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Research Article

RESEARCH ARTICLE

Sex in the cold: taxonomic reorganization of psychrotolerant yeasts in the order Leucosporidiales

Virginia de García[1,*], Marco A. Coelho[3], Teresa M. Maia[3], Luiz H. Rosa[2], Aline Martins Vaz[2], Carlos A. Rosa[2], José Paulo Sampaio[3], Paula Gonçalves[3], María van Broock[1] and Diego Libkind[1]

[1]Laboratorio de Microbiología Aplicada y Biotecnología, Centro Regional Universitario Bariloche, Universidad Nacional del Comahue, INIBIOMA-CONICET, Quintral 1250, San Carlos de Bariloche, Río Negro C.P. 8400, Argentina, [2]Departamento de Microbiologia, ICB, C.P. 486, Universidade Federal de Minas Gerais, Belo Horizonte, MG, 31270-901, Brazil. and [3]UCIBIO, REQUIMTE, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

*Corresponding author: Quintral 1250, San Carlos de Bariloche, Río Negro C.P. 8400, Argentina. Tel: +54-2944-428505 INT.:102; Fax: +54-2944-428505; E-mail: vikidegarcia@gmail.com

One sentence summary: Studies on different characteristics (from genetic to sexual behavior) of cold-adapted yeasts species, in order to clarify the evolutionary relationship.
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ABSTRACT

Species of Leucosporidiales are a group of psychrotolerant yeasts with biotechnological potential. In the present work, we studied the phenotypic, genetic and sexual characteristics of three species of this genus (*Leucosporidium scottii*, *Leucosporidiella creatinivora* and *Le. yakutica*) to clarify the evolutionary relationship among these closely related taxa. From the results obtained, it becomes clear that these yeasts can interbreed. Although genetic delimitation is possible for the three species, the extent of nucleotide substitutions and phenotypic differences observed between them are lower than that expected for species that have ended the speciation process. Our taxonomic conclusion is to maintain the three taxa until further genomic data are gathered. However, the concept of *L. scottii* species complex is proposed for this group of species. Finally, we transfer all *Leucosporidiella* and *Mastigobasidium* species to *Leucosporidium* (Leucosporidiales), and, in order to end the polyphyly condition of these taxa, we propose the new genus *Pseudoleucosporidium* gen. nov. and the new combination *Peudoleucosporidium fasciculatum* comb. nov.

Keywords: leucosporidiales; cold-adapted; sex

INTRODUCTION

Species of Leucosporidiales have been isolated predominantly from cold environments and are regarded as psychrotolerant yeasts, some of which are potential sources of extracellular enzymes that are active at low temperatures (cold-enzymes), antifreeze proteins, and have the ability to biodegrade phenol and phenol-related compounds (Lee *et al.* 2010; Golubev 2011; Sampaio 2011a, b; de Garcia, Brizzio and van Broock 2012). The order Leucosporidiales (Microbotryomycetes, Pucciniomycotina) accommodates two teleomorphic genera *Leucosporidium* and *Mastigobasidium*, and the asexual genus *Leucosporidiella* (Sampaio *et al.* 2003, 2011a). Within these, the genus *Leucosporidium* presently includes five species *Leucosporidium scottii* (type species), *L. golubevii*, *L. fellii*, *L. drummii* and the distantly related *L. fasciculatum* (Sampaio 2011a; Yurkov, Schäfer and Begerow 2012).

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Asexual species of the order are *Leucosporidiella creatinivora*, *Le. yakutica*, *Le. fragaria* and *Le. muscorum* (Sampaio *et al.* 2003; Golubev 2011; Sampaio 2011b). Recently, an anamorphic species was described as *Leucosporidium* based on the recent changes of the nomenclature code and named *L. escuderoi f.a.* (*f.a.*: *forma asexualis*, refers to asexual form) (Laich, Chávez and Vaca 2014).

Three species (*L. scottii*, *Le. creatinivora* and *Le. yakutica*) deserve further attention given their remarkable close relatedness, as shown by rRNA sequence analyses (Sampaio 2011a), which already led to the suggestion, not yet experimentally tested, that *Le. creatinivora* and *Le. yakutica* might represent anamorphs of *L. scottii* (Yurkov, Schäfer and Begerow 2012). *Leucosporidium scottii*, includes self-sterile (heterothallic) and self-fertile (homothallic) isolates, hyphae have clamp connections when formed after mating of sexually compatible strains. The species appears to have a bifactorial sexual incompatibility system also known as tetrapolar, given that four different mating types may arise after meiosis (Fell and Statzell-Tallman 1982). The tetrapolar system is exclusive of the basidiomycetes and is controlled by two independent molecular determinants of mating type (*MAT*) (Kothe 1996). Recognition between sexually compatible partners is initially determined by a pheromone/receptor (P/R) interaction system. After cell fusion, the progression through the sexual cycle requires the formation of heterodimeric homeodomain transcription factor HD1/HD2 that functions as a switch regulating the transition between pre- and post-mating development. The sexual cycle can only proceed when mating partners carry different alleles at both *MAT* loci. In *L. Scottii*, the presence of 5 A and 3 B *MAT*-specific factors has been suggested by Fell and Statzell-Tallman (1982), but these factors were not assigned to either of the two known molecular determinants of mating type in basidiomycetes (*P/R* or *HD*).

In the present work, we studied the phenotypic, genetic and sexual characteristics of *L. scottii*, *Le. creatinivora* and *Le. yakutica* from 12 new wild isolates (from Antarctica and Patagonia, Argentina) and 36 collection strains, to clarify the evolutionary relationship among these closely related species. Additionally, we propose taxonomic changes in the Leucosporidiales in order to meet the Article 59 of the *International Code of Nomenclature for algae, fungi and plants*, which states that only one valid name must be used for a fungus (McNeill *et al.* 2012).

MATERIALS AND METHODS

Yeast strains

Strains belonging to the species *L. scottii*, *Le. creatinivora* and *Le. yakutica* employed in this study were obtained from various international culture collections (Table 1), or isolated from natural sources such as glacier-related habitats in Patagonia (de Garcia *et al.* 2007; Libkind *et al.* 2009) and Antarctica (Vaz *et al.* 2011). Type strains of *L. golubevii* (CBS 9651^T), *L. scottii* (CBS 5930^T), *Le. creatinivora* (CBS 8620^T), *Le. yakutica* (CBS 8621^T), *Le. muscorum* (CBS 6921^T) and *Le. fragaria* (CBS 6254^T) were included in the analyses. The complete list of strains studied in this work is shown in Table 1.

Characterization

Physiological and biochemical characterization of the yeast strains without available data was carried out according to the techniques described by Kurtzman *et al.* (2011). For PCR fingerprinting, the microsatellite-primed PCR technique (MSP-PCR) was applied (Libkind *et al.* 2003). DNA extraction, primer M13 (GAGGGTGGCGGTTCT), PCR and electrophoretic conditions were performed as described by Libkind *et al.* (2003). Partial sequences of the D1/D2 domain of the LSU rRNA gene were sequenced with primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAGACG G-3′) (Boekhout, Fell and O'Donnell 1995), while the internal transcribed spacer region (ITS1, 5.8S and ITS2) was sequenced using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al.* 1990). Sequencing was carried out using an ET Dynamic Terminator Kit in a MegaBACE 1000/Automated 96 Capillary DNA sequencer (GE Healthcare, USA).

PCR detection and sequencing of pheromone receptor genes

PCR detection with degenerate primers for the two pheromone receptor alleles (*STE3.A1* or *STE3.A2*) was carried out to directly identify and assign the 'molecular mating-type' of selected strains representing different *Leucosporidium/Leucosporidiella* species, as previously described for several red yeasts (Sporidiobolales) (Coelho, Sampaio and Gonçalves 2010; Coelho, Gonçalves and Sampaio 2011). Partial sequences of *STE3.A1* were amplified and sequenced using primers pairs MC190 (5′-ACC YTG CTY CTC ATY TTY TGG) and MC191 (5′- ATG GCG CTY TTK GAC GAY TC), and the same procedures were carried out for *STE3.A2* using either primers MC166 (5′- AKC GTK CCK CTS TAC TGG CA) and MC125 (5′- GCS ACS ACC CGA YSA AAG TTG CG), or LsSTE3.A2˙53F (5′- TAC CGA AGC CGA AGG CGG CGA AGA A) and LsSTE3.A2˙1074R (5′- AAT GCC GGC CCG CTT TAC TGG CA) (Coelho, Sampaio and Gonçalves 2010; Coelho, Gonçalves and Sampaio 2011).

Sequence data and phylogenetic analyses

To infer the phylogenetic relationships of the different species, sequences of the D1/D2 domain of the LSU rRNA and ITS region (ITS1, 5.8S and ITS2) were aligned with ClustalW in MEGA5 software (Tamura *et al.* 2011). The final sequence alignment was subsequently used to construct a phylogenetic tree using two different methods: (1) the Bayesian Markov chain Monte Carlo method of phylogenetic inference, as implemented in the computer program MrBayes (Ronquist and Huelsenbeck 2003) and (2) the neighbor-joining algorithm and the Kimura two-parameter model to estimate the evolutionary distances. Branch supports were determined using bootstrap analysis from 1000 replicates. This analysis was conducted in the MEGA5 software (Tamura *et al.* 2011). Given that both methods showed similar results, the latter tree was chosen. Based on the same dataset, parsimony networks were constructed with the program TCS 1.21 (Clement, Posada and Crandall 2000), and gapped positions were excluded from this analysis.

Partial sequences of the pheromone receptors genes (*STE3.A1* and *STE3.A2*) were separately aligned using MUSCLE (Edgar 2004), and the corresponding phylogenetic trees were inferred by maximum likelihood (ML) using RAxML v7.2.8 and the GTRGAMMA model. *Sporobolomyces salmonicolor* and *S. johnsonii* sequences were used as outgroup. Branch supports for both phylogenetic trees were determined using 1000 rapid bootstrap replicates.

Table 1. Strains of *Leucosporidium* and *Leucosporidiella* species analyzed in these work, their origin, salient differential phenotypic characteristics, sexual and mating type and GenBank accession number.

Phylo	Species	Strain	MT	Sex	L-rhamnose	Cadaverine	5°C	30°C	Source/geographic location	ITS	D1D2	STE3-A1	STE3-A2
1	*L. creatinivora*	CBS 8620T	a2	As	−	+	+	−	Permafrost soil, Siberia, Russia	AF444629	AF189925	—	KP732343
1	*L. creatinivora*	CBS 9210	a1	Het	−	w	+	−	Soil, Iceland	KM213169	KM213196	KP732327	—
1	*L. creatinivora*	CBS 9305	a2	As	−	w	+	−	Alpine glaciers, permafrost rock, Russia	KM213172	EF643737	—	—
1	*L. creatinivora*	CRUB 1166	a1	Het	−	+	+	−	Verde Lake, Patagonia, Argentina	KM213173	EF595758	KP732328	—
1	*L. creatinivora*	CRUB 1214	a2	Het	−	+	+	−	Glacial melt-water, Patagonia Argentina	KM213174	DQ513291	—	KP732344
1	*L. creatinivora*	CRUB 1215	a1	Het	−	+	+	−	Glacial melt-water, Patagonia Argentina	KM213175	KM213199	KP732329	—
1	*L. creatinivora*	DBVPG 4794	a1	Het	−	+	+	−	Subglacial sediments, Forni glacier, Italian Alps	KM213170	EF643737	KP732330	—
1	*L. creatinivora*	JCM 10700	n.d.	n.d.	−	+	+	−	Permafrost rock, Russia	KM213171	KM213197	—	—
2	*L. yakutica*	UFMG-ANT 61	a1	Het	+	−	+	−	Antarctica	KM213180	KM213205	KP732331	—
2	*L. yakutica*	CBS 2300	a1	Het	+	−	+	−	Air, Norway	KM213178	KM213202	KP732332	KP732345
2	*L. yakutica*	CBS 4025	a2	Het	−	−	n.d.	−	Soil (therapeutic mud), Mecklenburg, Germany	KP732311	KP732295	—	KP732346
2	*L. yakutica*	CBS 4026	a2	Het	+	+	n.d.	−	Unknown location	KP732312	KP732296	—	—
2	*L. yakutica*	CBS 8248	a2	Het	−	+	+	−	Vanda Lake, Antarctica	KM213177	KM213201	—	KP732347
2	*L. yakutica*	CBS 8621T	a2	Het	+	−	+	−	Permafrost rock, Russia	AY212989	AY213001	—	KP732348
2	*L. yakutica*	CBS 9467	a1	Het	−	w	+	−	Soil, Oklahoma bromide, USA	KP732313	KP732297	KP732333	—
2	*L. yakutica*	JCM 10702	a2?	Het	+	w	+	−	unknown	KM213176	KM213200	—	—
3	*L. yakutica*	CBS 8040	a2	Het	+, d, w	−	+	−	Chesapeake bay, USA	KM213179	KM213204	—	KP732349
3	*L. yakutica*	PB 07	a2	As	−	−	+	−	Oil-shale mine, Austrian Alps	AJ853458	KM213203	—	KP732350
4	*L. scottii*	CBS 10581	a1	Het	n.d.	n.d.	n.d.	−	Soil, Otago peninsula, New zealand	KP732314	KP732298	—	—
4	*L. scottii*	CBS 614	a1	Het	+	+	+	−	Soil near meatworks, Queensland, Australia	KM213183	KM213207	KP732334	—
4	*L. scottii*	CBS 7673	a2	Het	+	+	n.d.	−	Seawater, unknown location	KP732315	KP732299	—	KP732351
4	*L. scottii*	CBS 8036	a1	Het	+	−	n.d.	−	Soil, unknown location	KP732316	KP732300	KP732335	—
4	*L. scottii*	CBS 8188	a1	Het	+	+	n.d.	+	Seaweed (Fucus thallus), Canada	KP732318	KP732302	—	—
4	*L. scottii*	CBS 9490	a2	Het	−	w	n.d.	−	Soil, The Neatherlands			—	—
4	*L. scottii*	CBS 9965	a1	Het	+	+	+	−	Rotten wood, The Neatherlands	KM213186	KM213210	KP732336	—
4	*L. scottii*	PB 20	a1	Het	+	+	+	−	Railway are, Austrian Alps	KM213185	KM213209	KP732337	—
4	*L. scottii*	PYCC 4508	a1	Het	+	+	n.d.	n.d.	Flower, Estoril, Portugal	KP732319	KP732303	—	—
4	*L. scottii*	PYCC 4509	a1	Het	+	+	n.d.	n.d.	Soil, Portugal	KP732320	KP732304	—	—
4	*L. scottii*	PYCC 4510	a1	Het	+	+	n.d.	n.d.	Leaf, Estoril, Portugal	KP732321	KP732305	—	—
4	*L. scottii*	PYCC 4696	a1	Het	+	+	n.d.	n.d.	Dry leaf, Oeiras, Portugal	KM213187	KM213211	KP732338	—
4	*L. scottii*	PYCC 4710	a2	Het	+	+	n.d.	n.d.	River, Oeiras, Portugal	KP732322	KP732306	—	—
4	*L. scottii*	PYCC 4751	a2	Het	+	+	n.d.	−	Wood, Setubal, Portugal	KP732323	KP732307	—	KP732352
4	*L. scottii*	PYCC 4754	a2	Het	+	+	n.d.	−	Moss, Oeiras, Portugal	KP732324	KP732308	—	—
4	*L. scottii*	PYCC 4755	a2	Het	−	d	n.d.	−	Soil, Oeiras, Portugal	KP732325	KP732309	—	KP732353
4	*L. scottii*	EXF-4013	a1?	Het	+	+	+	−	Seawater,Kongsfjorden glacier, Arctic	KM213182	KM213206	—	—
4	*L. scottii*	CBS 8162	n.d.	n.d.	+	+	+	−	Rotten trunk (Eucryphia cordifolia), Chile	KM213184	KM213208	—	—
5	*L. scottii*	UFMG-ANT 133	a2	Het	+	+	+	−	Botany Lake, Antarctica	KM213194	KM213218	—	—
5	*L. scottii*	UFMG-ANT 139	a1	Het	+	+	+	−	Ullman Lake, Antarctica	KM213188	KM213212	KP732339	—
5	*L. scottii*	UFMG-ANT 158	a2	Het	+	+	+	−	Botany Lake, Antarctica	KM213193	KM213217	—	KP732354
5	*L. scottii*	UFMG-ANT 160	a2	Het	+	+	+	−	Botany Lake, Antarctica	KM213192	KM213216	—	KP732355
5	*L. scottii*	UFMG-ANT 170	a2	Het	+	+	+	−	Ullman Lake, Antarctica	KM213191	KM213215	—	—

Table 1. continued.

Phylo	Species	Strain	MT	Sex	L-rhamnose	Cadaverine	5°C	30°C	Source/geographic location	ITS	D1D2	STE3-A1	STE3-A2
5	*L. scottii*	UFMG-ANT 166	a2	Het	+	+	+	–	Ullman Lake, Antarctica	KM213190	KM213214	—	—
5	*L. scottii*	CBS 5930T	a2	Het	+	+	+	–	Seawater, Antarctica	AF444495	AY213000	—	KP732356
5	*L. scottii*	CBS 5931	a1	Het	+	+	+	–	Seawater, Pacific sector of Antarctic Ocean	KM213181	KP732310	KP732340	—
5	*L. scottii*	CBS 5932	a1	Het	+	+	+	–	Seawater, Pacific sector of Antarctic Ocean	AF444496	AF189908	KP732341	—
5	*L. scottii*	CBS 8633	a2	Het	+	+	n.d.	–	Plant, Russia Kindo Peninsula, Kandalaksha	KP732326		—	—
5	*L. scottii*	UFMG-EACF 149	a1	Het	+	+	+	–	Ullman Lake, Antarctica	KM213189	KM213213	KP732342	—
	Le. muscorum	CBS 6921T							Rotting sphagnum moss, New Zealand	AF444527	AF070433		
	Le. fragaria	CBS 6254T							Strawberries, United Kingdom	AF444530	AF070428		
	L. golubevii	CBS 9651T							Water, River Olo, Portugal	AY212987	AY212997		
	Leucosporidium sp. *f.a.*	UFMG-ANT 91					+	–	Water (Machu Pichu Lake), Antarctica	KM213195	KM213219		

T, Type strain; CBS, Centraalbureau voor Schimmelcultures, the Netherlands; CRUB, Regional University Center of Bariloche (Centro Regional Universitario Bariloche), Argentina; DBVPG, Industrial Yeasts Collection, Department of Agricultural, Food and Environmental Science - University of Perugia; Perugia, Italy; EXF, Culture Collection of Extremophilic Fungi, Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia; JCM, Japan Collection of Microorganisms; UFMG-ANT and UFMG-EACF, Minas Gerais Federal University (Universidade Federal de Minas Gerais), Brazil; PYCC, Portuguese Yeast Culture Collection, FCT-UNL, Portugal. Growth tests: C-source: L-rhamnose, N-source: Cadaverine and growth at 30°C. –, negative; +, positive; w, weak; n.d.: not determined.

Sexual compatibility studies

For sexual compatibility determination, 2 day-old cultures were paired as well as maintained singly on SG agar (soytone 0.2% w/v, glucose 0.2% w/v and agar 1.5 w/v; Kurtzman *et al.* 2011), and incubated at room temperature (18°C) for up to 2 months. These plates were examined microscopically on a regular basis for production of mycelium and teliospores. Up to 900 paired crosses were performed to determine sexual compatibility between representative strains of the different species. These tests were carried out at least three times for each pair of strains and, when negative, they were increased up to five repetitions. Production of mycelium and teliospores was classified as extensive when the production of mycelium covered all colony surface (edge) and poor when the production of mycelium was observed in only a fraction of the colony surface (edge). Blocks of agar containing teliospores of selected crosses were transferred to distilled water and incubated at 5°C for 1 month. After this resting period, the agar blocks were placed into 2% water agar plates to induce germination of the teliospores. After 5 days at 18°C, the teliospores were examined for germination under a microscope.

RESULTS AND DISCUSSION

Leucosporidium scottii and closely related species

In order to test the hypothesis of conspecificity of the species *L. scottii*, *Le. creatinivora* and *Le. yakutica*, we studied a group of 47 collection strains and new isolates obtained from natural habitats. The strains were mostly (67%) isolated from cold environments around the world (either from terrestrial or aquatic substrates). All tested strains (31) grew well at 5°C and, with the only exception of CBS 8188, 42 strains tested for growth at 30°C were negative. These results reinforces the psychrotolerant condition of these taxa (Table 1), despite that a few isolates have been obtained from non-cold environments (terrestrial substrates from Portugal).

When tested for different physiological traits, the three species were in practice undistinguishable. The only salient pattern observed was that growth of *Le. creatinivora* strains was invariably negative in L-rhamnose and mostly positive in cadaverine, while *Le. yakutica* and *L. scottii* strains were variable and positive for both tests, respectively (Table 1). MSP-PCR fingerprinting (primer M13) employing a set of strains representative of each group provided the first evidence of genetic differentiation by sorting strains into three main groups that correlated with the three taxa (Fig. S1, Supporting Information). Strains PB20 and CBS 9965 showed a slightly different DNA profile from the remaining *L. scottii* strains suggesting genetic heterogeneity within this particular species.

To complement these results, and to try to establish species boundaries, an updated phylogenetic tree based on the rRNA gene sequences (ITS and D1/D2) of all 47 strains was constructed (Fig. 1). Of the 1008 bp analyzed we detected only 11 polymorphic sites and the maximum number of nucleotide differences observed between strains (*Le. yakutica* CBS 8621^T and *L. scottii* CBS 5930^T) was of seven substitutions (0.69%). For this pair of strains, four and three substitutions in the ITS and D1/D2 domain respectively were observed. Based on this phylogenetic analysis, the discrimination of the three taxa was not straightforward due to the fact that the studied strains were distributed along at least five subclades (Fig. 1). With the exception of the subclade including *L. scottii* CBS 5930^T, in which almost all strains come from Antarctica water samples, we were not able to find

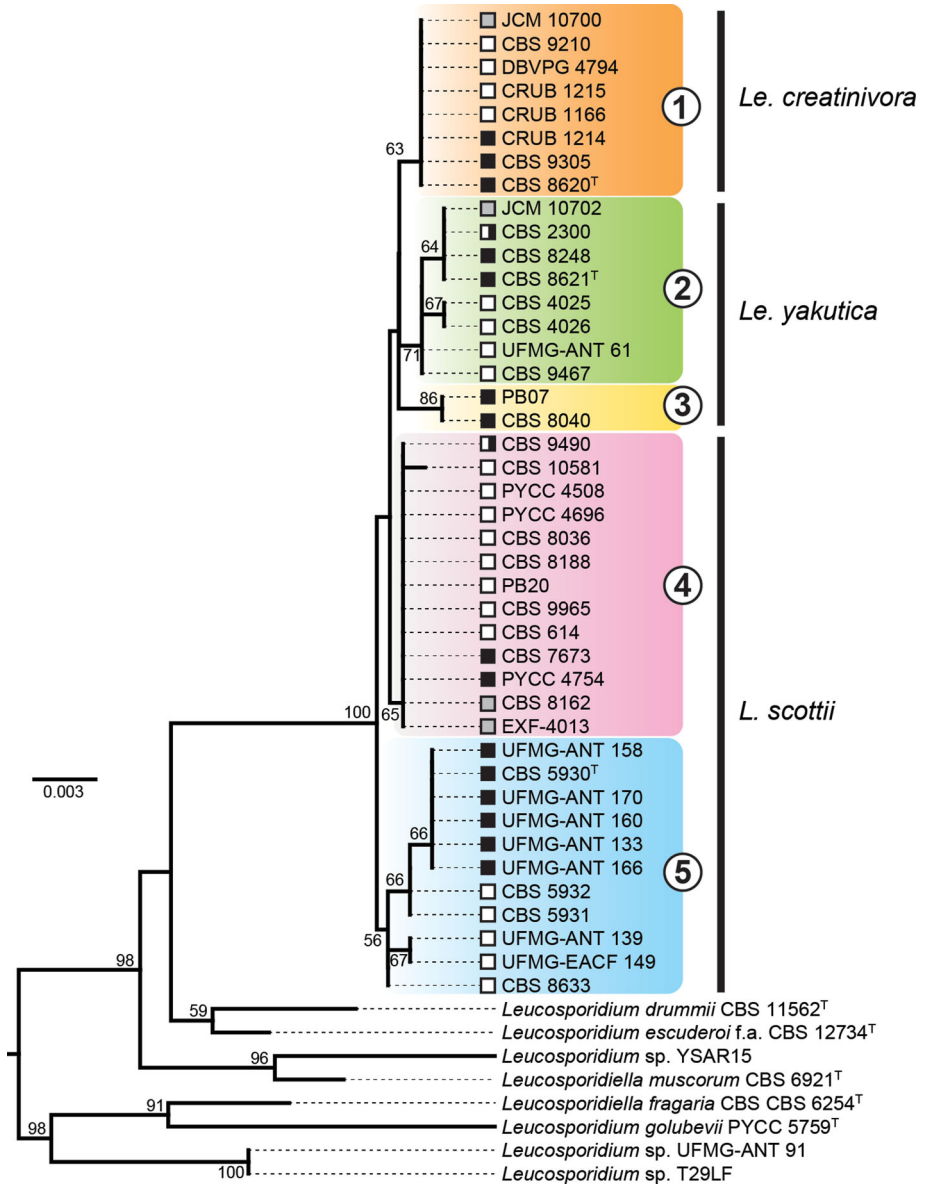

Figure 1. Phylogenetic placement of *L. scottii*, *Le. creatinivora* and *Le yakutica* strains based on the ITS and D1/D2 LSU rRNA sequences. Bar, substitutions accumulated every 100 nucleotides. Bootstrap values higher than 50% are shown (1000 replicates). ^{T}: Type strain. Square colors indicate mating alleles data: gray, n.d.; white, STE3.A1; black, STE3.A2; back and white, presence of both alleles. Shade colors (orange, green, yellow, pink and blue) and numbers (in circles) depict distinct phylogenetic subclades formed.

significant correlation between the geographical origin or type of substrate and the subclades formed. Seven strains, four from culture collections and three recent isolates, previously regarded as *Le. creatinivora* showed identical sequences and formed a uniform subclade together with the type strain of this species.

The type strain of *Le. yakutica* and three other collection strains, one of which is regarded as *L. scottii* (CBS 2300), were placed next to *Le. creatinivora* in the phylogenetic tree of Fig. 1, although with low statistical support. The recent isolate from Antarctica UFMG-ANT 61 was identical to CBS 9467 isolated from soil in Oklahoma (USA) and both showed one substitution towards the type strain of *Le. yakutica*. Similarly, strains CBS 4025 and CBS 4026 regarded as *L. scottii* differed from the type strain of *Le. yakutica* by only two substitutions, while seven substitutions were found towards the type strain of *L. scottii* (CBS 5930). Finally, two collection strains hitherto considered to belong to *L. scottii* (PB 07 and CBS 8040) were placed in a somewhat isolated position, but their growth responses were more similar to those of *Le. yakutica* isolates. The remaining 29 strains known as *L. scottii* were resolved into two major phylotypes, as previously suggested by MSP-PCR fingerprinting. The type strain of *L. scottii* grouped with five recent isolates of water samples from Antarctica. The Antarctic strain UFMG-ANT 91 was placed quite distantly from *L. scottii* and the other two related species, being phylogenetically closed to *Le. fragaria*. This strain probably represents a novel species together with strain T29LF (JQ857034) isolated from King George Island (Antarctica), and it was excluded from subsequent experiments and analyses.

Phylogenetic analysis showed only minor differences in the nucleotide sequences of ITS and D1/D2 (<0.4%, respectively) and a paraphyletic structure among strains of the three taxa. Thus, with the exception of *Le. creatinivora*, the phylogenetic

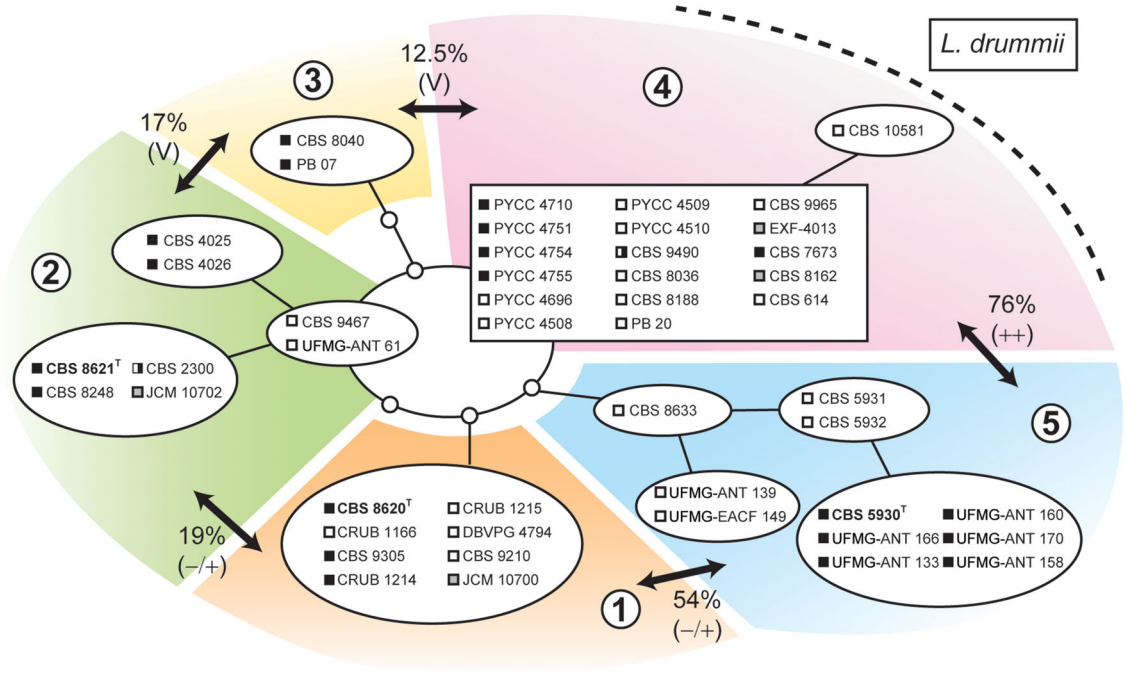

Figure 2. Parsimony network analysis of the combined ITS and D1/D2 domains of the LSU rRNA gene of strains of *L. scottii*, *Le. creatinivora and Le yakutica*. Each connecting line represents one substitution and each small circle represents a missing intermediate sequence. A rectangle indicates the sequence identified as ancestral by the analysis. *Leucosporidium drummii* was used as outgroup, the dashed line shows that was excluded from the network. Shade colors (orange, green, yellow, pink and blue) and numbers (in circles) indicate distinct phylogenetic subclades formed in Fig. 1. Arrows in numbers indicate percentage of positive crosses of the total expected if reproductive isolation was absent and mating types is considered. (++) Abundant production of mycelium and teliospores. (−+) Poor production of mycelium and teliospores, (v) variable, depending on strains crossed.

delineation at the species level becomes difficult due to the existence of genetic polymorphisms and intermediate phylotypes between *L. scottii* and *Le. yakutica*. Several authors consider that phylogenetic (bifurcating) trees are less appropriate models to represent the genealogy of alleles within a species, since intraspecific gene evolution is the combined product of a small number of relatively recent mutations and other processes such as recombination, hybridization and homoplasy (Clement, Posada and Crandall 2000; Posada and Crandall 2001). Hence, population genealogies may be better represented by methods that allow multifurcations, reticulation and the persistence of ancestral alleles in the population, which are expected to be sampled together with their descendants (Posada and Crandall 2001). In line with this, the alignment of concatenated ITS and D1/D2 sequences was used to construct a parsimony network with the program TCS, which assumes that sequences accepted in the same network represent alleles of a locus within a single species, while sequences that represent orthologs are expected to be assigned to distinct networks. Moreover, this method deals more adequately with sequences or haplotypes that may have arisen by recombination or homoplasy and such events are depicted as reticulated graphs. Results from this analysis revealed that all sequences were assigned to the same network at the 95% parsimony criterion *L. drummii* was used as outgroup (Fig. 2), and strains grouped similarly to the previous phylogenetic analyses. However, reticulated connections were observed between the different species as shown in Fig. 2. The ancestral rDNA haplotype as inferred by the program TCS coincided with the most frequent sequence sampled in our study. This in line with the expectations from the coalescent theory, which posits that within a population, the most ancestral allele tends to be the most abundant (Donnelly and Tavaré 1986; Crandall and Templeton 1993). Hence, the parsimony network generated from the available data suggests that all strains could be viewed as members of a relatively cohesive evolutionary unit.

When paired, strains of *L. scottii*, *Le. creatinivora* and *Le. yakutica* were able to undergo sexual reproduction with the formation of mycelium and teliospores (Fig. 3). Successful mating occurred both between strains of the same species and between strains of different species (all three possible combinations) (Fig. 3 and Table S1, Supporting Information). These results contrast with those reported in the original descriptions of *Le. creatinivora* and *Le. yakutica*, where negative results were obtained when these species were tested in mating experiments with available *L. scottii* strains (Golubev 1998). In addition, Statzell-Tallman and Fell (1998) stated that *L. scottii* does not mate with related species. Here, by comprehensibly studying a larger set of collection and wild isolates of the three species, we were able to establish that in fact these strains can interbreed and are able to form mycelium and teliospores that germinate to produce basidiospores. Negative results were invariably obtained when any of the studied strains were paired with the type strains of *Le. muscorum*, *Le. fragaria* and *L. golubevii* and the new isolate *Leucosporidium* sp. UFMG-ANT 91. A list of the results in which at least one inter- or intraspecies positive sexual cross was observed is depicted in Table S1 (Supporting Information). Within *L. scottii* strains, we found that the six isolates of the same phyloype as the type strain CBS 5930^T did not mate among one another, but mated in all crosses (100% mating success) with strains from the other *L. scottii* phylotype (Table 2). In these cases, mycelium and teliospores were abundant all around the colonies after 3–5 days of incubation. Additional *L. scottii* strains were also able to mate, either with strains of the same subclade (e.g. CBS 614 × CBS 7673) or with strains of a different subclade (e.g. CBS 5931 × CBS 7673 or CBS 5932 × CBS 7673). For *Le. yakutica* strains, we observed successful crosses between UFMG-ANT 61 and type

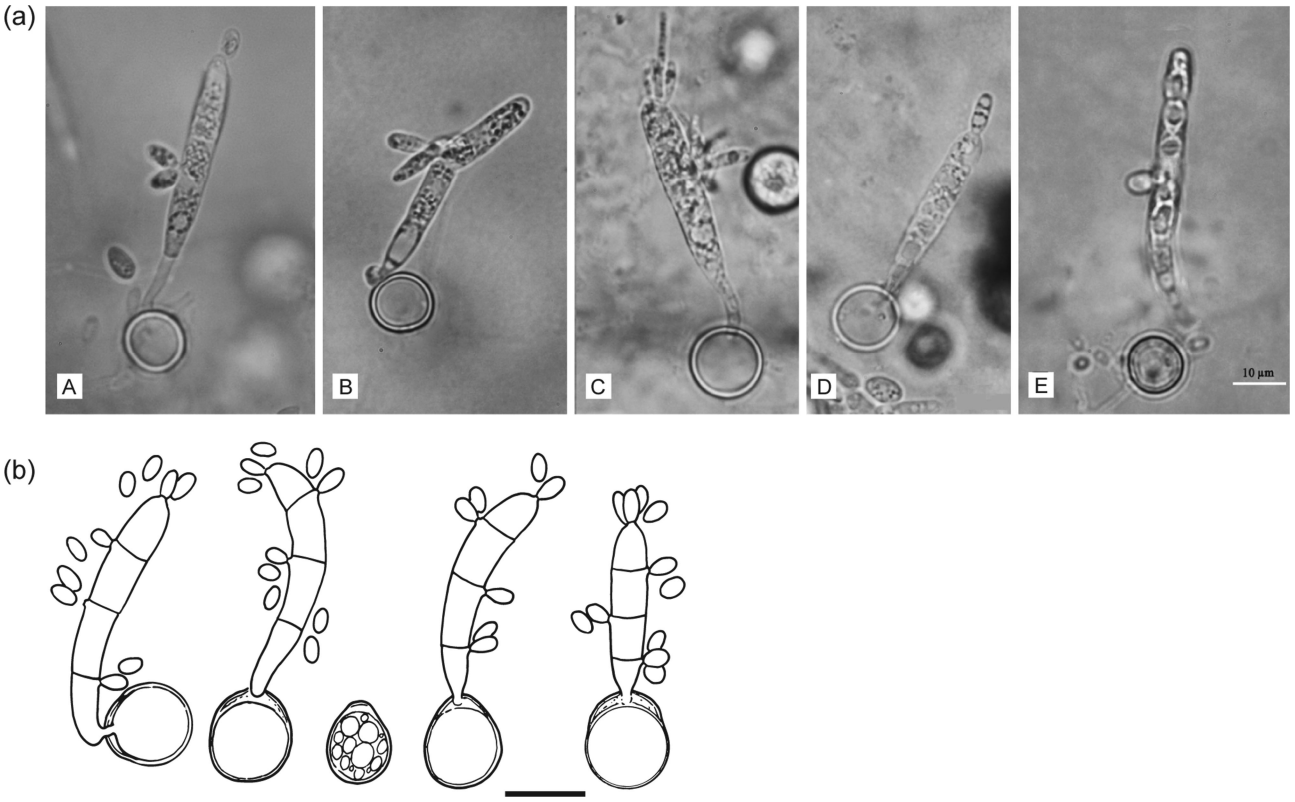

Figure 3. **(a)** Phase-contrast micrographs of germinated teliospores with transversally septate basidia and sessile basidiospores of: **(A)** *L. scottii* ANT 133 × *L. scottii* UFMG-ANT 139; **(B)** *L. scottii* UFMG-ANT 133 × *L. scottii* CBS 614; **(C)** *L. scotti* CBS 614 × *Le. yakutica* CBS 8248; **(D)** *L. scotii* CBS 5930^{T} × *Le. creatinivora* CRUB 1215; **(E)** *Le. creatinivora* DVBP 4794 × *Le. yakutica* CBS 8040. Bar: 10 μm. **(b)** Line drawing of germinated teliospores with transversally septate basidia and sessile basidiospores of *Le. creatinivora* CRUB 1214 × *Le. creatinivora* CRUB 1215. Bar: 10 μm.

Table 2. Percent mating success in inter and intra-species pairings in *L. scottii*, *Le. creatinivora* and *Le. yakutica*.

	MAT A1		
MAT A2	*Le. creatinivora* (4)	*Le. yakutica* (3)	*L. scottii* (9)
Le. creatinivora (4)	13% (2/16)	0% (0/12)	0% (0/36)
Le. yakutica (6)	17% (4/24)	22% (4/18)	38% (20/52)
L. scottii (7)	54% (13/24)	64% (9/14)	87% (52/60)

Numbers in brackets after species names refer to the number of strains used for the calculations, and numbers in brackets after percentages refer to absolute frequencies of positive pairings.

strain (CBS 8621^{T}), as well as JCM 10702, CBS 8248 and CBS 8040. With the exception of the latter cross, in these cases, the production of mycelium and teliospores was scarce and delayed. In *Le. creatinivora*, mating was observed when strains CRUB 1214 × CRUB 1215 and CRUB 1214 × DBVPG 4794 were crossed. Again, in these cases poor production of mycelium and teliospores was observed. Interspecies mating was observed mainly between subclades 2 (*Le. yakutica* MAT A2) and 5 (*L. scottii* MAT A1), which showed the highest rate of positive crosses (13 positive out of 16 combinations tested), followed by subclade 1 (*Le. creatinivora* MAT A1) and subclade 5 (*L. scottii* MAT A2) (13/24), and subclade 2 (*Le. yakutica* MAT A1) and 5 (*L. scottii* MAT A1) crosses (9/12). *Leucosporidiella creatinivora* (MAT A2) was the group that showed the lowest rate of crosses (Table 2). With a few exceptions (crosses involving *Le. yakutica* CBS 8248 or *L. scottii* CBS 5932), all interspecific crosses gave rise to poor production of mycelium and teliospores. A few cases are worth noting; for example, *L. scottii* UFMG-ANT 133, which was able to mate with *Le. creatinivora* strains CRUB 1215 and DVBPG 4794, also mated with two *Le. creatinivora* additional strains (CBS 9210 and CRUB 1166) that did not show sexual behavior with any other conspecific strain. In this case, the production of sexual structures (mycelium and teliospores) was scarce and restricted. Mating reactions among strains of the same subclade where not as frequent as expected for *Le. creatinivora* and *Le. yakutica* lineages given their values ranged 16–25%, while for *L. scottii* were much higher (50–100%) (data not shown).

Upon germination teliospores produced transversely septate basidia around which budding basidiospores and profuse cell development were observed (Figs 3 and 4). Basidia and basidiospore production was similar to that described for *L. scottii* by Statzell-Tallman and Fell (1998) and Sampaio *et al.* (2003). All strains used for mating experiments were checked for homothallic behavior. *Leucosporidium scottii* strains CBS 614 and CBS 5932, which had been previously regarded as self-fertile (Sampaio 2011a), did not behave as expected after five attempts but instead readily crossed with other conspecific strains (ex. CBS 5930^{T}). *Leucosporidium scottii* CBS 9965 was the only strain showing a homothallic sexual behavior with the development of scant mycelium and few teliospores after 2–3 month of incubation. These results could indicate that homothalism in this group of species is less frequent than previously assumed or that culture conditions needed for its occurrence are still not well known. Of the tested strains, only five strains did not show evidence of sexual activity in any of the above experiments, namely *L. scottii* CBS 8162, *Le. yakutica* PB 07 and *Le. creatinivora* strains CBS 8620^{T}, CSB 9305 and JCM 10700.

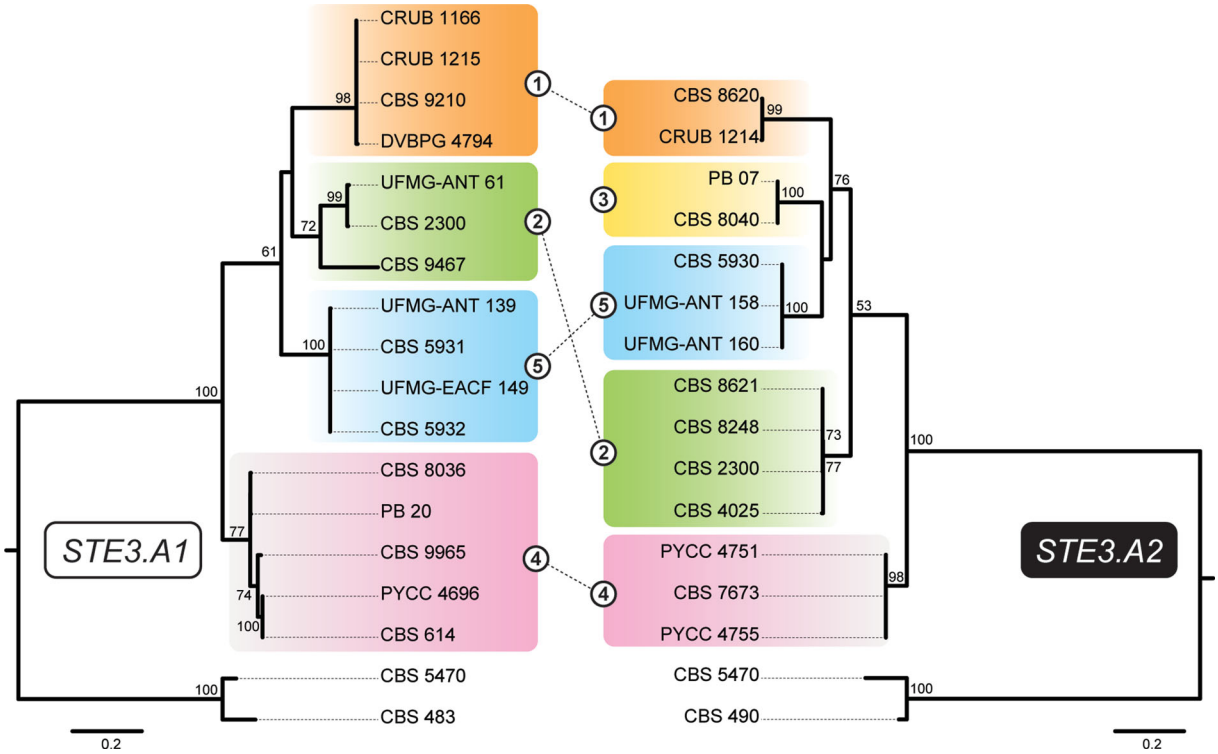

Figure 4. Phylogenetic tree of strains of *L. scottii*, *Le. creatinivora and Le yakutica* based on *STE3.A1* and *STE3.A2* alleles. Shade colors (orange, green, yellow, pink and blue) and numbers (in circles) indicate distinct phylogenetic subclades formed in Fig. 1.

In order to correlate the results of the mating tests with the molecular identification of the mating type, we investigated the presence of the mating pheromone receptor genes (*STE3.A1* or *STE3.A2*) in representative strains of the three species. To that end, we employed a PCR-based approach that specifically amplified *STE3.A1* or *STE3.A2* alleles. In all cases, the detection of *STE3.A1* and *STE3.A2* alleles was found to be perfectly correlated with the results of sexual crosses observed in this study (Fig. 4 and Table S1, Supporting Information), in line with the observations from other yeast species in the Pucciniomycotina lineage (Devier *et al.* 2009; Coelho, Gonçalves and Sampaio 2011). Interestingly, we did not find evidence for the existence of a strong mating type imbalance within the different groups since both alleles were always recovered. Strains CBS 2300 and CBS 9490 were found to carry both pheromone receptors, and the former was mating competent only when the mating partner was A2.

To shed light on the evolutionary history of the pheromone receptor genes in this group, we used partial gene sequences of *STE3.A1* and *STE3.A2* alleles obtained from several strains, some of which were not used in mating experiments (i.e. CBS 8036, PYCC 4696, CBS 4025, PYCC 4751 and PYCC 4755). Sequences from two red yeast species *S. salmonicolor* and *S. johnsonii* were also included given that several lines of evidence suggest that these two species are undergoing speciation although pre-zygotic barriers are not yet fully established (Valério, Gadanho and Sampaio 2008; Coelho, Sampaio and Gonçalves 2010; Coelho, Gonçalves and Sampaio 2011). The resulting *STE3* phylogenetic trees (Fig. 4) were not completely congruent among each other, although both showed the same *L. scottii* subclade as the basal lineage; a result in agreement with the parsimony network analysis (Fig. 2). The topologies of the trees were also different from the combined ITS and D1/D2 tree (Fig. 1). Notably, the divergence observed between *STE3* alleles of the different subclades was even higher than that found between *S. salmonicolor* and *S. johnsonii* (Fig. 4). Despite the divergence of *STE3.A1* and *STE3.A2* sequences between strains of *L. scottii*, *Le. creatinivora* and *Le. yakutica*, the fact that they are still able to mate suggests a 'functional plasticity' of the pheromone receptor system, a feature that was recently proposed to favor interspecific hybridizations in smut fungi (Kellner *et al.* 2011). The observation that pheromone precursor genes of the closely related red yeast species *S. salmonicolor* and *S. johnsonii* seem to encode identical peptides, possibly precluding pre-zygotic discrimination between these species, is also in line with this (Coelho, Gonçalves and Sampaio 2011).

Pheromone receptor genes proved to be advantageously used as markers for mating type at the molecular level across a broad range of red yeast species (Coelho, Gonçalves and Sampaio 2011). Our current work indicates that this might also be true for species within the Leucosporidiales. Nevertheless, since *L. scottii* seems to have a bifactorial or tetrapolar system, the identification of the *HD1/HD2* genes and their distribution among the studied species will be required as to fully define the mating type of a given strain.

In summary, we confirmed that *L. scottii*, *Le. creatinivora* and *Le. yakutica* are very closely related, with less than 1% divergence in the rDNA. Sequence-based delimitation of species is not clear, mainly for *L. scottii* and *Le. yakutica*, given that a paraphyletic structure is obtained regardless of the phylogenetic inference method applied. Furthermore, parsimony networks, which contemplate reticulate events during evolution, suggest that strains of the three currently recognized species could be viewed as members of a single evolutionary population. In line with this, our attempts to observe interspecies mating have demonstrated

that all three species are sexual since when compatible strains are crossed mating occurs. We also obtained evidence that genetic differentiation is taking place. The combined molecular approaches used in this work (MSP-PCR fingerprinting, rRNA and mating type genes phylogenetic analyses) allowed a better view of the genetic structure within the three species and permitted the discrimination of five main groups. The evolutionary relationships between such groups are not clear since different tree topologies were obtained with each molecular marker. Furthermore, evidence for the existence of incipient pre-zygotic reproductive barriers arises from the fact that the occurrence of interspecific crosses was less frequent than the intraspecific ones and, when achieved, the sexual structures produced were much less conspicuous than when compared with those of intraspecific mating. In this work, we were not able to assess the presence of post-zygotic reproductive barriers since the fitness and sexual capabilities of the interspecific offspring was not analyzed. However, based on the frequency of mating success and the extent of sexual structures produced (mycelium and teliospores) the degree of reproductive isolation within the three species could be estimated (Fig. 2 and Table 2). Within *scottii* lineages (subclades 4 and 5), the maximum ability to mate (76%) and the largest production of sexual structures was observed. All other combinations of subclades gave lower frequency of mating success (12.5–54%) and typically poor production of sexual structures (Fig. 2). These results indicate that despite a single exception, most studied species have difficulties to interbreed, suggesting that significant reproductive barriers exist among these.

Our results are in agreement with the previous work of Suh, Kuroiwa and Sugiyama (1993), which found three main groups of *L. scottii* strains on the basis of their nuclear DNA (nDNA) contents, and the major ubiquinone system (group 1 was not considered given it comprised a single strain which in the end was not *L. scottii*). Eight of these strains were also analyzed here and with the exception of CBS 5931^T, mating and grouping results were concordant. The strains of their group 2 belong to *Le. yakutica* (our groups 2 and 3), showed the lower nDNA content and shared with their group 3 (our *L. scottii* group 4) the ubiquinone Q10. The other ubiquinone found (Q9) was exclusive of their group 4 (our *L. scottii* group 5) which also showed the larger amount of DNA content. Based on nDNA results, the authors suggested the existence of aneuploidy strains within the group 2 (actually *Le. yakutica*). Aneuploidy may result in abnormal meiotic products, which would frequently impair mating and thus reducing mating success. This could represent an explanation to the overall lower sexual activity observed of *Le. yakutica* with respect to *L. scottii*. Unfortunately, equivalent information is missing for *Le. creatinivora* which in general showed even lower mating success.

It should be noted that the observation of mating and production of hyphae or even the germination of basidia are not sufficient proof of actual recombination, and could be that hybrid basidiospores are produced by hybridization process. However, the fact that all interspecific crosses gave rise to poor production of both mycelium and teliospores is a further indication that most likely post-zygotic isolation mechanisms are at work to keep species separate.

Taking together, our results possibly indicate a case of speciation in progress. Species delineation of very recently separated species is expected to be difficult given that a high degree of genetic relatedness is conserved (i.e. highly similar DNA sequences) and at the same time a residual ability to form hybrids with various degrees of offspring fertility is exhibited (Lachance and Fedor 2014). Examples of the latter have been previously reported for basidiomycetes and ascomycetes yeasts (Valério, Gadanho and Sampaio 2008; Lachance and Fedor 2014).

Therefore, taking into consideration that the data gathered so far do not point unequivocally to a single, continuous and close unit, we restrain from merging *Le. creatinivora* and *Le. yakutica* into *L. scottii*, and consider, instead, that they represent a species complex, with very similar phenotypes with unclear or cryptic species delimitations. We anticipate that future studies employing genome-based approaches will contribute to improve species delineation in this case.

Taxonomic reorganization at the genus level

In order to implement the elimination of the dual nomenclature for anamorphs and teleomorphs as advocated by McNeill *et al.* (2012) and Lachance and Kurtzman (2013), several asexual species have been reassigned to sexual genera being however referred to as *forma asexualis* (*f.a.*). Accordingly, and in order to standardize the species nomenclature within the order Leucosporidiales, we propose the reassignment of all anamorphic *Leucosporidiella* species to *Leucosporidium* as *forma asexualis*. *Leucosporidium creatinivorum* (Golubev) V. de Garcia, M. A. Coelho, T. Maia, L. H. Rosa, A. M. Vaz, C. A. Rosa, J. P. Sampaio, P. Gonçalves, M. R. van Broock and D. Libkind *f.a.* comb. nov. MycoBank No.: MB: 811712

Basionym: *Rhodotorula creatinivora* (Golubev 2011); MB: 373247; Synonym: *Leucosporidiella creatinivora* (Golubev) J. P. Sampaio; MB: 372908.

Leucosporidium fragarium (J.A. Barnett and Buhagiar) V. de Garcia, M. A. Coelho, T. Maia, L. H. Rosa, A. M. Vaz, C. A. Rosa, J. P. Sampaio, P. Gonçalves, M. R. van Broock and D. Libkind *f.a.* comb. nov. MycoBank No.: MB 810768.

Basionym: *Torulopsis fragaria* (Barnett and Buhagiar 1971); MB 324755. Synonym: *Leucosporidiella fragaria* (J. A. Barnett and Buhagiar) J. P. Sampaio; MB 372911.

Leucosporidium muscorum (Di Menna) V. de Garcia, M. A. Coelho, T. Maia, L. H. Rosa, A. M. Vaz, C. A. Rosa, J. P. Sampaio, P. Gonçalves, M. R. van Broock and D. Libkind *f.a.* comb. nov. MycoBank No.: MB 810769.

Basionym: *Candida muscorum* (Di Menna 1958); MB 294036. Synonym: *Leucosporidiella muscorum* (Di Menna) J. P. Sampaio; MB 372913.

Leucosporidium yakuticum (Golubev) V. de Garcia, M. A. Coelho, T. Maia, L. H. Rosa, A. M. Vaz, C. A. Rosa, J. P. Sampaio, P. Gonçalves, M. R. van Broock and D. Libkind *f.a.* comb. nov. MycoBank No.: 811713.

Basionym: *Rhodotorula yakutica* (Golubev 2011); MB: 456475; Synonym: *Leucosporidiella yakutica* (Golubev) J. P. Sampaio; MB: 372914.

Mastigobasidium intermedium, the single species of the genus *Mastigobasidium*, is phylogenetically very close to *Leucosporidium* and *Leucosporidiella* and is the only species of the Leucosporidiales that forms ballistoconidia and in which multiple basidia originate from a single teliospore (Sampaio *et al.* 2003; Golubev 2011). Such differences seem not sufficient to maintain the species in a separate genus and therefore, based on phylogenetic data, we propose to merge *Mastigobasidium* with *Leucosporidium*.

Leucosporidium intermedium (Golubev) V. de Garcia, M. A. Coelho, T. Maia, L. H. Rosa, A. M. Vaz, C. A. Rosa, J. P. Sampaio, P. Gonçalves, M. R. van Broock and D. Libkind *f.a.* comb. nov. MycoBank No.: MB 810770.

Basionym: *Bullera intermedia* (Nakase and Suzuki 1986); MB 133433. Synonym: *Bensingtonia intermedia* (Nakase and Suzuki)

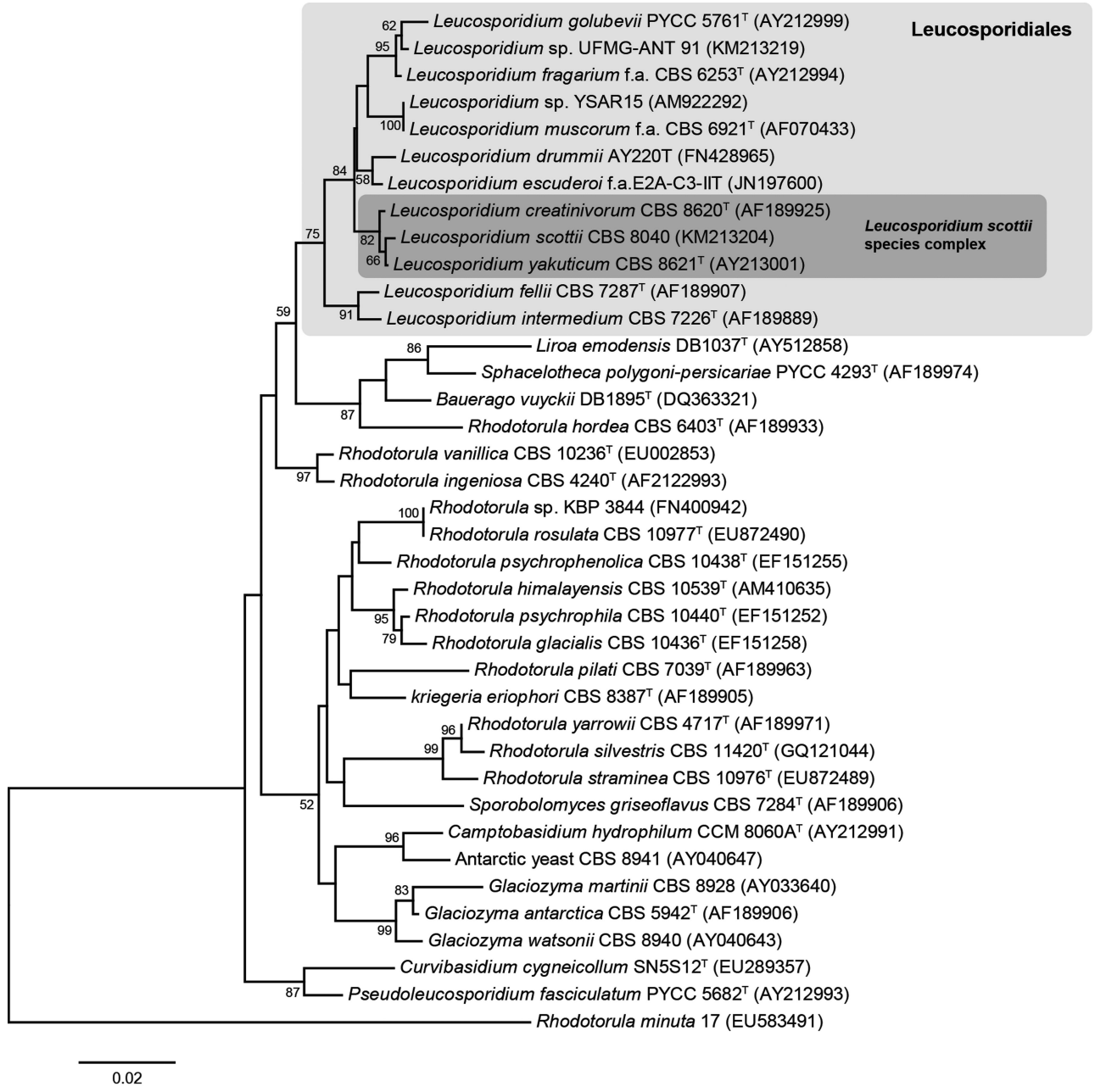

Figure 5. Phylogenetic tree of Leucosporidiales based on the ITS and D1/D2 LSU rRNA sequences. Bar, substitutions accumulated every 100 nucleotides. Bootstrap values higher than 50% are shown (1000 replicates). ^{T}: Type strain.

T. Nakase and T. Boekhout; MB 125785; *Sporobolomyces intermedius* (Nakase and Suzuki) T. Nakase and M. Suzuki; MB 133438; *Mastigobasidium intermedium* (Golubev) Golubev V. I.; MB 460068.

Based on the taxonomic changes proposed in the present paper that lead to an increase in the number of species included in the genus *Leucosporidium* (from five to eight), we modify accordingly the genus description. The phylogenetic analysis of all species of the order Leucosporidiales is shown in Fig. 5. Diagnosis of the genus *Leucosporidium* (Fell *et al.* 1969) Subphylum Pucciniomycotina, Class Microbotryomycetes, Order Leucosporidiales (Bauer *et al.* 2006) Species of this genus reproduce asexually by budding, cells are generally ovoid, ellipsoid or elongate, and ballistoconidia may be present. Cultures are smooth or delicately wrinkled and whitish, cream or yellowish-gray cream colored, and butyrous or mucoid; red, pink or orange pigments are not synthesized. Mycosporines are not produced. Pseudohyphae or true hyphae may be present. Sexual species are normally heterothallic but homothallic species occur, hyphae have clamp connections, and form globose teliospores. Teliospores germinate to produce transversely septate basidia. Basidiospores are ovoid, ellipsoid or bacilliform, form laterally or terminally in the basidia and are sessile or arise on pegs and are passively released. Basidiospores occur singly or in clusters and germinate by budding. Hyphal septa have 'simple' septal pores i.e. central pores with the cell wall attenuating towards the pore. Colacosomes (lenticular bodies) may be

present. Species of this genus do not ferment glucose, do not assimilate myo-inositol and do not produce amyloid compounds. D-glucuronate is assimilated and nitrate is utilized as sole source of nitrogen. The DBB reaction and production of urease are positive. Xylose is lacking in cell hydrolysates. Major coenzymes are CoQ-9 and CoQ-10. *Leucosporidium fasciculatum* is not related to *L. scottii* and does not belong to the order Leucosporidiales, it is in fact the closest relative of *Curvibasidium*; however, this species was not included in this genus due to considerably distinct structural morphologies. *Leucosporidium fasciculatum* differs from *Curvibasidium* spp. in the absence of clamp connections and the production of septate basidia, among other characteristics. Thus, and to avoid the polyphyletic nature of the genus *Leucosporidium,* we propose a new genus *Pseudoleucosporidium* to accommodate *L. fasciculatum* and a new combination: *Pseudoleucosporidium fasciculatum*. Description of *Pseudoleucosporidium* V. de Garcia, M. A. Coelho, T. Maia, L. H. Rosa, A. M. Vaz, C. A. Rosa, J. P. Sampaio, P. Gonçalves, M. R. van Broock and D. Libkind Gen. Nov. (MB 811849).Subphylum Pucciniomycotina, Class MicrobotryomycetesAsexual reproduction is by polar budding, cells are fusiform. Cultures are butyrous, smooth, semiglistening and the margin has a fringe of hyphae; red, pink or orange pigments are not synthesized. True hyphae and pseudohyphae are formed. The only known species is self-fertile, true hyphae devoid of clamp connections are present. The teliospores are large, spherical or pyriform, and occur terminally or intercalary. Basidia are stalked, transversely septate with four compartments. The basidiospores are bacilliform, and germinate by budding. Species of this genus do not ferment glucose, do not assimilate myo-inositol and do not produce amyloid compounds. D-glucuronate is not assimilated and nitrate is not utilized as sole source of nitrogen. The DBB reaction and production of urease are positive. *Pseudoleucosporidium fasciculatum* V. de Garcia, M. A. Coelho, T. Maia, L. H. Rosa, A. M. Vaz, C. A. Rosa, J. P. Sampaio, P. Gonçalves, M. R. van Broock and D. Libkind comb. nov. MycoBank No.: MB 811850.Basionym: *Leucosporidium fasciculatum* (Bab′eva and Lisichkina 2000) MB: 483153.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSYR online.

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Conflict of interest. None declared.

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