Inopinatum lactosum gen. & comb. nov., the first yeast-like fungus in Leotiomycetes

Danny Haelewaters1,*, Robyn A. Peterson2, Helena Nevalainen2 and M. Catherine Aime1,*

Abstract

Sporobolomyces lactosus is a pink yeast-like fungus that is not congeneric with other members of Sporobolomyces (Basidiomycota, Microbotryomycetes, Sporidiobolales). During our ongoing studies of pink yeasts we determined that S. lactosus was most closely related to Pseudeurotium zonatum (Ascomycota, Leotiomycetes, Thelebolales). A molecular phylogenetic analysis using sequences of the ITS region and the small and large subunit (SSU, LSU) rRNA genes, indicated that four isolates of S. lactosus, including three ex-type isolates, were placed in Thelebolales with maximum support. A new genus is proposed to accommodate S. lactosus, Inopinatum. This is the first pink yeast reported in Leotiomycetes.

Pink-pigmented yeasts in the order Sporidiobolales (Basidiomycota, Pucciniomycotina, Microbotryomycetes) produce lipid droplets with carotenoid pigments – mostly β-carotene and torulene – contributing to the pink to orange-red colour of colonies [1–3]. These pigments are thought to offer antimicrobial, anticancer, and anti-ageing activities and to protect against radiation [4, 5]. Because of these characteristics, pink-pigmented yeasts have gained interest from pharmaceutical, cosmetics, and biotechnology industries [6–8]. The pink yeasts were historically placed in two anamorphic basidiomycete genera, Rhodotorula and Sporobolomyces. In their traditional sense, both these asexual genera are polyphyletic, occurring in all three subphyla and several classes and orders of Basidiomycota [9–11].

Following the elimination of the use of dual naming systems for asexual and sexual morphs of fungi, Rhodotorula and Sporobolomyces are now retained only for those species within Sporidiobolales [12]. Efforts to reassign many of the species once classified into Rhodotorula and Sporobolomyces into natural genera are ongoing [8, 12, 13]. At present, the order Sporidiobolales is estimated at ca. 22 species [8, 14, 15]. These are reported from diverse habitats including freshwater and marine ecosystems, fruit must, surfaces of buildings, food, soil, air, and—the most common habitat from which they are isolated—leaf surfaces [3, 15–22].

During our studies of pink yeasts in the genus Sporobolomyces, we noted that the internal transcribed spacer (ITS) barcode sequence of Sporobolomyces lactosus [23] was not similar to other species in the genus. Moreover, S. lactosus is not treated in Kurtzman et al. [24]. A general Nucleotide blast search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of S. lactosus against ex-type sequences of all fungi resulted in Pseudeurotium zonatum CBS 329.36T (Ascomycota, Leotiomycetes, Thelebolales) as the closest match with 90.84% shared identity. An ex-type culture of S. lactosus was obtained from the Culture Collection of Yeasts (CCY:19-21-1T) [23] at the Slovak Academy of Sciences (Bratislava, Slovakia). Here we present the results of our phylogenetic analyses of S. lactosus and formally describe Inopinatum gen. nov. to accommodate it in the Thelebolaceae (Thelebolales), as the first known yeast-like species in the Leotiomycetes.

Author affiliations: 1 Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907, USA; 2 Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia. *Correspondence: M. Catherine Aime, maime@purdue.edu; Danny Haelewaters, danny.haelewaters@gmail.com

Keywords: ascomycetous yeast; pink yeast; Sporobolomyces lactosus; taxonomy.

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit rRNA gene; ML, maximum likelihood; nt, nucleotides; PDA, potato dextrose agar; SSU, small subunit rRNA gene.

The GenBank/EMBL/DDBJ accession numbers of the SSU and ITS sequences of Inopinatum lactosum gen. & comb. nov. CCY 19-21-1=JCM 8510 are AB021676 and AB038132. The GenBank/EMBL/DDBJ accession number of the ITS sequence of l. lactosum C4 is EU551181. The GenBank/EMBL/DDBJ accession numbers of the newly generated SSU, ITS, and LSU sequences of l. lactosum CCY 19-21-1T are MW471137, MW471138 (SSU), MW471139, MW471140 (ITS), MW471141, and MW471142 (LSU). The MycoBank accession numbers are MB835917 for Inopinatum gen. nov. and MB835918 for J. lactosum comb. nov. The aligned three-locus dataset used for ML phylogenetic inference is available from the figshare online repository with the URL https://doi.org/10.6084/m9.figshare.12495878.
Sporobolomyces lactosus, CCY:19-21-1 T, (Poland: Warsaw, Plock Refinery sewage treatment plant) [23], was grown on potato dextrose agar (PDA) with 2% agar, supplemented with 50 µg ml⁻¹ chloramphenicol and 100 µg ml⁻¹ ampicillin (BD, Franklin Lakes, New Jersey) to inhibit bacterial growth. Samples were removed for DNA isolation by using a J-hook to superficially scrape off pieces of fungal tissue. DNA was extracted using the Wizard Genomic DNA Purification kit (Promega Co., Madison, Wisconsin), following the manufacturer’s instructions. Next, we amplified the internal transcribed spacer, and nuclear small and large subunits of the ribosomal DNA repeat (ITS, SSU, and LSU, respectively). Primer combinations used were NS1/NS4 for SSU [25], ITS1f/ITS4 for ITS [25, 26], and LR0R/LR5 and LR0R/LR7 for LSU [27, 28]. PCR reactions consisted of 12.5 µl of Promega 2×PCR Master Mix, 1.25 µl of each 10 µm primer, 9.0 µl of H₂O, and 1.0 µl of template DNA. All amplifications were done in an Eppendorf Mastercycler ep Thermal Cycler (Hauppauge, New York, USA) for 30 cycles (1 minute at 94 °C, 1 minute at 50 °C, and 2 minutes at 72 °C). The PCR products were sequenced using the Sanger technique in the sequencing facility of the Molecular Biology and Biotechnology group of the Karolinska Institute, Sweden. Sequences were deposited in GenBank (Table 1).

Table 1. Sequences of Thelebolales used in phylogenetic analysis

<table>
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<tr>
<th>Species</th>
<th>Isolate/strain</th>
<th>SSU</th>
<th>ITS</th>
<th>LSU</th>
<th>Reference(s)</th>
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<td>NR_164245</td>
<td>NA</td>
<td>[62]</td>
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<td>Antarcomyces psychrotrophicus*</td>
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<td>NA</td>
<td>AJ133431</td>
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<td>[69]</td>
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<td>AB038132</td>
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<td>–</td>
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<td>NA</td>
<td>EU551181</td>
<td>NA</td>
<td>[64]</td>
</tr>
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<td>D. Haelew. F- 3088 (ex-CCY 19-21-1 T)</td>
<td>MW471137</td>
<td>MW471139</td>
<td>MW471141</td>
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</tr>
<tr>
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<td>MW471140</td>
<td>MW471142</td>
<td>This study</td>
</tr>
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<td>NR_164284</td>
<td>MH871738</td>
<td>[70]</td>
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<td>KJ755499</td>
<td>FJ176866</td>
<td>[71–73]</td>
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<td>KT225524</td>
<td>AY544680</td>
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<td>MH752062</td>
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<td>KF049206</td>
<td>FJ176884</td>
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<td>[78]</td>
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<td>JX270336</td>
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<td>[80]</td>
</tr>
<tr>
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</tr>
<tr>
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<td>AB015778</td>
<td>NR_165894</td>
<td>MH870271</td>
<td>[70, 74]</td>
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<td>Ramgea ozimeci</td>
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<td>NA</td>
<td>NR_164248</td>
<td>KY368753</td>
<td>[82]</td>
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<tr>
<td>Thelebolus balaustriformis</td>
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<td>NR_159056</td>
<td>NG_067559</td>
<td>[83]</td>
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<td>NG_062682</td>
<td>NR_138367</td>
<td>NG_067263</td>
<td>[60, 73]</td>
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<td>MH859396</td>
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<tr>
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<td>JGI Genome</td>
<td>NA</td>
<td>JGI genome</td>
<td>NA</td>
<td>–</td>
</tr>
</tbody>
</table>

*, Type species; †, ex-type; NA, Not available.
York) under the same cycling conditions as in Haelewaters et al. [13].

Maximum likelihood (ML) analyses were done using IQ-TREE [29], on a multi-locus dataset of all three amplified loci. Representative sequences for all genera in Thelebolales were downloaded from NCBI GenBank (Table 1). Sequences for each locus were aligned using muscle [30] available from the Cipres Science Gateway [31], and then trimmed using the command-line version of TrimAl 1.3 [32] with gap threshold of 0.6 and minimal coverage of 0.5. Substitution models were selected using ModelFinder [33] by considering the Akaike Information Criterion corrected for small sample size (AICc): TN+F+G4 for SSU (-lnL=2007.697), TIM2e+R2 for ITS (-lnL=2411.356), and TIM3 +F+R2 for LSU (-lnL=2300.016). ML was inferred for the concatenated SSU–ITS–LSU dataset under partitioned models, with rapid bootstrapping under 1000 replicates [34, 35].

Bayesian analyses were done using a Markov chain Monte Carlo (MCMC) approach implemented in the BEAST package [36], with a strict clock assuming a constant rate of evolution.
across the tree and a Yule Speciation tree prior [37, 38]. The nucleotide substitution models, as determined as by jModelTest 2.1.6 [39] under AICc, were as follows: HKY+G for SSU (lnL=-2008.6823), TIM2ef+G for ITS (lnL=-2406.8675), and TrN +I+G for LSU (lnL=-2300.4287). Two runs were undertaken from a random starting tree for 40 million generations, with a sampling frequency of 4000. Tracer 1.6 [40] was used to check MCMC trace plots. After removed of 10% as burn-in, trees files were combined, consensus trees were generated (with 0% burn-in), and the Maximum Clade Credibility (MCC) tree was inferred with the higher product of individual clade posterior probabilities (pp).

Intra- and interspecific divergence in the ITS and LSU regions was calculated using the Compute Pairwise Distances function in mega7 [41] with model/method set at ‘No. of differences’, gaps/missing data treatment set at ‘pairwise deletion’, and default settings for other parameters. The aligned, trimmed ITS sequences of our two ex- CCY 19-21-1 T isolates and of ex-type strain JCM 8510 were 100% identical. Isolate C4 differed in its ITS in four nucleotides (nt), followed by Pseudeurotium zonatum CBS 329.36 T =AFTOL-ID 1912 T with 42 nt differences in the ITS. The two ex-CCY 19-21-1 T isolates also shared 100% identity in their LSU sequences. The isolate from our dataset with least nt differences in the LSU region was Thelebolus balaustiformis MUT 2357 T (28 nt), followed by Leuconeurospora pulcherrima AFTOL-ID 1397 (29 nt), and Cleistothelebolus nipigonensis CBS 778.70 T and Pseudeurotium zonatum CBS 329.36 T (both 30 nt).

The phylogenetic reconstruction of Thelebolales based on the concatenated three-locus dataset is shown in Fig. 1. Crinula caliciformis and Holwaya mucida (Leotiomycetes incertae sedis) were chosen as outgroup taxa. All included genera except Ramgea were placed in either Pseudeurotiaceae or Thelebolaceae as currently accepted [42–44]. In our three-locus phylogenetic reconstruction, Ramgea ozimecii CNF 2/9997 T was retrieved as the earliest diverging clade in the order with maximum support, resulting in a paraphyletic family Thelebolaceae. Inopinatum lactosum gen. and comb. nov. was maximally supported as sister to other members of Thelebolaceae.

Leotiomycetes are a diverse class within subphylum Pezizomycotina [44, 45] comprising ca. 6500 described species in 630 genera. These fungi are often found as major components of environmental samples. Nonetheless, many taxa remain unnamed or incertae sedis within the class. Leotiomycetes species appear to be predominantly saprotrophic and parasitic, including economically and ecologically important pathogens such as the powdery mildews (Erysiphaceae) and the causal agent of white-nose syndrome in bats (Pseudogymnoascus destructans, only known from its asexual morph) [43, 44]. Other species, however, are mycorrhizal mutualists (ectomycorrhizae and ericoid mycorrhizae) and plant endophytes [46–49].

Ascomycetous yeasts and yeast-like taxa are primarily found in the subphyla Saccharomycotina (Saccharomycetes) and Taphrinomycotina (Neolectomycetes, Pneumocystomycetes, Schizosaccharomycetes, Taphrinomycetes) [24], but have also been revealed in other lineages: Arthoniomycetes, Dothideomycetes, Eurotiomycetes, Xylonomycetes (subphylum Pezizomycotina), and Gemmulina (Ascomycota incertae sedis) [50–56]. Only recently, the black yeast genus Phaeococcomyces was placed in a newly erected order Lichenostigmatales (Arthoniomycetes) along with taxa forming colonies of stromatic ascomata or conidiomata (Etayoa, Lichenostigma)—a lineage that is unique within this class, which is otherwise composed primarily of lichenized species [51].

Inopinatum lactosum is the first described yeast-like fungus in Leotiomycetes, adding to the body of work that is expanding the known morphological and ecological diversity in the class. For example, the perithecoid apothecial Annabellula australiensis (Leotiomycetes, Cordieritidaceae) was recently discovered from mangrove wood [57], a habitat that is otherwise mostly populated by Dothideomycetes and Sordariomycetes [58]. Inopinatum is placed in Thelebolaceae. This family includes many coprophilic and psychrophilic species [59–62] and, based on

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**Fig. 2.** Inopinatum lactosum gen. and comb. nov. Strain C4 from [64], growth on PDA supplemented with chloramphenicol (100 mg l⁻¹) and ampicillin (100 mg l⁻¹), after incubation of 5–7 days. (a) Colony, with (b) detail of colony with thick and ‘veiny’ undulating margin. (c) Vegetative cells, arrow pointing at daughter blastoconidium connected to its mother cell. Bar, 10 µm.
genomic-scale data, was recently determined to be the sister to Pseudourotiaceae—the order that contains the asexual fungus *P. destructans* [44, 63]. Although little is known of the ecology of *I. lactosum*, a coprophilous habit is likely given its isolation from animal faeces [64] and petrochemical wastewater [23].

*Inopinatum lactosum* is represented in NCBI GenBank by SSU, ITS, and LSU sequences of two ex-CCY 19-21-1 isolates (this study); SSU, ITS, LSU, and cytochrome b (cytb) sequences of strain JCM 8510 (unpublished data); and an ITS sequence of isolate C4 [64], which shares 99.01% identity with the ex-type sequences. The C4 isolate was screened for production of enzymes on agar plates containing different substrates. Protease, amylase, mannanase, and variable xylanase activity was observed at 25°C, while at 15°C and 39°C all enzymatic activity was either variable or absent [64].

**DESCRIPTION OF INOPINATUM HAELEW. & AIME, GEN. NOV.**

*Inopinatum* (Latin, meaning ‘unexpected’ and referring to the unexpected placement of this pink yeast genus in Leotiomycetes) MycoBank number: MB835917.

Type species: *Inopinatum lactosum* (E. Sláviková and Grab.-Lon.) Haelew. and Aime

Description: Yeast-like fungi belonging to Theobolaceae (Theobolales, Leotiomycetes). Teleomorph unknown. Anamorph pink-pigmented, forming pseudohyphae and hyphae; blastocnidia bilaterally symmetrical; no known fermentation. Isolated from animal faeces and wastewater.

*Inopinatum lactosum* (E. Sláviková and Grab.-Lon.) Haelew. and Aime, comb. nov. MycoBank number: MB835918. Fig. 2.


*Inopinatum lactosum* forms pink, glistening, ropey colonies on PDA (Fig. 2a). The colony margin is coarsely fimbriate, with a ‘veiny’ appearance (Fig. 2b) reminiscent of growth of some *Aureobasidium* Viala and G. Boyer and *Kabatiella* Bubák species in culture [65, 66]. Whereas *Aureobasidium* cultures become black with time, *I. lactosum* retains its pink pigmentation (Fig. 2a). Growth is dimorphic, producing short chains of pseudohyphae and a few true hyphae near margins, and blastoconidia from older growth in the center (Fig. 2c). CCY 19-21-1, the holotype strain of *I. lactosum*, was isolated from an activated sludge in Poland [23]. The conidia were described as ballistoconidia in the protologue but are blastoconidia [67], analogous to *Aureobasidium* [68]. The C4 strain was isolated from koala faeces [64], a habitat that is consistent with that of other members of Thelebolaceae that are mainly known from dung [59]. The holotype is CCY 19-21-1, from petrochemical wastewater in Warsaw, Poland, and is permanently preserved in a metabolically inactive state in the Culture Collection of Yeasts, Bratislava, Slovakia. Ex-type cultures are preserved as JCM 8510 and JCM 10082 in the Japan Collection of Microorganisms, Tsukuba, Japan; and as NGYC 2618 in the National Collection of Yeast Cultures, Norwich, UK.

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**Author contributions**

Conceptualization, Formal Analysis, Visualization, Writing — original draft: D.H. Methodology: D.H. and M.C.A.; Writing — review and editing: D.H., R.A.P., K.M.H.N., and M.C.A.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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