Leucosporidium drummii sp. nov., a member of the Microbotryomycetes isolated from soil

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Two strains of a novel teleomorphic basidiomycete were isolated from grassland soil. Standard phenotypic tests and phylogenetic analyses of 26S rRNA gene (D1/D2 domains) and ITS region sequences showed that the species belongs to the core group of the genus *Leucosporidium*. A novel species, *Leucosporidium drummii* sp. nov., is proposed to accommodate the two strains, with SEG-3-2-AY220^T (=CBS 11562^T=MUCL 52878^T) as the type strain. In addition, phylogenetic analysis revealed great genetic variability in the *Leucosporidium scottii* complex.

INTRODUCTION

One of the aims of the German Biodiversity Exploratories initiative (<http://www.biodiversity-exploratories.de/>) is to better understand biodiversity in relation to land use and management. In this context, we have been studying soil yeast communities (Yurkov *et al.*, 2012). During this survey, several novel species have been discovered, three of which, *Clavispora reshetovae*, *Barnettozyma vustinii* and *Holtermanniella takashimae*, were described recently (Yurkov *et al.*, 2009a, b; Wuczkowski *et al.*, 2011). Multiple cultures of non-pigmented yeasts with phenotypic characters resembling those of the basidiomycetous genus *Rhodotorula* (Pucciniomycotina, Microbotryomycetes) were used for experiments aiming to induce the teleomorphic stage. Of these, two cultures produced hyphae with teliospores. The absence of pigmentation, the lack of production of starch-like compounds, the inability to grow on inositol and the formation of hyphae suggested that they were related to members of the order Leucosporidiales (Microbotryomycetes, Pucciniomycotina). Phylogenetic analysis based on the D1/D2 domains of the 26S rRNA gene and the complete 5.8S–internal transcribed spacer (ITS) gene region confirmed the placement of the strains in the genus *Leucosporidium* and indicated that they represent a novel species for which the name *Leucosporidium drummii* sp. nov. is proposed.

METHODS

The two novel strains were isolated from grassland soil collected in the UNESCO Biosphere Reserve Schorfheide-Chorin (plot ID S526, SEG 3; approx. coordinates: 53.1028° N 13.9857° E), Germany, following a previously described procedure (Yurkov *et al.*, 2012).

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the LSU rRNA gene (D1/D2 domains) and ITS sequences of *Leucosporidium drummii* CBS 11562^T are FN428965 and FN908919, respectively.

Strain CBS 11561 was isolated as a separate colony and strain CBS 11562^T grew in a mixed culture with *Trichosporon porosum*. The latter culture was purified on modified Browns' nitrogen deficient media (Brown *et al.*, 1962) supplemented with imidazole (according to LaRue & Spencer, 1967) and cycloheximide (according to Danielson & Jurgensen, 1973). Cultures were maintained on MYP medium (Sampaio *et al.*, 2003). Phenotypic characterization of purified isolates was carried out according to Yarrow (1998) using both solid and liquid media. Plates were incubated at room temperature and checked after 7, 14 and 21 days. Growth tests in liquid media were performed in test tubes with 2.5 ml media, which were shaken on an orbital shaker at 200 r.p.m. All assimilation profiles of members of the genera *Leucosporidium* and *Leucosporidiella* were obtained from the CBS database (www.cbs.knaw.nl) except for that of *Leucosporidium golubevii*, which was described by Sampaio *et al.* (2003).

Both strains had a similar white and mucilaginous look, although CBS 11562^T displayed some hyphal growth. Formation of hyphae was induced in cultures after prolonged incubation (3 weeks) on MYP medium at 4 and 16 °C. Agar blocks containing hyphae were cut out, transferred to new plates and incubated at 16 °C in order to facilitate development of the hyphal stage. The last step was repeated four times. Teliospore germination was investigated after 3 to 6 months of incubation. Alternatively, agar blocks containing teliospores were cut out of a 1-year-old culture grown on MYP at 16 °C and transferred to a new plate. Germination of teliospores normally occurred on the day after the transfer. For observation of nuclei, a modified Giemsa staining protocol described by Sampaio *et al.* (2001) was used. For staining, agar blocks of a 1-year-old culture incubated on MYP agar at 16 °C for 7 days were used.

The DNA extraction protocol, PCR amplification, purification and sequencing were performed as described previously (Glushakova *et al.*, 2010; Yurkov *et al.*, 2012). The assembly and editing of sequence data were performed using Sequencher 4.8 (Gene Codes Corporation). Alignments were made using the MAFFT algorithm (Katoh *et al.*, 2002). The model of DNA substitution (GTR+G+I) and parameters for maximum-likelihood analysis were derived by using MODELTEST version 3.7 (Posada & Crandall, 1998). Maximum-likelihood analysis was performed using RAXML (-m GTRGAMMA option) version 7.0.3, with 1000 rounds of bootstrap replicates (Felsenstein, 1985; Stamatakis *et al.*, 2008). Additional sequences were retrieved from GenBank (www.ncbi.nlm.nih.gov) and CBS (www.cbs.knaw.nl) databases. All accession numbers and strain numbers are indicated on the phylogenetic tree (Fig. 1).

Fig. 1. Phylogenetic placement of *Leucosporidium drummii* obtained by maximum-likelihood analysis of the LSU rRNA gene (D1/D2 domains) and ITS region. The numbers given on branches are frequencies (>75%) with which a given branch appeared in 1000 bootstrap replications. Bar, number of expected substitutions accumulated per site. The tree is rooted with *Rhodosporidium toruloides* CBS 349^T (AF444489/AF070426) and *Sporidiobolus salmonicolor* CBS 490^T (AY015434/AF070439). Where no sequence accession numbers are given, data were retrieved from the CBS database; sequences determined in this study are given in bold. Mating types (MT) for *Leucosporidium scottii* and closely related species are given according to Fell & Statzell-Tallman (1982), Suh *et al.* (1993) and the CBS database; SF, self-fertile; ANA, anamorphic; ND, no data.

RESULTS

Ecology

Although the novel species was isolated from soil, its low incidence and abundance in this substrate suggest that soil is probably not the primary habitat of *Leucosporidium drummii*. Both strains of the novel species were isolated from the same mixed soil sample collected at a grassland plot in northern Germany (Brandenburg). We did not observe this species in central or southern Germany, i.e. in Thuringia or the Swabian Alps, respectively.

Members of the Leucosporidiales are psychrotolerant dimorphic fungi that occur in water, decaying plant material and soil (Fell *et al.*, 1969; Sampaio *et al.*, 2003). They are considered to be saprothrophs, although the possession of colacosomes suggests a parasitic nature (Sampaio *et al.*, 2003; Bauer *et al.*, 2006). Strain CBS 11562^T, recovered from the mixed culture with *Trichosporon porosum*, displayed vigorous hyphal growth. Interestingly, *Trichosporon porosum* was recently reported to produce cellobiose lipids, which are fungicidal agents with a very broad inhibition spectrum (Kulakovskaya *et al.*, 2010). However, these glycolipids did not inhibit growth of

any members of the Leucosporidiales (Kulakovskaya *et al.*, 2010). This finding is in agreement with our observations, i.e. the growth and development of *Leucosporidium drummii* was not suppressed during co-culture with *Trichosporon porosum*. Therefore, dimorphic fungi of the genus *Leucosporidium* seem to possess adaptations such as tolerance to some antifungal agents that probably facilitate their success as fungal parasites inhabiting litter or soil.

Phylogenetic placement

The genus *Leucosporidium* (Leucosporidiales, Microbotryomycetes, Pucciniomycotina) in its current state is polyphyletic and comprises five species (Sampaio *et al.*, 2003). Phylogenetic analysis based on partial 26S rRNA gene sequences demonstrated that the two species *Leucosporidium antarcticum* and *Leucosporidium fasciculatum* are not related to *Leucosporidium scottii*, *Leucosporidium golubevii* and *Leucosporidium fellii*, comprising the core group. The *Leucosporidium* core group also includes the teleomorphic species *Mastigobasidium intermedium* and several anamorphic species recently transferred to the genus *Leucosporidiella*, namely *Leucosporidiella creatinivora*, *Leucosporidiella fragaria*, *Leucosporidiella muscorum* and *Leucosporidiella yakutica* (Sampaio *et al.*, 2003). Phylogenetic analysis of the partial large subunit (LSU) rRNA gene (D1/D2 domains) and the ITS region sequences performed in the current study using the maximum-likelihood algorithm clearly suggests relatedness of *Leucosporidium drummii* with a species group that includes *Leucosporidium scottii*, *Leucosporidiella creatinivora*, *Leucosporidiella yakutica*, and *Leucosporidiella muscorum* (Fig. 1). The nearest sequence match among currently recognized species was obtained with the type strain of *Leucosporidiella muscorum*, which showed 10 nt substitutions and one indel in the D1/D2 domain of the LSU rRNA gene.

Additionally, our phylogenetic analysis revealed intraspecific variability of the *Leucosporidium scottii* strains deposited in the CBS culture collection. They showed 6–7 nt substitutions in the D1/D2 domains and 6–10 nt substitutions in the 5.8S–ITS region, respectively. Interestingly, sequences of two *Leucosporidiella yakutica* strains, CBS 8248 and CBS 8621^T, differed from those of *Leucosporidium scottii* strains CBS 2300 and CBS 9467 in 1 nt substitution each in the ITS region and showed identical D1/D2 domain sequences. In the CBS database, *Leucosporidium scottii* CBS 2300 is designated mating type A1B2; mating information for the other strain of this species is unavailable (Fig. 1). Similarly, *Leucosporidiella creatinivora* CBS 8620^T differed in 1 nt substitution in the D1/D2 domain from *Leucosporidium scottii* strains CBS 5930^T, 5932, 614, 8162 and 9490. Out of six variable positions in the ITS region among these cultures, *Leucosporidiella creatinivora* CBS 8620^T had only one unique substitution compared with these *Leucosporidium scottii* strains. This group of *Leucosporidium scottii* strains comprises both haploid mating-compatible strains and self-fertile cultures (Fell & Statzell-Tallman, 1982; CBS

database). According to the original description, both *Leucosporidiella creatinivora* and *Leucosporidiella yakutica* were tested in mating experiments with the mating types reported by Fell *et al.* (1969), namely strains CBS 5930^T and CBS 5931, corresponding to MT A2B2 and MT A1B1, respectively (Golubev, 1998). However, multiple allelic incompatibility of *Leucosporidium scottii*, containing at least 5 A and 3 B specificities, was demonstrated by Fell & Statzell-Tallman (1982). In summary, our observations imply that *Leucosporidiella creatinivora* as well as *Leucosporidiella yakutica* might represent anamorphs of *Leucosporidium scottii* or that D1/D2 and ITS sequence data are not sufficient to resolve these species. Therefore, we suggest using multigene phylogenies coupled with mating experiments to reassess anamorph/teleomorph relationships within the *Leucosporidium* core group.

Latin diagnosis of *Leucosporidium drummii* Yurkov, Schäfer et Begerow sp. nov.

Cultura in striis in agarum cum extracto malti, extracto levidinis et peptono (MYP) post unum mensem ad 20–22 °C cremaea, nitens, mucosa, laevis. In agarum MYP post 5 dies ad 16 °C, cellulae ovoideae vel ellipsoideae (3–4 × 15–35 µm), interdum elongatae simile hyphae, binae aut catenae breves (Fig. 2). Flosculi sunt polares. Ballistospores absunt. Status teleomorphicus post hebdomades tres ad 16 °C in agarum MYP observatus, homothallicus. Mycelium verum (2–3 µm in diametro) et teliospores sunt. Teliospores intercalares aut terminalis, sphaericae (8–11 µm in diametro), granulatae (Fig. 2). Teliospores basidia aut hyphae septata germinant. Basidia transversaliter septata, 3–5 × 35–45 µm. Basidiospores ovoideae, 3–4 × 25–35 µm. Teliospores interdum hyphis aseptatis in apicem cum metabasidia curvata et septata germinant.

Fermentatio (glucosum) nulla. D-Glucosum, D-galactosum (lente), D-glucosaminum, D-xylosum, L-rhamnosum, sucrosam, maltosum, trehalosum, cellobiosum, raffinose, melezitose, glycerolum, erythritolum, sorbitolum, D-mannitolum, acidum D-glucuronicum et acidum succinicum (lente) assimilantur at non L-sorbose, L-arabinosum, D-arabinosum, lactosum, inulinum, amyllum solubile, ribitolum, acidum DL-lacticum, acidum citricum nec inositolum. Assimilatio kalii nitrati, natrii nitrosi, L-lysini et ethylamini. Materia amyloidea iodophila non formantur. Ureum finditur. Diazonium caeruleum B est positivum. Temperatura maxima crescentiae: 25 °C. Ad crescentiam vitaminarum externarum non necessariae sunt.

Cultura typica SEG-3-2-AY220^T isolata ex solo prato in Brandenburg, Germania, viva et exsiccata numero CBS 11562^T (=MUCL 52878^T) in collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, Hollandia, sustentat.

Description of *Leucosporidium drummii* Yurkov, Schäfer et Begerow sp. nov.

Leucosporidium drummii (drum'mi.i. N.L. gen. masc. n. *drummii* of Drumm, named after the late German botanist

Fig. 2. Light microscopic images illustrating the different stages of *Leucosporidium drummii* sp. nov. (a) budding cells; (b) hyphal structures; (c) teliospores in chains; (d–f, i–k) various modes of teliospore germination; (g) teliospore germination showing monokaryotic cells (black arrows); (h) dikaryotic hyphal structures (white arrow); (l) fungal structures with mono- (black arrows) or dikaryotic (white arrows) cells. (a–c, e) Differential interference contrast images; (g–i) Giemsa-stained images. Bars, 10 μ m.

Dr Klaus Drumm for being an inspiring teacher and mentor in all fields of botany and mycology).

Streak culture after 1 month at 20–22 °C is cream coloured, shiny and mucilaginous with a smooth surface. Yeast cells

after 4 days on MYP agar (Sampaio *et al.*, 2003) are ovoid to elongate, 3–4 $\times$ 15–35 μ m (Fig. 2). Ballistospores are absent. Some hyphal strands are evident. The teleomorphic stage (homothallic, self-fertile cultures) was obtained independently for both strains after cultivation on MYP for 3 weeks at 16 °C.

Hyphae are 2–3 µm in diameter and devoid of clamp connections (Fig. 2). Teliospores are spherical, 8–11 µm in diameter, terminal or intercalary, single and in short chains of 2–3 spores (Fig. 2). Teliospore germination mode is variable. Teliospore germinates with basidium or hyphal segments. Basidia are transversally septate, measuring 3–5 × 35–45 µm. Basidiospores are ovoid, measuring 3–4 × 25–35 µm (Fig. 2). In some cases, teliospores germinate to produce several aseptate hyphae resulting in the formation of curved phragmometabasidia.

Sugars are not fermented. Assimilates the following carbon compounds: D-glucose, D-galactose (weak, delayed), D-glucosamine, D-xylose, L-rhamnose, sucrose, maltose, trehalose, cellobiose, raffinose, melezitose, glycerol, D-glucitol, D-mannitol, ethanol, D-glucuronic acid and succinic acid (delayed). No growth occurs on L-sorbose, L-arabinose, D-arabinose, lactose, inulin, starch, ribitol, *myo*-inositol, DL-lactic acid or citric acid. Assimilates the following nitrogen compounds: potassium nitrate, sodium nitrite, L-lysine and ethylamine. Grows in the presence of 0.01 % cycloheximide. Maximal growth temperature: 25 °C. Positive for urease activity. Diazonium blue B reaction is positive. Growth on vitamin-free medium is positive. Starch-like compounds are not produced.

The type strain is SEG-3-2-AY220^T (=CBS 11562^T=MUCL 52878^T), isolated from grassland soil in Brandenburg, Germany.

Additional remarks

In addition to the salient characters distinguishing currently recognized members of the Leucosporidiales (Sampaio *et al.*, 2003), the novel species differs from the other species in sorbose-utilizing ability.

Although this description is made based on homothallic self-fertile cultures, we believe that the respective mating types exist in nature. This assumption is based on the two different life cycles observed for *Leucosporidium scottii*, the type species of genus *Leucosporidium*, which has heterothallic and self-sporulating cultures (Fell *et al.*, 1969). The observed teleomorphic stage of the novel species clearly resembles the following morphological characters of *Leucosporidium scottii* self-fertile cultures: mycelium devoid of clamp connections and teliospores with bud-like projections developing into the new teliospores (Fell *et al.*, 1969). Unlike *Leucosporidium scottii*, the novel species displayed irregular and variable modes of teliospore germination. Teliospores of a 3- to 6-month-old culture germinated in agar with hyphal segments in addition to the typical basidia-like structures (Fig. 2e). Giemsa staining showed that yeast cells of *Leucosporidium drummii* contained one nucleus, whereas the hyphal stage was dikaryotic (Fig. 2h, i). Teliospore germination resulted in monokaryotic cells (Fig. 2g). An interesting observation was made after 1-year-old teliospores grown on MYP at 16 °C were harvested and transferred onto a new MYP plate. In addition to hyphal segments and basidia-like structures

(Fig. 2f, i–k), we observed teliospores producing several aseptate hyphae, which gave rise to curved phragmobasidia (Fig. 2d). This type of teliospore germination resembles the characteristic features of the genus *Mastigobasidium* (Golubev, 1999; Sampaio *et al.*, 2003).

DISCUSSION

In this study, we propose a novel species in the sexual genus *Leucosporidium*. This species produces hyphae without clamp connections and intercalary teliospores, which germinate with either typical basidia or hyphal segments. In the last revision of the genus *Leucosporidium*, Sampaio (2011) pointed out that the irregular mode of teliospore germination exemplified by *Leucosporidium antarcticum* should be considered as asexual, i.e. when the number of supposedly ‘basidial’ compartments varies within a culture. In our opinion, this irregular mode of germination does not contradict the presence of the teleomorphic stage. It was demonstrated that members of the Microbotryales, the sister lineage to Leucosporidiales, sometimes display variable modes of germination, e.g. the number of basidial compartments in *Microbotryum violaceum* varied between different samples (Hood *et al.*, 2001). In *Microbotryum lychnidis-dioicae*, the germination mode depended on environmental conditions, such as nutrient availability and temperature (Hood & Antonovics, 1998; Schäfer *et al.*, 2010). Similarly, depending on the conditions, teliospores of *Leucosporidium drummii* produced hyphae, which originated in curved metabasidia similar to that reported for *Mastigobasidium intermedium* (Golubev, 1999; Sampaio *et al.*, 2003). Ingold (1989) reported germination with multiple basidia-like structures for *Microbotryum succisae*, so-called metabasidium branching. Interestingly, the same type of germination was observed in *Sporidiobolus pararoseus* (Sporidiobolales, Microbotryomycetes) but, due to the absence of typical basidia, this species was considered to be asexual (Valério *et al.*, 2008). These examples imply that different modes of teliospore germination might be a common attribute of the Microbotryomycetes.

The examples mentioned above also reflect striking differences between criteria used to classify saprobic and parasitic taxa in the Microbotryomycetes. The parasitic fungi are commonly described on the basis of observed infection symptoms on their hosts and the morphology of the probasidia (e.g. Vánky, 1994) even though there is variance in teliospore germination patterns. Furthermore, many species of the genus *Microbotryum* (e.g. all parasites on the Polygonaceae) do not germinate on artificial media (Fischer & Holton, 1957). Because a parasitic origin of Leucosporidiales and Sporidiobolales has been suggested (Sampaio *et al.*, 2003), there is no serious reason to keep distinct approaches for classification of yeast-like and smut fungi. Here, we argue for a common solution for classification of dimorphic basidiomycetes and taxonomic simplicity. Basidiomycetous yeasts are spread around three phyla and thus should be classified together with allied taxa.

The placement of species producing teliospores in anamorphic genera would make the current state of systematics neither simple nor stable.

The use of a single name for the same fungus has been repeatedly necessitated in the last few years. As a result of past discussions during IMC9 nomenclature sessions, the use of one name for one fungus was accepted by the majority of delegates (Norvell *et al.*, 2010). In light of all these facts, further use of double nomenclature in a well-supported phylogenetic clade, like the *Leucosporidium* core group, is debatable and the use of anamorphic names should be limited to strictly asexual fungi. Thus, the placement of *Leucosporidium drummii*, which produces mycelium with teliospores, in the asexual genus *Leucosporidiella* due to the variance in germination mode is impractical and rather confusing for many mycologists. Additionally, the teleomorphic species *Mastigobasidium intermedium*, which is a member of the *Leucosporidium* core group (Fig. 1; Sampaio *et al.*, 2003), might be transferred to the genus *Leucosporidium*. Ballistoconidia production and teliospore germination mode morphologically distinguish *Mastigobasidium* from members of the genus *Leucosporidium* (Sampaio *et al.*, 2003). However, our observations suggest that teliospores of *Leucosporidium drummii* sometimes originate multiple aseptate hyphae and curved basidia. Additionally, formation of ballistoconidia is apparently a variable feature among the Microbotryomycetes and is exemplified with several closely related species assigned to the genera *Bensingtonia*, *Sporobolomyces* and *Rhodotorula* (e.g. *Sporobolomyces singularis* and *Rhodotorula lignophila*; *Bensingtonia yamatoana* and *Rhodotorula arctica*) based on this morphological attribute (Sampaio *et al.*, 2003; Vishniac & Takashima, 2010). Because the mode of teliospore germination, as well as other morphological attributes, might vary considerably within the Microbotryomycetes, the application of molecular markers provides a solid background for the classification of these dimorphic fungi. Notably, molecular markers are also consistent with ultrastructural markers, septal pore morphology and the presence of colacosomes (Sampaio *et al.*, 2003).

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