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Multi-gene analyses reveal taxonomic placement of *Scolicosporium minkeviciusii* in Phaeosphaeriaceae (Pleosporales)

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Abstract – *Scolicosporium minkeviciusii,* was newly collected in Italy, and subjected to morpho-molecular analyses. Morphological characters clearly indicate that this species is a coelomycete. Combined maximum-likelihood and maximum-parsimony analyses of LSU and SSU gene sequence data of *S. minkeviciusii* grouped it in Phaeosphaeriaceae with *Phaeosphaeria nodorum, P. oryzae* and *Stagonospora foliicola,* although the type species of *Scolicosporium*, *S. macrosporium*, which has not been sequenced, is considered to belong in the family Pleomassariaceae. In this study, we designate an epitype for *Scolicosporium minkeviciusii*. The placement of *S. macrosporium* and *Scolicosporium sensu stricto* remains uncertain and further morpho-molecular studies are necessary to confirm the taxonomic placement of this type species and to delimit this genus.

Asexual states / Classification / Coelomycetous fungi / Phylogeny

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INTRODUCTION

The term "coelomycete", introduced by Grove (1919), is used for asexually reproducing fungi which produce conidia "in a cavity lined by either fungal tissue, host tissue, or a combination of both" (Kirk *et al.,* 2008). Approximately 1000 genera of coelomycetes have been described (Wijayawardene *et al.,* 2012b) but most are not placed in a natural classification system, i.e. neither being linked to a sexual state nor to have a known familial affiliation (Wijayawardene *et al.,* 2012a). Most established links of sexual and asexual ascomycete states are based on co-occurrence of both forms in close proximity on the host (Spooner & Kirk, 1982), or through culture-based methods (Hyde *et al.,* 1996; Ramaley & Barr, 1996). Since the introduction of molecular techniques in fungal studies (White *et al.,* 1990), it has become possible to link sexual and asexual forms using DNA gene sequence analyses. It has, therefore, become necessary to re-collect species in order to extract DNA and to carry out morpho-molecular studies to confirm their natural taxonomic placements (Wijayawardene *et al.,* 2012c).

The genus *Scolicosporium* was introduced by Roumeguère (1880) for *S. fagi* Lib. ex Roum. Saccardo (1881) synonymized *Sporidesmium vermiforme* Riess and *Coryneum macrosporium* Berk. under *Scolicosporium fagi* (as *Scolecosporium fagi*). Sutton (1977, 1980) designated the older name, *Coryneum macrosporium*, as the type of *Scolicosporium* [as *S. macrosporium* (Berk.) B. Sutton] and synonymized *Coryneum macrosporium*, *Scolicosporium fagi* and *Sporidesmium vermiforme* with this species. In Index Fungorum (2013), there are 13 epithets listed under *Scolicosporium,* although most of these epithets have been placed in other genera (Sutton, 1975; Spooner & Kirk, 1982). Only three species are presently accepted in *Scolicosporium*, i.e. *S. macrosporium* (Sutton, 1977)*, S. pauciseptatum* Constant. (≡ *Hendersonia fusarioides* Sacc.) (Constantinescu, 1991) and *S. minkeviciusii* Treigienė (Treigienė & Mel'nik, 2002).

Scolicosporium has been treated as a coelomycete (Nag Raj & DiCosmo, 1980; Sutton, 1980), but Spooner & Kirk (1982) considered the genus to have hyphomycetous affinities. Spooner & Kirk (1982) said that *Asteromassaria macrospora* (Desm.) Höhn is the sexual state of *Scolicosporium macrosporium*, based on association of both species on the same host. This link has not been proven by molecular analysis and no *Scolicosporium* species DNA sequences have so far been deposited in GenBank.

We studied a recent specimen of *Scolicosporium minkeviciusii* (Treigienė & Mel'nik, 2002) on *Quercus pubescens* collected in Italy. The aim of this paper is to epitypify, re-describe and illustrate this fungus. Single conidial culture was sequenced using LSU and SSU genes to infer the phylogenetic placement of this species within the Ascomycota.

MATERIALS AND METHODS

Collection and isolation: Conidiomata were observed by hand lens during the collection of saprobic fungi associated with dead plant material in Buggiana (Suasia Valley), Italy. These were collected, placed in paper bags and returned to

the laboratory. The specimens were observed under a stereoscope. Conidiomata were removed, placed in a droplet of distilled water on a clean slide, neatly squashed and examined under a compound microscope to observe the conidial characters. Single spore isolation was carried out following the method described in Chomnunti *et al.* (2011). Germinating conidia were transferred aseptically to potato dextrose agar (PDA) plates and grown at 18-20°C. Colony colour and morphological characteristics were assessed after 5 days and 1 week. The specimens were deposited in the Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand. Living cultures were also deposited in Landcare Research, New Zealand (ICMP), Mae Fah Luang University Culture Collection (MFLUCC) and Department of Plant Pathology, Agriculture College, Guizhou University, China (GHUP).

DNA extraction, PCR amplification and sequencing: A Biomiga Inco. kit (GD2416, San Diego, CA 92121) was used to extract genomic DNA from fresh fungal mycelia. For the amplification of internal transcribed spacers (ITS), small subunit nuclear rRNA gene region (SSU) and large subunit nuclear rRNA gene region (LSU), ITS5 and ITS4, NS1 and NS4 (White *et al.,* 1990) and LROR and LR5 (Vilgalys & Hester, 1990) primers were used respectively. DNA amplification was carried out as previously described by Liu *et al.* (2012). The amplified genes were then sent to SinoGenoMax Co., Beijing, China for DNA sequencing. The nucleotide sequence data obtained were deposited in GenBank (Table 1).

Phylogenetic analyses: Generated sequences of LSU (KF 366382) and SSU (KF 366383) nuclear rRNA were used for molecular phylogenetic analyses and the sequence alignments were deposited in TreeBASE (http://www.treebase.org/; submission ID 14584). A BLAST search in GenBank for LSU and SSU sequences which followed alignments for Dothideomycetes in Schoch *et al.* (2006) and Zhang *et al.* (2009, 2012) revealed the closest taxa to our strain. Different sequences from the closest taxa in different families (Coniothyriaceae, Cucurbitariaceae, Didymellaceae, Leptosphaeriaceae, Phaeosphaeriaceae and Pleosporaceae) were selected. These sequences were downloaded and aligned separately using Bioedit (Hall, 2004) and ClustalX (Kohli & Bachhawat, 2003). Alignments were checked and manual adjustments made where appropriate. Individual datasets were concatenated into a combined dataset. The ITS gene sequence was excluded in the phylogenetic analyses, but are deposited in GenBank as it is the preferred locus for use in fungal barcoding (Schoch *et al.*, 2011).

Maximum likelihood (ML) analysis was performed in RAxML (Stamatakis, 2006) implemented in raxmlGUI v.0.9b2 (Silvestro & Michalak, 2012). Fifty thorough maximum likelihood (ML) tree searches were done in RAxML under the general time reversible model (GTR), with each one starting from a separate randomised tree and the best scoring tree selected. One thousand non parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution (Liu *et al.*, 2012).

Maximum-parsimony analysis was carried out using PAUP v. 4.0b10 (Swofford, 2003), and was performed using the heuristic search option with 1000 random taxa additions and tree bisection and reconnection (TBR) as the branch swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Max trees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability was assessed using a bootstrap (BT) analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa (Hillis & Bull, 1993). Trees were visualized with Tree View (Page, 1996).

Table 1. Sequence data used in this study. Newly produced sequences in bold

Taxon	*Culture collection number*	*GenBank Accession number*	
		LSU	*SSU*
Ampelomyces quisqualis	CBS 129.79	EU754128	EU754029
Cochliobolus heterostrophus	AFTOL-ID 54	AY544645	AY544727
Cochliobolus sativus	AFTOL-ID	DQ678045	DQ677995
Coniothyrium palmarum	CBS 400.71	EU754153	EU754054
Coniothyrium palmarum	CBS 758.73	EU754154	EU754055
Cucurbitaria berberidis	CBS 394.84	GQ387605	GQ387544
Didymella exigua	CBS 183.55	EU754155	EU754056
Didymella pisi	CBS 126.54	GU237968	EU754038
Leptosphaeria doliolum	CBS 541.66	JF740284	
Leptosphaeria doliolum	CBS 155.94	JF740282	
Leptosphaeria doliolum	CBS 125979	JF740283	
Leptosphaeria slovacica	CBS 389.80	JF740315	JF740101
Leptosphaerulina australis	CBS 317.83	GU301830	GU296160
Montagnula anthostomoides	CBS 615.86	GU205223	GU205246
Ophiosphaerella herpotricha	AFTOL-ID 1595	DQ767656	DQ767650
Paraphoma fimeti	CBS 170.70	GQ387584	GQ387523
Peyronellaea zeae-maydis	CBS 588.69	EU754192	EU754093
Phaeosphaeria nodorum	CBS 110109	EU754175	EU754076
Phaeosphaeria oryzae	CBS 110110	GQ387591	GQ387530
Phoma herbarum	CBS 615.75	EU754186	EU754087
Pleospora calvescens	CBS 246.79	EU754131	EU754032
Pleospora herbarum	CBS 191.86	GU238160	GU238232
Pyrenochaeta acicola	CBS 122789	EU754204	EU754204
Pyrenochaeta nobilis	CBS 407.76	EU754206	EU754107
Pyrenochaeta quercina	CBS 115095	GQ387619	GQ387558
Pyrenochaetopsis decipiens	CBS 343.85	GQ387624	GQ387563
Pyrenochaetopsis leptospora	CBS 101635	GQ387627	GQ387566
Pyrenophora phaeocomes	AFTOL-ID 283	DQ499596	DQ499595
Scolicosporium minkeviciusii	MFLUCC 12-0089	**KF366382**	**KF366383**
Setophoma sacchari	CBS 333.39	GQ387586	GQ387525
Stagonospora foliicola	CBS 110111	EU754217	EU754118
Wojnowicia hirta	CBS 160.73	EU754222	EU754123
Wojnowicia viburni	MFLUCC 120733	KC594287	KC594288

RESULTS

Phylogenetic analyses: The combined gene data set of LSU and SSU consists of 32 sequences of 29 taxa with the out group taxon. The dataset consists of 2376 characters including coded alignment gaps; 1660 are constant, while 194 are variable and 125 are parsimony uninformative in the MP and ML analyses. A best scoring RAxML tree is shown in Fig. 1. Bootstrap support (BS) values of ML and MP (equal to or above 50% based on 1000 replicates) are shown above branches.

Our strain of *Scolicosporium minkeviciusii* (ICMP 19881; MFLUCC 12-0089) clustered in Phaeosphaeriaeae, however, the phylogenetic relationship of this species towards the various genera accepted within the family remains uncertain. The phylogenetic tree also suggested that this strain might be close to *Phaeosphaeria, Setophoma* and *Stagonospora* as they formed a subclade, although without support.

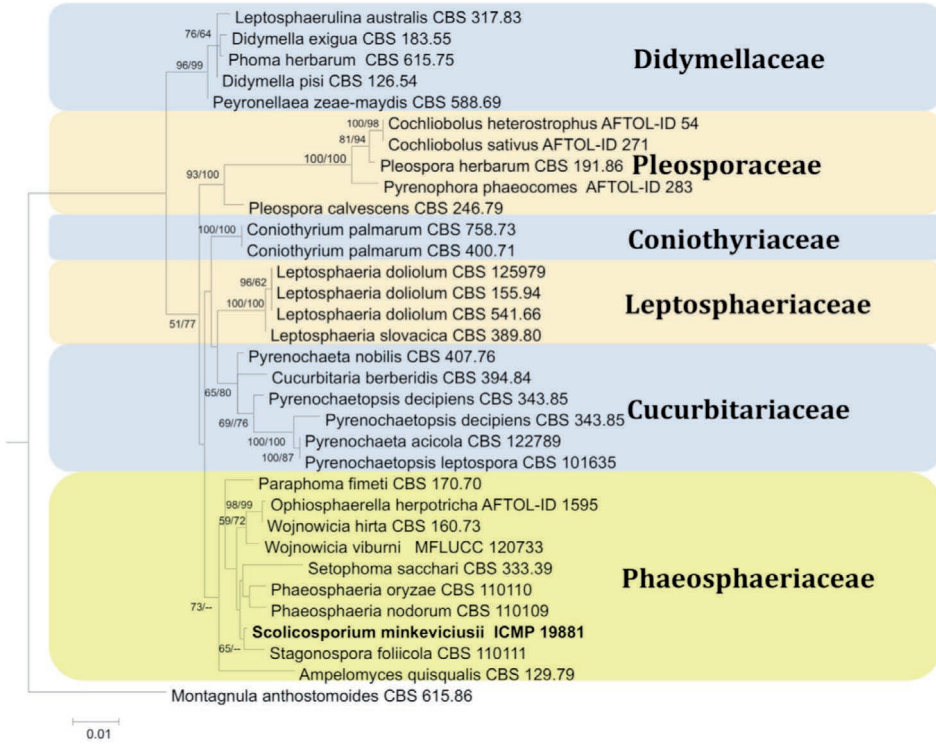

Fig. 1. RAxML tree generated by the analysis of combined data set of LSU and SSU nrDNA sequences. Bootstrap support values greater than 50% for maximum likelihood (ML) and maximum parsimony (MP) analyses are given above the nodes respectively. The epitype strain of *Scolicosporium minkeviciusii* (MFLUCC 12-0089) (Phaeosphaeriaceae) is in bold. The tree is rooted to *Montagnula anthostomoides* (CBS 615.86) (Montagnulaceae).

Taxonomy

Scolicosporium minkeviciusii Treigienė, in Treigienė & Mel'nik, *Mikol. Fitopatol.* 36(6): 45 (2002) **Figs 2-5**

Saprobic on bark of *Quercus pubescens* Willd. and *Q. robur* L. *Conidiomata* 100-120 µm high × 120-180 µm wide, pycnidial, solitary to gregarious, elongate-globose, dark brown, uniloculate. *Ostiole* central, sometimes towards one side, papillate when young, opening longitudinally at maturity. *Pycnidial wall* comprising 3-4 layers, outer layers dark brown, 8–10 µm wide, cells of *textura angularis*, inner layers hyaline, 5-40 µm wide. *Conidiophores* 13-30 × 2-3 µm, 1-2-septate, branched at the base, hyaline, cylindrical. *Conidiogenous cells* annelledic, hyaline, cylindrical. *Conidia* 60-65 × 7-9 µm ($\bar{x}$ = 62.2 × 7.7 µm, n = 20), curved to sigmoid, pale to moderately dark brown, with hyaline end cells, 6-7-transverse eusepta, smooth-walled, tapered to the obtuse apex, base truncate.

Culture characteristics: Conidial germination starts from basal hyaline cell or first lower median cell. Cells at first swell and produce germ tubes from any point. The germ tubes may eventually emerge from all cells. On PDA, pale brown on surface and white at margin, slow growing, attaining a diam. of 1-2 cm after 14 days at 20°C, with thin mycelium, zonate, circular. Reverse of the colony reddish orange after 2 weeks.

Material examined: Italy, Emilia-Romagna, Forlì-Cesena Province, Santa Sofia, Buggiana (Suasia Valley), on bark of *Quercus pubescens,* 19 November 2011, Erio Camporesi, It NNW 40 (MFLU 13-0089, epitype designated here; ex-

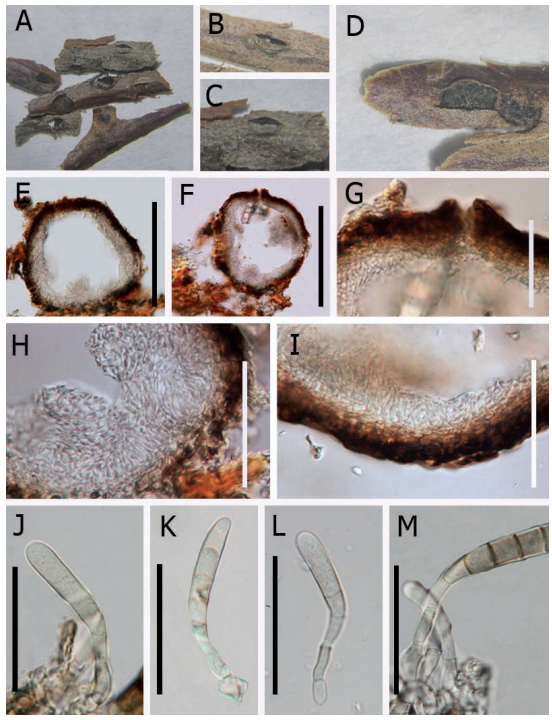

Fig. 2. *Scolicosporium minkeviciusii* on *Quercus pubescens* (MFLU 13-0089, epitype). **A.** Conidiomata on host. **B, C.** Immature conidiomata. **D.** Mature sporodochia-like conidiomata. **E, F.** Cross sections of immature conidiomata. **G.** Ostiolar opening. **H, I.** Wall of the conidioma. **J-M.** Different stages of developing conidia with conidiophores. Scale bars: **E** 130 µm; **F** 150 µm; **G** 40 µm; **H-M** 30 µm.

epitype culture ICMP 19881 = HGUP N53 = MFLUCC 12–0089); Lithuania, Vilnius, in silave Tartokiai dicto, hab. in *ramis emortuis siccis Quercus robur,* 15 November 1996, A. Treigienė (LE 212415, holotype).

GenBank accession numbers of ex-epitype: for LSU (KF366382) and SSU (KF366383).

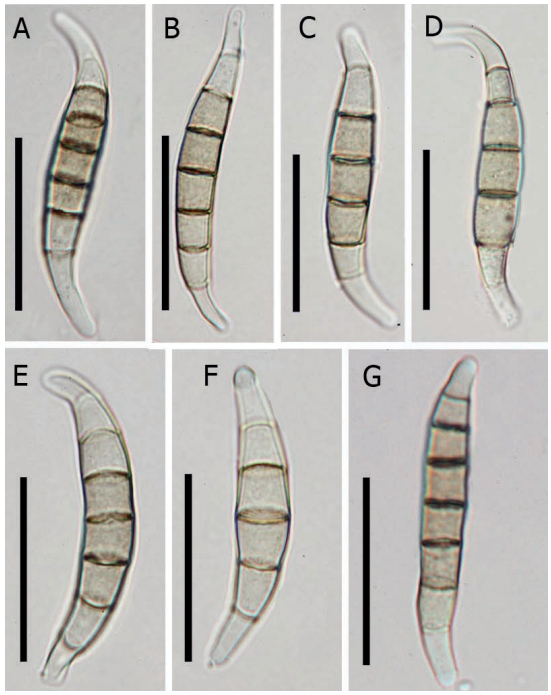

Fig. 3. Conidia of *Scolicosporium minkeviciusii*. Scale bars: **A-G.** 30 µm.

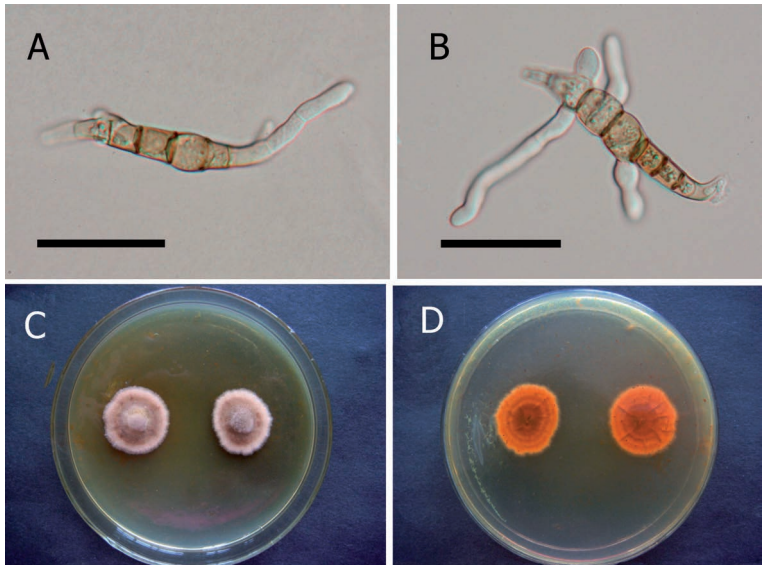

Fig. 4. *Scolicosporium minkeviciusii* (epitype). **A, B.** Germinating conidia. **C.** Cultural characters on PDA from above after 7 days. **D.** Reverse culture. Scale bars: **A, B.** 30 µm.

DISCUSSION

There have been only a few studies on *Scolicosporium* (Saccado, 1881; Sutton, 1975, 1980; Spooner & Kirk, 1982; Constantinescu, 1991; Treigienė & Mel'nik, 2002) since introduction of the genus by Roumeguère (1880). Several names have been included in the genus (Index Fungorum, 2013); although Sutton (1975) concluded that they are not congeneric with *S. macrosporium,* the type species of the genus. Sutton (1975) and Spooner & Kirk (1982) excluded several taxa as they have different morphological characters. These species and the reasons for their exclusion are listed in Table 2.

Constantinescu (1991) synonymized *Hendersonia fusarioides* with *S. pauciseptatum* and mentioned that three other *Scolicosporium* species (*S. barringtoniae* Viennot-Bourgin, *S. gei* Chona *et al*. and *S. lactucae* Munjal & Kapoor.) were not discussed by Sutton (1975) and Spooner & Kirk (1982). Constantinescu (1991) then proceeded to remove these species from *Scolicosporium* because, in most cases, conidia were uniseptate (but *S. barringtoniae* varies with 1-2-septa). Treigienė & Mel'nik (2002) introduced *S. minkeviciusii*, hence there are three accepted species in *Scolicosporium* i.e. *S. macrosporium, S. barringtoniae* and *S. minkeviciusii.*

Sutton (1975, 1977, 1980) and Nag Raj & DiCosmo (1980) considered *Scolicosporium* as coelomycetous, while Spooner & Kirk (1982) discussed its hyphomycetous affinity. Seifert *et al.* (2011) listed *Scolicosporium* as a hyphomycete. This contrasting understanding is primarily due to the nature of conidiomata. In *S. minkeviciusii*, conidiomata are immersed in the substrate when immature and typically coelomycetous (Fig. 2 B, C). As the conidiomata mature they become sporodochia-like (Fig. 2 D). The nature of conidioma is very similar with the definition of acervulus (Kirk *et al.,* 2008). However, the conidioma of *S. minkeviciusii* is subglobose and much more typical of a pycnidium than an acervulus. The longitudinal ostiolar opening found in the conidioma of *Scolicosporium minkeviciusii* also agrees with the definition of a pycnidium in Kirk *et al.* (2008). Considering these characters, we conclude that *S. minkeviciusii* is coelomycetous fungus.

A link between *Scolicosporium* and its sexual state was reported by Saccardo (1883) who connected *S. macrosporium* (as *Coryneum macrosporum*

Table 2. Species excluded from *Scolicosporium*
(based on Sutton, 1975 and Spooner & Kirk, 1982)

Species	*Sutton (1975)*	*Spooner & Kirk (1982)*
Scolicosporium betulae Rostr.	? *Scolicosporium fagi* Lib. ex Roum.	*Excipularia fusispora* (Berk. & Broome) Sacc.
S. coryli Dearn. & House	Doubtful	*Seimatosporium* Corda or *Monochaetia* (Sacc.) Allesch.
S. fusarioides (Sacc.) B. Sutton	*Scolicosporium fusarioides* (Sacc.) B. Sutton	*Excipularia fusispora* (Berk. & Broome) Sacc.
S. pedicellatum Dearn. & Overh.	*Seiridium* Nees	*Seiridium* Nees
S. phoebes T.S. Ramakr	Doubtful	Doubtful
S. syzygii Ciccar.	Doubtful	Doubtful
S. transversum Fairm.	*Seiridium* Nees	?*Seimatosporium* Corda
S. typhae Höhn.	Not mentioned	*Scolecosporiella* Petr.

Berk.) with *Asteromassaria macrospora* (Desm.) Höhn. (as *Massaria macrospora* (Desm.) Sacc.). Höhnel (1917), Spooner & Kirk (1982) and Sivanesan (1984) accepted this connection as the taxa were observed in close proximity on the same host. In our analyses of combined LSU and SSU gene regions, *Scolicosporium minkeviciusii* groups in Phaeosphaeriaceae (Fig. 1) along with other well-established genera, i.e. *Ampelomyces*, *Paraphoma*, *Phaeosphaeria, Setophoma, Stagonospora* and *Wojnowicia* (de Gruyter *et al.,* 2009, 2010; Wijayawardene *et al.,* 2013). We therefore suggest that *S. minkeviciusii* is placed in Phaeosphaeriaceae, although better support is needed to resolve relationships with other genera. As the type species of *Scolicosporium*, *S. macrosporium* has not been recollected and sequenced, its family affinities require further studies.

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