Accepted Manuscript

The 'species complex' issue in clinically relevant fungi: A case study in *Scedosporium apiospermum*

Min Chen, Dr. Jingsi Zeng, G. Sybren De Hoog, Benjamin Stielow, A.H.G. Gerrits Van Den Ende, Wanqing Liao, Mag. Dr. rer. nat. Michaela Lackner

PII: S1878-6146(15)00169-5

DOI: 10.1016/j.funbio.2015.09.003

Reference: FUNBIO 632

To appear in: *Fungal Biology*

Received Date: 21 July 2015

Revised Date: 3 September 2015

Accepted Date: 7 September 2015

Please cite this article as: Chen, M., Zeng, J., De Hoog, G.S., Stielow, B., Gerrits Van Den Ende, A.H.G., Liao, W., Lackner, M., The 'species complex' issue in clinically relevant fungi: A case study in *Scedosporium apiospermum*, *Fungal Biology* (2015), doi: 10.1016/j.funbio.2015.09.003.

3-September-2015 *Fungal Biology*

The 'species complex' issue in clinically relevant fungi: a case study in *Scedosporium apiospermum*

Min CHEN[a,b,c], *Jingsi ZENG*[d,*], *G. Sybren DE HOOG* [a,b,c,e], *Benjamin STIELOW*[b], *A. H. G. GERRITS VAN DEN ENDE*[b], *Wanqing LIAO*[a], *Michaela LACKNER*[f,*]

[a]*Shanghai Institute of Medical Mycology, Shanghai Key Laboratory of Molecular Medical Mycology, Department of Dermatology, Shanghai Changzheng Hospital, Shanghai, China*

[b]*CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands*

[c]*Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands*

[d]*Department of Dermatology and Venereology, Union Hospital, Tongji Medical College, Huazhong Science and Technology University, Wuhan, China*

[e]*Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510630, China; Department of Dermatology, Peking University First Hospital, Research Center for Medical Mycology, Beijing, 100034, China; Biological Sciences Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia; Department of Basic Biology, University of Paraná, Curitiba, Brazil*

[f]*Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria*

*Corresponding authors:

Mag. Dr. rer. nat. Michaela Lackner, Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Schoepfstrase 41, 6020 Innsbruck, Austria. Phone: +43 512 9003 70725; Fax: +43 512 9003 73700; E-mail: Michaela.Lackner@i-med.ac.at.

Dr. Jingsi Zeng, Department of Dermatology and Venereology, Union Hospital, Tongji Medical College, Huazhong Science and Technology University, Jiefang Dadao 1277, 430022 Wuhan, China. Phone: +0086-27-85726195; Fax: +0086 27 85776343; E-mail: wuhanzjs@aliyun.com.

Abstract

The genus *Scedosporium* currently comprises six species, *S. apiospermum*, *S. boydii*, *Pseudallescheria angusta*, *S. minutisporum*, *S. dehoogii*, and *S. aurantiacum*, most of which can be distinguished with the primary fungal DNA barcode, the ITS1/2 region of the rDNA gene cluster. In the present study, four additional genetic loci were explored from a phylogenetic point of view enabling a barcoding approach based on K2P pairwise distances to resolve the taxa within the genus *Scedosporium*. We included partial γ-actin (*ACT*), β-tubulin (*BT2*), elongation factor 1α (*TEF1*) and the small ribosomal protein 60S L10 (L1) (*RP60S*) genetic loci. Phylogenetic inference of each marker individually showed that four out of six species in the genus *Scedosporium* can be distinguished unambiguously, while strains of *S. apiospermum*, *S. boydii*, and *P. angusta* showed occasional recombination, and accordingly, no genealogical concordance between markers was obtainable. We defined *S. apiospermum*, *S. boydii* and *P. angusta* as the '*S. apiospermum* species complex' since observed differences were not consistent between lineages, and no clinical differences are known between entities within the complex. While *BT2* revealed the best performance among the genetic loci tested at the lineage level, barcoding of the ITS region is sufficient for distinction of all entities in *Scedosporium* at the species or 'complex' level.

Keywords: *Scedosporium*; species complex; populations; primary barcode; ITS rDNA; secondary barcode

Introduction

The term ‘species complex’ is suggestive to cover groups of organisms or lineages that are taxonomically closely related or even difficult to distinguish. However, no clear definition of the category ‘species complex’ exists so far and some clarity in the use of the term is urgently needed. The best-known use of ‘species complex’ for the kingdom Fungi is in the genus *Fusarium*, where ‘species complexes’ were introduced (O’Donnell et al. 2012) as an alternative to the subgeneric ‘sections’ as currently in use in genera like *Aspergillus* (Geiser et al. 2007) or *Trichoderma* (Bisset 1991; Druzhinina & Kubicek 2005); in *Fusarium* the older, phenotypic sections did not match with phylogeny. The current species complexes in *Fusarium* are monophyletic, together encompass all species known in the respective genera, and hence such species complexes can be viewed as taxonomic categories. However, in other cases a ‘species complex’ just describes a selected group of entities that are difficult to distinguish from each other and/or classification of such groups is yet unclear. For example, some genetically diverse strains with unclear taxonomic status were listed as ‘*Aspergillus viridinutans* species complex’ (Hong et al. 2005). Bensch et al. (2012) grouped series of closely related molecular siblings in *Cladosporium* as ‘species complexes’ under the name of their original phenotype name such as ‘*C. herbarum* complex’ or ‘*C. cladosporioides* complex’; only few of the siblings within these complexes revealed deviant ecological characteristics.

In addition to taxonomic criteria, species complexes have also been defined for divergent practical reasons, one of which may be clinical or industrial significance. Howard et al. (2011) suggested to list all well-described clinical species of the *Aspergillus* section *Nigri* as ‘*Aspergillus niger* complex’ due to absence of differences in antifungal susceptibility profiles. Some authors even united groups of unrelated fungi (Reedy et al. 2009) that were as yet unclassified (Manamgoda et al. 2012).

A further reason to aggregate species as a ‘species complex’ is unsettled taxonomy. For example, *Cryptococcus neoformans*, a potentially fatal pathogenic yeast, was initially divided into two varieties, var. *neoformans* and var. *gattii*. Katsu et al. (2004) united separate lineages within *C. neoformans* as the ‘*C. neoformans* complex’ using the primary barcoding ITS locus of rDNA. Subsequently, Kwon-Chung et al. (2006) brought the var. *gattii* to species level due to the significant divergence of ecological, biochemical, and molecular characteristics. After a long debate, these molecular siblings recently have been proposed as seven separate species in the ‘*C. neoformans* complex’ (Hagen et al. 2015). This is an example of a species aggregate with entities that are closely related but appear to differ in some clinically relevant parameters. This was also the case in the ‘*Candida parapsilosis* complex’, where the original species proved to have higher antifungal susceptibility than more recent molecular siblings (Treviño-Rangel et al. 2012).

Scedosporium (being preferred over its sexual state name, *Pseudallescheria*; Lackner et al. 2014b) is a genus of ubiquitous ascomycetous fungi causing a wide array of human infections. Among the

genus *Scedosporium*, *S. apiospermum* and *S. boydii* are clinically relevant, being the second most common clinical molds in cystic fibrosis, after *Aspergillus fumigatus*. Currently, an increasing incidence of infections caused by these species has been noticed, presently mainly in immunocompromised hosts (Tammer et al. 2011). Two prevalent species are currently recognized, *S. apiospermum* and *S. boydii*, for which as yet not unambiguous diagnostic parameters are available and which are often taken together as a 'complex'.

Thus, the term 'species complex' may (a) stand for a fixed taxonomic category below the genus level, (b) indicate some closely related strains with uncertain taxonomic status, or it may (c) stand for divergent species that for practical reasons are not precisely identifiable. The aim of the present study is to provide clarity and consistency for the term 'species complex' in medical mycology. Cases (a) and (c) are conceptually clear, just differing in their practical bias: taxonomically valid groups, which are either identified or are not distinguished. Here we focused on the most problematic situation (b), where data as yet obtained is insufficient to describe entities within the 'species complex' properly, and use *Scedosporium* as an example.

Materials and methods

Strains

Members of the genus *Scedosporium* were studied by the analysis of five gene fragments and compared with previously published AFLP profiles (Lackner et al. 2014a); the same set of strains was used in all partitions. The 10 populations distinguished by AFLP were used as reference, in accordance with Lackner et al. (2014a), including *P. minutispora*, *S. dehoogii*, and *S. aurantiacum*. Thus, a total of 65 strains were analyzed, including 19 strains of *S. apiospermum*, 23 strains of *S. boydii*, 9 strains of *S. dehoogii*, 7 strains of *P. angusta*, 3 strains of *S. minutisporum* and 3 strains of *S. aurantiacum*. A single isolate of *Pseudallescheria desertorum* (CBS 489.72) was used as outgroup. All of them were obtained from the reference collection of the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (CBS-KNAW), Utrecht, the Netherlands. All available type strains were included. Stock cultures were maintained on slants of 2 % malt extract agar (MEA) at 24 ℃. Meta data on origin and sources of isolation are listed in Supplementary Table 1.

DNA extraction

DNA was extracted following the CTAB protocol that was described previously (Lackner et al. 2014a). Quality of genomic DNA was verified by running 2 µL DNA sample in a 1.0 % agarose gel. DNA sample was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher, Wilmington, DE, U.S.A.), and was stored at –20 ℃ until further use.

DNA amplification and sequencing

Five gene regions were amplified for inclusion in the multi-locus sequence data analysis, i.e. partial the γ-actin (*ACT*) gene, β-tubulin (*BT2*), elongation factor 1α (*TEF1*), the small ribosomal protein 60S L10 (L1) (RP60S L10) and the ITS region. DNA from each isolate was amplified by PCR in 12.5 µL reaction volumes using the primers and protocols described in Table 1 (Stielow et al. 2015). PCR reactions (12.5 µL final vol) were performed in a mixture containing 1.25 µL 10× PCR buffer, 7.0 µL ddH_2O, 1.25 µL dNTP mix (1.0 mM), 0.25 µL of each primer (10 pmol), 0.5 µL Taq polymerase (0.4 U/µL), 1.0 µL DMSO (Sigma), 0.5 µL $MgCl_2$ (50 mM), and 0.5 µL template DNA (100 ng/µL). The ABI Prism BigDye Terminator v. 3.1 (Thermo Fisher) was applied in a quarter its suggested reaction volume. Reaction products were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was performed on an ABI 3770XL capillary sequencer (Thermo Fisher). Bidirectional reads were edited and adjusted by Lasegene Seqman (DNASTAR, Madison, WI, U.S.A.). The length and guanine-cytosine content (G+C %) of each gene were analyzed by BIOEDIT v. 7.0.5.2 (Hall 1999) and MEGA6.1 (Tamura et al. 2011).

Phylogenetic analyses

The sequences of ITS and the *ACT*, *BT2*, *TEF1* and *RP60S* genetic loci were aligned using the server version of the MAFFT v. 7.0 (www.ebi.ac.uk/Tools/msa/mafft/), followed by manually adjustment of 5' and 3' primed ends in BIOEDIT v. 7.0.5.2. Gene sequences of *Pseudallescheria desertorum* CBS 489.72 were used to root the tree. The best-fit model of sequence evolution was determined by MODELTEST v. 2.3 (Nylander 2004). All sequences determined in this study were deposited in GenBank and the accession numbers were listed in Supplementary Table 1. After verifying the best models, phylogenetic trees were inferred using maximum likelihood with 1000 rounds of re-sampling in MEGA6.1, and bootstrap branch support was regarded conclusive when exceeding 80 %. Topological congruency was performed using MRBAYES v. 3.1.2. on the CIPRES portal (http://www.phylo.org/). Two parallel runs of four chains were run for 10,000,000 generations and trees were sampled every 1,000 generations. TRACER version 1.5 was used to verify that the mean likelihood value, effective sample size (ESS) and other parameters reached a plateau. For each run, 10 % of the trees were discarded as they were obtained during the burnin phase. Trees were viewed and edited with FIGTREE v. 1.1.2 and MEGA 6.1 software.

Inter-species distances, intra-species heterogeneity and barcoding gaps

Pairwise distances between species were calculated using estimation of evolutionary divergence over sequence pairs between species. Each genetic locus dataset analysis was conducted in MEGA6.1 using the best model of sequence evolution (Table 1). Regarding inter-species distance calculation, the evolutionary distances were derived from numbers of base substitutions per site. The average

barcoding gaps between species also were calculated by MEGA6.1. As for the intra-species distances (heterogeneity), each gene dataset was calculated by estimation of average evolutionary divergence over sequence pairs within species and conducted in MEGA 6.1 using the best model. Average distances resulted from number of base substitutions per site. Average intra-species heterogeneity of each dataset was also calculated by MEGA 6.1. The barcoding gap is defined as 'the lowest inter-species distance' minus 'the highest intra-species heterogeneity'.

Sliding window analysis

Sliding windows for each genetic locus were inferred via the SPIDER (Species identity and evolution in R; http://spider.r-forge.r-project.org/) package in R statistical software, employing the pairwise distance (K2P as default) functions 'slideAnalyses' and 'slideBoxplots' as implemented in the package; with 'library' dependencies 'ape', 'pegas', 'adegenet' and 'ade4'. The sliding window 'walk' was pre defined with 100 bp over all five markers to retrieve per window, per marker informativeness representation (Brown 2011). Function 'slide analyses' infers a comprehensive set of graphical overviews to visualize the 'barcode' quality of a given marker, e.g. topological tree consistency, mean K2P distances, zero-cell K2P distances and species monophyly per window. We extracted the plot 'species monophyly' and presentation of intra- *vs.* inter-species distances (= the barcoding gap; via function 'slide boxplots') per 100 bp window for each marker to determine/visualize the overall 'barcode' quality for each gene.

Results

Profiles of each gene dataset

Using primers and PCR conditions according to Stielow et al. (2015), a vast majority of the five genetic loci of all investigated strains could be amplified and sequenced, ranging from 98.0 % in *TEF1* and *BT2* to 100 % in ITS, *ACT* and *RP60S* (Table 2). Multiple copies were detected in *S. apiospermum* using *TEF1* and in *P. angusta* using *BT2*. Among *S. apiospermum* and related species, the lengths of the gene regions showed a significant variability, ranging from 328 bp (*ACT*) to 565 bp (*TEF1*). G+C% of the gene regions varied significantly, ranging from approximately 50 % (*BT2*) to approximately 58 % (*RP60S*). *ACT* showed the highest sequence variation (53/328, variable sites / gene length), followed by *RP60S* (41/393), *BT2* (20/425), *TEF1* (12/553) and ITS (9/512). Profiles of these genetic loci are shown in detail in Table 1.

Inter-species distances

The ITS region, and *BT2* and *RP60S* genes showed higher inter-specific values among the *S. apiospermum* species complex and related species than *ACT* and *TEF1*. For the protein coding loci, *BT2* had the highest inter-species distances (0.065 to 0.118), followed by *RP60S* (0.044 to 0.075), *ACT* (0.021 to 0.060) and *TEF1* (0.017 to 0.044). Inter-species distances were significantly lower among *S. apiospermum*, *S. boydii* and *P. angusta* within the *S. apiospermum* species complex when sister taxa were compared to members of the species complex. The *BT2*, *RP60S* and *ACT* genetic loci had higher inter-species distances in the *S. apiospermum* species complex than ITS and *TEF1*, which is concordant with their numbers of variable sites. In the *S. apiospermum* species complex, the genetic loci *BT2* (0.022–0.040), *RP60S* (0.025–0.037) and *ACT* (0.037–0.056) showed a higher inter-specific value than *TEF1* (0.006–0.007) and ITS (0.003–0.010). Details are shown in Table 1.

Intra-species heterogeneity and barcoding gaps

Intra-species heterogeneities and barcoding gaps were calculated in MEGA6.1 and are shown in Table 1. In the *S. apiospermum* species complex, ITS showed the highest average intra-species heterogeneity (0.034), while *ACT* had the lowest average value of 0.005. The highest intra-species variability was found in ITS (0.034) in *S. dehoogii*, and the lowest intra-species variability was also found in ITS (0.000) in *S. minutispora*.

All datasets were calculated for barcoding gaps among the members of the *S. apiospermum* species complex and related species in the present study. The highest barcoding gap was with ITS (0.042), followed by *BT2* (0.038), *RP60S* (0.019), *ACT* (0.009) and *TEF1* (0.008).

Sliding window analysis

Five datasets were statistical analyzed using the R-package SPIDER as shown in (Fig 3A, B). No absolute separation between inter- and intraspecific distances could be retrieved for any of the five gene markers based on K2P pairwise distances. However, high proportions of monophyletic species even in the absence of perfect distance separation were retrieved for a number of genetic loci when entities were merged prior analysis into the '*S. apiospermum* complex'. If not merged, all taxa became indistinguishable based on a standard K2P distance matrix (data is available upon request). The *BT2* dataset revealed the optimal 'barcode' characteristics among all investigated genetic loci, as some very short sections (between ~1–40 and 135–185 bp) indicated perfect inter- and intra-species distance separation, but not over the whole length of the gene. *BT2* also ranked first with respect to rendering taxa as monophyletic, even in the presence of high average intraspecific heterogeneity (see evaluation above). *ACT* indicated a similar potential as *BT2*, since intra- and interspecific distances were separated in one section (~140–200 bp), but not as clearly as for *BT2*; proportions for inferring taxa as monophyletic ranged between 0.60 and 0.75. *TEF1* indicated some local optima equal to *BT2* in ITS region performance to separate within and between species distances (between ~50–55 and 150–230

bp), but also the opposite, particularly for the 3' primed end of the sequence. The *RP60S L10* (L1) 5' primed end (~1–100 bp) had perfectly separated distances but this, interestingly, did not coincide with a high proportion of taxonomic entities being inferred as monophyletic. In the ribosomal cluster, ITS showed relatively poor performance with respect to resolving all entities as monophyletic, but ability to clearly separate inter- and intra-specific distances was observed, particularly in ITS. Barcoding performance of genetic loci, ranked from optimal to poor, was: *BT2* > *ACT* > *TEF1* > ITS = *RP60S*. All five genetic locus datasets had the ability to infer the investigated taxa as monophyletic entities with window proportions ranging from 0.00 to 0.80, for each analyzed compartment as mentioned above. Ranking for the largest proportions of monophyletic taxa is equal to barcoding performance.

Phylogenetic analyses

Single-locus analyses were performed for ITS, *BT2*, *ACT*, *RP60S* and *TEF1* in order to investigate the phylogenetic relationships among members of the *S. apiospermum* species complex and related species. Similar to the selection of Lackner et al. (2014a), *P. desertorum* was defined as out-group species. The *S. apiospermum* species complex could be unambiguously segregated from all related species such as *S. dehoogii* based on Bayesian and maximum likelihood inference using separate *BT2*, *ACT*, *RP60S* and the concatenated 5 genetic loci sequences (Fig 1; Supplementary Fig 3). Conversely, isolates of the *S. apiospermum* species complex were more distantly split in phylogenetic trees based on ITS and *TEF1* (Supplementary Fig 1). No major conflicts were detected among the single genetic locus phylogenies, thus confirming lineage assignment of all *Scedosporium* species and the *S. apiospermum* species complex.

Midpoint-rooted phylogenies were explored to analyze the diversity within the *S. apiospermum* species complex. The supported clades in ITS comprise type or authentic strains of *S. apiospermum*, *S. boydii* and *P. angusta*, respectively (Supplementary Fig 2). Strains listed as *P. ellipsoidea*, which were listed as such because of affiliation to group AFLP1, formed clusters with bootstrap support below 80 % for most of the genetic loci and were therefore not differentiated from the *S. boydii* clusters. In the *RP60S* top-ranked tree (Fig 2), *S. apiospermum* and *P. angusta* clades were subdivided into 4 and 2 clusters, respectively. In the *ACT* top-ranked tree, *S. boydii* had 4 clusters, and *S. apiospermum* and *P. angusta* had 2 clusters and 1 cluster, respectively. In the *BT2* top-ranked tree, *S. boydii* and *S. apiospermum* contained 4 and 2 clusters, respectively; *P. angusta* contained 2 clusters. Thus the *S. boydii* clusters had the largest degree of heterogeneity, followed by *S. apiospermum* and *P. angusta*.

Additionally, taking the three main clades *S. apiospermum*, *S. boydii* and *P. angusta* as reference, group members were mostly identical, except for five strains (CBS 115829, CBS 987.73, CBS 101719, CBS 117432 and CBS 117436) which were interchanged between *S. apiospermum* and *S. boydii* (Table 3) and thus could be regarded as *in silico* recombinants.

Discussion

The indication 'species complex' is frequently used in medical mycology, with rather diverse connotations. In the present paper, 'species complexes' are defined as aggregated taxonomic entities (cryptic siblings), i.e. species that cannot confidently be distinguished by standard diagnostic tools, forming a clear monophyletic group. In our example dataset the standard barcoding genetic locus ITS functions well for most species, except for *S. apiospermum*, *S. boydii* and *P. angusta*. Thus, secondary barcodes other than those investigated would be necessary for routine diagnostic application. Species complexes under a stringent barcoding concept may be attributed to the following: (1) Groups of individuals which appear as a single monophyletic clade when the primary barcoding gene is applied, and where application of a secondary barcode is judged irrelevant for practical reasons depending on the area of interest; (2) Groups of individuals with unconfirmed species delimitation, even when secondary barcodes are applied. As outlined by Al-Hatmi et al. (2015), species complexes of type (1) are found in *Fusarium*, where classical phenotypic species have been split up into smaller molecular entities. There is no need to illustrate 'complexes' e.g. in *Sporothrix*, where species can be distinguished phenotypically and clearly differ in clinical behavior, i.e. virulence, antifungal susceptibility and distribution (Zhang et al. 2015). For these reasons it is less appropriate to amalgamate *Sporothrix* species in a 'complex', as it is frequently done (e.g. Tellez et al. 2014).

Complexes of type (1) and (2) thus refer to clusters of species were distinction is either (1) judged irrelevant, or (2) are as yet impossible. Hong et al. (2005) listed an '*Aspergillus viridinutans* complex' for a variable cluster of unnamed strains. *S. apiospermum* is another example of the second type (2) of species complexes, i.e. a monophyletic group showing diversity with undefined species delimitation. The diversity of *S. apiospermum* and all its relatives known to date, are affiliated to the same clade defining the genus *Scedosporium*, here investigated with five genetic markers, namely ITS, *ACT*, *BT2*, *TEF1*, and *RP60S*, and data were compared with previously published AFLP patterns of the same strains (Lackner et al. 2014a). In our study, we found a barcoding gap in ITS region between the '*S. apiospermum* complex' and remaining species. A recent barcoding paper (Irinyi et al. 2015) reported the absence of barcoding gap in ITS within *Scedosporium* species that we here treat as 'complex'. The barcoding gap of Irinyi and colleagues was calculated using *S. apiospermum*, *S. boydii*, *S. aurantiacum*, *S. dehoogii*, and more remote species. In contrast, we compared *S. apiospermum*, *S. boydii*, and *P. angusta* on the one hand with remaining species on the other. Although the highest barcoding gap was found in ITS (0.042), a continuous region (approximately ranging from 130 to 160 bp) in *BT2* (barcoding gap = 0.038) showed a high proportion (> 0.8) of species that are monophyletic (Fig 3) and this area also showed a distinct barcoding gap between the closest species. Thus, we think *BT2* is the best barcoding gene among the genetic loci tested in the present study, the region 130–160 being sufficient for unambiguous distinction as a barcode identifier (Heinrichs et al. 2012). All entities in *Scedosporium* (at the 'complex' level) can be distinguished by

other the remaining four genetic markers, and including primary barcoding ITS. In addition, a high phylogenetic resolution between entities was achieved using *BT2*, *P60S* and *ACT*, thus partially coinciding with results from a puristic K2P distance matrix. *Scedosporium dehoogii*, *S. minutisporum* and *S. aurantiacum* are phenotypically distinguishable and differ in clinical relevance (Kaltseis et al.2009) and antifungal susceptibility (Lackner et al. 2014a) and therefore it is not useful to include these in a complex; these are simply closely related species. The complex under consideration comprises *Scedosporium* (*Pseudallescheria*) *boydii* and *S. apiospermum* with the inclusion of *P. ellipsoidea* and *P. fusoidea* as synonyms, and with *P. angusta* as a doubtful intermediate taxon (Gilgado et al. 2005). A debate concerning the distinction of these species has been ongoing ever since (Lackner et al. 2014b).

The barcode ITS, as well as *TEF1* just allow approximate distinction of *S. apiospermum* and *S. boydii*, with in our dataset 5 strains deviating, i.e. with a predictive power of about 78 %. Higher degrees of resolution with 7–12 well-supported clades are found with secondary barcodes *ACT*, *RP60S* and *BT2*. The type strain of *P. ellipsoidea* was member of a cluster which was not inferred different from *S. boydii* and thus should not be recognized as such; *P. fusoidea* was earlier proven to be a synonym of *S. boydii* (Lackner et al. 2014a). In contrast, *P. angusta* comprised a cluster with high bootstrap branch support that was recognized in all loci, eventually being composed of two supported sub-clusters (Fig. 2) and taking an intermediate position between *S. apiospermum* and *S. boydii* (Lackner et al. 2014a).

Although differences in re-sampling support were found between clusters united as *S. apiospermum* and *S. boydii*, five putative *in silico* recombinants were detected. Whether these recombinants form fertile offspring or are hybrids remains to be verified with *in vitro* crossing experiments. Gilgado et al. (2005) noted a difference between homothallism (observed in *S. boydii*) and heterothallism (observed in *S. apiospermum*), but given the limited number of strains tested it is likely that these sexual traits were not following clear species limits; the molecular mechanism behind this has as yet not been revealed. No recombination event was observed with any of the clusters in *P. angusta*, but this may be due to the limited number of strains available. We conclude that the three species clusters (*S. apiospermum*, *P. angusta*, and *S. boydii*) are genetically different, but given the occurrence of recombination in on average 13.5 % of the strains of *S. apiospermum* and *S. boydii* analyzed, these taxa are probably better recognized at the level of populations rather than at the species level. Increased efforts in genome sequencing will likely shed more light on these lineages.

All three entities could be further subdivided into sub-populations, with the help of secondary barcoding markers used here. Higher diversity was observed in *S. boydii* than in *S. apiospermum* and *P. angusta*; this was particularly the case for *BT2*. When antifungal susceptibility patterns were plotted on any of the clusters, no significant difference was found in frequency of azole-, echinocandin- or polyene-resistance (Lackner et al. 2014a). Also no difference was found in severity or location of infection linked to any of the clusters. Discrimination of *S. apiospermum*, *P. angusta*, and *S. boydii*

does not imply a changed therapeutically management or information on the severity of infection or the potential to disseminate, the delimitation of current species within the *S. apiospermum* species complex does not play a significant role in medical mycology and routine laboratory diagnostics.

In conclusion, the term 'species complex' should be primarily used to indicate some closely related strains with uncertain taxonomic or species status in medical mycology. We may state that for reasons of absence of genetic separation, as well as absence of clinical relevance of individual lineages, the species *S. apiospermum*, *P. angusta*, and *S. boydii* should be referred to as the '*Scedosporium apiospermum* species complex'. Their distinction in clinical practice is redundant, and use of ITS region is sufficient. Highest resolution is achieved with *BT2*, where a small region of 30 bp is sufficient for distinction of all relevant entities and is particularly suited for probe development.

Acknowledgments

This study was funded in part with the grants from National Natural Science Foundation of China (No. 81201269), the 973 Program (2013CB531601 and 2013CB531606), Severe Infectious Diseases from National Health Department (2013ZX10004612) and Shanghai Science and Technology counsel projects of 14DZ2272900.

References

Al-Hatmi AMS, Gerrits VDE, Stielow AHG, Diepeningen BV, Seifert AD, Mccormick KA, Assabgui W, Gräfenhan RT, Sybren De Hoog, André Levesque GC, 2015. Evaluation of two novel barcodes for species recognition of opportunistic pathogens in *Fusarium*. *Fungal Biology*. (Accepted).

Bensch K, Braun U, Groenewald JZ, Crous PW, 2012. The genus *Cladosporium*. *Studies in Mycology* **72:** 1–401.

Bissett J, 1991. A revision of the genus *Trichoderma*. III. Section *Pachybasium*. *Canadian Journal of Botany* **69:** 2373–2417.

Brown SDJ. Spider: Species Identity and Evolution in R. A Tutorial. http://spider.r-forge.r-project.org/tutorial/tutorial.pdf.

Druzhinina IS, Kopchinskiy AG, Komoń M, Bissett J, Szakacs G, Kubicek CP, 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genetics and Biology* **42:** 813–828.

Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA, 2007. The current status of species recognition and identification in *Aspergillus*. *Studies in Mycology* **59:** 1–10.

Gilgado F, Cano J, Gené J, Guarro J, 2005. Molecular phylogeny of the *Pseudallescheria boydii* species complex: proposal of two new species. *Journal of Clinical Microbiology* **43:** 4930–4942.

Hagen F, Khayhan K, Theelen B, Kolecka A, Polacheck I, Sionov E, Falk R, Parnmen S, Lumbsch HT, Boekhout T, 2015. Recognition of seven species in the *Cryptococcus gattii*/*Cryptococcus neoformans* species complex. *Fungal Genetics and Biology* **78:** 16–48.

Hall TA, 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

Heinrichs G, de Hoog GS, Haase G, 2012. Barcode identifiers as a practical tool for reliable species assignment of medically important black yeast species. *Journal of Clinical Microbiology* **50:** 3023-3030.

Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA, 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* **97:** 1316–1329.

Howard SJ, Harrison E, Bowyer P, Varga J, Denning DW, 2011. Cryptic species and azole resistance in the *Aspergillus niger* complex. *Antimicrobial Agents and Chemotherapy* **55:** 4802–4809.

Irinyi L, Serena C, Garcia-Hermoso D, Arabatzis M, Desnos-Ollivier M, Vu D, Cardinali G, Arthur I, Normand AC, Giraldo A, da Cunha KC, Sandoval-Denis M, Hendrickx M, Nishikaku AS, de Azevedo Melo AS, Merseguel KB, Khan A, Parente Rocha JA, Sampaio P, da Silva Briones MR, e Ferreira RC, de Medeiros Muniz M, Castañón-Olivares LR, Estrada-Barcenas D, Cassagne C, Mary C, Duan SY, Kong F, Sun AY, Zeng X, Zhao Z, Gantois N, Botterel F, Robbertse B, Schoch C, Gams W, Ellis D, Halliday C, Chen S, Sorrell TC, Piarroux R, Colombo AL, Pais C, de Hoog S, Zancopé-Oliveira RM, Taylor ML, Toriello C, de Almeida Soares CM, Delhaes L, Stubbe D, Dromer F, Ranque S, Guarro J, Cano-Lira JF, Robert V, Velegraki A, Meyer W, 2015. International Society of Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database--the quality controlled standard tool for routine identification of human and animal pathogenic fungi. *Medical Mycology* **53:** 313-337.

Kaltseis J, Rainer J, De Hoog GS, 2009. Ecology of *Pseudallescheria* and *Scedosporium* species in human-dominated and natural environments and their distribution in clinical samples. *Medical Mycology* **47:** 398-405.

Katsu M, Kidd S, Ando A, Moretti-Branchini ML, Mikami Y, Nishimura K, Meyer W, 2004. The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. *FEMS Yeast Research* **4:** 377–388.

Kwon-Chung KJ, Varma A, 2006. Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS Yeast Research* **6:** 574-587.

Lackner M, Hagen F, Meis JF, Gerrits van den Ende AH, Vu D, Robert V, Fritz J, Moussa TA, de Hoog GS, 2014a. Susceptibility and diversity in the therapy-refractory genus *scedosporium*. *Antimicrobial Agents and Chemotherapy* **58**: 5877–5885.

Lackner M, de Hoog GS, Yang LY, Moreno LF, Ahmed SA, Fritz A, Kaltseis J, Nagl M, Lass-Flörl C, Risslegger B, Rambach G, Speth C, Robert V, Buzina W, Chen S, Bouchara JP, Cano-Lira JF, Guarro J, Gené J, Silva FF, Haido R, Haase G, Havlicek V, Garcia-Hermoso D, & Jacques F. Meis & Hagen F, Kirchmair M, Rainer J, Schwabenbauer K, Zoderer M, Meyer W, Gilgado F, Schwabenbauer K, Vicente VA, Piecková E, Regenermel M, Rath PM, Steinmann J, de Alencar XW,

Symoens F, Tintelnot K, Ulfig K, Velegraki A, Tortorano AM, Giraud S, Mina S, Rigler-Hohenwarter K, Hernando FL, Ramirez-Garcia A, Pellon A, Kaur J, Bergter EB, de Meirelles JV, da Silva ID, Delhaes L, Alastruey-Izquerdo A, Li RY, Lu QY, Moussa T, Almaghrabi O, Al-Zahrani H, Okada G, Deng SW, Liao WQ, Zeng JS, Issakainen J, Lopes LCL, 2014b. Proposed nomenclature for *Pseudallescheria*, *Scedosporium* and related genera. *Fungal Diversity* **67:** 1–10.

Manamgoda DS, Cai L, McKenzie EHC, Crous PW, Madrid H, Chukeatirote E, Shivas RG, Tan YP, Hyde KD, 2012. A phylogenetic and taxonomic re-evaluation of the *Bipolaris – Cochliobolus – Curvularia* complex. *Fungal Diversity* **56:** 131–144.

Nylander J, 2004. MrModeltest v2. Program distributed by the author. *Evolutionary Biology Centre Uppsala University* **2:** 1–2.

O'Donnell K, Humber RA, Geiser DM, Kang S, Park B, Robert VA, Crous PW, Johnston PR, Aoki T, Rooney AP, Rehner SA, 2012. Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via FUSARIUM-ID and *Fusarium* MLST. *Mycologia* **104:** 427–445.

Reedy JL, Floyd AM, Heitman J, 2009. Mechanistic plasticity of sexual reproduction and meiosis in the *Candida* pathogenic species complex. *Current Biology* **19:** 891–899.

Stielow JB, Lévesque CA, Seifert KA, Meyer W, Irinyi L, 2015. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia* (submitted).

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28:** 2731–2739.

Tammer I, Tintelnot K, Braun-Dullaeus RC, Mawrin C, Scherlach C, Schlüter D, König W, 2011. Infections due to *Pseudallescheria*/*Scedosporium* species in patients with advanced HIV disease – a diagnostic and therapeutic challenge. *International Journal of Infectious Diseases* **15:** e422–429.

Téllez MD, Batista-Duharte A, Portuondo D, Quinello C, Bonne-Hernández R, Carlos IZ, 2014. *Sporothrix schenckii* complex biology: environment and fungal pathogenicity. *Microbiology* **160:** 2352–2365.

Treviño-Rangel Rde J, Garza-González E, González JG, Bocanegra-García V, Llaca JM, González GM, 2012. Molecular characterization and antifungal susceptibility of the *Candida parapsilosis* species complex of clinical isolates from Monterrey, Mexico. *Medical Mycology* **50:** 781–784.

Zhang Y, Hagen F, Stielow B, Rodrigues AM, Samerpitak K, Zhou X, Feng P, Yang L, Chen M, Deng S, Li S, Liao W, Li R, Li F, Meis JF, Guarro J, Teixeira M, Al-Zahrani HS, Camargo ZP, Zhang L, de Hoog GS, 2015. Phylogeography and evolutionary patterns in *Sporothrix* spanning more than 14,000 human and animal case reports. *Persoonia* **35:** 1–20.

Legends

Table 1 Phylogenetic distance analysis of the *Scedosporium apiospermum* species complex, *S. dehoogii*, *S. minutisporum*, *S. aurantiacum* and *Pseudallescheria desertorum* based on ITS, *BT2*, *ACT*, *RP60S* and *TEF1*.

Table 2 Success rates of sequencing for each genetic locus.

Table 3 Number of clades and recombinants (species association) per gene marker compared with AFLP clusters.
SA, *Scedosporium apiospermum*; SB, *S. boydii*; nd, no data; *AFLP results from Lackner et al. (2014a).

Table S1 Isolates of the *S. apiospermum* species complex and related species included in the study.

Fig. 1 Phylogenetic tree inferred from maximum likelihood (ML) and bayesian (BI) statistics based on separate *ACT*, *BT2* and *RP60S* sequences of the *Scedosporium apiospermum* species complex, *S. dehoogii*, *S. minutisporum*, *S. aurantiacum* and *Pseudallescheria desertorum* by the outgroup method. The *S. apiospermum* species complex was marked using broken line. Bootstrap and posterior probabilities values were added to respective branches (ML/BI). Branches with bootstrap support values higher than 80% and/or 0.95 are indicated in bold.

Fig. 2 Midpoint-rooted phylogenetic analysis inferred from ML and BI statistics based on separate *ACT*, *BT2* and *RP60S* sequences of *Scedosporium apiospermum*, *S. boydii* and *Pseudallescheria angusta*. Bootstrap and posterior probabilities values were added to respective branches (ML/BI). The strains considered as in silico recombinants were marked using coloured geometric figure.

Fig. 3 Pairwise distance sliding window analysis of five genetic loci alignments (analyzed individually) showing closest inter-specific (orange whiskers) and intra-specific (blue whiskers) distances (Column A) and proportion of monophyletic species over a 100 bp sliding window (Column B).
Hypervariable alignment sections were automatically excluded, as indicated by 'gaps' for the plot 'proportion of species that are monophyletic' per gene (section ~140-200 bp in *BT2* and ~150-230 bp in ITS region).

Fig. S1 Phylogenetic relationship inferred from maximum likelihood (ML) and bayesian (BI) statistics based on separate ITS and *TEF1* sequences of the *Scedosporium apiospermum* species complex, *S.*

575 *dehoogii*, *S. minutisporum*, and *S. aurantiacum*, with *Pseudallescheria desertorum* as outgroup.
576 Bootstrap and posterior probabilities values were added to respective branches (ML/BI). Branches
577 with bootstrap support values higher than 80% and/or 0.95 are indicated in bold.
578
579 Fig. S2 Midpoint-rooted phylogenetic analysis inferred from ML and BI statistics based on separate
580 ITS and *TEF1* sequences of *Scedosporium apiospermum*, *S. boydii* and *Pseudallescheria angusta*.
581 Bootstrap and posterior probabilities values were added to respective branches (ML/BI).
582
583 Fig. S3 Phylogenetic tree inferred from maximum likelihood (ML) and bayesian (BI) statistics based
584 on concatenated *ACT*, *BT2*, *RP60S*, *TEF1* and ITS sequences of the *Scedosporium apiospermum*
585 species complex, *S. dehoogii*, *S. minutisporum*, and *S. aurantiacum*, with *Pseudallescheria desertorum*
586 as outgroup. Bootstrap and posterior probabilities values were added to respective branches (ML/BI).
587 The species complex was marked using broken line. Branches with bootstrap support values higher
588 than 80% and/or 0.95 are indicated in bold.
589
590
591
592

Table 1. Phylogenetic distance analysis of the *S. apiospermum* species complex, *S. dehoogii*, *S. minutisporum*, *S. aurantiacum* and *P. desertorum* based on ITS, *BT2*, *ACT*, *RP60S* and *TEF1*.

Gene	**ITS**	***BT2***	***ACT***	***TEF1***	***RP60S***
Length (bp)	521	487-495	326-328	565	383-392
Aligned sites	521	495	328	565	393
Best model	K2	K2+G	K2+G	T92+G	T92
Conserved sites	512	425	275	553	352
Variable sites	9	20	53	12	41
Parsimony-informative sites	9	20	45	11	34
Singleton sites	1	20	8	1	9
G+C%					
*S. apiospermum*species complex	53.2–56.1%	49.7–51.6%	51.4–53.0%	56.7–57.5%	56.8–58.3%
S. dehoogii	53.4–55.6%	48.8–49.5%	52.5–53.5%	56.5–57.3%	57.0–58.0%
S. minutisporum	54.8%	49.3–49.4%	51.9–52.1%	57.2–57.3%	57.1–57.4%
S. aurantiacum	51.4–52.2%	48.0–48.2%	52.8–53.4%	57.4–57.6%	57.4–57.7%
Intra-specific heterogeneity					
*S. apiospermum*species complex	0.034	0.024	0.005	0.006	0.025
S. dehoogii	0.042	0.027	0.007	0.009	0.002
S.minutisporum	0.000	0.016	0.003	0.001	0.013
S. aurantiacum	0.002	0.003	0.012	0.001	0.002
Distances in *S. apiospermum*species complex					
S. apiospermum vs. *S. boydii*	0.007	0.040	0.055	0.007	0.028
S. apiospermum vs. *P. angusta*	0.010	0.036	0.056	0.006	0.037
S. boydii vs. *P. angusta*	0.003	0.022	0.037	0.007	0.025
Inter-specific distances					
S. apiospermum species complex vs. *S. dehoogii*	0.084	0.065	0.024	0.017	0.044
S. apiospermum species complex vs. *S. minutisporum*	0.108	0.076	0.021	0.017	0.065
S. apiospermum species complex vs. *S. aurantiacum*	0.125	0.107	0.034	0.040	0.066
Barcoding gap	0.042	0.038	0.009	0.008	0.019

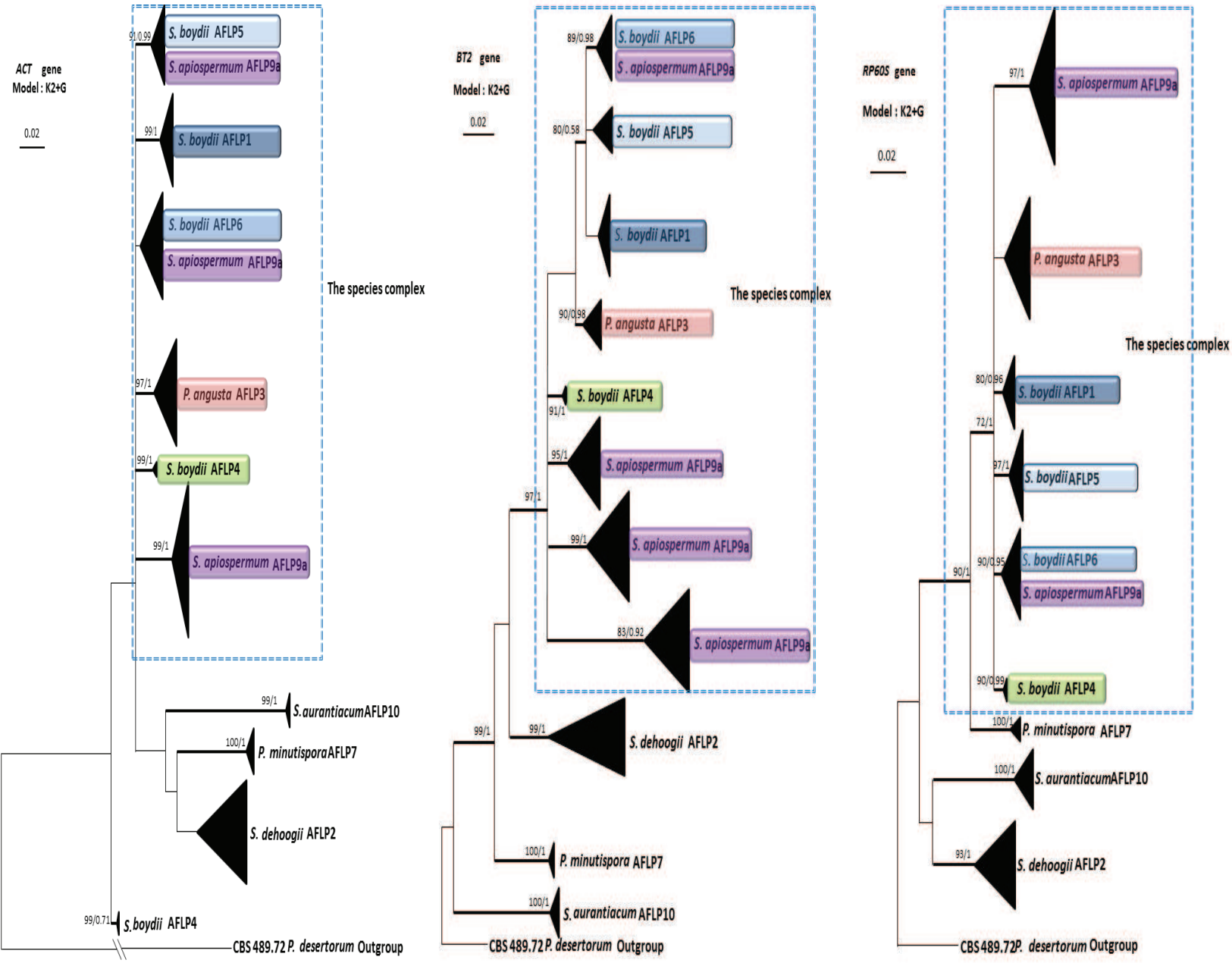
Table 2. Success rates of sequencing for each genetic locus.

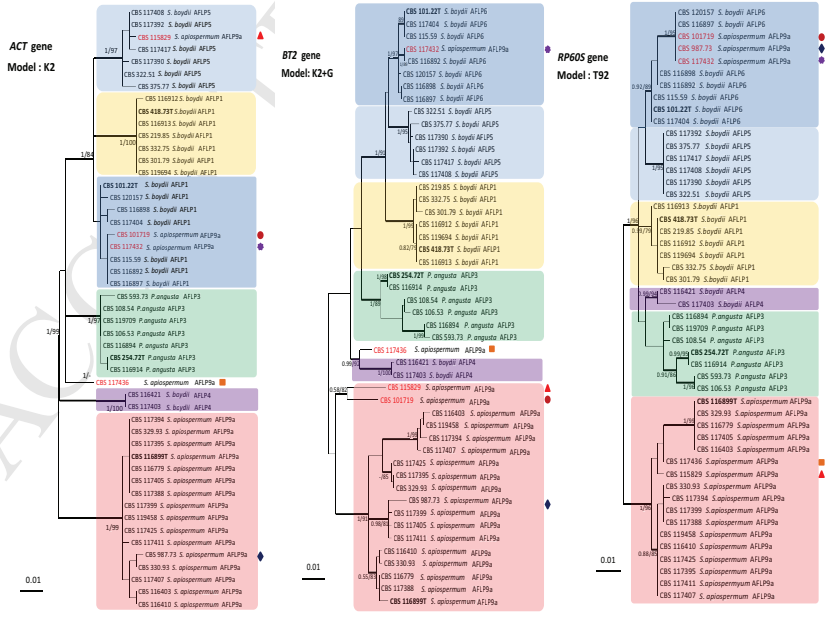
Contigs (%)	*S. apiospermum* (n=19)	*S. boydii* (n=23)	*P. angusta* (n=7)	*S. dehoogii* (n=9)	*S. minutisporum* (n=3)	*S. aurantiacum* (n=3)	*P. desertorum* (n=1)
ITS (%)	100% (19/19)	100% (23/23)	100% (7/7)	100% (9/9)	100% (3/3)	100% (3/3)	100% (1/1)
BT2 (%)	100% (19/19)	100% (23/23)	85.7% (6/7)	100% (9/9)	100% (3/3)	100% (3/3)	100% (1/1)
ACT (%)	100% (19/19)	100% (23/23)	100% (7/7)	100% (9/9)	100% (3/3)	100% (3/3)	100% (1/1)
RP60S (%)	100% (19/19)	100% (23/23)	100% (7/7)	100% (9/9)	100% (3/3)	100% (3/3)	100% (1/1)
TEF1 (%)	94.7% (18/19)	100% (23/23)	100% (7/7)	100% (9/9)	100% (3/3)	100% (3/3)	100% (1/1)

Table 3. Number of clades and recombinants (species association) per gene marker compared with AFLP clusters.

Strain ID	Gene marker: number of clades					
	TEF1: 3	ITS: 3	*RP60S*: 7	*ACT:* 9	*BT2*: 12	AFLP*: 7
CBS 115829	SA	SA	SA	SB	nd	SA
CBS 987.73	SA	SA	SB	SB	nd	SA
CBS 101719	SB	SA	SB	SB	SA	SA
CBS 17432	SB	SB	SB	SB	SB	SA
CBS 117436	SA	SB	SA	deviating	SA	SA

SA, *Scedosporium apiospermum*; SB, *Scedosporium boydii*; nd, no data; *AFLP results from Lackner *et al*. (2014a).

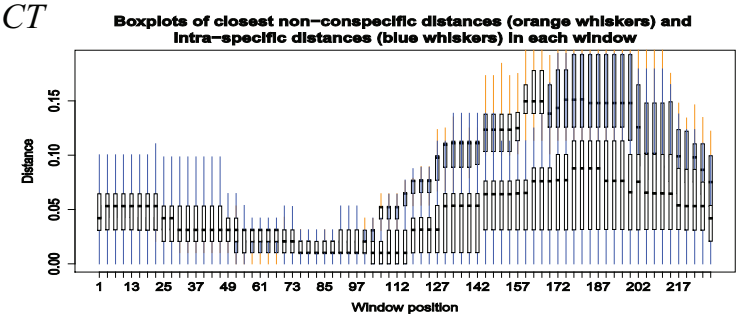

ACT gene
Model : K2+G
0.02
S. boydii AFLP5
S. apiospermum AFLP9a
S. boydii AFLP1
S. boydii AFLP6
S. apiospermum AFLP9a
P. angusta AFLP3
S. boydii AFLP4
S. apiospermum AFLP9a
The species complex
S. aurantiacum AFLP10
P. minutispora AFLP7
S. dehoogii AFLP2
S. boydii AFLP4
CBS 489.72 P. desertorum Outgroup
BT2 gene
Model : K2+G
0.02
S. boydii AFLP6
S . apiospermum AFLP9a
S. boydii AFLP5
S. boydii AFLP1
P. angusta AFLP3
S. boydii AFLP4
S. apiospermum AFLP9a
S. apiospermum AFLP9a
S. apiospermum AFLP9a
The species complex
S. dehoogii AFLP2
P. minutispora AFLP7
S. aurantiacum AFLP10
CBS 489.72 P. desertorum Outgroup
RP60S gene
Model : K2+G
0.02
S. apiospermum AFLP9a
P. angusta AFLP3
S. boydii AFLP1
S. boydii AFLP5
S. boydii AFLP6
S. apiospermum AFLP9a
S. boydii AFLP4
The species complex
P. minutispora AFLP7
S. aurantiacum AFLP10
S. dehoogii AFLP2
CBS 489.72 P. desertorum Outgroup

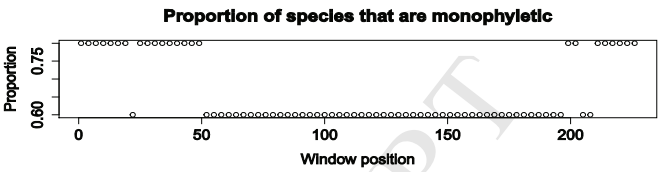


A

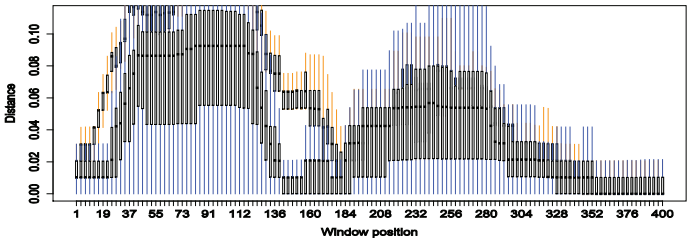
B

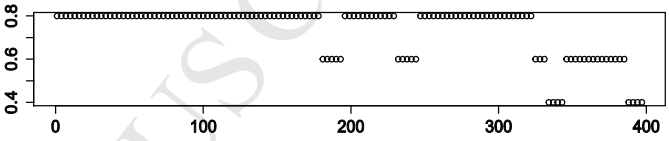
ACT



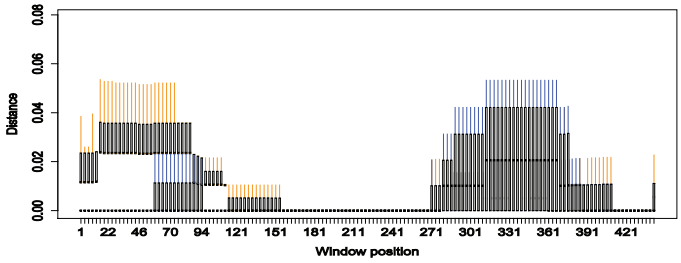


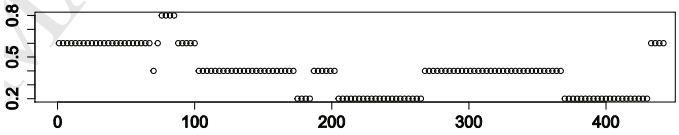
BT2



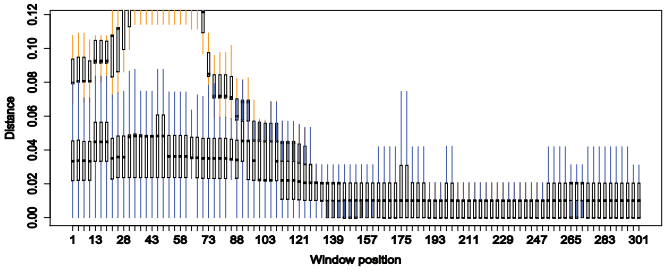


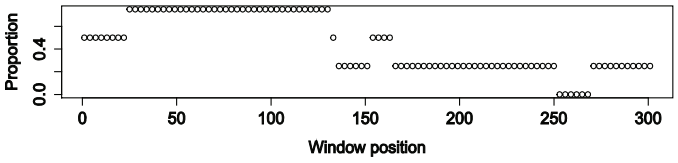
ITS



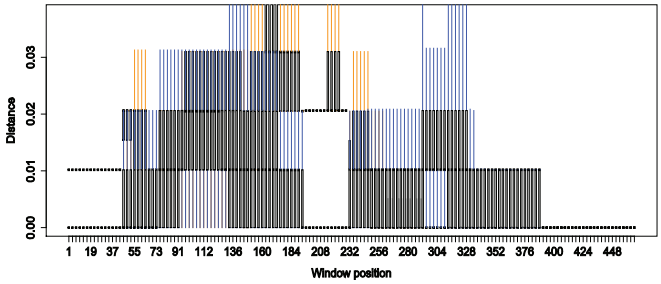


RP60S





TEF1



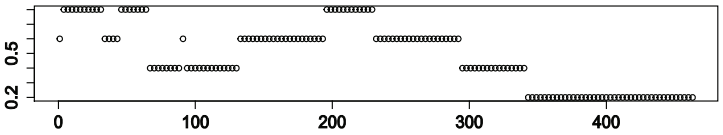


Table S1. Isolates of the *S. apiospermum* complex and related species included in the study.

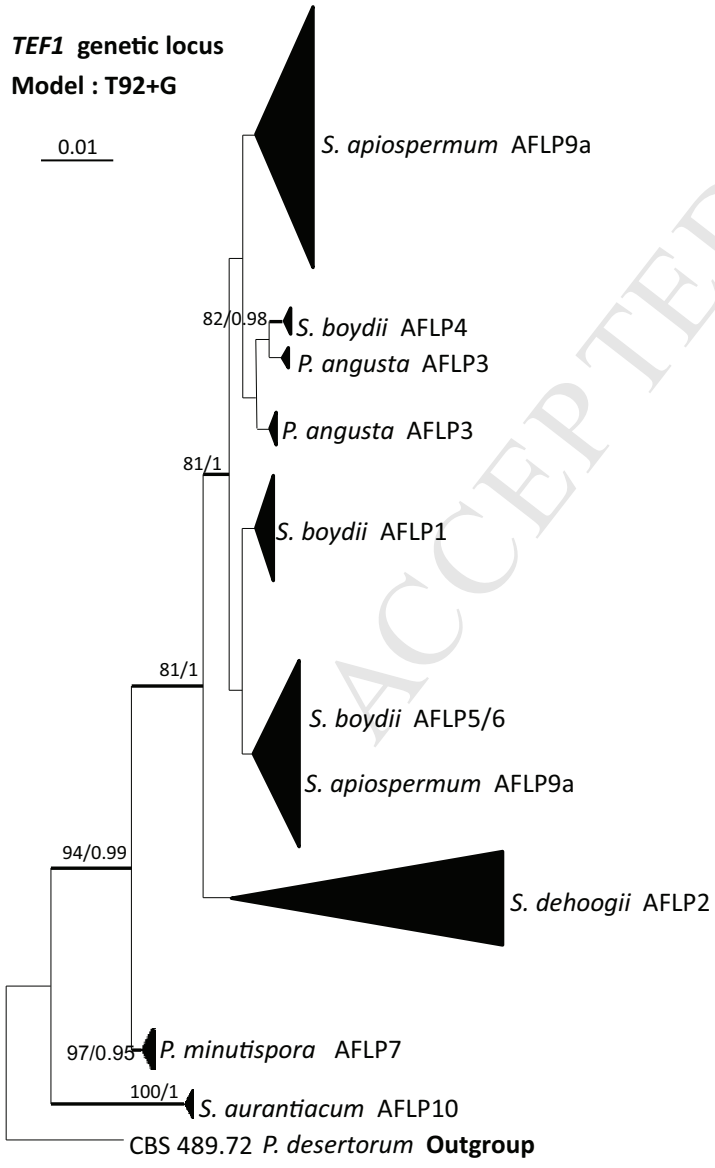
Strains	Species	*In silico* recombinant	AFLP genotype (Lackner *et al*. 2014a)	Genbank accession no.				
				ITS	*BT2*	*ACT*	*TEF1*	*RP60S*
CBS 117388	*S. apiospermum*	No	AFLP9	KT008498	KT008479	KT072637	KT069564	KT070568
CBS 117432	*S. apiospermum*	Yes	AFLP9	KT008516	KT008456	KT072660	KT069586	KT070585
CBS 116403	*S. apiospermum*	No	AFLP9	KT008508	KT008469	KT072648	ND	KT070580
CBS 987.73	*S. apiospermum*	Yes	AFLP9	KT008499	KT008478	KT072652	KT069572	KT070586
CBS 117405	*S. apiospermum*	No	AFLP9	KT008514	KT008483	KT072638	KT069565	KT070581
CBS 116779	*S. apiospermum*	No	AFLP9	KT008500	KT008480	KT072639	KT069566	KT070582
CBS 116410	*S. apiospermum*	No	AFLP9	KT008501	KT008481	KT072649	KT069573	KT070571
CBS 117425	*S. apiospermum*	No	AFLP9	KT008502	KT008475	KT072644	KT069574	KT070572
CBS 117436	*S. apiospermum*	Yes	AFLP9	KT008517	KT008487	KT072636	KT069567	KT070578
CBS 116899	*S. apiospermum*	No	AFLP9	KT008509	KT008473	KT072640	KT069575	KT070583
CBS 117399	*S. apiospermum*	No	AFLP9	KT008503	KT008485	KT072645	KT069568	KT070569

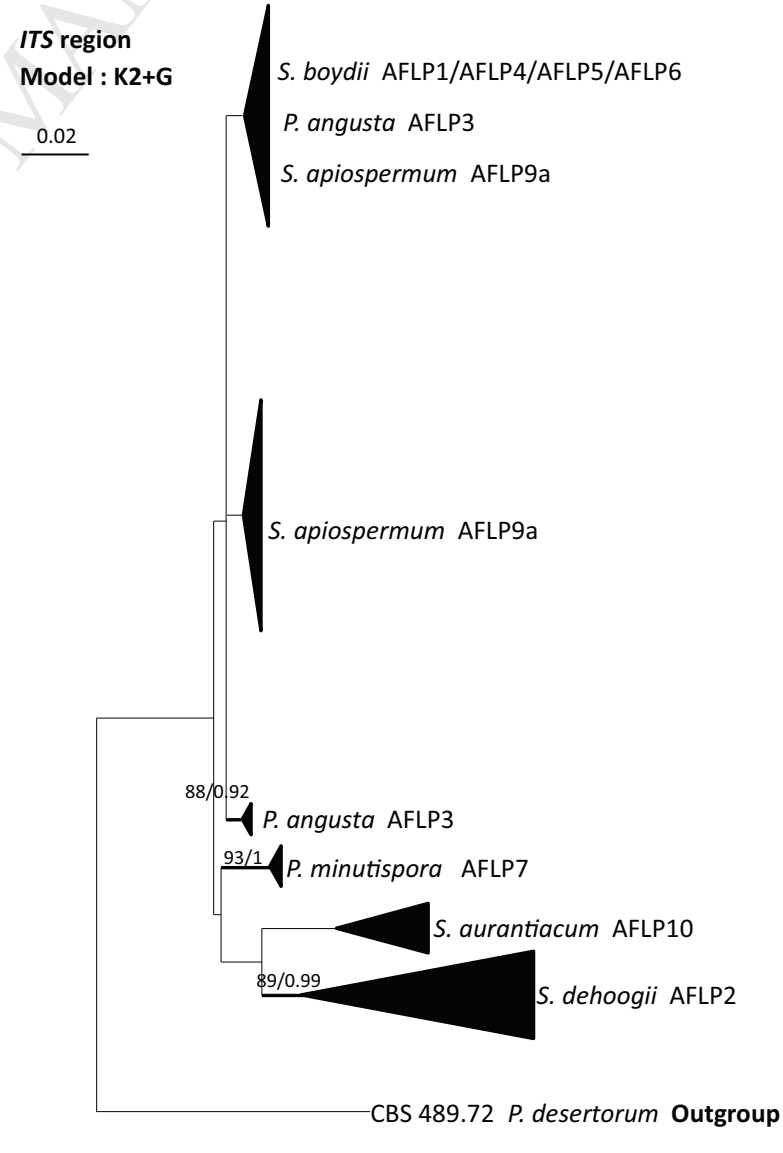
CBS 115829	*S. apiospermum*	Yes	AFLP9	KT008510	KT008474	KT072669	KT069576	KT070579
CBS 117395	*S. apiospermum*	No	AFLP9	KT008515	KT008476	KT072641	KT069577	KT070573
CBS 101719	*S. apiospermum*	Yes	AFLP9	KT008504	KT008486	KT072668	KT069587	KT070587
CBS 330.93	*S. apiospermum*	No	AFLP9	KT008505	KT008482	KT072651	KT069569	KT070570
CBS 117411	*S. apiospermum*	No	AFLP9	KT008513	KT008484	KT072647	KT069570	KT070574
CBS 117394	*S. apiospermum*	No	AFLP9	KT008506	KT008472	KT072642	KT069571	KT070577
CBS 329.93	*S. apiospermum*	No	AFLP9	KT008511	KT008477	KT072643	KT069578	KT070584
CBS 117407	*S. apiospermum*	No	AFLP9	KT008512	KT008471	KT072650	KT069579	KT070575
CBS 101.22	*S. boydii*	No	AFLP6	KT008518	KT008455	KT072664	KT069589	KT070590
CBS 116898	*S. boydii*	No	AFLP6	KT008520	KT008458	KT072666	KT069590	KT070593
CBS 115.59	*S. boydii*	No	AFLP6	KT008522	KT008454	KT072661	KT069591	KT070592
CBS 116897	*S. boydii*	No	AFLP6	KT008524	KT008459	KT072663	KT069593	KT070589
CBS 120157	*S. boydii*	No	AFLP6	KT008519	KT008460	KT072665	KT069588	KT070588
CBS 117404	*S. boydii*	No	AFLP6	KT008521	KT008453	KT072667	KT069600	KT070591

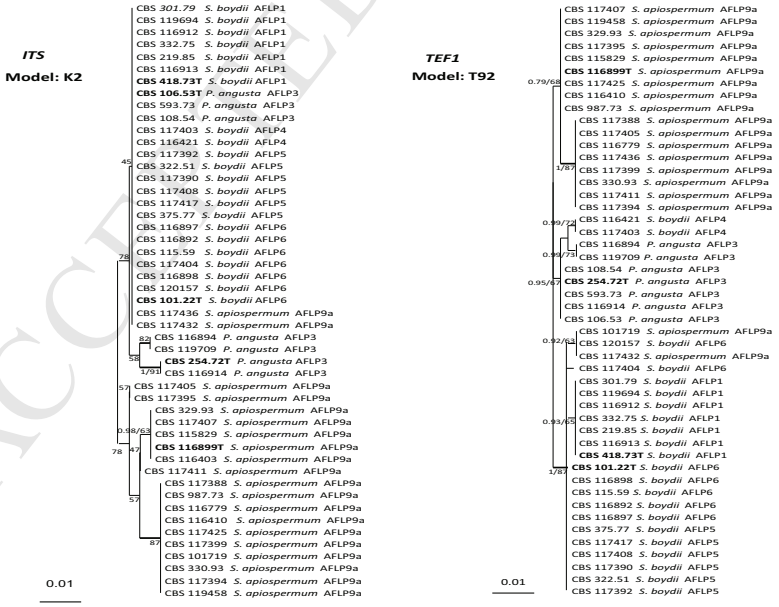
CBS 116892	*S. boydii*	No	AFLP6 unassigned	KT008523	KT008457	KT072662	KT069592	KT070594
CBS 117408	*S. boydii*	No	AFLP5 unassigned	KT008527	KT008462	KT072670	KT069596	KT070606
CBS 375.77	*S. boydii*	No	AFLP5	KT008525	KT008463	KT072675	KT069594	KT070604
CBS 117392	*S. boydii*	No	AFLP5	KT008530	KT008466	KT072671	KT069599	KT070609
CBS 322.51	*S. boydii*	No	AFLP5	KT008529	KT008461	KT072673	KT069598	KT070608
CBS 117417	*S. boydii*	No	AFLP5	KT008526	KT008464	KT072674	KT069595	KT070605
CBS 117390	*S. boydii*	No	AFLP5	KT008528	KT008465	KT072672	KT069597	KT070607
CBS 116421	*S. boydii*	No	AFLP4	KT008531	KT008488	KT072683	KT069582	KT070602
CBS 117403	*S. boydii*	No	AFLP4	KT008532	KT008489	KT072684	KT069583	KT070603
CBS 119458	*S. boydii*	No	AFLP unnamed	KT008507	KT008470	KT072646	KT069580	KT070576
CBS 418.73	*S. boydii*	No	AFLP1	KT008540	KT008449	KT072676	KT069601	KT070595
CBS 116913	*S. boydii*	No	AFLP1	KT008541	KT008446	KT072677	KT069602	KT070599
CBS 219.85	*S. boydii*	No	AFLP1	KT008542	KT008450	KT072678	KT069603	KT070596
CBS 332.75	*S. boydii*	No	AFLP1	KT008543	KT008451	KT072679	KT069604	KT070600

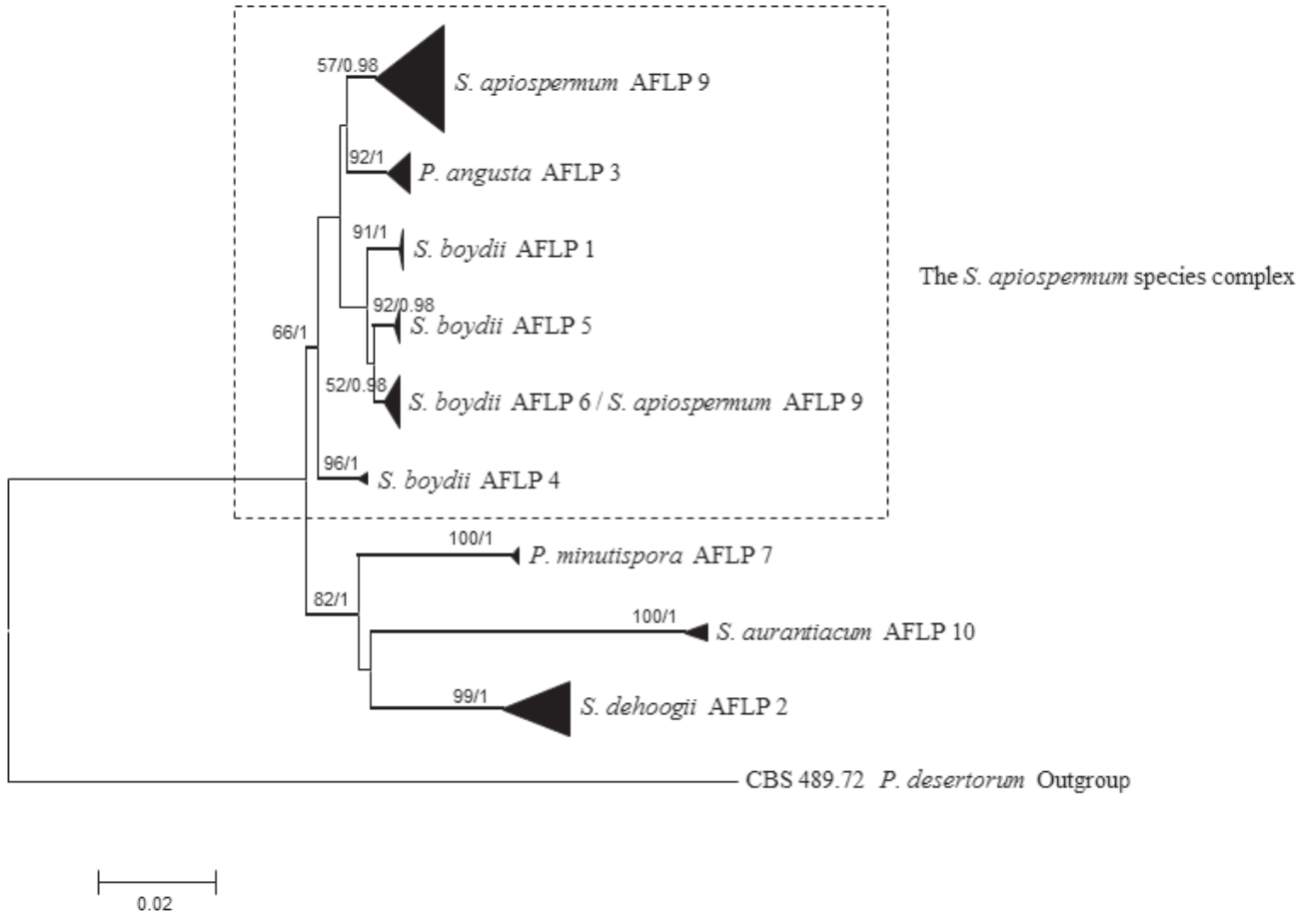
CBS 116912	*S. boydii*	No	AFLP1	KT008544	KT008447	KT072682	KT069605	KT070597
CBS 301.79	*S. boydii*	No	AFLP1	KT008545	KT008452	KT072680	KT069606	KT070601
CBS 119694	*S. boydii*	No	AFLP1	KT008546	KT008448	KT072681	KT069607	KT070598
CBS 108.54	*P. angusta*	No	AFLP3	KT008533	KT008442	KT072653	KT069581	KT070610
CBS 116894	*P. angusta*	No	AFLP3	KT008536	KT008444	KT072654	KT069584	KT070611
CBS 254.72	*P. angusta*	No	AFLP3	KT008538	KT008467	KT072657	KT069619	KT070613
CBS 593.73	*P. angusta*	No	AFLP3	KT008534	KT008445	KT072659	KT069620	KT070615
CBS 119709	*P. angusta*	No	AFLP3	KT008537	ND	KT072655	KT069585	KT070612
CBS 116914	*P. angusta*	No	AFLP3	KT008539	KT008468	KT072658	KT069621	KT070614
CBS 106.53	*P. angusta*	No	AFLP3	KT008535	KT008443	KT072656	KT069622	KT070616
CBS 117415	*S. dehoogii*	No	AFLP2 unnamed	KT008547	KT008490	KT072687	KT069623	KT070620
CBS 101720	*S. dehoogii*	No	AFLP2	KT008554	KT008496	KT072686	KT069611	KT070623
CBS 499.90	*S. dehoogii*	No	AFLP2	KT008548	KT008497	KT072685	KT069608	KT070621
CBS 117406	*S. dehoogii*	No	AFLP2	KT163400	KT163401	KT072689	KT069615	KT070625

CBS 101721	*S. dehoogii*	No	AFLP2	KT008550	KT008492	KT072692	KT069613	KT070627
CBS 101723	*S. dehoogii*	No	AFLP2	KT008551	KT008491	KT072690	KT069612	KT070626
CBS 117393	*S. dehoogii*	No	AFLP2	KT008553	KT008495	KT072688	KT069610	KT070624
CBS 117387	*S. dehoogii*	No	AFLP2	KT008552	KT008494	KT072691	KT069609	KT070622
CBS 101724	*S. dehoogii*	No	AFLP2	KT008549	KT008493	KT072693	KT069614	KT070628
CBS 100396	*S. minutisporum*	No	AFLP7	KT008555	KT008440	KT072694	KT069616	KT070617
CBS 116911	*S. minutisporum*	No	AFLP7	KT008556	KT008441	KT072695	KT069617	KT070618
CBS 116595	*S. minutisporum*	No	AFLP7	KT008557	KT008439	KT072696	KT069618	KT070619
CBS 117414	*S. aurantiacum*	No	AFLP10	KT008558	KT008436	KT072697	KT069624	KT070629
CBS 103.44	*S. aurantiacum*	No	AFLP10	KT008559	KT008437	KT072698	KT069625	KT070630
CBS 117426	*S. aurantiacum*	No	AFLP10	KT008560	KT008435	KT072699	KT069626	KT070631
CBS 489.72	*S. desertorum*	No	Outgroup	KT008561	KT008438	KT072700	KT069627	KT070632








57/0.98
S. apiospermum AFLP 9
92/1
P. angusta AFLP 3
91/1
S. boydii AFLP 1
92/0.98
S. boydii AFLP 5
66/1
52/0.98
S. boydii AFLP 6 / S. apiospermum AFLP 9
96/1
S. boydii AFLP 4
The S. apiospermum species complex
100/1
P. minutispora AFLP 7
82/1
100/1
S. aurantiacum AFLP 10
99/1
S. dehoogii AFLP 2
CBS 489.72 P. desertorum Outgroup
0.02