 MH041569 | Ordynets et al. (2018) |
| *S. meridense* | Brazil: Rondónia | O:F: KHL 15322 | K.-H. Larsson | MH041540 MH041602 | Ordynets et al. (2018) |
| *S. meridense* | Brazil: São Paulo | GB: Hjm 16,400 | D. Pegler; K. Hjortstam; L. Ryvarden | MH041538 MH041604 | Ordynets et al. (2018) |
| *S. meridense* | Costa Rica: Guanacaste | GB: KHL 11355 | K.-H. Larsson | na | MH041583 Ordynets et al. (2018) |
| *S. meridense* | Costa Rica: Guanacaste | GB: KHL 11365 | K.-H. Larsson | MH041523 MH041584 | Ordynets et al. (2018) |
| *S. meridense* | Réunion: Saint-Benoît | LY 12816 | G. Gilles | na | MH041597 Ordynets et al. (2018) |
| *S. meridense* | Taiwan: Nantou | KAS: GEL 3520 | E. Langer; G. Langer; C.-J. Chen | MH041548 | na Ordynets et al. (2018) |
| *S. meridense* | Argentina: Misiones | O:F: LR 19581 | L. Ryvarden | MH041551 MH041578 | Ordynets et al. (2018) |
| *S. meridense* | Brazil: Rondónia | O:F: KHL 15325 | K.-H. Larsson | na | MH041585 Ordynets et al. (2018) |
| *S. meridense* | Colombia: Magdalena | O:F: 918846 | L. Ryvarden | MH041554 MH041575 | Ordynets et al. (2018) |
| *S. meridense* | Puerto Rico: Cerro Alto | GB: KHL 9561 | K.-H. Larsson | MH041524 MH041581 | Ordynets et al. (2018) |
| *S. meridense* | Puerto Rico: Luquillo | GB: KHL 10397 | K.-H. Larsson | MH041519 MH041582 | Ordynets et al. (2018) |
| *S. nikau* | Réunion: Saint-Pierre | KAS: L 1296 | J. Riebesehl; M. Schroth | MH041513 MH041565 | Ordynets et al. (2018) |
| *S. Oberwinkleri* (holotype) | Réunion: Saint-Pierre | KAS: L 1860 | J. Riebesehl | MH041511 MH041562 | Ordynets et al. (2018) |
| *S. obtusisporum* | Germany: Hesse | FR: Piepenbrink & Lotz-Winter W213-3-1 | O. Koukol | MH041521 MH041566 | Ordynets et al. (2018) |
| *S. obtusisporum* | Jamaica: Cornwall | GB: KHL 10622 | K.-H. Larsson | MH041559 MH041606 | Ordynets et al. (2018) |
| *S. parvisporum* (holotype) | Réunion: Saint-Pierre | KAS: L 0140 | E. Langer | MH041529 MH041590 | Ordynets et al. (2018) |
| *S. parvisporum* | Réunion: Saint-Benoît | KAS: L 1226 | J. Riebesehl | MH041528 MH041587 | Ordynets et al. (2018) |
| *S. parvisporum* | Réunion: Saint-Pierre | KAS: GEL 5032 | E. Langer; E. Hennen | MH041530 MH041591 | Ordynets et al. (2018) |
| *S. parvisporum* | Réunion: Saint-Pierre | LY 12750 | G. Gilles | na | MH041589 Ordynets et al. (2018) |
| *S. rarerocrystallinum* (holotype) | Colombia: Cundinamarca | O:F: 918488 | L. Ryvarden | MH041512 MH041564 | Ordynets et al. (2018) |
| *S. robustus* (holotype) | Jamaica: Cornwall | GB: KHL 10813 | K.-H. Larsson | MH041514 MH041608 | Ordynets et al. (2018) |
| *S. robustus* | Jamaica: Cornwall | GB: KHL 10780 | K.-H. Larsson | AY463468 AY586714 | Larsson (unpublished) Ordynets et al. (2018) |
| *S. robustus* | Puerto Rico: Luquillo | GB: KHL 10039 | K.-H. Larsson | MH041515 na | Ordynets et al. (2018) |
| *S. robustus* | Puerto Rico: Río Grande | GB: KHL 10272 | K.-H. Larsson | MH041516 MH041607 | Ordynets et al. (2018) |
| *S. tedersooi* (holotype) | Vietnam: Ninh Bình | TU 110894 | L. Tedersoo | UDB014161 na | Tedersoo (unpublished) |
| *S. tedersooi* | Vietnam: Ninh Bình | TU 110895 | L. Tedersoo | UDB014162 na | Tedersoo (unpublished) |
| *S. tropicum* (holotype) | China: Hainan | BJFC 022470 | S.H. He | MK204531 MK204544 | Liu et al. (2019) |
| *S. tropicum* | China: Hainan | BJFC 022083 | S.H. He | MK204530 MK204542 | Liu et al. (2019) |
| *S. tropicum* | Papua New Guinea: Morobe | TU 110416 | L. Tedersoo | UDB013052 UDB013052 | Tedersoo (unpublished) |
Fig. 1 Phylogenetic relationship of *Subulicystidium* based on concatenated ITS+28S nc rDNA alignment. 50% majority-rule consensus tree from Bayesian analysis is shown, with posterior probabilities above the branches and supports from approximated likelihood ratio test from the maximum likelihood estimation below the branches, both in the range from 0 to 1. NA means the absence of branch support. Tips of the tree include GenBank/UNITE accession numbers for the ITS region followed by 28S region, voucher specimen and country of collection. Tree tips are coloured according to the species name. Scale bar shows the number of substitutions per site.
The smaller clade contained two specimens from temperate Europe, two from the Mediterranean region in...
Europe and one specimen from the Southern Hemisphere (La Réunion).

Within the larger clade of \textit{S. perlongisporum}, further labelled as clade 1 (aLRT = 1), two subclades on short basal branches were distinguishable (Fig. 2a). Subclade 1.1 (aLRT = 0.8) included DNA sequences merely from the Eastern Hemisphere while subclade 1.2 (aLRT = 0.67) was dominated by DNA sequences from the Western Hemisphere. The sequences from La Réunion and Madagascar were found in both subclades.

Genetic and morphological diversity within \textit{S. perlongisporum}

Between the ITS rDNA sequences of \textit{S. perlongisporum}, there were signatures in the multiple sequence alignment that allowed to differentiate clade 2 from clade 1. The most striking feature of clade 2 was the insertion at positions 107–118 (Fig. 2a). Subclade 1.1 (aLRT = 0.8) included DNA sequences merely from the Eastern Hemisphere while subclade 1.2 (aLRT = 0.67) was dominated by DNA sequences from the Western Hemisphere. The sequences from La Réunion and Madagascar were found in both subclades.

Pairwise genetic distances were lowest between members of clade 1 (maximum 0.037, median 0.021; Fig. 3) and slightly higher between members of clade 2 (maximum 0.052, median 0.042). The distances between the members of clades 1 and 2 were always distinctly higher (maximum 0.122, median 0.109).

Between the clades 1 and 2 of \textit{S. perlongisporum}, there were no differences in the mean length, width and length-to-width ratio of spores (Supplementary fig. S4.1a and table S4.2). The mean length of cystidia per specimen was significantly higher in clade 2 compared with that in clade 1 (Wilcoxon \(W = 18, p = 0.019\), Fig. 4). There were no differences in the mean width and length-to-width ratio of cystidia between clades 1 and 2.

Morphological differences between long-spored \textit{Subulicystidium} species

Morphological comparison based only on specimens for which DNA sequences were obtained showed that \textit{S. cochleum}, \textit{S. longisporum} and \textit{S. perlongisporum} differed in basidiospore length (Fig. 5a). Basidiospore width and length-to-width ratio distinguished \textit{S. longisporum} from \textit{S. perlongisporum} and \textit{S. cochleum} but did not separate the two latter species. \textit{S. perlongisporum} had shorter and narrower cystidia than the two other species (Fig. 5b).

Discussion

In this study, we assessed the range of molecular (nc rDNA) and morphological variation in the long-spored species of \textit{Subulicystidium}: \textit{S. cochleum}, \textit{S. longisporum} and \textit{S. perlongisporum}. We found that each of these species was monophyletic. However, none of them was sister to any of the other. For \textit{S. cochleum} and \textit{S. perlongisporum}, we confirmed a transoceanic distribution pattern.

We included the holotype of \textit{S. perlongisporum} in our study (LY11631) and successfully obtained ITS and 28S DNA sequences from this 35-year-old specimen. We generated first DNA sequence data for the species \textit{S. cochleum}. We successfully sequenced collections made in Costa Rica, Jamaica and Madagascar, but failed to obtain sequences from those made in Réunion and Zimbabwe. We provided additional morphometric and genetic data for \textit{S. longisporum}.

Furthermore, phylogenetic analyses allowed us to re-identify the specimen from Papua New Guinea (TU110416) as \textit{S. tropicum} (previous identification: \textit{S. brachysporum}). The new identification was added to the specimen and DNA sequence records in the PlutoF platform (https://plutof.ut.ee/#/specimen/view/651339; Abarenkov et al. 2010).

With the support of molecular data, we demonstrated a rather broad intraspecific variation in studied \textit{Subulicystidium} species, especially in the length of spores and cystidia. Following the recommendations of Parmasto...
et al. (1987), we provided more accurate morphological data for *S. perlongisporum*, *S. cochleum* and *S. longisporum* by separating intra-individual, intraspecific and interspecific size variation. Our data showed that basidiospores of *S. cochleum* and *S. perlongisporum* can be considerably shorter than stated in the protologues of these species (Punugui et al. 1980; Boidin and Gilles 1988). Previous authors also faced this problem and apparently attributed more importance to the values of spore width and length-to-width ratio for identification (Boidin and Gilles 1988; Duhem and Michel 2001). We confirm that spore length is more variable than the spore width or length-to-width ratio in the studied species of *Subulicystidium*.

Despite the similarity in the shape and size of spores, the three long-spored *Subulicystidium* species were not closely related. Therefore, the spore shape can be considered a homoplastic character in this genus. On the other hand, we recovered a sister relationship for *S. cochleum* and *S. acerosum*. They share cystidia that are sheathed by needle-like crystals in the middle part. This crystal arrangement is different from most species in *Subulicystidium* that have rectangular crystals arranged in longitudinal rows. In *S. oberwinkleri*, a peculiar cystidium ornamentation (crystal plates) correlates with an isolated phylogenetic position. The cystidial encrustation deserves more attention in further studies on *Subulicystidium*.

We found that *S. perlongisporum* includes two sympatric lineages. Morphologically these lineages differed slightly, but significantly, in the mean length of cystidia (longer in clade 2) but not in other characters of cystidia and spores. The genetic distance between ITS nrDNA sequences from these two lineages was as high as are usually the distances between separate species within the same fungal genus, i.e. well over 3% (Schoch et al. 2012; Köljalg et al. 2013).

Clades 1 and 2 of *S. perlongisporum* differed strongly in the number of available specimens, viz. 22 versus 5. The holotype of *S. perlongisporum* from La Réunion Island (LY 11631) was recovered in clade 1. The DNA sequences within clade 1 were more similar and were connected by shorter branches on the phylogenetic tree than the members of clade 2. We see a need for additional data for clade 2, in order to test whether it represents more than one cryptic lineage, and in order to confirm with a more balanced sampling whether the observed difference in the length of cystidia compared to clade 1 is real. Until then, we are reluctant to introduce any new species name.

Using nc ITS rDNA data, other authors showed that a single corticioid species may represent numerous phylogenetic lineages. Allopatric speciation was found within *Hyphoderma setigerum* and *Xylocladon raduloides* species complexes (Nilsson et al. 2003; Fernández-López et al. 2019). In contrast, the clades of *Peniophorella praetermissa* species complex contained specimens of very distant geographic origin suggesting sympatric speciation (Hallenberg et al. 2007). Morphological differences between the members of the clades in the above-mentioned species complexes were often missing. Our finding for *S. perlongisporum* is congruent with the pattern for *P. praetermissa*, although the former includes only two clades while the latter has eight. It remains a challenge to explain why allopatric speciation prevails in some species complexes while genetically deviating populations may occur sympatrically in others, even when all share a saprotrophic lifestyle and live in strongly decayed wood.

Struck et al. (2018) consolidated the concept of cryptic species and stated that crypts may represent a substantial fraction of biodiversity. They emphasized the need for a quantitative assessment of morphological disparity versus genetic divergence and comparing them with those for non-cryptic taxa. However, methods for quantitative assessment of morphological variation should become more standardized. Our protocols for morphometric analysis used for *Subulicystidium* (Ordynets et al. 2018; Ordynets and Denecke 2018) can be applied to all other fungi. We hope these protocols will enhance the reproducibility of the morphometric analysis in mycology and facilitate the correlation of morphometric data with genomic-scale DNA data.

Acknowledgements We would like to thank the curators of herbaria and mycologists who provided loan specimens for our study: Ibai Olariaga (ARAN), Markus Scholler (KR), Mélanie Thiébaut (LY), Ira Saar (TU), Cornelia Díger-Endrulat and Franz Oberwinkler (TUB). Ulrike Frieling and Sylvia Heinemann (University of Kassel) and Rasmus Pussepp (University of Tartu) are deeply acknowledged for the assistance with the molecular laboratory work. We thank the reviewers and editors for their comments that helped to improve the manuscript.

Author contributions A. Ordynets conceived the idea. K.-H. Larsson, E. Langer, A. Saitta, S. Volobuev, S. Bolshakov, B. Buyck and E. Yurchenko collected and identified fungal specimens. R. Liebisch, A. Ordynets, A. Saitta, D. Scherf, L. Lysenko, S. Volobuev and S. Bolshakov obtained morphometric data. R. Liebisch, A. Saitta, D. Scherf, L. Lysenko, S. Volobuev, S. Bolshakov and K.-H. Larsson performed molecular lab work and generated DNA sequences. A. Ordynets, R. Liebisch and D. Scherf analyzed the data. A. Ordynets wrote the first version of the manuscript and all authors read, contributed to and agreed on the final version.

Funding information Open Access funding provided by Projekt DEAL. The work of A. Ordynets and E. Langer was partly funded by the LOEWE excellence initiative of the state of Hesse (Germany) within the framework of the Cluster for Integrative Fungal Research (IPF). K.-H. Larsson received support from SIU, Norwegian Centre for International Cooperation in Education (projects CAPES-SIU-2013/10057 and CAPES-SIU-2015/10004). The work of S. Volobuev and S. Bolshakov was carried out within the framework of the institutional research project AAAA-A19-11902080079-6 using equipment of The Core Facilities Center “Cell and Molecular Technologies in Plant Science” at the Komarov Botanical Institute RAS (St.-Petersburg, Russia).

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