Graphiola fimbriata: the first species of Graphiolaceae (Exobasidiales, Basidiomycota) described only based on its yeast stage

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Graphiola fimbriata sp. nov. is the first anamorphic species of Graphiolaceae (Exobasidiales, Basidiomycota)

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Abstract

The systematic position of three yeast strains isolated from a plant cell culture, a piece of termite nest, and as a foliar endophyte of *Coffea arabica*, respectively, is evaluated using morphological, physiological, and phylogenetical analyses. In culture, all three isolates produced white, pale orange to pink colored colonies of cylindrical cells with monopolar budding and pseudohyphae. Standard phenotypic, biochemical, physiological characterization and phylogenetic analyses of the combined 26S rRNA gene (D1/D2 domains) and ITS region sequences showed the conspecificity of these isolates and suggest their placement within the Exobasidiales (Ustilaginomycotina) as a sister lineage of the sampled and sequenced *Graphiola* species. Here, we describe this species as *Graphiola fimbriata* sp. nov. MycoBank MB 825077 (holotype: PC1^{T}; ex-type cultures: IBRC-M 30158^{T} = CBS 13945^{T} = DSM 104832^{T}). This is the first anamorphic saprobic species described in the genus *Graphiola*. The description of the genus *Graphiola* is therefore emended to allow species known only from a saprobic state.

Key words: 1 new taxon, *Graphiola fimbriata* sp. nov., Asexual state, Morphological characterization, Phylogenetic analyses, Yeast

INTRODUCTION

Fungi with yeast and yeast-like morphology are present within all three main lineages of Basidiomycota, namely the subphyla Agaricomycotina, Pucciniomycotina, and Ustilaginomycotina (Sampaio 2004, Boekhout et al. 2011, Kurtzman & Boekhout 2017). Yeast and filamentous taxa are often intermixed as revealed by recent phylogenetic studies (Liu et al. 2015a, 2015b, Wang et al. 2015a, 2015b, 2015c). With a few exceptions, a transition from filamentous to yeast stage is often associated with the change from a parasitic to saprobic life style (Begerow et al. 2017). The subphylum Ustilaginomycotina comprises a highly diverse assemblage of fungi, including teliosporic plant pathogens, non-teliosporic plant pathogens, anamorphic plant pathogens, as well as endophytic, lipophilic, saprobic, and zoophilic yeasts (Begerow et al. 2014). The orders Malasseziales, Monilielalles, and Violaceomycetales include exclusively yeast species of the genera *Malassezia*, *Moniliella*, and *Violaceomyces*, respectively (Wang et al. 2014, Albu et al. 2015), while the remaining yeasts and yeast-like fungi are scattered amongst the remaining orders of Ustilaginomycotina, with the exception of the members of Doassansiales, Tilletiales and Uleiellales for which no yeasts have been discovered so far.

The anamorphic saprobic members of Ustilaginomycotina have previously been classified mainly in the polyphyletic genera *Pseudozyma* and *Tilletiopsis* (Begerow et al. 2000, Sampaio 2004, Kurtzman et al. 2011, 2015), and later in several monophyletic genera *Acaromyces*, *Farysizyma*, *Fereydounia*, *Jaminaea*, *Meira*, and *Sympodiomycopsis* (Sugiyama et al. 1991, Boekhout et al. 2003, Inácio et al. 2008, Sipiczki & Kajdacsi 2009, Nasr et al. 2014). Sexual and asexual morphs in Ustilaginomycotina were recently grouped together in order to unify the taxonomy of plant parasites and species known only from their yeast states (Piątek et al. 2015,

Wang et al. 2015a, Kijpornyongpan & Aime 2016). As the result, several monophyletic genera (*Dirkmeia*, *Golubevia*, *Kalmanozyma*, and *Robbauera*) were erected to accommodate species previously classified in the genera *Pseudozyma* and *Tilletiopsis* (Wang et al. 2015a). The current classification scheme recommends that each novel lineage not linked with a teleomorphic genus or recognized anamorphic genus should be assigned to a new genus. The subphylum Ustilaginomycotina comprises the least number of yeast and yeast-like species (71) compared to the two other subphyla of Basidiomycota (Wang et al. 2015a). However, studies that involved environmental sequencing suggest a much greater, unexplored diversity of fungi in this group (e.g., Richards et al. 2012, Nasanit et al. 2015, Dunthorn et al. 2017, Jimu et al. 2017).

In the course of independent studies two yeast strains have been isolated. The first culture (PC1) was isolated in the Iranian Biological Resource Center (IBRC) from a contaminated plant culture, and the second isolate (SNB-CN72) was obtained from a nest of the termite species *Nasutitermes corniger* harvested in Rémire-Montjoly, French Guiana. Phylogenetic analyses of the ITS region and the D1/D2 domains of the LSU rRNA gene placed the strains within Graphiolaceae (Exobasidiomycetes, Exobasidiales) close to plant parasites of the genus *Graphiola,* and revealed relatedness to a third yeast isolate (IBL 03150) reported by Posada et al. (2007) and deposited in the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. Species in the plant pathogenic genus *Graphiola* are traditionally circumscribed based on their host spectrum and symptoms, and nucleotide sequence data for these fungi are rare (Wang et al. 2015a).

The aim of the study was to characterise the yeast-like isolates and determine their phylogenetic placement, incorporating morphological, physiological, and molecular data yet available for members of the genus *Graphiola*.

MATERIALS AND METHODS

Sample collection and isolation

A plant cell culture of an unidentified plant species mixed with a yeast strain was obtained from the plant bank section of the Iranian Biological Resource Center (IBRC) in 2013 (strain designation: PC1). Another strain was isolated from a piece of *Nasutitermes corniger* nest collected in Rémire-Montjoly, (Cayenne, French Guiana) in July 2011 (strain designation: SNB-CN72; Nirma et al. 2013, 2015). A third strain was obtained from the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (original strain designation: IBL 03150). It was isolated as foliar endophyte of *Coffea Arabica* (Posada et al. 2007). Designations and GenBank accession numbers of the yeast strains used in this study are given in Table 1.

Cultures were maintained on YPG agar medium (0.5% yeast extract, 1% peptone, 2% glucose, 2% agar, w/v) at 25 °C during experiments.

To expand the ITS sequence sampling for molecular phylogenetic analyses a specimen of the plant parasitic genus *Graphiola* was additionally used and newly sequenced: *Graphiola phoenicis* (Moug. ex Fr.) Poit. on *Phoenix reclinata* Jacq., South Africa, Kwa Zulu-Natal, Durban, Yellowwood Park, Kenneth Stainbank Nature Reserve, 14 Feb. 2012, leg. A.R. Wood 883, KR-M-0042315.

Morphological examination

Colony morphology images were taken using a stereo microscope coupled with the Nikon zoom digital camera. In addition, scanning electron microscopy was performed on the isolate using a

VEGA3-TESCAN SEM instrument (Van Wyk & Wingfield 1994). Briefly, the cells were fixed in 3.0%, 0.1 M, pH 7.0 sodium phosphate-buffered glutaraldehyde for 3 h at room temperature, followed by 1 h fixation in 2% osmium tetroxide. The cells were dehydrated by increasing ethanol concentrations (30%, 50%, 70%, 90%, and 96%) for 30 min and two 30 min washes in 100% ethanol. The standard characterization of the yeast isolates was performed according to methods described earlier (Barnett et al. 2000, Kurtzman et al. 2011). Assimilation of carbon and nitrogen sources was carried out on solid and in liquid media, respectively.

DNA extraction, PCR, and sequencing

Nuclear DNA was extracted by the method of Hanna & Xio (2006). For the *Graphiola phoenicis* specimen, the genomic DNA was isolated directly from the herbarium specimen. The 5'-end (D1/D2 region) of the nuclear large subunit ribosomal DNA (LSU) and the ITS 1 and ITS 2 regions of the nuclear rDNA including the 5.8S rDNA (ITS) were amplified and sequenced using primer pairs NL1 (5'GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG3') (Kurtzman & Robnett 1998) and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), respectively. DNA sequences determined for this study were deposited in GenBank (accession numbers are given in Figs 1 & 2 and in Table 1. Additional sequences of *Graphiola cylindrica* and *G. phoenicis* were obtained from GenBank and the public catalogue of the NITE Biological Resource Center collection (NBRC), Japan.

Phylogenetic analyses

144 To elucidate the phylogenetic position of the isolated strains their sequences were analysed
145 within a LSU and a concatenated ITS + LSU dataset. Since preliminary analyses and Blast
146 searches (Altschul et al. 1997) revealed an affinity of the stains to the order Exobasidiales (class
147 Exobasidiomycetes), the LSU dataset was reduced to members of the Exobasidiales and some
148 representatives of the Ustilaginomycetes were used as an outgroup. Isolates and clones, for
149 which only ITS sequences were available, were analysed in the LSU + ITS dataset. If present in
150 GenBank sequences of the respective type species were used. Additionally sequences of all
151 available brachybasidiaceous species and all available sequences clustering within the
152 Graphiolaceae including sequences from the Biological Resource Center, NITE, Japan were
153 added.
154 GenBank accession numbers of the sequences used for both the LSU and ITS+LSU dataset
155 (Begerow et al. 1997, 2001, 2002, Guo et al. 2001, Boekhout et al. 2003, Castlebury et al. 2005,
156 Stoll et al. 2005, Yasuda et al. 2005, 2006, Posada et al. 2007, Tanaka et al. 2008, Piepenbring et
157 al. 2010, 2012, Yuan et al. 2011, Jusino et al. 2015, Urbina et al. 2016) are given in Figs 1 & 2.
158 Sequence alignment was obtained independently for both the LSU dataset and the ITS and LSU
159 part of the ITS+LSU dataset using MAFFT 7.313 (Katoh & Standley 2013) using the L-INS-i
160 option. To obtain reproducible results, manipulation of the alignments by hand as well as manual
161 exclusion of ambiguous sites were avoided as suggested by Gatesy et al. (1993) and Giribet and
162 Wheeler (1999), respectively. Instead, highly divergent portions of the alignments were omitted
163 using GBlocks 0.91b (Castresana 2000) with the following options for the LSU dataset:
164 'Minimum Number of Sequences for a Conserved Position': 20, 'Minimum Number of
165 Sequences for a Flank Position': 20, 'Maximum Number of Contiguous Non-conserved
166 Positions': 8, 'Minimum Length of a Block': 5, and 'Allowed Gap Positions' to 'With half', for

the ITS part of the ITS+LSU dataset: 11/11/8/5/'With half' and for the LSU part of the ITS+LSU dataset: 12/12/8/5/'With half'. After alignment the ITS and LSU part of the ITS+LSU dataset were concatenated. The resulting alignments [LSU dataset: new number of positions: 591 (32% of the original 1801 positions), number of variable sites: 279; ITS+LSU dataset: new number of positions: 1121 (34% of the original 3264 positions), number of variable sites: 468] were used for phylogenetic analyses using a Maximum Likelihood (ML) and a Bayesian Approach (BA) following Vasighzadeh et al. (2014).

For the LSU dataset trees were rooted with the ustilaginomycetous species *Urocystis ficariae* and *Ustilago hordei,* for the ITS+LSU dataset trees were rooted with the brachybasidiaceous species *Dicellomyces gloeosporus* and *Meira geulakonigii*.

Metabolite profiling analysis was carried out for the three yeast strains and the results are presented as supplementary data.

RESULTS

Morphological examination

Morphology of the strains IBL 03150, PC1, and SNB-CN72 showed no significant differences, results are included in the species description.

Metabolite profiling with UHPLC-MS was carried out for the three yeast strains and the results are provided in supplementary data. In the absence of metabolic data from closely related species, the analysis of UHPLC-MS profiles was restricted to the tree strains. We observed that the culture medium impacted more significantly on the profiles than the nature of the strain (Supplementary data). Hierarchical clustering analyses (HCA) conducted on the 3 (N) × 29 (X)

matrix of merged profiles showed that strains PC1 and IBL 03150 are more alike than SNB-CN72 (Supplementary data).

Phylogenetic analyses

The different runs of the BA that were performed and the ML analyses yielded consistent topologies. To illustrate the results the consensus trees of one run of the BA of the LSU and the concatenated ITS+LSU dataset are presented (Figs 1 & 2). Using the ustilaginomycetous species as outgroup in the LSU analysis, the clades in the phylogenetic tree were congruent to the families discussed in Begerow et al. (2014). In all analyses the sequences of the yeast isolates clustered within the Exobasidiales together with the sequence MF334501 from an uncultured fungus clone forming the sister lineage of the sampled *Graphiola* spp. of which the *G. phoenicis* cluster included several sequences of yeast isolates and uncultured fungus clones, respectively. The clade comprising *Graphiola cylindrica, G. geonomae, G. phoenicis*, and the yeast isolates received good statistical support (BA: 100%, ML: 93%). The yeast isolates branched first in the *Graphiola* clade and were placed sister to *G. cylindrica, G. geonomae*, and *G. phoenicis*. Combined ITS+LSU analyses (Fig. 2) revealed the same groups and relations including more sequences from *Graphiola* spp. as well as from the studied yeast isolates and uncultured fungus clones. The three strains shared identical LSU sequences and showed 5 variable positions (2 substitutions and 3 indels) in the ITS region.

TAXONOMY

***Graphiola* Poit., Ann. Sci. Nat. (Paris): 473 (1824) emend. "**S. Nasr, M. Lutz, D. Stien & A. Yurkov**"**

According to the current genus concept of *Graphiola* (Piepenbring et al. 2012), the genus comprises plant pathogens on palms (Arecaceae). Tubaki & Yokohama (1971) and Oberwinkler et al. (1982) obtained and studied *Graphiola phoenicis* in culture. They provided also the diagnosis of this species, including morphological and physiological properties observed in culture. Emendation of the diagnosis of the genus *Graphiola* is proposed to allow classification of asexual, known only from a saprobic state yeast species in the genus.

Sexual reproduction is observed in some species. Fungi are dimorphic with a filamentous sexual form parasitizing on plants and free-living saprobic yeast states. On malt extract, colonies are white, pale orange to pink in color. Budding cells are present in culture. Colonies are white, pale orange to pink in color. Ballistoconidia are not produced. Pseudohyphae may be present in cultures. Fermentation is absent. DBB reaction is positive. Urease activity is positive.

Graphiola fimbriata S. Nasr, M. Lutz, D. Stien & A. Yurkov, sp. nov. (Fig. 3)

MycoBank MB 825077

Etymology: Referring to the margin of the colonies.

Description: After three days on YPG agar at 25 ºC, the cells are cylindrical, 1-2.5 × 4-8.5 µm, and occur single or in small chains. Budding is monopolar. After five days, the colony is white, convex, and the margin is filiform. Pellicles are formed on liquid media. After one week at 25 ºC on MEA and Cornmeal agar, the slide culture undifferentiated pseudohyphae are formed. Fermentation is negative. The following compounds are assimilated: glucose, sucrose, maltose, trehalose, melezitose, D-xylose, raffinose, L-arabinose, D-mannitol, myo-inositol, D-ribose and arbutin; assimilation of salicin, glycerol and cellobiose is weak. No growth occurs on galactose,

lactose, L-rhamnose, melibiose, inulin, D-arabinose, soluble starch, ethanol, methanol, DL-lactate, succinate, citrate and n-hexadecane. Sodium nitrate, potassium nitrate, L-lysine, Ethylamine hydrochloride (variable) and cadaverine dihydrochloride (variable) are assimilated. No growth occurs on glucosamine, imidazole, creatine or creatinine. Growth in vitamin-free medium is positive. Growth at 15 °C, 25 °C, 30 °C, and 34 °C is positive but not at 4 °C, 37 °C and 40 °C. Growth occurs on YM agar supplemented with 5% (w/v) NaCl, 10 % (w/v) NaCl but not on YM agar with 16% (w/v) NaCl. Starch-like compounds are not produced. No growth occurs on media supplemented with 0.01% and 0.1% cycloheximide, and 1% acetic acid. The diazonium blue B reaction is weakly positive. Urease activity is positive.

Molecular characteristics: nucleotide sequences of ITS and LSU (D1/D2 domains) rDNA sequences are deposited in GenBank (Table 1.)

Deposits: holotype, PC1^{T} (= IBRC-M 30158^{T}) isolated as a contaminant of an unidentified plant culture, preserved in a metabolically inactive state at the Iranian Biological Resource Centre, Teheran (holotype), Iran. Ex-type cultures are deposited in the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (= CBS 13945^{T}) and in the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSM 104832^{T}).

Strain examined: PC1^{T}, SNB-CN72 (= DSM 104833), IBL 03150

DISCUSSION

Morphological and physiological characteristics of the three yeast isolates examined in this study are uniform, differences in ITS and LSU sequences are low all that suggesting assignment to a single species. Molecular phylogenetic analyses reveal the three yeast isolates within the

Graphiolaceae as a sister lineage of *Graphiola cylindrica, G. geonomae,* and *G. phoenicis.* Thus, we propose *Graphiola fimbriata* sp. nov. to accommodate the three yeast strains. On the basis of the pair-wise sequence similarities (ITS and D1/D2 regions) and subsequence phylogenetic analyses (Figs 1 & 2), the novel species differs from all other *Graphiola* species.

We also emend the description of the genus *Graphiola* to allow the inclusion of species known from saprobic state only (see Taxonomy section).

The ecology and distribution range of the novel species is unknown. All described *Graphiola* species are plant pathogens on palms (Arecaceae). We hypothesize that the novel species is probably associated with plants, as also suggested by the origin of the studied isolates and closely related culture Exobasidiomycetidae sp. AUMC 10262 (KX011608) from the palm weevil *Rhynchophorus ferrugineus* that infests palms, which is a primary host of *Graphiola*. Although the novel species is known from its asexual state, it is most likely that it has a host like all other *Graphiola* species. Widespread transcontinental transfer of plants results in a global dissemination of plant pathogens and crop pests (Bebber 2015, Hurley et al. 2016). Among them, fungal pathogens currently lead the global invasion of agriculture, despite their more restricted host range (Bebber et al. 2014, Wingfield et al. 2017). In our study, a new species of the plant pathogenic genus *Graphiola* was identified over a broad geographic range that includes the Old World (Iran and Egypt), tropical Asia and the Americas (French Guiana and USA). It is very likely that the ongoing transport of plants and pests promoted dissemination of *Graphiola fimbriata* between continents, localities, and habitats.

It cannot be precluded that *Graphiola fimbriata* represents the anamorphic stage of a palm pathogen of which no sequence data are available. Of the 12 *Graphiola* species described (Piepenbring et al. 2012) only three are represented by sequence data in GenBank. Moreover

281 species diversity seems underestimated in the genus *Graphiola*. Assuming that its host
282 specificity developed to the same degree shown for other plant pathogens (Piątek et al. 2013,
283 Savchenko et al. 2014, Vasighzadeh et al. 2014, Choi & Thines 2015, Scholler et al. 2016), the
284 number of 12 *Graphiola* species on 38 palm species from 21 plant genera (Farr & Rossman n.d.,
285 Piepenbring et al. 2012) is lower than in other known plant-parasite systems. Intensive sampling
286 and molecular analyses were used successfully in other groups to link anamorph and teleomorph
287 stages (Sampaio 2004, Boekhout et al. 2006, Inácio et al. 2008, Wang et al. 2015a, Kruse et al.
288 2017, Piątek et al. 2017). However, both sexual and asexual species are yet strongly under
289 sampled in many lineages (Liu et al. 2015a, Wang et al. 2015a, Wang et al. 2015b). Although
290 these studies unified systematics of plant parasites and yeast taxa, the results strongly suggest
291 that the available genetic data is insufficient to resolve anamorph-teleomorph relationships in
292 sister lineages (e.g., Boekhout et al. 2006, Kruse et al. 2017).

293 The lack of sequence data of sexual species also complicates biodiversity assessments and new
294 species discovery. With this study we provide the first overview on *Graphiola* diversity from
295 sequence data available in public databases. We propose new species *Graphiola fimbriata* to
296 accommodate yeasts from several localities and habitats. Our results show that sequences related
297 to *Graphiola* species are rapidly accumulating in public databases as taxonomically unassigned
298 isolates and clones. In our opinion, it is important to describe *Graphiola fimbriata* to provide a
299 proper name for these isolates to communicate it in the future. We agree that taxonomic
300 redundancy cannot be ruled out in the genus *Graphiola* considering a very few sequenced
301 species. Rediscovery and description of already known species as new may happen when stable
302 and informative morphological characters for species differentiation are limited, both in plant
303 material and in culture. Thus, future studies should be addressed on resampling and sequencing

of new and already existing material in order to better understand genetic diversity and taxonomy in this group of fungi.

There is little known about physiological properties of dimorphic plant parasites, whereas fungi traditionally recognized as "yeasts" were intensively studied in this respect. Undersampling and the lack of genetic (including housekeeping genes) and physiological data do not allow delimitation of many genera (e.g., Wang et al. 2015a). In this study, we decided not to propose a new genus for the novel fungus, but to accommodate it in the genus *Graphiola* in order to reduce the taxonomic complexity in this group. Neither phylogenetic analyses nor the analysis of physiological data revealed a basis to distinguish the new species from other *Graphiola* species. Therefore, we modified the description of the genus *Graphiola* to include information about its asexual form considering own results and previous reports by Tubaki & Yokohama (1971) and Oberwinkler et al. (1982). Despite limited genetic data, our study identified several fungal isolates and metabarcoding clones as *Graphiola fimbriata* and *G. phoenicis* (Figs. 1 & 2). These fungi were found in Indomalayan and Neotropical realms (e.g., Takashima et al. 2012, Urbina et al. 2016), which are the regions where palms are widely distributed (Kissling et al. 2012).

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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TABLES

Table 1. Designations and GenBank accession numbers of the yeast strains used in this study.

Yeast strains	Strain designation		GenBank accession numbers	
	IBRC-M	CBS	LSU	ITS
PC1	30158^T	13945^T	KM403453	KM403454
SNB-CN72	30163	–	KP229360	KJ023736
IBL 03150	-	14052	KP308195	DQ682574

FIGURE LEGENDS

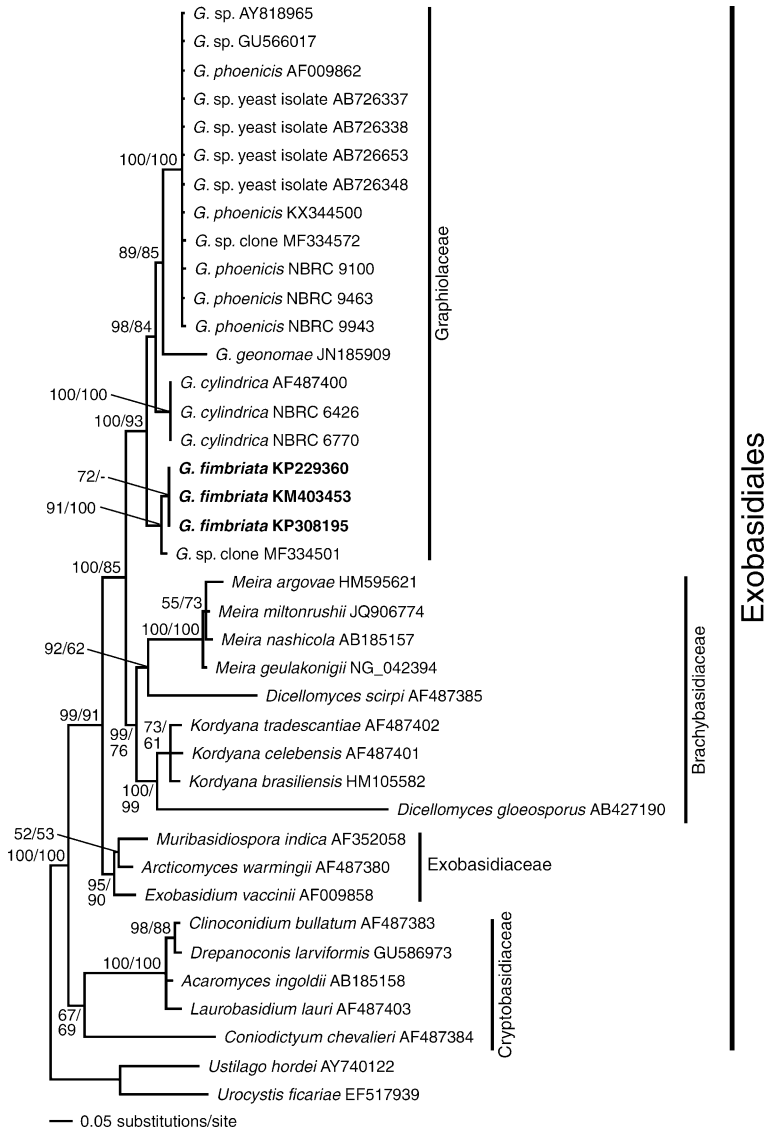


Fig. 1. Bayesian inference of phylogenetic relationships within the sampled Exobasidiales: Markov chain Monte Carlo analysis of an alignment of LSU base sequences using the GTR+I+G model of DNA substitution with gamma distributed substitution rates and estimation of invariant sites, random starting trees, and default starting parameters of the DNA substitution model. A 50% majority-rule consensus tree is shown computed from 75 000 trees that were sampled after

the process had become stationary. The topology was rooted with the ustilaginomycetous species *Urocystis ficariae* and *Ustilago hordei*. Numbers on branches before slashes are estimates for a posteriori probabilities; numbers on branches after slashes are ML bootstrap support values. Branch lengths were averaged over the sampled trees. They are scaled in terms of expected numbers of nucleotide substitutions per site. The taxonomical concept applied corresponds to Begerow et al. (2014).

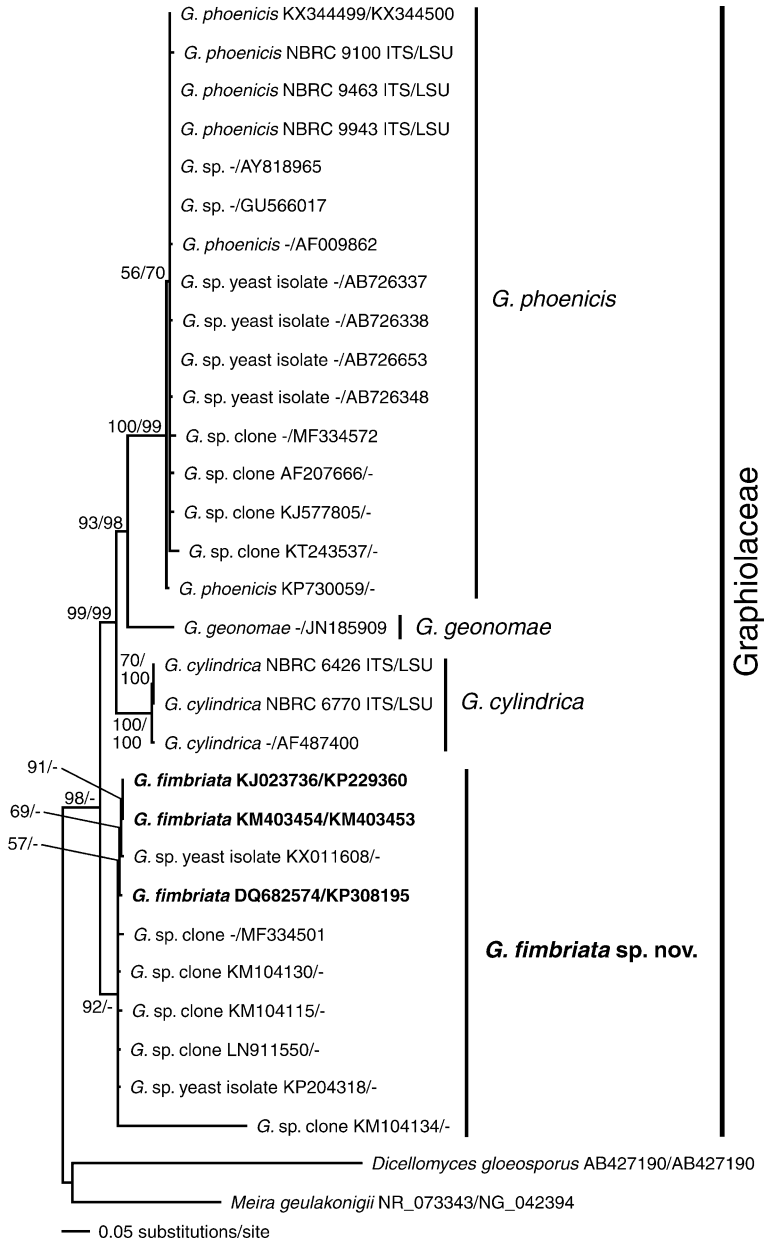
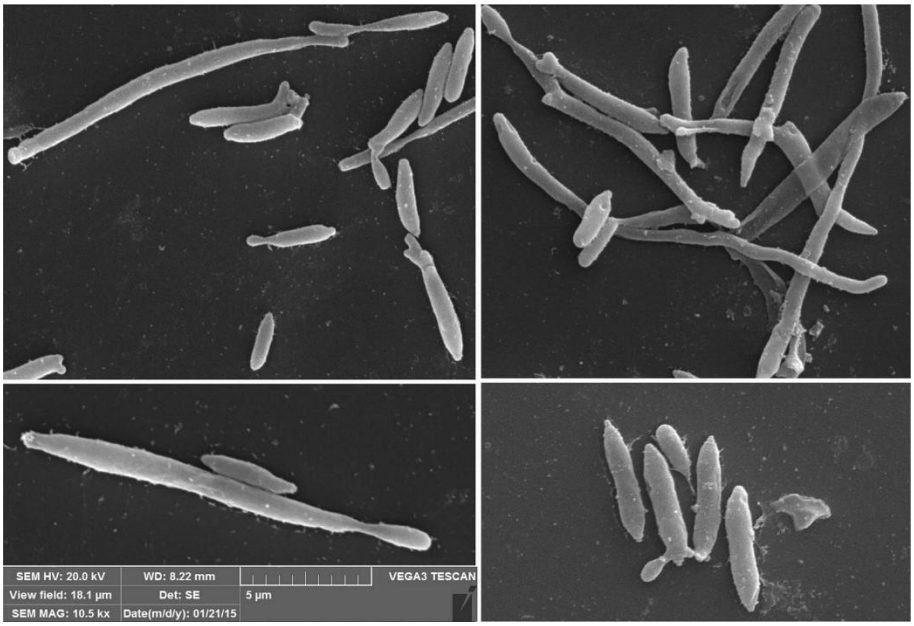


Fig. 2. Bayesian inference of phylogenetic relationships within the sampled Exobasidiales: Markov chain Monte Carlo analysis of an alignment of ITS+LSU base sequences using the GTR+I+G model of DNA substitution with gamma distributed substitution rates and estimation of invariant sites, random starting trees, and default starting parameters of the DNA substitution model. A 75% majority-rule consensus tree is shown computed from 75 000 trees that were sampled after the process had become stationary. The topology was rooted with the

545 cryptobasidiaceous species *Acaromyces ingoldii* and *Clinoconidium* sp. Numbers on branches
546 before slashes are estimates for a posteriori probabilities; numbers on branches after slashes are
547 ML bootstrap support values. Branch lengths were averaged over the sampled trees. They are
548 scaled in terms of expected numbers of nucleotide substitutions per site. The taxonomical
549 concept applied corresponds to Begerow et al. (2014).
550



551

552 **Fig. 3.** Scanning electron micrographs of the strain IBRC-M 30158^{T} showing budding cells and
553 bud scars. Scale bars: 5 µm.
554