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Relationships among the genera *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora* determined from rDNA sequence divergence

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SUMMARY

Species of the genera *Ashbya*, *Eremothecium*, *Holleya*, and *Nematospora* were compared from extent of divergence in a 580-nucleotide region near the 5′ end of the large subunit (26S) ribosomal DNA gene. The four genera are closely related and comprise a subclade of the hemiascomycetes. Because the taxa show little divergence, it is proposed that all be placed in the genus *Eremothecium*. The family Eremotheciaceae, fam. nov., is proposed.

INTRODUCTION

The genera *Ashbya* Guilliermond, *Eremothecium* Borzi, *Holleya* Yamada, *Metschnikowia* Kamienski, and *Nematospora* Peglion are characterized by needled-shaped ascospores that may be linear or falcate. Ascospores of *Nematospora* differ further and are appended with a long flagellum-like terminal extension of cell wall material. With the exception of *Metschnikowia*, the taxa are pathogenic to a variety of plant species [3], and *Ashbya* and *Eremothecium* are used worldwide for production of riboflavin (vitamin B_2) [10,33].

Batra [3] and von Arx et al. [29] discussed the phenotypic similarities of *Ashbya*, *Eremothecium* and *Nematospora* and suggested that the three taxa might be congeneric. Another genus in this complex is *Spermophthora* Ashby & Nowell. There is no extant type material from *Spermophthora*, and strains of the taxon have not been isolated since its original description. Von Arx et al. [29] suggested that *Spermophthora gossypii* Ashby & Nowell, the only known species of *Spermophthora*, represented a contaminated specimen of *Eremothecium* (*Crebrothecium* Routien) *ashbyi* (Guilliermond ex Routien) Batra. *Holleya sinecauda* (Holley) Yamada was first described as *Nematospora sinecauda* Holley [9]. Yamada [34] transferred *N. sinecauda* to the new genus *Holleya* because, unlike *Nematospora coryli* Peglion, which has ascospores with long terminal appendages and coenzyme Q with either five or six isoprene units, *N. sinecauda* forms shorter ascospores without terminal appendages and produces coenzyme Q with a mixture of eight (minor component) and nine (major component) isoprene units.

Classification of the foregoing taxa has been complicated by the perception [22] that genera which commonly form budding yeast cells (*Holleya*, *Metschnikowia*, *Nematospora*) are phylogenetically separate from genera that do not ordinarily form budding cells (*Ashbya*, *Eremothecium*). Phylogenetic analysis of ribosomal RNA/ribosomal DNA (rRNA/rDNA) nucleotide sequence divergence in selected genera of ascomycetous yeasts and yeastlike fungi has indicated that the budding yeasts and yeastlike fungi represent a group of taxa that is separate from the filamentous ascomycetes [2,4,7,13,14,31]. In an extension of these studies, Kurtzman and Robnett [17] compared partial sequences of large and small subunit rRNAs from the type species of all cultivatable genera of ascomycetous yeasts and yeastlike fungi. Results from this study demonstrated that all budding yeasts and yeastlike fungi are members of a clade separate from the filamentous ascomycetes. The analysis also demonstrated that *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora* represent closely related members of a subclade that is phylogenetically separate from the subclade that includes the genus *Metschnikowia*. While the separation of the two subclades from one another and from all other yeast genera was strongly supported (bootstrap values = 100%), there were insufficient phylogenetically informative sites to resolve branching order within the subclade comprised of *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora*.

In the present study, these four genera were reexamined from comparisons of additional nucleotide sequences. The resulting analysis has been used as the basis for redefining the systematics of this group.

This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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MATERIALS AND METHODS

Organisms and culture conditions

The strains studied are listed in Table 1, and all are maintained in the Agricultural Research Service Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL, USA. Cells for DNA extraction were grown for approximately 48 h at 25 °C in 50 ml YM broth [32] on a rotary shaker at 200 r.p.m. and harvested by centrifugation. After the cells were washed once with distilled water, they were resuspended in 2 ml distilled water and 1 ml of the suspension was pipetted into a microfuge tube. After centrifugation, excess water was decanted and the packed cells were lyophilized overnight and stored in a freezer until used.

DNA isolation, PCR and sequencing reactions

DNA isolation for PCR was performed using a modified version of the SDS protocol of Raeder and Broda [26]. The lyophilized cell mass was broken apart in a 1.5-ml microfuge tube with a pipette tip, and ca. 0.5 ml of 0.5-mm glass beads were added to the microfuge tube. The tube was shaken for 20 min on a wrist-action shaker at maximum speed. This treatment fractured about 25% of the cells. The cells were suspended in 1 ml of extraction buffer (200 mM Tris-HCl, pH 8.4; 200 mM NaCl; 25 mM EDTA, pH 8.0; 0.5% SDS) and extracted with phenol-chloroform and chloroform. DNA was precipitated from the aqueous phase by adding 0.54 volume isopropanol and pelleted for ca. 2 min in a microfuge at 10 000 r.p.m. The pellet was washed gently with 70% ethanol, resuspended in 100 μl TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) and dissolved by incubation at 55 °C for 1 h to overnight. Dilute DNA samples for PCR were prepared by adding 4 μl of the genomic stocks to 1 ml TE/10 buffer.

A divergent domain at the 5′ end of the 26S rDNA gene [5] was symmetrically amplified using primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) [24]. Amplification was for 36 PCR cycles with annealing at 52 °C, extension at 72 °C for 2 min, and denaturation at 94 °C for 1 min. The amplified DNA was purified with Geneclean II (Bio 101, La Jolla, CA, USA) according to the manufacturer's instructions. Visualization of the amplified DNA was performed following Geneclean treatment by electrophoresis in 1.5% agarose in 1 × TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) and then staining with ethidium bromide (8×10^{-5} μg μl^{-1}).

Both strands of the rDNA regions compared were sequenced using the ABI Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA). Four sequencing reactions were required for each DNA. Primers for these reactions were the external primers NL-1 and NL-4, and the internal primers NL-2 (5′-CTCTCTTTTCAAAGTTCTTTTCATCT) and NL-3 (5′-AGATGAAAAGAACTTTGAAAAGAGAG) [24].

Sequence data were visually aligned using QEdit 2.15 (SemWare, Marietta, GA, USA). The sequences of *Saccharomyces cerevisiae* Meyen ex Hansen (a non-mycelial species) and *Cephaloascus fragrans* Hanawa (a mycelial species) were used as references. Phylogenetic relationships were determined from the program PAUP, version 3.1.1 [28] using the branch-and-bound search option followed by bootstrap analysis (1000 replications). An alternative analysis used the heuristic search option of PAUP.

RESULTS AND DISCUSSION

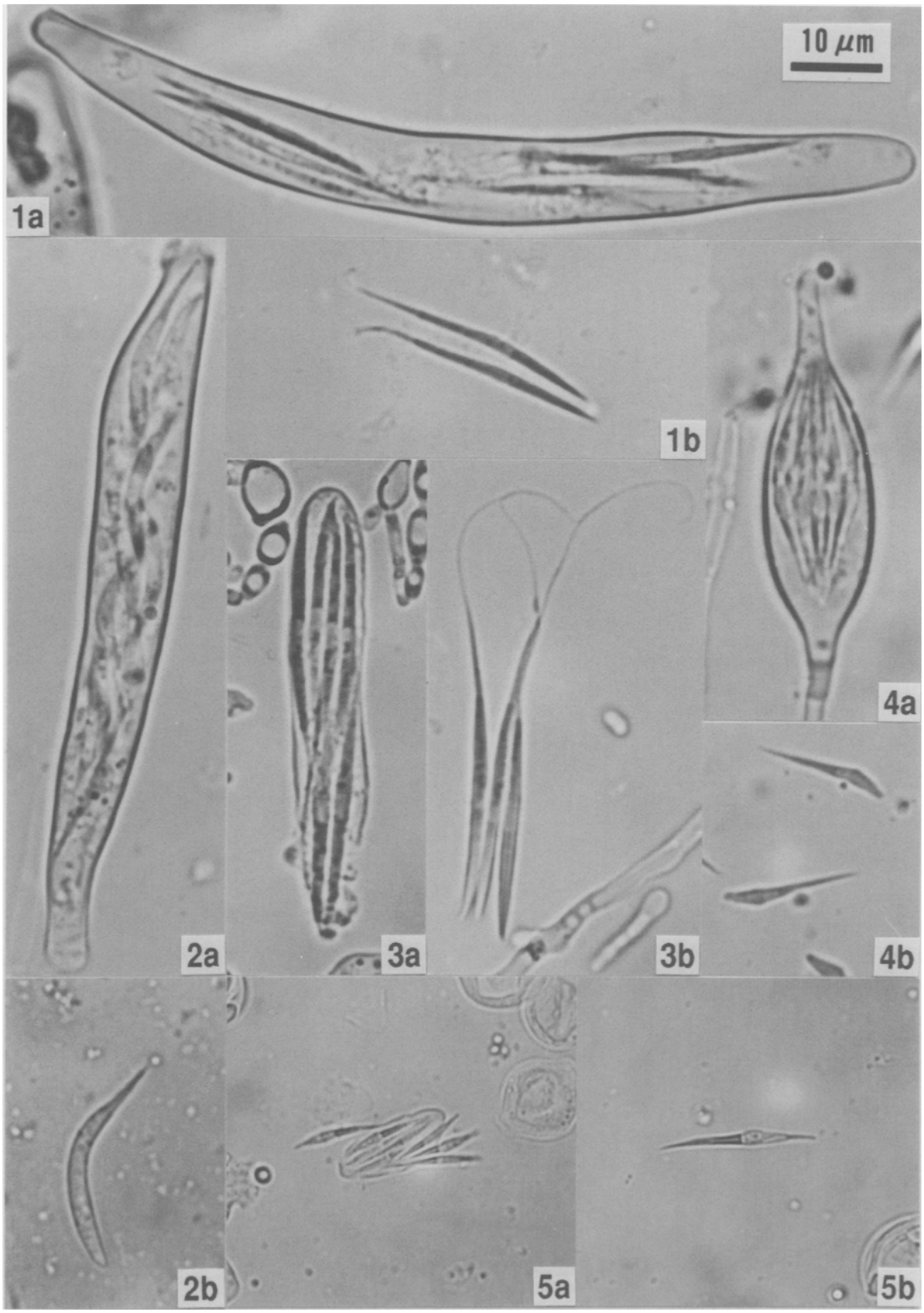
Asci and ascospores of *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora* are illustrated in Figs 1–5. Ascospores often form as two interleaved groups within the asci and, at maturity, ascus walls deliquesce. The ascospores of all taxa are elongated and needle-like; those of *E. ashbyi* are notably falcate whereas ascospores of *N. coryli* are appended with a long, whip-like extension of wall material. Ascospores of *Eremo-*

TABLE 1

Key characteristics of the strains examined

Species	Strain no.		Coenzyme Q (major component)	Bud formation	Source of isolate
	NRRL	CBS			
Ashbya gossypii	Y-1056[a]	109.51	6	−	Unknown
	Y-1810			−	Cotton boll, Trinidad
Eremothecium ashbyi	Y-1363[a]		6	−	Cotton boll?
	Y-7249			−	Cotton boll, South Africa
Eremothecium cymbalariae	Y-17582[a]	270.75	7	−	*Brachynema germari*, Iran
Holleya sinecauda	Y-17231[b]	8199	9	+	Oriental mustard, Canada
Nematospora coryli	Y-12970[b]	2608	5	+	Hazel nut
	Y-1618	2599	6	+	Cotton boll, as *N. phaseoli*
Saccharomyces cerevisiae	Y-12632[b]	1171	6	+	Beer
Cephaloascus fragrans	Y-6742[b]	121.29	7	+	Unknown

[a] Authentic strain. There is no known type strain.
[b] Type strain.



Figs 1–5. Asci and ascospores. a) Asci with immature ascospores. b) Mature ascospores released following deliquescence of ascus walls. 1) *Ashbya gossypii*. 2) *Eremothecium ashbyi*. 3) *Nematospora coryli*. 4) *Eremothecium cymbalariae*. 5) *Holleya sinecauda*. The 10-μm marker bar applies to all Figures.

thecium are one-celled, but those of *Ashbya*, *Holleya* and *Nematospora* are usually two-celled. *Holleya* typically produces eight ascospores per ascus but the number may be double that for *Nematospora*. The other taxa usually form in excess of eight ascospores per ascus with as many as 32 sometimes occurring in the asci of *A. gossypii*. Holley at al. [9] provided electron micrographs that show the posterior half of ascospores from *H. sinecauda* to have prominent spiral ridges whereas the anterior end is smooth.

Aligned sequences from the 5′ end of the 26S rDNA (positions 63–642 of *S. cerevisiae*) from the type or authentic strains of *A. gossypii*, *E. ashbyi*, *E. cymbalariae*, *H. sinecauda* and reference species *S. cerevisiae* and *C. fragrans* are presented in Fig. 6. Of the 580 nucleotides sequenced, there were 147 variable sites of which 42 were phylogenetically informative. A phylogenetic analysis implemented by the branch-and-bound option of PAUP found four equally parsimonious trees of 188 steps [consistency index (CI) = 0.862, homoplasy index (HI) = 0.138, retention index (RI) = 0.552, rescaled consistency index (RC) = 0.475]. The ingroup tree topologies were identical when using either *S. cerevisiae* or *C. fragrans* as the outgroup. Application of the general heuristic search option of PAUP also resulted in four equally parsimonious trees (188 steps, CI = 0.862, HI = 0.138, RI = 0.552, RC = 0.475) that were identical to those from the branch-and-bound search.

The strict consensus tree resulting from the branch-and-bound analysis is presented in Fig. 7. Monophyly of the *Ashbya/Eremothecium/Holleya/Nematospora* clade is strongly supported by bootstrap analysis (100%), as was the case in a previous analysis when the four taxa were compared from partial sequences of 18S and 26S rRNAs from the type species of all cultivatable ascomycetous yeast genera [17]. However, as with the previous work, branching order within the clade is unresolved, although an expectation of the present study was that the additional ca. 300 nucleotides sequenced from the variable 5′ end of the 26S rDNA might provide increased phylogenetic resolution among the species. As implied from the strict consensus tree (Fig. 7), branching order among the four equally parsimonious trees is random. For example, in one tree, *N. coryli* and *H. sinecauda* share a branch, but in another tree, *N. coryli* pairs with *A. gossypii*.

Ashbya gossypii, *E. ashbyi*, *E. cymbalariae*, *H. sinecauda* and *N. coryli* differ from one another by 3–6% nucleotide substitutions (Table 2), which suggests that they represent separate biological species. It has been shown for a variety of yeasts that strains of a species either have the same sequence in the 5′ end of the 26S rRNA/rDNA molecule or that they differ from one another by generally no more than 1% substitutions [12,14,16,25]. Divergence in this region is generally sufficient to recognize sibling species. Consequently, extent of nucleotide divergence has provided a reliable prediction of whether or not strains are members of the same species. These studies also have shown that the substitutions among closely related species usually do not represent synapomorphies and thus offer no resolution of branching order. For example, in the genus *Debaryomyces* Lodder & Kreger-van Rij, branching order is unresolved among four of the six species in the subclade to which *D. hansenii* (Zopf) Lodder & Kreger-van Rij is assigned [16,18]. Hoelzer and Melnick [8] noted a lack of phylogenetic resolution among closely related animal species and suggested that this may reflect that cladogenesis does not always occur through a series of bifurcations.

The close relatedness between species of the *Ashbya/Eremothecium/Holleya/Nematospora* clade raises the question of whether the species should be retained in the present four genera or placed in a single genus. The genera are separated from one another on coenzyme Q composition, presence or absence of budding cells and morphology of ascospores. Genera are often defined from the presence of unique morphological or physiological properties, but the genetic basis of these properties is frequently unknown. For example, *Hansenula* H. & P. Sydow was separated from *Pichia* Hansen because species of the former genus assimilated nitrate as a sole source of nitrogen whereas species of the latter genus did not. Nitrate assimilation proved to be a strain-specific character and *Hansenula* is now regarded as a synonym of *Pichia* [11].

Ascospore morphology has proven to be an uncertain predictor of kinship. The monotypic genera *Schwanniomyces* Klöcker and *Wingea* van der Walt were defined because of the unique appearance of their ascospores; otherwise, they are phenotypically indistinguishable from species of the genus *Debaryomyces*. Comparisons of rRNA sequences placed both genera within the circumscription of *Debaryomyces* [16,18]. In contrast, rRNA sequence analysis showed *Williopsis* Zender and *Saturnospora* Liu & Kurtzman to be phylogenetically divergent even though both genera are characterized by saturnoid ascospores [21].

The occurrence of budding has been used to separate taxonomically ascomycetous yeasts from the nonbudding members of the hemiascomycetes [22]. Analysis of rRNA sequences has shown that budding as well as nonbudding hemiascomycetes represent members of the same clade and that this clade is a sister group to the filamentous ascomycetes, some of whose members are also dimorphic [6,13,14,17,27]. Consequently, budding must be regarded as homoplasic.

The preceding examples demonstrate that the defining characters for many genera are without phylogenetic significance. Although there are no genetic or phylogenetic definitions for genera, most systematists believe that genera should be defined along natural lines, and this requires assigned species to be monophyletic. Do species assigned to monophyletic genera typically exhibit a particular range of genetic divergence? Analysis of the extent of nucleotide substitutions at the 5′ end of 26S rRNA (positions ca. 300–630) for species of eight ascomycetous yeast genera believed to be monophyletic showed 3–23% divergence [14]. The range in substitutions seen among the genera can be attributed to differences in divergence times and to possible differences in rates of substitution. Extent of divergence alone is not adequate to define genera, but it does provide perspective when evaluating the taxonomic status of monophyletic groups of species. The nucleotide divergence exhibited among species of the *Ashbya/Eremothecium/Holleya/Nematospora* clade is 3–6%

(Table 2), and this is at the lower end of the range found for ascomycetous yeast genera.

The history of the genus *Holleya* requires some comment. Yamada [34] transferred *N. sinecauda* to the newly described genus *Holleya* primarily for two reasons: 1) *N. sinecauda* forms coenzyme Q-9 (with Q-8 as a minor component) whereas strains of *N. coryli*, the only other accepted species of the genus, form either Co Q-5 or Co Q-6; and 2) ascospores of *N. sinecauda* are ornamented differently from those of *N. coryli* and are two to three times smaller. Congeneric species often have coenzyme Q molecules with the same number of isoprene units in the major component [summarized in 1,30]. Exceptions, other than among strains of *N. coryli*, are found for species of *Schizosaccharomyces* Lindner (Q-9, Q-10) [16] and *Lipomyces* Lodder & Kreger-van Rij (Q-9, Q-10) [15], and for *Pichia methanolica* Makiguchi (equimolar amounts of Q-7 and Q-8 [20].

Yamada and Nagahama [35] compared partial 18S (1 region) and 26S (2 regions) rRNA sequences from *H. sinecauda* and *N. coryli*. The analysis of these data was based on the number of nucleotide differences between strains of these two species. The two 26S regions showed *H. sinecauda* and *N. coryli* are closely related, but the 18S region suggested the two species are more divergent. Analysis of the 18S region formed the basis for maintaining the genus *Holleya*. An examination of the data of Yamada and Nagahama [35] showed no phylogenetically informative sites for regions 18S-(1451–1618) and 26S-(1685–1835), and just four phylogenetically informative sites for region 26S-(493–625).

Phylogenetic analysis of rDNA sequences from the present study shows that species of the genera *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora* are monophyletic and exhibit little interspecific divergence. These criteria argue for assignment of the species to a single genus. At odds with this interpretation is the disparity in coenzyme Q values found among the species. As discussed earlier, some genera show no differences in coenzyme Q contents whereas other genera, and even some species, produce two forms, but none has the wide range seen among species of the *Ashbya/Eremothecium/Holleya/Nematospora* clade. Because rRNA/rDNA comparisons have been reliable predictors of kinship among other groups of yeasts [12], the sequence data are given precedence in the present study. It should also be noted that ascus size varies considerably among the species, but ascospores range no more than two- to three-fold in size and thus parallel the variation in ascospore size found for species of the genus *Metschnikowia* [19].

For the reasons given above, it is proposed to assign all species of the *Ashbya/Eremothecium/Holleya/Nematospora* clade to *Eremothecium*, the genus of taxonomic priority. The following is an emended diagnosis of *Eremothecium*: colonies yellow to yellow-orange from the presence of riboflavin or white to faintly dark tan, and smooth and butyrous to floccose. Budding cells are present or absent. When present, budding is multipolar and often on a narrow base. Cells range from globose to cylindroidal and may show various shapes. Pseudomycelium may be produced. True hyphae are formed and are often sparsely septate. Asci may be free or show terminal or intercalary attachment to hyphae. Asci are deliquescent. Asci form 8–32 ascospores which are often arranged in two interleaving fascicles. Ascospores are acicular or falcate, one- or two-celled, and may have a terminal appendage which can be elongated and whiplike.

The following five species are accepted in the emended genus *Eremothecium*. Complete citations for synonyms have been given by Batra [3], Miller and Phaff [23] and von Arx et al. [29].

(1) *Eremothecium ashbyi* (Guilliermond ex Routien) Batra (1973)
Synonyms:
Eremothecium ashbyi Guilliermond (1935, invalid, Art. 38, ICBN)
Crebrothecium ashbyi Guilliermond ex Routien (1949)
?*Spermophthora gossypii* Ashby & Nowell (1926)

(2) *Eremothecium coryli* (Peglion) Kurtzman, comb. nov.
Basionym:
Nematospora coryli Peglion. *Atti della Reale Accademia dei Lincei, Serie Quinta. Rendiconti, Classe di Scienze, Fisiche, Matematiche e Naturali* 6: 276. 1897.
Synonyms:
?*Nematospora lycopersici* Schneider (1917)
?*Nematospora phaseoli* Wingard (1922)
Nematospora nagpuri Dastur (1930)

(3) *Eremothecium cymbalariae* Borzi (1888)

(4) *Eremothecium gossypii* (Ashby & Nowell) Kurtzman, comb. nov.
Basionym:
Nematospora gossypii Ashby & Nowell. *Annals of Botany* 40: 69. 1926.
Synonyms:
Ashbya gossypii (Ashby & Nowell) Guilliermond (1928)
Ashbia gossypii (Ashby & Nowell) Ciferri & Fragoso (1928)

(5) *Eremothecium sinecaudum* (Holley) Kurtzman, comb. nov.
Basionym:
Nematospora sinecauda Holley. *Antonie van Leeuwenhoek* 50: 309. 1984.
Synonym:
Holleya sinecauda (Holley) Yamada (1986)

Species of the genus *Eremothecium* can be separated by the appearance of their ascospores under the microscope (Figs 1–5). The nucleotide sequence 5′-GCTTCGGCCA (positions 604–613, Fig. 6) may be genus-specific because it distinguishes the five species of *Eremothecium* from the type species of all other genera of the ascomycetous yeast clade [17].

Eremothecium and its synonyms have been variously assigned to the Spermophthoraceae Guilliermond, Nematosporaceae Novak & Zsolt, and Metschnikowiaceae Kamienski. In view of the proposed taxonomic changes, the following new family is described:

Eremotheciaceae Kurtzman, fam. nov.

Cellulae globosae vel cylindricae, gemmatione multilaterali propagantes; hyphae septatae praesentes; asci ovoidei vel elongati, deliquescentes, 8–32 spori; ascosporae aciculares, septa-

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(63-162)
Sac. cerevisiae  AAACCAACCG GGATTGCCTT AGTAACGGCG AGTGAAGCGG CAAAAGCTCA AATTTGAAAT CTGGTACCTT CGGTGCCCGA GTTGTAATTT GGAGAGGCA
Cep. fragrans    .....A.... .....A.... .......... .....T..G. .......... .......... ....C..T.. .A...T.... .......... .A...A..T.
Ash. gossypii    .....A.... .....A.... .......... .....T..G. .......... .......... ....CG.... ...C.T.... .......... .A...AA.T.
Ere. ashbyi      .....A.... .....A.... .......... .....T..G. .......... .......... ....C..... ....T..... .......... .A...A.CT.
Ere. cymbalariae .....A.... .....A.... .......... .....T..G. .......... .......... ....CG.... ...C.T.... .......... .A...A.CT.
Hol. sinecauda   .....A.... .....A.... .......... .....T..G. .......... .......... ....CGT... ...C.T.T.. .......... .A...A.CT.
Nem. coryli      .....A.... .....A.... .......... .....T..G. .......... .......... ....CGT... ..AC.T.... .......... .A...A.TA.

(163-262)
Sac. cerevisiae  ACTTTGGGGC CGTTCCTTGT CTATGTTCCT TGGAACAGGA CGTCATAGAG GGTGAGAATC CCGTGTGGCG AGGAGTGC-- GGTTCTTTGT AAAGTGCCTT
Cep. fragrans    .......AT T.GCT..... .......... .......... .....C.... .......... .....C.AT. .-.-...CC AA.C..A... .......T..
Ash. gossypii    C...G.TT.. TAG...C... .......... .......... .....C.... .......... ....C..... -..G....-T A..G.CA.C. ..G..T.T..
Ere. ashbyi      CTC.G.A-.T TAG...C... .......... .......... .....C.... .......... ....C..... -..G....-T AA........ .G.....T..
Ere. cymbalariae CTC.G.A-A. TAG...C... .......... .......... .....C.... .......... ....C..... -..G....-T A....CA... .G.....T..
Hol. sinecauda   CTC.G.T-.. TAG...C... .......... .......... .....C.... .......... ....C..... -..G....-T A....CA... .G.....T..
Nem. coryli      CTC.GAT-.. TAG...C... .......... .......... .....C.... .......... ....C..... -..G....-T A..GTCA... .G.....T..

(263-362)
Sac. cerevisiae  CGAAGAGTCG AGTTGTTTGG GAATGCAGCT CTAAGTGGGT GGTAAATTCC ATCTAAAGCT AAATATTGGC GAGAGACCGA TAGCGAACAA GTACAGTGAT
Cep. fragrans    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Ash. gossypii    ...C...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Ere. ashbyi      ...C...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Ere. cymbalariae ...C...... .......... .......... .......... .....C.... .......... .......... .......... .......... ..........
Hol. sinecauda   ...C...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Nem. coryli      ...C...... .......... .......... .......... .......... .......... .......... .......... .......... ..........

(363-462)
Sac. cerevisiae  GGAAAGATGA AAAGAACTTT GAAAAGAGAG TGAAAAAGTA CGTGAAATTG TTGAAAGGGA AGGGCATTTG ATCAGACATG GTGTTTTGTG CCCTCTGCTC
Cep. fragrans    .......... .......... .......... .......... .......... .......... .....T.GA. .......T.. .......... TA.A..TTC.
Ash. gossypii    .......... .......... .......... .......... .......... .......... .......... .......... .....C. G..CTC....
Ere. ashbyi      .......... .......... .......... .......G.. .......... .......... .......... .......... .....C. G...A.A.C.
Ere. cymbalariae .......... .......... .......... .......... .......... .......... .......... .....G.... .....C. G..CTC....
Hol. sinecauda   .......... .......... .......... .......... .......... .......... .......... .....C.... .....CGC GG.A..TAC.
Nem. coryli      .......... .......... .......... .......... .......... .......... .......... .....G.... .....C. G.ACTC....

(463-562)
Sac. cerevisiae  C-TTGTGGGT AGGGGAATCT C-GCATTTCA CTGGGCCAGC ATCAGTTTTG GTGGCAGGAT AAATCCATAG GAATGTAGCT TGC-CT-CGG T--AAGTATT
Cep. fragrans    T-.CT...A. T.T.-CT.TC GCATTAC.-. .C........ ...G....G. ....T..... ..TGA..T.. ...C..G.... CTA-..T... .AG.-..G..
Ash. gossypii    -.-.....-. .C...G.... .......... .......... .....T.... .......C.G ...C.T.... .T.T..T... AGG....G..
Ash. gossypii    -.-.....-. .C...G.... .......... .......... .....T.... .......C.G ...C.T.... .T.T..T... AGG....G..
Ere. ashbyi      -.T..-..A .C...GC... .......... .......... .....T.... .......C.G ...C.T.... .CTT..T... AGG....G..
Ere. cymbalariae -.C..-..A .C...GC... .......... .......... .....T.... .......C.G ...C.T.... .T.T..T... AGG....G..
Hol. sinecauda   -.C..-..A .C...GC... .......... .......... .....T.... .......C.. ...C.T.T.. .T.T..T... AGG....G..
Nem. coryli      -.C..-..A .C...GC... .......... .......... .....T.... .......C.. ...C.T.G.. .TGT..T... AGG....G..

(563-642)
Sac. cerevisiae  ATAGCCTGTG GGAATACTGC CAGCTGGGAC TGAGGACTGC GACGTAAGTC AAGGATGCTG GCATAATGGT TATATGCCGC
Cep. fragrans    .....T.... TTG..G..... .TA.CTA... C.......... .T.T.TT.A. T.......... ..G.....A. CT..A..... .....
Ash. gossypii    .....AA... .TT..G..... ..A.C..... .......... .G.T.CG.C. .......... .......... ..A..... .....
Ere. ashbyi      .....AA... .TC..G..... ..A.C..... .......... .G.T.CG.C. .......... .......... ..A..... .....
Ere. cymbalariae .....A.... .AC..G..... ..A.C..... .......... .G.T.CG.C. .......... .......... ..A..... .....
Hol. sinecauda   .....TA... .TC..G..... ..A.C....T .......... .G.T.CG.C. .......... .......... ..A..... .....
Nem. coryli      .....CAA.. .TC..G..... ..A.C..... .......... .G.T.CG.C. .......... .......... ..A..... .....
```

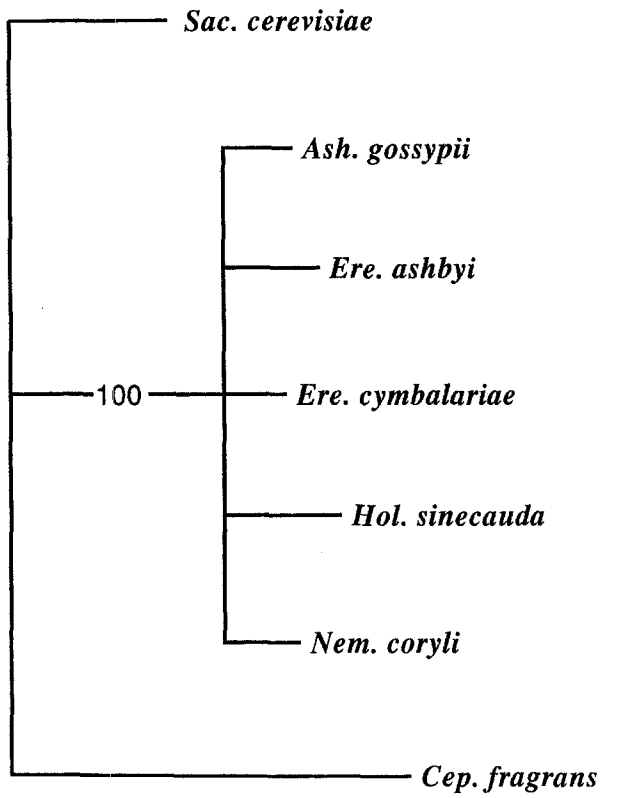

Fig. 7. Strict consensus tree calculated from the branch-and-bound option of PAUP using the sequences from Fig. 6. Parameters from the analysis are given in the text. Branch lengths are proportional to the numbers of steps in the calculation. *S. cerevisiae* was the designated outgroup in the analysis. *Sac.* = *Saccharomyces*, *Cep.* = *Cephaloascus*, *Ash.* = *Ashbya*, *Ere.* = *Eremothecium*, *Hol.* = *Holleya*, *Nem.* = *Nematospora*.

tae vel aseptatae, glabrae vel ornamentatae. Genus typicum: *Eremothecium* Borzi. *Bolletino della Societa di Botanico Italiana* 20: 455. 1888.

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Fig. 6. Alignment of 26S rDNA sequences from positions 63–642 from species of *Saccharomyces*, *Cephaloascus*, *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora*. The sequence of *S. cerevisiae* is given as a reference. Dots indicate nucleotides identical to the reference species and dashes indicate deletions.

TABLE 2

Matrix of differences between species of *Ashbya*, *Eremothecium*, *Holleya*, *Nematospora*, *Saccharomyces*, and *Cephaloascus* for nucleotides 63–642 of 26S rDNA

Species	Nucleotide differences/580 positions [a]					
	S. cer.	*A. gos.*	*E. ash.*	*E. cym.*	*H. sin.*	*N. cor.*
Ash. gossypii	65					
Ere. ashbyi	66	30				
Ere. cymbalariae	67	22	20			
Hol. sinecauda	77	36	33	30		
Nem. coryli	76	25	31	22	28	
Cep. fragrans	98	106	100	107	100	109

[a]Nucleotide differences between conspecific strains:
Ash. gossypii NRRL Y-1056/NRRL Y-1810 = 0;
Ere. ashbyi NRRL Y-1363/NRRL Y-7249 = 1;
Nem. coryli NRRL Y-12970/NRRL Y-1618 = 0.

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