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SHORT COMMUNICATION

Intraspecific variation and emendation of *Hannaella kunmingensis*

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Abstract In order to investigate the intraspecific variability in *Hannaella kunmingensis*, 11 isolates, including the type strain, were analyzed for their morphological and biochemical traits. The combined internal transcribed spacer region (ITS), D1/D2 domains of the large subunit rDNA (LSU), and cytochrome *b* gene were examined using phylogenetic and parsimony network analyses. Our investigations revealed differences in colony morphology as well as differences in 31 out of 64 phenotypic characteristics examined, including growth in lactose, vitamin free medium, xylitol, L-arabinitol, and nitrite. Growth in the presence of 0.1 % cycloheximide was also highlighted in *H. kunmingensis*. All the 11 strains were conspecific in the LSU; however, variations of about 2.5 % were found in the ITS while isolate CBS 8356 exhibited a 27.3 % divergence from the other strains in the cytochrome *b* gene. Parsimony network analysis revealed the existence of three haplotypes among the *H. kunmingensis* strains studied but excluded CBS 8356 from the network connecting these haplotypes. This study contributes to the knowledge of the intraspecific diversity of *H. kunmingensis*. To accommodate such intraspecific variations, an emendation of the species diagnosis is proposed.

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Introduction

Long before the current era of species identification by DNA comparisons, yeasts were delineated based on differences in morphological and phenotypic attributes (Kurtzman 2011). Most of these characteristics are variable, hence, the need for more accurate diagnostic tools for yeast species identification soon became apparent (Kurtzman and Fell 2006; Kurtzman 2011). The use of DNA sequence comparisons for yeast identification was widely embraced after Kurtzman and Robnett (1998) predicted that strains showing six or more nucleotide differences (1 % substitution) in the D1/D2 domains of the large subunit rDNA (LSU) are likely to represent different species particularly for ascomycetous yeasts. Complementary D1/D2 (Fell et al. 2000) and internal transcribed spacer (ITS) databases (Sugita et al. 1999, 2000; Scorzetti et al. 2002) were established for basidiomycetous yeasts and are continually being updated by describers of new taxa.

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The extent of phenotypic and genetic variations among basidiomycetous yeast strains that share a common gene pool have been continuously revealed by several studies (Scorzetti et al. 2002; Kurtzman et al. 2011a). Typical examples include intraspecific diversity exhibited by some *Cryptococcus* species. Based on their serological variances, strains of *C. neoformans* (San Felice) Vuill. and *C. gattii* (Vanbreus. & Takashio) Kwon-Chung & Boekhout have been classified into serotypes A, D and AD for the former, and B and C for the latter (Boekhout et al. 2001; Kwon-Chung et al. 2002; Bovers et al. 2006, 2008). Although with no genetic variation, heterogeneity in colony morphology and physiological characteristics were observed in species of *C. victoriae* M.J. Montes et al., with the color ranging from cream, pale yellowish-brown to pinkish and the surface ranging from smooth, somewhat warty or reticulate (Montes et al. 1999; Thomas-Hall et al. 2002). Rhode (2006) reported differences in the carbon assimilation profiles and growth temperature requirements of 35 strains of *C. laurentii* (Kuff.) C.E. Skinner isolated from a soil sample while Sugita et al. (2000) also reported intraspecific ITS diversity among some other strains of this species.

Intraspecific variations have also been reported for mating strains of the same species. For example, sexually compatible strains of the heterothallic species *Sporidiobolus salmonicolor* Fell & Tallman (CBS 490 and CBS 2630) were found to differ by seven and 26 nucleotide positions in the LSU and ITS, respectively (Sampaio 2011).

Inference of evolutionary relationships between different yeasts has, over the years, relied mostly on trees derived from phylogenetic analyses of rDNA sequence data. However, studies have revealed that phylogenetic trees sometimes do not accurately measure gene genealogies of haplotypes resulting from intraspecific polymorphisms (Clement et al. 2000; Posada and Crandall 2001). Parsimony network analysis (TCS) on the other hand provides the advantage of a network that discriminates better among haplotypes than a tree with dichotomic splits (Hart and Sunday 2007). Studies applying parsimony network analysis for the estimation of gene genealogies in basidiomycetous yeasts are scanty. However, by analyzing the LSU and ITS of multiple isolates of the ascomycetous yeast *Candida apicola* (Hajsig) S. A. Mey. & Yarrow, 28 out of 30 strains which could have otherwise been classified into separate species if the criterion predicted by Kurtzman and Robnett (1998) were adopted, were retained in this species (Lachance et al. 2010). Applying similar analysis to 81 strains formerly classified in *Candida azyma* (van der Walt, Johannsen & Yarrow) S.A. Mey. & Yarrow, Lachance et al. (2010) reassigned 57 isolates to the new species *C. parazyma* Lachance while only 18 isolates were retained in *C. azyma*. Besides yeast taxonomy, parsimony network analysis has been used to determine mitochondrial haplotypes in *Phytophthora cinnamomi* Rands (Martin and Coffey 2012) as well as species boundaries in cephalotrichid nemerteans (Chen et al. 2010). According to Kurata et al. (2008) parsimony analysis of the chloroplast DNA of the pitcher plant *Nepenthes vieillardii* Hook. F. (Nepenthaceae) in New Caledonia revealed the presence of 17 haplotypes within this species.

Recent phylogenetic revision of the *Luteolus* lineage in Tremelalles based on sequences of the nuclear rRNA (18S rDNA, ITS and LSU) and mitochondrial cytochrome *b* genes led to the creation of two genera, namely *Derxomyces* and *Hannaella* (Wang and Bai 2008). While the genus *Derxomyces* (type species: *Derxomyces mrakii* (Hamam. & Nakase) F.Y. Bai & Q.M. Wang) was proposed to accommodate species in the *Bullera mrakii* Hamam. & Nakase clade, the genus *Hannaella* accommodates species in the *Bullera sinensis* M.X. Li clade (formerly classified in *Bullera* and *Cryptococcus*). Therefore, yeast species formerly classified in the genera *Cryptococcus* and *Bullera* within these clades have been re-classified as *Derxomyces* or *Hannaella* (Wang and Bai 2008).

Here we report results of intraspecific diversity within the taxon *Hannaella kunmingensis* F.Y. Bai, M. Takash. & Nakase ex F.Y. Bai & Q.M. Wang, anamorphic basidiomycetous yeast formerly described as *Bullera kunmingensis* F.Y. Bai, M. Takash. & Nakase. We subjected 11 strains (including the type strain CBS 8960^T) isolated from different substrates and geographic locations, to detailed physiological tests and genetic characterization using phylogenetic and parsimony network analysis (TCS). Based on our findings, an emendation of *Hannaella kunmingensis* is proposed.

Materials and methods

Strains used

A total of 11 strains of *H. kunmingensis* were used in this study (Table S1). Strains ATT 066, ATT 082 and ATT 265 were isolated from fungus gardens of the leafcutter ant *Atta texana* Buckley at Hornsby Bend Environmental Research Center, Austin, Texas, USA. Two strains, ATT 082 and ATT 066, were isolated during the winter season from nests at two different locations (30°13′97″N, 97°39′10″W and 30° 13′94″N, 97°39′18″W for nests UGM060121-01 and UGM060121-02, respectively); while strain ATT 265 was isolated during fall season from nest UGM060121-01. For yeast isolation, 1 g of each garden sample was initially diluted ten-fold in 0.05 % Tween 80 and 0.2 % peptone water and plated in YMA with antibiotic (Rodrigues et al. 2009).

Five strains originating from Brazil (BI 96, BI 203, BI 254, BI 279 and BI 319) were isolated from three bromeliad plants (Landell et al. 2006). Leaves and flowers of the

bromeliads were collected in May and November 2004, January 2005 and August 2006 in Itapuã Park, South of Brazil (50°50′ to 51°05′W and 30° 20′ to 30°27′S). Strains BI 96 and BI 279 were isolated from the flowers and strain BI 203 from leaves of *Tillandsia gardneri* Lindl. Strains BI 254 and BI 319 were obtained from the leaves of *Vriesea friburgensis* Mez and *Aechmea recurvata* (Klotzsch) L.B. Smith, respectively. Furthermore, strains CBS 8356 and CBS 8367 were discovered in Rio de Janeiro from a bee (*Trigona* sp.) and snail (*Helix* sp.), respectively. These two strains and their phenotypic data were kindly provided by Jack W. Fell (Rosenstiel School of Marine and Atmospheric Science, University of Miami). *H. kunmingensis* strain CBS 8960^{T} was also freely donated by Feng-Yan Bai (Systematic Mycology and Lichenology Laboratory, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China) and was also included in our analyses.

Phenotypic and morphological characterization

Phenotypic characteristics of the yeast isolates were carried out on solid media by replica plating according to tests described by Kurtzman et al. (2011b). A total of 64 phenotypic tests were conducted, including tests of assimilation of various carbon and nitrogen sources and other tests such as growth at various temperatures, formation of extracellular amyloidal compounds as well as cycloheximide resistance and the results are provided in the emendation. Tests in which discrepancies in results in comparison with the type strain occurred were repeated in liquid medium in glass tubes (16×180 mm) each containing 5 ml of the test substrate (such results are provided in Table 1). All tests were performed at 25 °C and results were checked at 7 days interval for 3 weeks.

For macro-morphological characterization, cultures were grown on YM medium (1.0 % glucose, 0.5 % peptone, 0.3 % yeast extract, 0.3 % malt extract, with and without 2.0 % agar) and studied with phase contrast microscope (Leica DM-1000). Mating experiments were performed by mixing all the strains in all combinations on cornmeal agar, 5 % malt extract agar and potato dextrose agar (PDA) for at least 3 months and checked microscopically at weekly intervals.

DNA extraction, amplification and sequencing

Genomic DNA from 2 to 3 day-old cultures was extracted according to methods described by Almeida (2005). The diluted (1:750) crude genomic DNA was used to amplify the domains D1/D2 of the LSU gene, the complete ITS region (ITS1 - 5.8S rDNA - ITS2) and the mitochondrial cytochrome *b* gene. DNA amplification and cycle sequencing reactions were carried out using primers NL1 and NL4 for the LSU; and primers ITS1 and ITS4 for the ITS (White et al. 1990). Mitochondrial cytochrome *b* gene amplification was carried out using the forward primer E1M4 (5′-TGR GGW GCW ACW GTT ATTACT A-3′) and the reverse primer E2mr3 (5′-GGW ATA GCA CGT ARA AYW GCR TA-3′) according to Biswas et al. (2001). Amplification scheme for the LSU and ITS followed Pagnocca et al. (2008) while the protocol described by Wang and Bai (2008) was used for the mitochondrial cytochrome *b* gene. Primers used for the cycle sequencing reactions of cytochrome *b* gene were both the forward primers E1M4 and CytBf (5′-TAA CAA TCA CCA TCT AC-3′) and the reverse primer E2mr3 (Biswas et al. 2001). PCR amplicons were purified using *illustra PCR DNA and Gel Band Purification Kit* (GE Healthcare UK Limited, Buckinghamshire, UK). Sequencing reactions were performed in a 10 µl reaction using BigDye® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) and analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems).

Phylogenetic and parsimony network analyses

Obtained sequences were compared against GenBank database and the MycoID database (Mycobank). Sequences of *Hannaella* species were retrieved from the GenBank and aligned in Muscle v. 3.8 (Edgar 2004) followed by trimming in Bioedit (Hall 1999) to remove primer sequences. Phylogenetic relationships were inferred using concatenated sequences of the ITS, LSU and cytochrome *b* gene in MEGA 5 (Tamura et al. 2011). Neighbor-joining tree was generated using the Kimura 2-parameter to correct for genetic distances (Kimura 1980). Gaps were excluded from the analysis. The robustness of trees was calculated by using 1,000 bootstrap pseudoreplicates (Felsenstein 1985). Alignment and tree are available in TreeBASE under submission ID 12815. Pair-wise intra- and interspecific genetic distances among *H. kunmingensis* strains (n=10); and *Hannaella* species, respectively, were calculated using the Kimura 2-parameter model of nucleotide substitution in MEGA 5.

Parsimony network analysis was carried out in TCS v. 1.21 (Clement et al. 2000) using 95 % connection limit. The concatenated dataset of the ITS, LSU, and cytochrome *b* were used and gaps were excluded from the alignments. *Hannaella luteola* (Saito) F.Y Bai & Q.M. Wang, the closest phylogenetic neighbor of *H. kunmingensis* (Fig. 1) was also included in the network analysis to test if it will be retained in the same network. Overall, parsimony network analysis revealed four haplotypes using concatenated data set of the LSU, ITS and cytochrome *b* with an alignment length of 1,420 base pairs. All sequences generated in the present study are deposited at GenBank and accessions are denoted in Fig. 1 and Table S1.

Table 1 Micromorphological and phenotypic characteristics of 11 isolates of *Hannaella kunmingensis*

Characteristics	CBS 8960^T	ATT 082	ATT 066	ATT 265	BI 96	BI 203	BI 254	BI 279	BI 319	CBS 8356	CBS 8367
Morphological characteristics											
Shape	Cylindrical to ellipsoidal	Oval	Ellipsoidal	Oval	Cylindrical to ellipsoidal	Subglobose	Elongated oval	Oval	Elongated oval	Globose	Oval
Cell size (μm)	5–7.5×3–4	3.4–5.7×2.1–4.3	3.46×2–4.1	3.8–6.1×2.7–4.1	3–5.7×1.6–4.1	2.9–4.6×2.3–3.5	1.8–5.9×1.4–3.6	3.4–6.1×2.7–3.7	3.2–6.1×1.6–2.9	4–9.2×3.2–7.5	4.6–6.5×3.4–4.9
Ballistoconidia formation	+	−	+	+	−	−	+	−	−	−	−
Assimilation of:											
L-Sorbose	+	s	s	s	−	w	+	+	+	−	l
Cellobiose	+	+	+	+	−	+	+	+	+	+	+
Trehalose	+	+	+	+	−	+	+	+	+	+	+
Lactose	−	−	l	lw	−	−	+	+	w	+	l
Soluble starch	+	w	w	w	+	+	+	+	+	+	−
D-glucosamine	+	−	−	−	−	lw/−	+	−	−	−	+
Ethanol	+	+	+	+	+	+	−	+	+	+	+
Galactitol	+	+	+	+	−	+	+	+	−	+	+
D-glucitol	s/w	−	−	−	+	+	+	+	+	+	l
Salicin	+	−	−	−	−	lw	l	−	+	+	+
D-gluconate	+	v	+	+	lw	+	+	+	−	+	+
DL-lactate	+	+	+	+	+	+	−	+	+	+	+
Succinate	+	+	w	+	+	+	−	+	+	+	+
Citrate	+	+	+	+	l	−	+	+	w	+	+
Xylitol	–[a]	+	+	+	w	w	+	+	w	+	+
L-arabinitol	–[a]	+, s	+	s	w	w	+	w	w	+	l
D-Glucono-1,5-lactone	+	+	+	+	−	+	+	+	+	+	+
Propane 1,2 diol	–[a]	lw	lw	lw	−	lw	−	lw	lw	lw	+
Nitrite	+	−	−	−	−	−	−	−	−	+	−
Creatine	–[a]	−	−	lw	−	−	−	−	−	−	−
Cadaverine	+	+	+	+	−	+	+	+	+	+	+
Imidazole	–[a]	−	lw	−	−	lw	−	lw	lw	−	−
Creatinine	l[a]	−	−	−	−	−	−	−	−	−	−
Ethylamine	+	−	−	−	+	−	+	+	−	+	+
10 % NaCl/Glucose 5 %	−	lw	lw	w	w	w	l	lw	w	w	w
Starch synthesis	+	+	+	+	−	+	−	+	+	+	+
Additional tests:											
Growth in 0.01 % cycloheximide	–[a]	−	−	−	−	−	−	−	−	+	+
Growth in 0.1 % cycloheximide	–[a]	−	−	−	−	−	−	−	−	+	s

Table 1 (continued)

Characteristics	CBS 8960^T	ATT 082	ATT 066	ATT 265	BI 96	BI 203	BI 254	BI 279	BI 319	CBS 8356	CBS 8367
Growth in vitamin-free medium	–	+	+	+	+	+	+	+	+	+	+
Growth at 30 °C	v	+	+	+	–	–	–	–	–	–	–
Growth at 32 °C	–	w	w	w	–	–	–	–	–	–	–

+ positive growth; – no growth; *w* weak growth; *l* latent (rapid positive growth after 7 days); *s* positive but slow; *lw* latent but weak (rapid development of a weak growth after days); *v* positive or negative results; *n* not tested

[a] Physiological tests carried out by our laboratory because were not included in the original description of *H. kunmingensis* (Bai et al. 2001)

Results

Morphological characteristics

Except for strain CBS 8960^T which forms white colonies, all isolates formed mucoid or fluidy cream colonies after 5 days at 25 °C in YM agar. Micro-morphology of the isolates showed different cell sizes and shapes ranging from oval, ellipsoidal, globose to elongate. Cells of strain CBS 8356 (4–9.2×3.2–7.5 μm) followed by CBS 8960^T (5–7.5×3–4 μm) were the largest (Table 1). Ballistoconidia were confirmed in the type strain and three other isolates (Table 1). Mating reactions were not observed in the crossing experiments performed with the 11 strains. Moreover only strain CBS 8356 produced pseudohyphae which are visible as filaments around the colonies after 1 week growth on cornmeal agar incubated at 15 °C.

Physiological variations

Differences were observed in 31 phenotypic tests namely 18 and six carbon and nitrogen sources, respectively, and seven additional tests (Table 1). Except for strain CBS 8960^T, all strains grew in vitamin free, xylitol and L-arabinitol media (Table 1). The latter two tests and additional ones (see Table 1) were not included in the original species diagnosis of *H. kunmingensis*. Conversely, only strains CBS 8960^T and CBS 8356 assimilated nitrite (Table 1). Growth in 0.1 % cycloheximide was observed in two strains CBS 8356 and CBS 8367.

Sequence analyses

The 11 strains of *H. kunmingensis* showed 100 % sequence similarity in the LSU (597 bp), but 97.5 % similarities were observed in the ITS (437 bp) when the type strain was compared with the remaining strains. Pair-wise comparisons of the cytochrome *b* gene revealed 100 % similarities in all the strains except for strain CBS 8356 which exhibited a similarity of 72.7 % with the other strains. Because of unsuccessful alignment of the cytochrome b gene of strain BI 254 with sequences of the other strains, it was not included in phylogenetic and parsimony network analyses. Phylogenetic analysis revealed that all except strain CBS 8356 are conspecific with high bootstrap support (Fig. 1). The phylogram showed that strain CBS 8356 occupies a somewhat separate position in the tree in comparison with the other strains (Fig. 1). Genetic distances data revealed that among *H. kunmingensis* strains, values ranged from 0 to 0.019 in the ITS and 0–0.339 in the cytochrome *b* gene; moreover, no variation was found in the LSU. Interspecific genetic distance ranged from 0.033 to 0.068 in the LSU, 0.055–0.173 in the ITS and 0.029–0.094 in cytochrome *b*

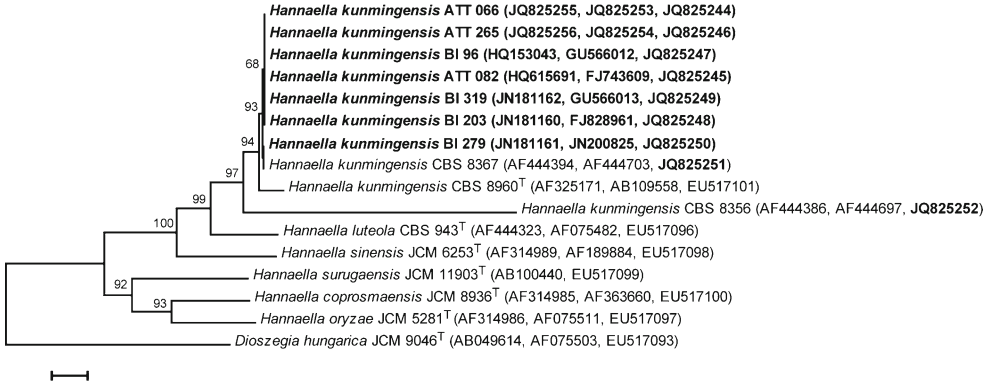

Fig. 1 Neighbour-joining tree obtained from combined ITS, LSU and cytochrome b gene of *Hannaella kunmingensis* and related species. The Kimura two-parameter substitution model was used to calculate genetic distances. Numbers on the tree branches indicate bootstrap support values from 1,000 pseudoreplicates (values less than 50 % are not shown). *Dioszegia hungarica* was used as outgroup. Numbers in *parentheses* indicate GenBank accessions. Sequences with accessions in bold were generated in this study. *Scale bar* represents 0.01 substitutions per site

gene. Mean ± standard error intraspecific and inter-specific divergence were 0.005±0.002 and 0.064±0.007 in the ITS, respectively; while values of 0.079±0.007 and 0.068±0.007, respectively, were obtained for the cytochrome *b* gene. Under the 95 % connection limit, parsimony network analysis generated three connected haplotypes, and two distinct haplotypes consisting of CBS 8356 and *H. luteola* CBS 943^T, that were separated from the network (Fig. 2).

Emendation of *Hannaella kunmingensis*

Description of taxa

Bai et al. (2001) provided the following description of *Hannaella kunmingensis* formerly *B. kunmingensis*. After 5 days at 17 °C on YM broth, cells are oval, ellipsoidal, or long ellipsoidal, 2.5–10×5–12 μm. Budding is polar with sympodial proliferation and cells may adhere in short chains. Culture on YM agar after 1 month at 17 °C is grayish white to grayish yellow, mucous to fluid and shining with entire margin. Globose, napiform or turbinate ballistoconidia are formed on cornmeal agar. The colony is low convex, pale yellowish-brown, shiny, smooth and mucoid, and has an entire margin. Ballistoconidia on cornmeal agar are globose, napiform to turbinate, 4–12×3.5–10 μm. Glucose is not fermented. Glucose, sucrose, raffinose, melibiose, galactose, trehalose, maltose, melezitose, methyl-α-D-glucoside, soluble starch, cellobiose, salicin, L-sorbose, L-rhamnose, D-xylose, L-arabinose, D-arabinose, D-ribose, ethanol, glycerol, erythritol, ribitol (s), galactitol, D-mannitol, D-glucitol (s/w), myo-inositol, L-lactate, succinate, citrate, D-gluconate, D-glucosamine, 2-keto-D-gluconate, D-glucuronate are assimilated. Growth on inulin, lactose, methanol, nitrate, 50 % glucose, 10 % NaCl/5 % glucose is negative, while maximum growth temperature requirement is between 28 °C and 29 °C.

To accommodate variations observed in additional strains, we propose to emend the description of the species *H. kunmingensis* as follows:

Growth in YM broth: After 5 days at 25 °C, cells are oval, ellipsoidal, elongated, cylindrical, globose or subglobose

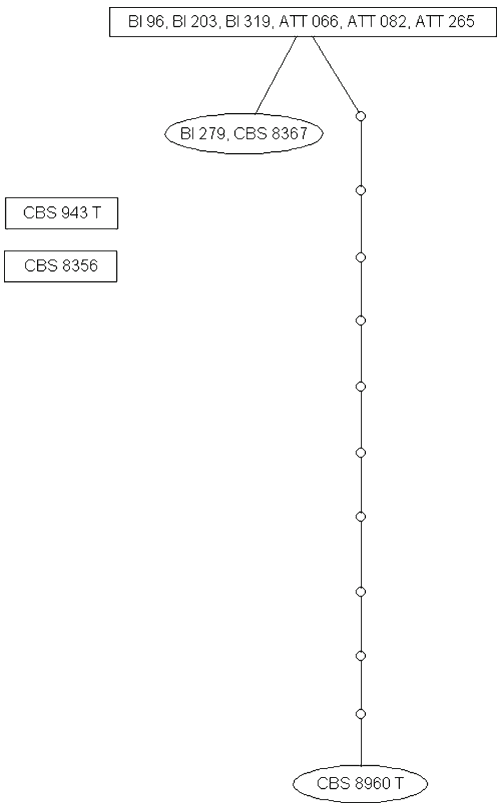

Fig. 2 Parsimony network analysis of the ITS, LSU rRNA and cytochrome *b* gene of strains identified as *H. kunmingensis*. Each *connecting line* represents one substitution and each *small circle* represents a missing intermediate sequence. The *rectangle* connected in the network identifies the haplotype considered as ancestral by the analysis. Isolated haplotype represents strain excluded from the network

and with sizes ranging from 3.2–6.1 × 1.6–2.9 μm to 4–9.2 × 3.2–7.5 μm. Asexual reproduction is by polar budding with sympodial proliferation or by multilateral budding. Ballistoconidia may or may not be formed and pseudohyphae may or may not be present. Colonies on YM agar after 5 days are convex, whitish-cream, yellowish-cream, shiny, smooth, mucoid with entire margin.

Fermentation is absent. Growth on the following carbon and nitrogen compounds are variable, i.e. positive or negative, depending on strain: L-sorbose, cellobiose, trehalose, lactose, soluble starch, D-glucosamine, ethanol, galactitol, D-glucitol, salicin, D-gluconate, DL-lactate, succinate, citrate, xylitol, L-arabinitol, D-glucono-1,5-lactone, propane 1,2 diol, nitrite, creatine, 10 % NaCl/5 % glucose, imidazole, creatinine, ethylamine, cadaverine (Table 1). Starch synthesis, growth on cycloheximide (0.01 % and 0.1 %) and vitamin-free medium are also variable. *H. kunmingensis* grew at 15 °C and the maximum temperature at which growth was recorded is 32 °C.

Systematics

The LSU showed no variation for the strains of *H. kunmingensis*. From analysis of the complete ITS, variation of nine nucleotide differences (and two gaps) were observed between strains ATT 082, ATT 066, ATT 265, B1 96, BI 203, BI 279, BI 319, CBS 8356, CBS 8367 and the type strain CBS 8960^T. Analysis of the cytochrome b gene also revealed intraspecific variation; Pairwise comparison of the sequence of CBS 8356 with the type strain CBS 8960^T revealed 104 base-pair substitutions i.e., 27.3 % nucleotide difference in the cytochrome *b* gene.

Discussion

Morphological, phenotypic as well as genetic properties based on the ITS, LSU and cytochrome *b* gene of strains of *H. kunmingensis* were compared. Our findings showed that all the strains examined in this study, including the type strain, demonstrated wide variations in their capacity to assimilate various carbon and nitrogen sources. Some phenotypic characteristics that were previously under-estimated in *H. kunmingensis*, such as assimilation of lactose and ability to grow in the presence of 10 % NaCl/glucose 5 % and 0.1 % cycloheximide, were shown after the inclusion of additional strains to this species. All the studied strains were comparable in the LSU suggesting that this genetic marker is non-variable in this species, but the ITS and cytochrome *b* gene regions were found to be heterogeneous in *H. kunmingensis*. Pair-wise genetic distances of the ITS region revealed the existence of variation among *H. kunmingensis* strains. However, when considering the cytochrome *b* gene, intraspecific variation within *H. kunmingensis* (0.000–0.339) was found to exceed interspecific variation (0.029–0.094). The reason for this observation was due to the sequence of strain CBS 8356 which highly varied from the other strains. In a study investigating potential DNA barcodes for fungi, ITS was rated superior to the LSU for the delimitation of basidiomycetous species based on its high species resolving power and more clearly defined barcode gap (Seifert 2009; Schoch et al. 2012). Our findings showed that there was no overlap between intraspecific (among *H. kunmingensis*) and interspecific (between *Hannaella* species) distances in the ITS.

The high discriminative power of ITS have assisted in solving many taxonomic and systematic problems relating to separation of species; hence, ITS was selected as the DNA-barcode of fungi (Begerow et al. 2010, Schoch et al. 2012). The ITS region is often used for delineating yeast basidiomycetous species that are too closely related to be distinguished using the D1/D2 domains of the LSU (Scorzetti et al. 2002). Cytochrome *b* gene has also proven useful in the phylogenetic analysis of basidiomycetous yeasts (Biswas et al. 2005) and was used to resolve phylogenetic relationships among the *B. mrakii*, *Hannaella sinensis* (M.X. Li) F.Y. Bai & Q.M. Wang (= *B. sinensis*) and *Dioszegia* clades (Wang and Bai 2008); however, this genetic region is often associated with introns (Biswas et al. 2001, 2005) which could make amplification and sequencing difficult. Both ITS and cytochrome *b* gene confirmed to be useful for species delineation in *H. kunmingensis* except for strain CBS 8356 (see below).

Nine strains clustered together with a high bootstrap support on the phylogenetic tree confirming they belong to the same lineage (Fig. 1). Strain CBS 8356 did not cluster with the other strains of *H. kunmingensis* due to the high variability in cytochrome *b* gene. Ninety-five percent parsimony criterion also accepted the nine strains in a single network (Fig. 2), thus affirming that they were related to each other while at the same time revealing the existence of three haplotype groups within these strains (Fig. 2). The haplotype containing sequences of ATT 082, ATT 066, ATT 265, BI 96, BI 203, and BI 319 was considered ancestral according to the criterion of Posada and Crandall (2001). Parsimony network analysis further reinforced the distinct status of the strain CBS 8356 by excluding it as well as *H. luteola* (closest phylogenetic neighbor of *H. kunmingensis*) from the main network (Fig. 2). Based on the outcomes of phylogenetic and parsimony network analyses, two scenarios could be deduced. The first possibility is that strain CBS 8356 is a variety of *H. kunmingensis* which suffered mutational changes in the cytochrome *b* gene. Another possibility could be a divergent strain tending

towards speciation. Hart and Sunday (2007) discussed the accuracy of the TCS analysis for differentiating species. They reported the method as having a high positive rate to identify known species boundaries as well as for discovering new species from sequence data especially where such taxa were adequately sampled. They were however cautious to establish an edge for species boundaries for taxa with low sampling effort as observed in this study.

With respect to phenotypic characteristic, strain CBS 8356 differed from the type strain by inability to assimilate L-sorbose and D-glucosamine and its positive growth in xylitol and vitamin-free medium (Table 1). Some remarkable traits exhibited by strain CBS 8356, however, are its ability to grow rapidly in the presence of 0.1 % cycloheximide and to produce pseudohyphae. Ability to grow in the presence of cycloheximide is infrequent among yeasts (Kurtzman et al. 2011a). Resistance or sensitivity to cycloheximide can be used for the taxonomic separation of species. For example, separation of the two ascomycetous species *Candida apicola* and *Starmerella bombicola* C.A. Rosa & Lachance is possible based on their negative and positive growth, respectively, in the presence of 0.001 % cycloheximide while *C. ambrosiae* Kurtzman and *C. atmosphaerica* Santa María are phenotypically distinguishable only in resistance to 0.01 % cycloheximide and growth at 37 °C (Lachance et al. 2011). Media supplemented with cycloheximide can also be used to selectively isolate yeasts with the ability to grow in the presence of this antibiotic as demonstrated by Yurkov et al. (2012) who used medium containing 5 mg/L of cycloheximide to isolate species of *Rhodotorula, Rhodosporidium, Mastigobasidium, Cryptococcus, Cystofilobasidium, Holtermanniella*, *Trichosporon* and *Kluyveromyces*. Phenotypic characteristics such as growth in the presence of cycloheximide could be of important value if additional strains with these same traits are discovered.

Considering the fact that the taxonomic status of CBS 8356 could not be clearly resolved, the isolation of more strains and their full characterization in relation to known strains of *H. kunmingensis* could shed light into this taxon.

Conclusion

This work demonstrated the sequence variability (ITS and cytochrome *b* gene) and phenotypic traits among strains identified as *H. kunmingensis*. Application of TCS network analysis demonstrated the relationship among the strains confirming the usefulness of such approach. The diagnosis of *H. kunmingensis* was emended based on these new observations.

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Table S1. Strains examined in this study

Strain	Source	Location	GenBank accession[1]			Reference
			ITS	LSU	Cyto b	
CBS 8960^T	Leaves of *Parthenocissus* sp.	Yunnan, China	AF325171	FJ743609	EU517101	Bai et al. (2001), Wang and Bai (2008).
ATT 066	Fungus garden of *Atta texana* (Fall, 2006)	Hornsby Bend Environmental Research Center, Austin, Texas, USA	JQ825255	JQ825253	JQ825244	Rodrigues et al. (2009)
ATT 082 (= CBS 11954)	Fungus garden of *Atta texana* (Winter, 2006)	Hornsby Bend Environmental Research Center, Austin, Texas, USA	HQ615691	FJ743609	JQ825245	Rodrigues et al. (2009)
ATT 265	Fungus garden of *Atta texana* (Winter, 2006)	Hornsby Bend Environmental Research Center, Austin, Texas, USA	JQ825256	JQ825254	JQ825246	Rodrigues et al. (2009)
BI 96	Flowers of *Tillandsia gardneri* (May 2004)	Pedreira Beach, Itapuã park, Rio Grande do Sul, Brazil	HQ153043	FJ743609	JQ825247	Landell et al. (2006)
BI 203	Leaves of *Tillandsia gardneri* (November, 2004)	Pedreira Beach, Itapuã park, Rio Grande do Sul, Brazil	JN181160	FJ828961	JQ825248	Landell et al. (2006)
BI 254	Leaves of *Vriesea friburgensis* (January, 2005)	Pedreira Beach, Itapuã park, Rio Grande do Sul, Brazil	HQ153045	FJ828963	ND[2]	Landell et al. (2006)
BI 279	Flowers of *Tillandsia gardneri* (August, 2006)	Pedreira Beach, Itapuã park, Rio Grande do Sul, Brazil	JN181161	JN200825	JQ825250	Landell et al. (2006)
BI 319	Leaves of *Aechmea recurvata* (August, 2006)	Pedreira Beach, Itapuã park, Rio Grande do Sul, Brazil	JN181162	GU566013	JQ825250	Landell et al. (2006)
CBS 8356	Bee (*Trigona* sp.),	Botanical Garden, Rio de Janeiro, Brazil	AF444386	AF444697	JQ825252	Kurtzman et al. (2011a)
CBS 8367	Snail (*Helix* sp.)	Tijuca forest, Rio de Janeiro, Brazil	AF444394	AF444703	JQ825251	Kurtzman et al. (2011a)

[1] ITS: internal transcribed spacer region (ITS1 – 5.8S rDNA – ITS2); LSU: large subunit (28S) rDNA; Cyto b: cytochrome b gene.

[2] ND: sequence not determined. Due to alignment failure with other *Hannaella* sequences, this strain was not included in molecular analyses