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Research Article

Molecular and morphological studies of *Paecilomyces sinensis* reveal a new clade in clavicipitaceous fungi and its new systematic position

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The type materials of *Paecilomyces sinensis*, including herbarium specimen and ex-type strain, were re-examined to clarify its relationships with other species. Morphological observations on the strain grown in various culture media revealed that the fungus was morphologically related to *Polycyphalomyces*, since it produced conidial mass and lanceolate or narrowly lageniform phialides. Six genes, including ITS, *nrSSU*, *nrLSU*, *tef1*, *rpb1* and *rpb2*, were amplified from the type materials and used in phylogenetic analyses to determine the systematic position of the fungus in the framework of clavicipitaceous fungi. The results place *P. sinensis* with *Polycyphalomyces formosus*, the type species of *Polycyphalomyces*, and *Cordyceps ramosapulvinate* forming a clade unaffiliated with the known families of clavicipitaceous fungi. Based on both morphological and molecular data, a new combination, *Polycyphalomyces sinensis*, is proposed for *Paecilomyces sinensis*. The new clade found in this study is designated as *Polycyphalomyces* clade and expands the phylogenetic diversity for clavicipitaceous fungi. The teleomorph–anamorph connection between *Berkeleylla* and *Polycyphalomyces* previously conceived cannot be retained as the type species of *Polycyphalomyces*, *P. formosus*, is closely linked to species of *Cordyceps s.l.* in the new clade.

**Key words:** clavicipitaceous fungi, *Cordyceps s.l.*, *Ophiocordyceps sinensis*, *Paecilomyces*, phylogeny, *Polycyphalomyces sinensis*

Introduction

*Paecilomyces sinensis* C.T. Chen, S.R. Xiao & Z.Y. Shi was described as a new species, different from the asexual stage of *Cordyceps militaris* (L.) Link, with respect to conidial features, phialide size, host and habit characters (Chen et al., 1984; Liang et al., 2005). The type specimen of *P. sinensis* (HMAS 43720) was derived from a strain CN 80-2, which was isolated from the sclerotium of *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, one of the most important Chinese medicinal fungi, collected from Sichuan, China in 1980. *Paecilomyces sinensis* was, therefore, considered as the possible anamorph (asexual stage) of *O. sinensis* (Chen et al., 1984), and the name was subsequently accepted and cited in many articles on the anamorph of *O. sinensis* (Jiang & Yao, 2003), but was excluded by several molecular investigations (Jiang & Yao, 2002). Pharmacological studies on *P. sinensis* were carried out on immunomodulatory (e.g. Zhang et al., 1998, 1999; Cheng et al., 2005), antitumour (e.g. Huang et al., 1984; Wu et al., 1986), anti-estrogenicity (e.g. Lin et al., 1988) and anti-oxidation (e.g. Liu et al., 1989, 1991) effects, etc. The similarity in pharmacological effects between *P. sinensis* and *O. sinensis* generated the idea of using the former as a substitute of the latter (Huang et al., 1984; Li et al., 1991; Zeng et al., 2000, 2001).

The genus *Paecilomyces* Bainier was established with a single species, *P. variotii* Bainier (Bainier, 1907), and restricted to species with verticillate conidiophores bearing divergent whorls of branches and phialides by Samson (1974) in his monographic study. Phylogenetic studies of this genus revealed that *Paecilomyces* was polyphyletic at the ordinal level (Obornik et al., 2001), involving three ascomycete orders: *Eurotiales*, *Hypocreales* and *Sordariales* (Luangsa-ard et al., 2004, 2005; Inglis & Tigano, 2006). Sung et al. (2007a) dispersed *Paecilomyces s.l.* species among three families in the reclassification of *Cordyceps s.l.* and clavicipitaceous fungi. Many species of *Paecilomyces*...
have been included in those studies, but *P. sinensis* was not examined and the current systematic position of the species remains unknown.

*Polycephalomyces* Kobayasi was erected with *P. formosus* Kobayasi as the type (Kobayasi, 1941). Species in *Polycephalomyces* are synnematous, having awl-shaped phialides and producing single-celled conidia in a viscous matrix at the apex of the synnema (Bischoff et al., 2003). Phylogenetic analyses of 28S rDNA were conducted and all the three *Polycephalomyces* species used were recovered in the clade of *Clavicipitaceae* s.l. (Bischoff et al., 2003). Host substrates, phialide shape and conidial mass are the characters distinguishing *Polycephalomyces* from *Paecilomyces* (see Brown & Smith, 1957; Samson, 1974 for *Paecilomyces*; and Seifert, 1985 for *Polycephalomyces*).

The type materials of *Paecilomyces sinensis*, including the type specimen (HMAS 43720) and the ex-type strain (CN 80-2), were re-examined both morphologically and molecularly in the present study. The systematic position of the species was re-determined based on morphological features and molecular evidence with the discovery of a new major clade in the clavicipitaceous fungi.

**Materials and methods**

**Fungal materials**

A type specimen of *Paecilomyces sinensis* (HMAS 43720), from dried colonies of CN 80-2, was deposited in the Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences (HMAS) and was made available to the present study. The ex-type strain (CN 80-2) was isolated from a sclerotium of *O. sinensis* collected in Kangding County, Sichuan Province in 1980 and maintained at 4 °C on Potato Dextrose Agar (PDA) slants in the dark. The stock of the strain was transferred to new PDA slants and incubated at 25 °C for 14–20 days for this study.

**Culture and observation**

Cultures were inoculated with a 1 cm diameter agar disc from a colony of 7 cm diameter in a Petri dish and grown on PDA, Czapek Agar, 2% Malt Extract Agar (MA) and Oatmeal Agar (OA) at 25 °C. Morphological examinations were performed every 2–5 days. For characterizing the strain, microscope slide cultures were prepared by inoculating a small amount of mycelium on a desired nutrient agar medium block of 1 cm² overlaid by a cover slip. Microscopic observations were carried out and photographed with a Zeiss Axioscope microscope equipped with AxioCam MRC. Microscopic measurements were made with AxioVision Rel. 4.6 software (Zeiss, Welwyn Garden City, UK).

**DNA isolation, PCR amplification, cloning and sequencing**

The fresh mycelium of CN 80-2 was obtained from the strain grown on PDA at 25 °C for 10 days. Total genomic DNA was then extracted with the E.Z.N.A.™ Fungal DNA Mini Kit (OMEGA, Bio-tek, USA) following the manufacturer’s instructions. A method using Chelex-100 (Bio-Rad, Hercules, CA, USA), which needs the least material, was employed to extract DNA from HMAS 43720 to avoid damaging the type specimen. A small amount of dried mycelium was taken from the specimen, crushed in 60 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and heated at 95 °C for 10 min in the presence of 20 µl 20% Chelex-100. Liquid suspension was cooled on ice for approximately 1 min and centrifuged at 12 000 rpm for 5 min. The supernatant was aspirated to a new microfuge tube and kept at −20 °C or directly used as the DNA template for PCR amplification.

Six nuclear genes, including ITS, *nrSSU*, *nrLSU*, *tef1*, *rpb1*, and *rpb2*, were amplified and sequenced. For ITS region, primers ITS5 and ITS4 (White et al., 1990) were used and the PCR amplification was conducted as described in Jiang & Yao (2005). The *nrLSU* and the *nrSSU* were amplified with primers of LROR and LR5 (Vilgalys & Sun, 1994), and NS1 and NS4 (White et al., 1990) respectively, following the PCR procedures of Sung et al. (2001). A touchdown PCR was employed for *tef1* using primers 983F and 2218R according to Rehner (2001). The *rpb1* was amplified with primers CRPB1 and RPB1Cr under the conditions of Castlebury et al. (2004). Amplification of *rpb2* with primers fRPB2-5F and fRPB2-7cR was carried out on the PCR System 9700, Applied Biosystems, USA). The 50 µl PCR reactions contained 25 µl 2 × Taq PCR Master Mix (Tiangen Biotech Co., LTD, China), 0.5 µl of each primer (10 pM) and 1 µl template DNA. The oligonucleotides of the primers used in this study are listed in Table S1 (see supplementary material, which is available on the Supplementary tab of the article’s Taylor & Francis Online page at http://dx.doi/10.1080/14772000.2012.690784). PCR products of *nrSSU*, *nrLSU*, *tef1*, *rpb1* and *rpb2* were purified using PCR cleanup plates (Millipore Corporation, USA) and directly subjected to sequencing, whilst the products of ITS were cloned into pUCm-T vector with DNA Cloning Vector Kit (Bio Basic Inc. Canada) before sequencing, owing to high GC contents which caused the failure of direct sequencing. Sequences were analysed on a capillary sequencer (Applied Biosystems 3730 Analyzer, Foster City, California) by the Beijing Genomics Institute (Beijing, China).

ITS sequences were obtained from both the type specimen and the ex-type strain and submitted to
GenBank with accession numbers of HQ832885 and HQ832884 (both are identical, see Table S2, supplementary material, which is available on the Supplementary tab of the article’s Taylor & Francis Online page at http://dx.doi/10.1080/14772000.2012.690784). Sequences of nrSSU, nrLSU, tef1, rpb1 and rpb2 genes were also obtained from CN 80-2 with GenBank accession numbers of HQ832887, HQ832886, HQ832890, HQ832888 and HQ832889, subsequently (Table S3, see supplementary material, which is available on the Supplementary tab of the article’s Taylor & Francis Online page at http://dx.doi/10.1080/14772000.2012.690784).

**Taxon sampling**

Eleven sequences retrieved from GenBank (nrLSU, tef1 and rpb1 for both Polycyphalomyces formosus and Cordyceps ramosopulvinata Kobayasi & Shimizu, submitted by Bischoff et al. (2003) and Chaverri et al. (2005); and nrSSU, nrLSU, tef1, rpb1 and rpb2 for Ophiocordyceps sinensis, submitted by Sung et al. (2007a)) were appended to the 5-gene dataset (including nrSSU, nrLSU, tef1, rpb1 and rpb2 from 91 taxa) of Sung et al. (2007b), together with the sequences of nrSSU, nrLSU, tef1, rpb1 and rpb2 from Paecilomyces sinensis obtained in this study. The outgroup taxa, Glomerella cingulata (Stoneman) Spauld. & H. Schrenk (Glomerellaceae) and Verticillium dahliae Kleb. (Plectosphaerellaceae) designated in previous phylogenetic analyses of Spatafora et al. (1998), Castlebury et al. (2004) and Sung et al. (2007b) were followed here. A total of 95 taxa with 3–5 genes were included in the dataset (see Table S3, supplementary material, which is available on the Supplementary tab of the article’s Taylor & Francis Online page at http://dx.doi/10.1080/14772000.2012.690784).

The ITS sequence from the type materials of P. sinensis was used as a query in a BLAST search (Altschul et al., 1997) in GenBank on 2 December 2011. Thirty-one sequences with a maximum score higher than 500 were retrieved. A name search using Polycyphalomyces in GenBank was also performed and only one ITS sequence (AJ786598) was found and retrieved. In GenBank, only 15 ITS sequences from the same vouchers of the species included in Sung et al. (2007b) were available and they were retrieved for the ITS analyses. Cosmospora coccinea Rabenh. was chosen as an outgroup based on the availability of ITS regions from vouchers included in Sung et al. (2007b). A total of 50 ITS sequences, including the two obtained from the type specimen and ex-type strain of P. sinensis and one from a specimen of O. sinensis submitted by this laboratory before were assembled (see Table S2, supplementary material, which is available on the Supplementary tab of the article’s Taylor & Francis Online page at http://dx.doi/10.1080/14772000.2012.690784).

**Phylogenetic analyses**

Sequences of the 5-gene dataset used in this study were aligned in Clustal X Version 1.83 (Thompson et al., 1997) and then edited manually in BioEdit Version 7.0.9.0 (Hall, 1999). ITS alignments were performed by Simultaneous Alignment and Tree Estimation (SATé, Liu et al., 2009).

Maximum likelihood (ML) analyses were performed with RAXML-7.0.3-WIN using a GTR-GAMMA model of evolution (Stamatakis, 2006). Nodal support was assessed with nonparametric bootstrapping using 500 replicates. For multi-gene analyses, the model was separately applied to each of the 11 partitions, which consisted of nrSSU, nrLSU and the nine codon positions of three protein-coding genes (tef1, rpb1 and rpb2).

Bayesian analyses were run in MRBAYES v3.1.2 (Ronquist & Huelsenbeck, 2003) for $5 \times 10^6$ generations using two independent runs with four chains until the average standard deviation of the split frequencies between the simultaneous runs was below 0.01 and the log-likelihood had reached stationarity. General time reversible (GTR) model of DNA substitution with gamma-distributed rate variation across invariant sites as determined by jModelTest version 0.1.1 (Posada, 2008) was used for the 5-gene analyses, whilst the default F81 model was used for the ITS analyses. Trees were sampled every 100 generations. The first 12,500 trees were discarded and the remaining 37,500 were used for calculating posterior probabilities (PP) in the majority-rule consensus tree. For 5-gene analyses, independent models were applied for each of the 11 partitions (nrSSU, nrLSU and nine codon positions of the three protein-coding genes). Both the analyses of 5-gene and ITS datasets were repeated three times.

Maximum parsimony (MP) analyses were performed using the program PAUP* 4.0b8 (Swofford, 2001) with the following settings: heuristic search, gaps treated as ‘missing data’, 1000 replicates of random sequence addition, TBR branch swapping and MulTrees ON. Bootstrap proportions (BP) were calculated by analysis of 1000 replicates, each with five replicates of random sequence addition, with other settings identical to the above.

**Results**

**Morphological observations**

**Colony.** Colonies grew fairly well on PDA, attaining a diameter of 2 cm in 10 days, which was round, flat, short floccose and white at first; becoming denser in age, white tinged with pink in the centre, finally orange yellow, reverse dull buff and then becoming pale brown or reddish brown. Colonies grew fairly rapidly on MA, attaining a diameter of 2 cm in 10 days, which was round, flat, floccose, and white all the time and becoming reverse dull buff. Colonies grew slowly on Czapek agar, attaining a diameter of around 1 cm in 10 days, which was similar to those on PDA, but
without a change in colour. It was white all of the time, reverse dull buff. Colonies grew slowly on OA, attaining a diameter of around 1 cm in 10 days, which was round, flat, loosely cottony, white all the time, and reverse dull buff.

**Hypha.** On the four media, hyphae were delicate, hyaline, septate, branched, smooth-walled, 1.2–4.5 µm in width (Fig. 1a–c).

**Synnema.** Synnemata arose after 13 days on PDA, 15 days on Czapek, and 22 days on MA, but did not form on OA (observed up to 38 days); 5–6 cm long, clavate, at first white, overcolouring with age to orange yellow; radiating the ring-like distribution and consisting of parallel hyphae (unbranched or branched) with phototropism (Fig. 2). It was usually with opaque, slimy, yellow-orange conidial mass at the terminal portion of the synnema (Fig. 1d).

**Phialide.** Phialides on the four media were the same: acropleurogenous, monothetic, opposite or alternate, lanceolate or narrowly lageniform, with a narrow neck and occurring directly on the aerial hyphae and the peripheral hyphae around the coremium, sometimes on simple conidiophores; 12.5–66 µm in length, tapering gradually from 1.4–3.5 µm at the base to 0.6–1.8 µm at the tip, basipetally yielding the catenulate conidia (Fig. 1a–c).

**Conidium.** Conidia were produced in the four media, which were one-celled, smooth-walled and hyaline. α-conidia were ovoid, 1.7–2.6 × 1.3–2.0 µm, in viscous pools located on the agar and at the terminal portion of synnema forming a conidial mass (Fig. 1d). β-conidia were fusiform, 3.3–4.5 × 1.3–2.0 µm, produced along stipe of the synnema as well as on surface mycelium of the colony, single or often in chains on phialide, slimmed down completely to form irregular spore balls (Fig. 1a–c).
New clade in clavicipitaceous fungi and systematic position of *Paecilomyces sinensis* 225

At least seven major terminal clades were recognized from the 5-gene analyses (Fig. 3), with an additional new clade found by Sung et al. (2007a). The nomenclature of the clades followed the convention of former phylogenetic studies (Sung et al., 2007a, 2007b): *Bionecratiaceae, Nectriaceae, Hypocreaceae, Clavicipitaceae s.s.*, *Ophiocordycipitaceae* and *Cordycepaceae*. The new clade found in this study is designated as *Polycephalomyces* clade (Fig. 3). All the clades received support of 1.00 PP in Bayesian analyses except *Hypocreaceae* (0.96), of 100% BP in ML analyses except *Clavicipitaceae s.s.* (98%), and of over 90% BP in MP analyses except *Ophiocordycipitaceae* (59%). Three species added in this study, *C. ramosopulvinata*, *Polycephalomyces formosus* and *Paecilomyces sinensis*, formed a new monophyletic clade, strongly supported by all MP, ML and Bayesian analyses (MP-BP = 100%, ML-BP = 100%, PP = 1.00). The monophyletic group including *Clavicipitaceae s.s.*, *Ophiocordycipitaceae* and the *Polycephalomyces* clade received weak to strong statistical support from MP, ML and Bayesian analyses (MP-BP = 52%, ML-BP = 89%, PP = 1.00) and was sister to the monophyletic group of *Cordycepaceae* and *Hypocreaceae*. However, internal relationships among *Clavicipitaceae s.s.*, *Ophiocordycipitaceae* and the *Polycephalomyces* clade were not resolved.

**Phylogenetic analyses**

**Multi-gene.** The combined 5-gene dataset with 95 taxa consisted of 4667 base pairs (*nrSSU* 1088 bp, *nrLSU* 887 bp, *tef1* 996 bp, *rpb1* 651 bp, *rpb2* 1045 bp) after the exclusion of ambiguously aligned sites, 1814 of which were parsimony informative (*nrSSU* 196 bp, *nrLSU* 266 bp, *tef1* 409 bp, *rpb1* 567 bp, *rpb2* 376 bp). A total of 82 taxa were complete for all the five genes partitions. Eleven taxa were complete for four genes and two taxa for three genes (see Table S3, supplementary material, which is available on the Supplementary tab of the article’s Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2012.690784). The number of taxa for each gene was as follows: *nrSSU* 93 taxa, *nrLSU* 95 taxa, *tef1* 95 taxa, *rpb1* 92 taxa and *rpb2* 85 taxa (see Table S3, supplementary material, which is available on the Supplementary tab of the article’s Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2012.690784). MP analyses of the dataset resulted in 144 equally parsimonious trees with 1283 steps (CI = 0.5271, RI = 0.7955). ML analyses produced a tree with a log-likelihood (–ln) of 6962.457750. In the Bayesian analyses, triple repeated analyses converged on a stationary phase and the three 50% majority rule consensus trees were topologically identical. There is no significant difference in the tree topology produced by MP, ML and Bayesian analyses. The Bayesian consensus tree is provided here with BP values of MP and ML analyses, and PP values from one of the Bayesian analyses (Fig. 4).

**ITS.** The ITS sequence data matrix included 820 characters after the exclusion of ambiguous sites at both ends. Of the characters, 294 were parsimony informative. The sequence alignment was deposited in TreeBase (http://www.treebase.org) under the accession number S12637. MP analyses of the dataset resulted in 144 equally parsimonious trees with 1283 steps (CI = 0.5713, RI = 0.7955). ML analyses produced a tree with a log-likelihood (–ln) of 83432.05. Bayesian analyses (MP-BP = 100%, ML-BP = 92%, PP = 1.00; Fig. 4). This new clade comprised two subclades designated as subclade I (MP-BP = 100%, ML-BP = 100%, PP = 1.00; Fig. 4). This new clade included the ITS sequences from the type materials of *O. crinalis* (Ellis ex Lloyd) G.H. Sung et al., *C. kanzashiana*.
Fig. 3. Phylogenetic tree from Bayesian analyses of 5-gene (nrSSU, nrLSU, tef1, rpb1 and rpb2) showing the relationships within Hypocreales. Bootstrap proportions (equal to or above 70%) of MP analyses (MP-BP), ML analyses (ML-BP) and posterior probabilities (PP) (equal to or above 95%) are shown above internodes before backslash, after backslash and below internodes, respectively.
New clade in clavicipitaceous fungi and systematic position of *Paecilomyces sinensis*

**Fig. 4.** Phylogeny of the *Polycephalomyces* clade from Bayesian analyses using ITS sequences. Bootstrap proportions (equal to or above 70%) of MP analyses (MP-BP), ML analyses (ML-BP) and posterior probabilities (PP) (equal to or above 95%) are shown above internodes before backslash, after backslash and below internodes, respectively.

Kobayasi & Shimizu, *C. militaris* cf. var. *sphaerocephala* J.C. Schmidt, *C. ramosopulvinata* Kobayasi & Shimizu and *O. neovolkiana* (Kobayasi) G.H. Sung et al., apart from those un-named sequences, while subclade II consisted of sequences from *O. cuboidea* (Kobayasi & Shimizu) S. Ban, Sakane & Nakagiri, *O. ryogamiensis* (Kobayasi & Shimizu) G.H. Sung et al., *O. paracuboidea* S. Ban, Sakane & Nakagiri and *O. prolifica* (Kobayasi) S. Ban, Sakane & Nakagiri (Fig. 4).

**Taxonomy**

**New combination.** Although the fungus was described in *Paecilomyces*, *P. sinensis* shares many characters with *Polycephalomyces*. Species in *Paecilomyces* and *Polycephalomyces* have verticillately branched conidiophores while *Paecilomyces* includes a number of monophialidic species lacking conidiophores. The two genera have phialidic conidiogenous cells arranged in terminal whorls with cylindrical base and long neck in common,
but conidiogenous cells in Paecilomyces tapering often abruptly into a long distinct neck while those of Polycephalomyces can also be awl-shaped, acropleurogenous, with slightly ellipsoidal base and lacking flared collarette due to a long tapering neck. Conidia in Paecilomyces are one-celled (rarely two-celled), hyaline or slightly pigmented, smooth-walled or echinulate, of various shapes, in basipetal chains, compared with those small conidia, less than 5 μm long, with oblong, ellipsoidal, subglobose or globose shapes and produced singly or in chains in Polycephalomyces. Notably, thick-walled, single or in short chains, smooth-walled or ornate chlamydospores are sometimes produced in Paecilomyces, and there are also some ornate cells in Polycephalomyces, but all the species in the latter can produce yellow or yellow-orange, slimy, terminal, opaque conidial mass, which has not been recorded in Paecilomyces. Among these morphological characters (for Paecilomyces see Bainier, 1907; Onions & Barron, 1967; Samson, 1974; for Polycephalomyces see Seifert, 1985), Polycephalomyces may be distinguished from Paecilomyces by the phialides with the base tapering gradually into a long distinct neck and by the conidial mass.

Observations on the ex-type culture of Paecilomyces sinensis, CN 80-2, revealed that phialides were born directly on the vegetative hyphae, sometimes on simple conidiophores (Fig. 1a–c). Paecilomyces sinensis overlaps the species of Polycephalomyces in all the characters of the conidiogenous cells: acropleurogenous, phialidic, tapering gradually from the base to the tip, with a narrow neck, distinguishing the fungus from Polycephalomyces (Fig. 1a–c). The fungus was found to produce two types of conidia in culture (Fig. 1), very similar to that of Polycephalomyces formosus and P. ramosus (Peck) Mains (Bischoff et al., 2003). It can also be distinguished from other species of Paecilomyces by lacking chlamydospores, but producing yellow or yellow-orange, slimy, opaque conidial mass at the terminal portion of synnema as members of Polycephalomyces (Fig. 1d). In summary, Paecilomyces sinensis is morphologically more close to Polycephalomyces than to Paecilomyces.

The phylogenetic analyses of both 5-gene and ITS datasets conducted here assigned Paecilomyces sinensis along with Polycephalomyces formosus, the type species of the genus, and C. ramosopulvinata to a new Polycephalomyces clade among clavicipitaceous fungi. The molecular link between anamorphic species P. formosus and teleomorphic species C. ramosopulvinata was established by Chaverri et al. (2005) in their analyses of Clavicipitaceae s.l. It is clear that the genus Paecilomyces is not a suitable generic position for the fungus named P. sinensis. Its correct generic assignment should be in Polycephalomyces. Therefore, a new combination for the species is proposed below.

**Polycephalomyces sinensis** (Q.T. Chen, S.R. Xiao & Z.Y. Shi) W.J. Wang, X.L. Wang, Y. Li, S.R. Xiao & Y.J. Yao, comb. nov. (Figs 1–2)


Index Fungorum number: IF550007.

**Remarks.** Seifert (1985) accepted four species in Polycephalomyces, including *P. cylindrosporus* Samson & H.C. Evans, *P. formosus*, *P. ramosus* and *P. tomentosus* (Schrad.) Seifert, and treated *P. orbicularis* (Berk. & Broome) Ing as synonymous with *Stilbella hyssizeda* (Pers.) Seifert and *P. paludosus* Mains with *P. ramosus* although the name *P. paludosus* was still used for the anamorph of *O. paluda* Mains in Sung et al. (2007a). Polycephalomyces tomentosus was then transferred back to *Blistum* based on the 28S rDNA phylogenetic analyses and its myxomyceticolous habit (Bischoff et al., 2003). Polycephalomyces ramosus and *P. formosus* are considered as anamorphs of *Cordyceps* s.l. or hyperparasites of a variety of entomogenous fungi (Massee, 1895; Petch, 1924, 1933; Samson et al., 1984; Samson & Evans, 1985; Seifert, 1985). *Polycephalomyces cylindrosporus* is on a broad range of insect hosts (Samson et al., 1981). A new species, *P. ditmarii* Van Vooren & Audibert, was later described as the anamorph of *C. ditmarii* on *Hymenoptera* insects (Van Vooren & Audibert, 2005). In total, there are currently six species in Polycephalomyces, associated with insects or entomogenous fungi. They can be distinguished by the following key based on morphological characters.

**Key to species of Polycephalomyces**

1. Phialides in terminal whorls only .................. 2
2. Conidia acropleurogenous and terminal ......... 4
3. Conidia formed singly, obovoid, subellipsoid to subclavate ........................................ 3
4. Conidia formed in chains, obovoid to ellipsoid or oblong-ellipsoid ................................ 5
5. Phialides subulate, scattered on hyphal branches; conidia obovoid ........................................ 6
6. Phialides cylindrical, palisade-like arranged; conidia subellipsoid to subclavate ........... *P. ditmarii* 4
7. Conidia cylindrical .................. *P. cylindrosporus* 4
8. Conidia oval, obovoid to broadly obpyriform, ellipsoid to fusiform .......................... 5
9. Phialides on conidiophores, with a basal verticillate branch or in terminal whorls ........ *P. ramosus* 5
10. Phialides directly on aerial hyphae and peripheral hyphae around synnema, sometimes on simple conidiophore .................. *P. sinensis*
Discussion

The species *Polycephalomyces sinensis* (previously named *Paecilomyces sinensis*) was re-evaluated morphologically and phylogenetically based on the type materials. ITS sequences from both the dried specimen (HAMAS 43720) and the living strain (CN 80-2) were identical. Morphological observations on CN 80-2 showed the same characters as described by Chen et al. (1984), especially in synnemata, conidia and phialides, suggesting that the fungus shared conidial mass, two types of conidia and all the characters of the phialides with species in the genus *Polycephalomyces*. Molecular analyses of both 5-gene and ITS datasets also supported the placement of this species in *Polycephalomyces* rather than in *Paecilomyces*. As an important fungus in both pharmaceutical and systematic studies as shown above, taxonomic revision will cast the light on further investigations on bio-active compounds of this species and on phylogenetic relationships of clavicipitaceous fungi.

Previous phylogenetic analyses (Sung et al. 2007a) divided the family *Clavicipitaceae s.l.* into three monophyletic groups, i.e., *Clavicipitaceae s.s.*, *Ophiocordycipitaceae* and *Cordycipitaceae*. The present study revealed more molecular variations of clavicipitaceous fungi with an additional new clade, *Polycephalomyces* clade (Figs 3 and 4). The multi-gene analyses have proved to be effective in resolving phylogenetic classification of the clavicipitaceous fungi at the family level (Sung et al., 2007a, 2007b). A broader taxon sampling in this study discovered additional phylogenetic diversity, the *Polycephalomyces* clade, even though the additional taxa, *C. ramosopulvinata* and *Polycephalomyces formosus*, were not complete with all the 5-gene partitions. Compared with the 5-gene analyses, ITS analyses resolved the relationships within the *Polycephalomyces* clade by recognizing two well-supported subclades owing to a more intensive sampling in this clade and to higher sequence variations of the ITS region (Fig. 4).

Evidence of the connection between *Polycephalomyces* and *Cordycips* *s.l.* can be found in some previous studies. *Polycephalomyces formosus* and *P. ramosus* were grouped together with *C. prolific* *Kobayasi* (≡ *Ophiocordycips* *prolifica*) and *Cordycipitoides bisporus* Stifter (≡ *O. bispora* (Stifter) G.H. Sung et al.), forming a main clade in the phylogenetic analyses of 28S rDNA (Bischoff et al., 2003). Further, *P. formosus* and *Cordycips ramosopulvinata* were clustered together in a separate clade in a phylogenetic analysis using three loci (nrLSU, tef1 and rpb1) from 24 clavicipitaceous species (Chaverri et al., 2005). In the phylogenetic analyses of *Cordycips* *s.l.*, using SSU rDNA and ITS by Nikoh & Fukatsu (2000; as ‘the nuclear LSU rDNA’ in the paper), major clades similar to that of the three *Clavicipitaceae* *s.l.* clades in Sung et al. (2007a) can be found. Although, the long branch effect of *C. tricentri* Yasuda (≡ *O. tricentri* (Yasuda) G.H. Sung et al.) distorted the member inclusion of *Clavicipitaceae s.s.* and *Ophiocordycipitaceae*. In Nikoh & Fukatsu (2000), *C. kanzashiana* (AB027371) was clustered with *C. prolific* (≡ *O. prolific*, AB027370) and *C. ramosopulvinata* (AB027372) in a monophyletic group embedded in members of *Ophiocordycipitaceae* in Sung et al. (2007a). The same sequences from *C. kanzashiana*, *O. prolific* and *C. ramosopulvinata* were used in the ITS analyses of *Cordycips* s.l. by Stensrud et al. (2005) and formed a separate clade to the other clades compatible to those of Sung et al. (2007a), with the long branch of *O. tricentri* group emerging with members of *Clavicipitaceae s.s.* In an ITS analysis (Ban et al., 2009), 14 samples of *O. cuboidea*, *O. paracuboidea*, *O. prolific* and *O. ryo-gamiensis* aggregated into one cluster consisting of four small clades, a result confirmed here as shown in Fig. 4, subclade II. However, the *Polycephalomyces* clade revealed in this study was embedded within *Ophiocordycips* in the phylogenetic tree of LSU D1/D2 (Ban et al., 2009), which might have been caused by different taxon sampling and the insufficient resolution of LSU D1/D2 regions.

Species of *Cordycips* s.l. have been separated into three major clades in Sung et al. (2007a) with many assigned to genera in the classification. Five residual species in Sung et al. (2007a), *C. ramosopulvinata*, *C. albornithetica* Kobayasi & Shimizu (≡ *O. cuboidea*), *C. cuboidea* (≡ *O. cuboidea*), *C. kanzashiana* and *C. prolific* (≡ *O. prolific*), were grouped in the new *Polycephalomyces* clade from further study. Ban et al. (2009) suggested two new combinations in *Ophiocordycips*: *O. cuboidea* and *O. prolific*, with *C. albornithetica* reduced to a synonym of *O. cuboidea*. In addition, a new species, *O. paracuboidea*, was also proposed by Ban et al. (2009) for materials previously misidentified as *O. cuboidea*. Apparently, these species are molecularly different from *Ophiocordycips* based on the results of this study and will require taxonomic revision after further study.

In revising the classification of *Cordycips* *s.l.* and clavicipitaceous fungi (Sung et al., 2007a), most of the diagnostic characters used in the previous taxonomy of *Cordycips* *s.l.*, e.g. arrangement of perithecia, ascospore fragmentation, etc., were found phylogenetically uninformative based on the multi-gene analyses; whilst the texture, pigmentation and morphology of stromata were considered most consistent with the molecular phylogeny. Members of *Clavicipitaceae* have brightly coloured, fleshy stromata, whilst the majority of species in *Ophiocordycipitaceae* produce darkly pigmented, tough to pliant stromata, and the family *Clavicipitaceae* *s.s.* have darkly or brightly coloured, fleshy or tough stromata. However, exceptions and overlaps of these characters (rarely brightly coloured and rarely fleshy stromata) were described for *Ophiocordycipitaceae* (Sung et al., 2007a). From the description of families described in Sung et al.
(2007a), Cordycipitaceae can be distinguished by pallid or brightly pigmented, fleshy stromata with perithecia oriented at right angles to the surface of the stroma, while both Clavicipitaceae s.s. (darkly or brightly coloured, fleshy or tough stromata) and Ophiocordycipitaceae (darkly pigmented or rarely brightly coloured, tough, fibrous to plant, rarely fleshy stroma) are not easy to separate. Species in Polycephalomyces clade share characters with Clavicipitaceae s.s. and Ophiocordycipitaceae in darkly or brightly coloured and tough or fleshy stromata. More morphological characters may become available to separate Polycephalomyces clade from those two families if more materials are examined and analysed.

In the phylogenetic analyses of 28S rDNA conducted by Bischoff et al. (2003), P. ramosus and P. formosus were clustered together and formed a monophyletic clade with C. proliferum (≡ O. prolifica) and Cordycepioides bispuris (≡ O. bispora), in correspondence with the Polycephalomyces clade defined in the present study. However, the only ITS sequence found in the name search of Polycephalomyces in GenBank, AJ786598 (P. ramosus), was grouped in Ophiocordycipitaceae clade, rather than in Polycephalomyces clade in the ITS analyses (Fig. 4). Further examination showed that the sequence AJ786598 was identical to that of O. entomorrhiza (Dick.s.) G.H. Sung et al., probably referable to the anamorph of O. entomorrhiza, Hirsutella eleutherorum (Nees) Petch, rather than to a member of Polycephalomyces. Moreover, there are six ITS sequences referred to the name Paecilomyces sinensis in GenBank. Among them, AJ243771 was from a strain, HMIGB Zhw02, isolated from O. sinensis and identified as P. sinensis by Zhao et al. (1999) and also cited by Chen et al. (2001). The sequence was later used as the correct identification for re-determination of strains CBS 113409 and CBS 115145 with ITS sequences AY857543 and AY857544, respectively, by Prenafeta-Boldú et al. (2006). Three additional ITS sequences were submitted to GenBank without evaluation of any reports. Of all these six ITS sequences, unfortunately, only HQ918290 showed almost the same sequence as those from the type materials of P. sinensis, while the other five were distantly related to all the ITS sequences included in the present analyses. These five ITS sequences are not the true sequences of Paecilomyces sinensis (≡ Polycephalomyces sinensis) and require further determination.

The teleomorph–anamorph (sexual and asexual) connection between Berkelella and Polycephalomyces, listed in the Dictionary of the Fungi (Kirk et al., 2008) based on the linkage of P. tomentosus to the teleomorph Byssostrilbe stilbigera (Berk. & Broome) Petch (Seifert, 1985) and on nomenclature priority of Berkelella over Byssostrilbe (Rossman et al., 1999), cannot be retained, because the segregation of P. tomentosus from Polycephalomyces was supported by both the phylogenetic analyses of 28S rDNA sequences and the myxomyceticolous habit (Bischoff et al., 2003). As the genus Blistum is typified by P. tomentosus (≡ Blistum tomentosus (Schrad.) B. Sutton), the anamorph of Berkelella should be linked to Blistum. The linkage between Polycephalomyces and Cordyceps s.l. (Polycephalomyces clade in particular), was established by Bischoff et al. (2003) and supported by this study. In addition, P. orbicularis was treated as a synonym of Stilbella byssiseda (Seifert, 1985). Both Blistum tomentosus (≡ P. tomentosus) and Stilbella byssiseda (≡ P. orbicularis) were reported as myxomycete pathogens (Seifert, 1985), and the latter was regarded as the only species in Stilbella Lindau, which is associated with myxomycetes and did not produce verrucose ornamented cells (Bischoff et al., 2003). Further investigation is desirable to determine if both Blistum and Stilbella have the same telemorph, Berkelella. Although Berkelella was not sampled by Sung et al. (2007a) and left as Clavicipitaceae incertae sedis in their new classification, it was accepted as a genus of Clavicipitaceae (Kirk et al., 2008). Apparently, species of Polycephalomyces, either linked to Berkelella or Cordyceps s.l., are among clavicipitaceous fungi, belonging to Hypocreales, Hypocreomycetidae, Sordariomycetes (Kirk et al., 2008). The teleomorph–anamorph connections between Byssochlamys Westling and Paecilomyces was made by Stolk & Samson (1971) and supported by recent studies (Houbraken et al., 2006; Samson et al., 2009). The teleomorph genus Byssochlamys belongs to Trichocomaceae, Eurotiomycetes, Eurotiales (Kirk et al., 2008) in a different class of Ascomycota.

Both the 5′-gene (Fig. 3) and ITS (Fig. 4) analyses revealed that O. sinensis was clustered in Ophiocordycipitaceae, consistent with the results of Sung et al. (2007a). As Polycephalomyces sinensis (≡ Paecilomyces sinensis) belonged to the new Polycephalomyces clade, it is distantly related to O. sinensis. Considering that some Polycephalomyces species were reported as possible hyperparasites of entomogenous fungi, further investigation is needed to determine whether P. sinensis is parasitic on O. sinensis or not.

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