Heitmania gen. nov., a new yeast genus in Microbotryomycetes, and description of three novel species: Heitmania litseae sp. nov., Heitmania castanopsis sp. nov. and Heitmania elacocarpi sp. nov.

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Abstract

Nine anamorphic yeast strains isolated from various plant leaves collected in southern China were phylogenetically characterized based on sequences of the internal transcribed spacer (ITS) region, the D1/D2 domains of the large subunit (LSU) rRNA gene, the small subunit (SSU) rRNA gene, the two subunits of the RNA polymerase II gene (RPB1 and RPB2) and the translation elongation factor 1-α (TEF1). Phylogenetic analysis of the combined sequences of the six genes showed that the new strains formed a distinct clade in the class Microbotryomycetes but could not be assigned to any of the existing genera, families or orders of the class. Three separate groups were consistently resolved from the nine new strains based on the combined sequences of the six genes and single gene sequences of ITS, RPB1, RPB2 and TEF1. The results suggest that the nine yeast strains compared represent three novel species in a novel genus. The names Heitmania gen. nov. (Mycobank registration number MB819987), Heitmania litseae sp. nov. (MB820112, type strain CGMCC 2.5697®CBS 14756®), Heitmania castanopsis sp. nov. (MB819988, CGMCC 2.5698®CBS 14750®) and Heitmania elacocarpi sp. nov. (MB820113, CGMCC 2.5695®CBS 14752®) are proposed for the new taxa.

The class Microbotryomycetes was proposed based on the small- and large-subunit (SSU and LSU) rRNA gene sequence analysis and ultrastructural features for a group of simple-septate basidiomycetes in Pucciniomycotina [1]. This class harbours more than 200 described species with diversified morphological characters and ecological distribution, including mycoparasites, phytopathogens and putative saprotrophs which can inhabit the phylloplane, deep sea, bark, mushrooms and soil [2–4]. Many species contain colacosomes, which are special organelles usually associated with mycoparasitism [5–7]. The monophyletic nature of this class was confirmed by multiple gene sequence analyses and more than 110 yeast species were assigned to this class [4, 8]. At present four orders (Kriegeriales, Leuco sporidiales, Microbotryales and Sporidiobolales) containing yeast species and one order (Heterostigmatidae) consisting partly of mycoparasites have been proposed in this class [1–4, 8–10]. However, two other families (Chrysozymaceae and Colacogloeaceae), and 12 genera in the class could not be assigned to any higher ranks because of their phylogenetic ambiguity [4, 8]. Furthermore, a considerable number of the described genera are monotypic [3, 4, 8], implying that the diversity of this class remains to be fully revealed. The discovery of the hidden diversity and the addition of new taxa to the phylogenetic tree will also be helpful for a better understanding of the phylogeny of the taxa within the class, because the phylogenetic ambiguity is probably caused by insufficient sampling. During a survey of yeast diversity in the phylloplane in a subtropical, evergreen, broad-leaved forest in southern China, approximately 800 strains were isolated from 49 samples of plant leaves. Based on the sequence analyses of the ITS and LSU D1/D2 domain, these strains were classified into 52 known species of 31 genera. In addition, nine strains were found to represent three novel species belonging to a novel genus within the class Microbotryomycetes based on multiple gene sequence analysis and physiological and biochemical characterization.

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Keywords: Heitmania; Microbotryomycetes; yeast; novel genus; novel species.

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DBJ accession numbers for the ITS region, LSU rRNA gene D1/D2 domain, SSU rRNA gene, RPB1, RPB2 and TEF1 sequences determined in this study are KY450662-KY450670, MF163542-MF163550, KY614366-KY614374, KY614375-KY614382 and MF163551-MF163559.

Two supplementary tables and two supplementary figures are available with the online Supplementary Material.
The yeast strains studied are listed in Table 1. They were isolated from plant leaves collected on Gutian Mountain (29° 10' 19.44” – 29° 17' 41.4’” N, 118° 03' 49.77” – 118° 11’ 12.2” E; annual precipitation 1963.7 mm; annual average temperature 15.3 °C), Zhejiang Province, China in July and August 2010 using the improved ballistoconidia-fall method described by Nakase and Takashima [11]. The phenotypic and physiological characters were examined according to standard methods used in yeast taxonomy [12].

Genomic DNA was extracted from yeast cells that were actively growing on YPD medium following the protocol described by Makimura et al. [13]. The E.Z.N.A. Yeast DNA Kit (Omega Bio-tek, USA) was used when high-quality genomic DNA was required for PCR amplification of protein-coding genes. The ITS region (including the 5.8S rRNA gene) and D1/D2 domain of the LSU rRNA gene were amplified using the protocols described previously [14]. The SSU rRNA gene, two subunits of the RNA polymerase II gene (RPB1 and RPB2) and the translation elongation factor 1-α (TEF1) were amplified and sequenced according to Liu et al. [15].

Phylogenetic analyses were performed as described previously [4, 8] with modifications. Multiple sequence alignment was performed using MAFFT version 7 and the G-INS-I option [16]. Maximum likelihood (ML) and Bayesian analyses were conducted for separate and combined nucleotide sequences using RAxML v8.1.X [17] with 1000 bootstrap replicates and MrBayes 3.2.2 [18] with 5 million generations, respectively. The best-fit evolution model of separate and combined nucleotide sequences was analysed with jModeltest [19]. The TIM2 +I+G model was suggested as the best model for the ITS region, protein-coding genes RPB1 and RPB2, and the combined sequences. The TIM1 +I+G model was suggested as the best model for the protein-coding gene TEF1 sequences. Intra- and inter-group sequence divergences and the numbers of synonymous and non-synonymous substitutions in the protein coding genes were calculated using DnaSP v5.10 [20]. Phylogenetic network analysis was performed to further resolve relationships among the strains compared. Single-gene ML tree files were combined into a single file and analysed with SplitsTree 4.13.1 using the SuperNetwork algorithm (Z-closure method, tree-size-weighted mean, maximum dimension=4, number of runs=100) [21]. The GenBank accession numbers for the sequences of the ITS region, LSU rRNA gene D1/D2 domain, SSU rRNA gene, RPB1, RPB2 and TEF1 used in this study are listed in Table S1 (available with the online Supplementary Material).

**MOLECULAR PHYLOGENY**

A total of nine yeast strains with similar cream-coloured colonies isolated from various plant leaves (Table 1) were studied and their ITS and D1/D2 sequences determined. The BLAST search through GenBank against the rDNA sequences from the new strains did not find identical or similar sequences. The top matches were from taxa in the class Microbotryomycetes, including Bannozyyma yamatoana, Colacogloea diffiusus, Hamamotoa singularis, Phenolifera glacialis and Sampaiozyma ingeniosa and a few environmental sequences designated as uncultured fungal clones from soil. They showed 85% and 94% or less sequence identities with the new strains studied in the ITS region and D1/D2 domain, respectively. Because the environmental sequences were not closely related to the new strains, they were not included in subsequent phylogenetic analyses.

In the trees constructed respectively from the D1/D2 domain (Fig. S1a) and the ITS region (Fig. S1b), sequence data sets containing all the taxa in Microbotryomycetes as employed in Wang et al. [4, 8], newly described species [22] and a few undescribed species, the nine new strains formed a distinct clade. However, the phylogenetic position of the new clade was uncertain (Fig. S1a, b). In order to obtain a more robust topology and to definitely determine the phylogenetic relationships of these new strains with other taxa in Microbotryomycetes, four more genes including the SSU rRNA gene and three protein coding genes, RPB1, RPB2 and TEF1, were sequenced. Phylogenetic analyses were performed based on the concatenated sequences of these four genes together with the ITS and D1/D2 sequences from the new strains and type strains of described species representing all orders, families and genera in Microbotryomycetes.

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**Table 1. List of phyllosphere strains from Gutian Mountain, Zhejiang Province, China**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Other designation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heitmania litsea sp. nov.</td>
<td>GT-187t</td>
<td>CGMCC 2.5697, CBS 14756</td>
<td>Litsea coreana var. sinensis</td>
</tr>
<tr>
<td>Heitmania castanopsis sp. nov.</td>
<td>GT-158</td>
<td>CGMCC 2.5694, CBS 14754</td>
<td>Castanopsis eyrei</td>
</tr>
<tr>
<td></td>
<td>GT-185</td>
<td>CGMCC 2.5696, CBS 14757</td>
<td>Castanopsis tibetana</td>
</tr>
<tr>
<td></td>
<td>GT-342</td>
<td>CGMCC 2.5700, CBS 14758</td>
<td>Vaccinium bracteatum</td>
</tr>
<tr>
<td></td>
<td>GT-346</td>
<td>CGMCC 2.5701, CBS 14755</td>
<td>Castanopsis tibetana</td>
</tr>
<tr>
<td></td>
<td>GT-936</td>
<td>CGMCC 2.5702, CBS 14753</td>
<td>Litsea coreana var. sinensis</td>
</tr>
<tr>
<td>Heitmania castanopsis sp. nov.</td>
<td>GT-233t</td>
<td>CGMCC 2.5698, CBS 14750</td>
<td>Castanopsis tibetana</td>
</tr>
<tr>
<td></td>
<td>GT-257</td>
<td>CGMCC 2.5699, CBS 14751</td>
<td>Castanopsis tibetana</td>
</tr>
<tr>
<td>Heitmania elacocarpi sp. nov.</td>
<td>GT-160t</td>
<td>CGMCC 2.5695, CBS 14752</td>
<td>Elacocarpus japonicus</td>
</tr>
</tbody>
</table>
The phylogenetic relationships of the novel genus *Heitmania* with related taxa in Microbotryomycetes. The tree backbone was constructed using maximum-likelihood analysis of the combined sequences of the ITS region (including 5.8S rRNA), LSU rRNA D1/D2 domains, SSU rRNA gene, *RPB1, RPB2* and *TEF1* genes. Bootstrap percentages (BP) over 50% from 1000 replicates and posterior probabilities (PP) of Bayesian inference above 0.9 are shown respectively from left to right. NS, not supported (BP <50% or BP <0.9).
using the ML and Bayesian inference algorithms. The new strains formed a strongly supported clade neighbouring monotypic genera *Heteroagriostidium*, *Reniforma*, *Kriegeria* and *Libkindia*, and the two species of *Yunzhangia*. However, the phylogenetic relationships of the new clade with the described taxa were still not statistically supported (Fig. 1). This result suggests that the new strains represent a distinct genus which cannot be assigned to any existing order or family in the class *Microbotryomycetes* at present.

The nine strains possessed identical or similar D1/D2 sequences with no more than four nucleotide differences (Fig. S1a), however, they were clustered into three groups represented by strains GT-160$^T$ (one strain), GT-187$^T$ (six strains) and GT-233$^T$ (two strains), respectively, based on the combined sequences of the six genes sequenced (Fig. 1). The three groups were also concordantly resolved in the single gene trees drawn from the ITS, *RPB1*, *RPB2* and *TEF1* sequences (Fig. S1b–e), implying that recombination in these genes is lacking among the three groups and that reproductive isolation has been established among them. The result suggests that the three groups represent three separate species using 4 of 6 genealogies for the GT-187$^T$ group and the GT-233$^T$ group and 3 of 6 genealogies for the GT-160$^T$ group according to the genealogical concordance phylogenetic species recognition concept [23]. Although strain GT-160$^T$ was closely related to the GT-187$^T$ group and located basal to the latter in the ITS, *RPB1* and *RPB2* gene trees (Fig. S1b–d), it was located basal to the GT-233$^T$ group in the *TEF1* gene tree (Fig. S1e) with 75 % bootstrap and 1.0 posterior probability supports. Phylogenetic network analysis of the single-gene trees of the six genes also showed that the three groups were clearly separated (Fig. S2). The strong reticulation in the network of the GT-187$^T$ group was consistent with the incongruence of phylogenetic relationships among the strains in this group as resolved in different gene trees (Fig. S1b–e), suggesting frequent recombination among the strains in this group. In contrast, intergroup reticulation was rarely observed, supporting the reproductive isolation of the three groups from each other (Fig. S2).

Significant sequence differences between the three groups were observed in the ITS region and the three protein coding genes. The six strains within the GT-187$^T$ group and the two strains within the GT-233$^T$ group showed 98.8–100% and 99.8 % sequence identities, respectively, in the ITS region. In this region, these two groups differed from each other by 7–8 % mismatches. Similarly, strain GT-160$^T$ differed from the GT-233$^T$ group by 7 % mismatches in the ITS region. The data clearly support the distinction between the GT-187$^T$ and the GT-233$^T$ groups and between strain GT-160$^T$ and the GT-233$^T$ group at the species level.

In the ITS region, strain GT-160$^T$ differed from the strains in the GT-187$^T$ group by 3 % mismatches, being significantly higher than the maximum sequence difference (1.2 % mismatches) among the strains within this group. The protein coding genes sequenced showed greater sequence divergence than strain GT-160$^T$ and the GT-187$^T$ group (Table S2, Fig. S1c–e). The sequence differences among the strains within the GT-187$^T$ group were less than 3, 6 and 3 % substitutions in the *RPB1*, *RPB2* and *TEF1* genes, respectively; and no non-synonymous substitution was detected in the former two genes and two non-synonymous substitutions were observed in *TEF1*. However, strain GT-160$^T$ differed from the GT-187$^T$ group by 15–16, 20–22 and 12–13 % substitutions in the *RPB1*, *RPB2* and *TEF1* genes, respectively; and four, 14 and five non-synonymous substitutions were found in the inter-group sequence differences in the three genes, respectively (Table S2). The sequence differences between strain GT-160$^T$ and the GT-233$^T$ group and between the GT-187$^T$ and the GT-233$^T$ groups in the three protein genes were more significant (Table S2). None of the new strains exhibited identical sequences in the protein coding genes compared (Fig. S1c–e), suggesting each of them represents a separate strain.

**MORPHOLOGY AND PHYSIOLOGY**

All the strains studied formed cream-coloured colonies and ovoid, ellipsoidal and cylindrical vegetative cells (Fig. 2a, c, e). Ballistoconidia were formed and are allantoid and falcate in shape (Fig. 2b, d, f). Strain GT-160$^T$ produced more abundant ballistoconidia than the strains in the other two groups. Physiologically, the three groups differed from each other slightly in the ability to utilize D-galactose, L-sorbose, D-glucitol and α-methyl-D-glucoside (Table 2). Sexual cycles were not observed in the cultures of single strains or intra-group mixed strains on YM and corn meal agar [12].

The data obtained from this study suggest that the nine yeast strains compared represent three new species within a new genus. A new genus name *Heitmania* gen. nov. and

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**Fig. 2.** Vegetative cells (a, c, e) grown in YM agar for 7 days at 17 °C and ballistoconidia (b, d, f) produced on YM agar for 10 days at 17 °C of new species *Heitmania litseae* (a, b); *Heitmania castanopsis* GT-233$^T$ (c, d); and *Heitmania elacocarpi* GT-160$^T$ (e, f), bars 10 µm. The pictures were taken using a microscope with DIC.
three new species names, *Heitmania litseae* sp. nov., *Heitmania castanopsis* sp. nov. and *Heitmania elacocarpi* sp. nov., are therefore proposed and described below.

**DESCRIPTION OF HEITMANIA X.-Z. LIU, F.-Y. BAI, M. GROENEW. AND T. BOEKHOUT GEN. NOV., MYCOBANK MB 819987**

*Heitmania* (Heit.ma’ni.a. N.L. fem. n. *Heitmania* named in honour of Joseph Heitman for his contributions to molecular mycology and fungal genetics).

Colonies are cream-coloured, butyrous, with a smooth surface and an entire margin. Yeast cells are ovoid, ellipsoidal and cylindrical, 2.0–6.0 µm × 6.0–24.0 µm. Pseudohyphae are not formed. Ballistoconidia are produced on YM agar and are allantoids and faculate. This genus is phylogenetically well separated from other genera in *Microbotryomycetes*. The type species is *Heitmania litseae*.

**DESCRIPTION OF HEITMANIA LITSEAE X.-Z. LIU, F.-Y. BAI, M. GROENEW. AND T. BOEKHOUT SP. NOV., MYCOBANK MB 820112**

*Heitmania litseae* (lit. se’ae. N.L. gen. n. *litseae* referring to *Litsea*, the genus of the plant from which the type strain was isolated).

After 5 days of growth on YM agar at 17°C, cells are ovoid, ellipsoidal and cylindrical, 2.0–6.0 µm × 6.0–19.0 µm (Fig. 2a), and occur singly. Budding is polar. The streak culture is cream-coloured, butyrous, with a smooth surface and an entire margin. In Dalmau plate culture on corn meal agar, pseudohyphae are not formed. Ballistoconidia are produced on YM agar and are allantoids, 2.5–3.0 µm × 7.5–9.0 µm (Fig. 2b). Physiological properties are listed in Table 2. Sexual structures were not observed on YM and CMA agar. The type strain, GT-187T, was isolated from a leaf of *Litsea coriacea* var. *sinensis* collected on Gutian Mountain, Zhejiang Province, China in July 2010. This strain has been deposited in the China General Microbiological Collection Centre (CGMCC), Beijing, China (CGMCC 2.5697T) and in the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands (CBS 14756T) as lyophilized culture.
DESCRIPTION OF HEITMANIA CASTANOPSIS
X.-Z. LIU, F.-Y. BAI, M. GROENEWALD AND T. BOEKHOUT SP. NOV., MYCOBANK MB 819988

Heitmania castanopsis (cas.tan.op’sis. N.L. gen. n. castanop-sis referring to Castanopsis, the genus of the plant from which the type strain was isolated).

After 5 days of growth on YM agar at 17 °C, cells are ellipsoidal and cylindrical, 2.0–4.0 μm × 7.0–20.0 μm (Fig. 2c), and occur singly. Budding is polar. The streak culture is cream-coloured, butyrous, with a smooth surface and an entire margin. In Dalmau plate culture on corn meal agar, cells are ellipso-oids and cylindrical, 2.0–3.0 μm × 7.5–9.0 μm (Fig. 2d). Physiological properties are listed in Table 2. Sexual structures were not observed on YM and CMA agar. The type strain, GT-233T, was isolated from a leaf of Casta-nopsis tibetana collected on Gutian Mountain, Zhejiang Province, China in July 2010. This strain has been deposited in the China General Microbiological Collection Centre (CGMCC), Academia Sinica, Beijing, China (CGMCC 2.5698T) and in the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands (CBS 14750T) as lyophilized culture.

DESCRIPTION OF HEITMANIA ELACOCARPI
X.-Z. LIU, F.-Y. BAI, M. GROENEWALD AND T. BOEKHOUT SP. NOV., MYCOBANK MB 820113

Heitmania elacocarpi (e.la.co.ca’ri. N.L. gen. n. elacocarp referring to Elacocarpus, the genus of the plant from which the type strain was isolated).

After 5 days of growth on YM agar at 17 °C, cells are ellipsoidal, 2.5–6.0 μm × 15.0–24.0 μm (Fig. 2e), and occur singly. Budding is polar. The streak culture is cream-coloured, butyrous, with a smooth surface and an entire margin. In Dalmau plate culture on corn meal agar, pseudohyphae are not formed. Ballistoconidia are produced on YM agar and are allantoids, 2.5–3.0 μm × 7.5–9.0 μm (Fig. 2d). Physiological properties are listed in Table 2. Sexual structures were not observed on YM and CMA agar. The type strain, GT-233T, was isolated from a leaf of Elacocarpus japonicus collected on Gutian Mountain, Zhejiang Province, China in July 2010. This strain has been deposited in the China General Microbiological Collection Centre (CGMCC), Academia Sinica, Beijing, China (CGMCC 2.5695T) and in the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands (CBS 14752T) as lyophilized culture.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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