Bullera cylindrica sp. nov., *Bullera hubeiensis* sp. nov. and *Bullera nakasei* sp. nov., ballistoconidium-forming yeast species from plant leaves

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Among yeasts isolated from plant leaves collected in different regions of China that form whitish or yellowish colonies and symmetrical ballistoconidia, four strains were shown to represent three novel *Bullera* species by conventional and molecular taxonomic characterization. The novel species are described as *Bullera cylindrica* sp. nov. (type strain CB 169^T = AS 2.2308^T = CBS 9744^T), *Bullera hubeiensis* sp. nov. (type strain HX 19.3^T = AS 2.2466^T = CBS 9747^T) and *Bullera nakasei* sp. nov. (type strain HX 15.5^T = AS 2.2435^T = CBS 9746^T). These three species, and another eight previously described *Bullera* species represented by *Bullera mrakii*, formed a strongly supported distinct clade among the hymenomycetous yeasts in each of the phylogenetic trees drawn from the 26S rDNA D1/D2 domain and the internal transcribed spacer region sequences.

INTRODUCTION

In the *Cryptococcus luteolus* lineage of hymenomycetous yeasts defined on the basis of 18S rDNA sequence analysis (Takashima & Nakase, 1999), two species isolated from plants collected in New Zealand, *Bullera mrakii* and *Bullera huiaensis* (Hamamoto & Nakase, 1996), form a distinct clade. Three additional *Bullera* species isolated from the Ogasawara Islands, Japan, were added to the clade by Sugita *et al.* (1999). The number of species in the clade was increased to eight by Bai *et al.* (2001), who identified three novel *Bullera* species among strains originally assigned to *Bullera variabilis* and assigned these species to the *B. mrakii* clade by 18S rRNA gene and internal transcribed spacer (ITS) region sequence analyses. None of the species in this clade were studied by Fell *et al.* (2000) or Scorzett *et al.* (2002).

Recently, three novel *Bullera* species belonging to the *B. mrakii* clade were found among ballistoconidium-forming yeast strains isolated from plant leaves collected in China. The

present study supports the distinct nature of the *B. mrakii* clade in basidiomycetous yeasts of the Tremellales group.

METHODS

Yeast strains and phenotypic characterization. Four strains were examined in the present study. Strain CB 169^T was isolated from a wilting leaf of *Brachybotrys paridiformis* collected on Changbai Mountain, Jilin Province, in October 1998. Strains HX 15.5^T and HX 19.3^T were isolated from wilting leaves of *Litsea* sp. and *Tilia* sp., respectively, collected in Hubei Province in October 2002. Strain ST 19.14 was isolated from a wilting leaf of *Pinus armandii* collected in Shan'xi Province in October 2002. Yeast strain isolation was performed using the improved ballistoconidia-fall method (Nakase & Takashima, 1993).

Morphological, physiological and biochemical characteristics were examined according to standard methods (Yarrow, 1998). Assimilation of nitrogen compounds was investigated on solid media with starved inocula (Nakase & Suzuki, 1986). Extraction, purification and identification of ubiquinones were carried out according to Yamada & Kondo (1973).

Sequence analysis. Nuclear DNA was extracted by the method of Makimura *et al.* (1994). Sequencing of the ITS (including 5.8S rDNA) and 26S rDNA D1/D2 domain and molecular phylogenetic analysis were performed using methods described previously (Bai *et al.*, 2002a).

RESULTS AND DISCUSSION

Morphology and chemotaxonomy

The four strains studied formed whitish colonies and reproduced asexually by polar or multilateral budding and

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Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the ITS region and 26S rDNA D1/D2 domain sequences determined in this study are AY487563–AY487570, AB118870 and AB118871.

A phylogenetic tree showing relationships between the three novel *Bullera* species and related taxa is available as supplementary material in IJSEM Online.

production of rotationally symmetrical ballistoconidia. Stalked conidia were not produced. The major ubiquinone was Q-10. Sexual cycles were not observed in single strains or mixed cultures. According to the current taxonomy of basidiomycetous yeasts (Boekhout, 1998; Boekhout & Nakase, 1998), these strains were assigned to genus *Bullera*.

rDNA sequence analysis

Three taxa were recognized among the four strains studied from the rDNA sequence comparisons. Strains HX 15.5^T and ST 19.14 had identical D1/D2 and ITS sequences. CB 169^T and HX 19.3^T differed markedly from each other and from the other two strains in the same rDNA regions.

In the phylogenetic tree drawn from the D1/D2 sequence alignment, the three taxa formed three distinct branches in the *B. mrakii* clade (Fig. 1). This clade was strongly supported (100%) by bootstrap analysis. In the tree drawn from ITS sequences, the *B. mrakii* clade was also resolved with 100% bootstrap support (phylogenetic tree available as supplementary material in IJSEM Online). Strains CB

169^T and HX 15.5^T clustered in a subclade with *B. mrakii* and four other described species in both the D1/D2 and ITS trees. Strain CB 169^T differed from the other taxa in the subclade by 7–19 nt (1.4–3.1%) and 29–40 nt (>7%) in the D1/D2 and ITS regions, respectively. Strain HX 15.5^T differed from the other taxa in the same subclade by 13–19 nt (2.1–3.1%) and 39–58 nt (>10%) in the D1/D2 and ITS regions, respectively.

Strain HX 19.3^T occupied a basal position in the *B. mrakii* clade in the D1/D2 tree (Fig. 1). It differed from the other taxa in this clade by 19–24 nt (3.0–4.0%). In the ITS tree, the position of HX 19.3^T was slightly different. This strain formed a less well supported subclade together with *Bullera komagatae*, *Bullera schimicola* and *Bullera pseudoschimicola* in the ITS tree (available as supplementary material in IJSEM Online) and differed from these species by more than 100 nt in the ITS region.

These data indicated that strains CB 169^T, HX 15.5^T and HX 19.3^T represent three novel *Bullera* species, for which the names *Bullera cylindrica* sp. nov., *Bullera nakasei* sp. nov. and *Bullera hubeiensis* sp. nov. are proposed.

Fig. 1. Phylogenetic tree drawn from neighbour-joining analysis of the 26S rDNA D1/D2 domain sequences, depicting the relationships of the three novel *Bullera* species with closely related taxa. Bootstrap percentages over 50% from 1000 bootstrap replicates are shown. Reference sequences were retrieved from GenBank/DBJ; accession numbers are indicated.

Characteristics of the *B. mrakii* clade

The *B. mrakii* clade recognized in the present study belongs to the Tremellales in the Hymenomycetes (Fell *et al.*, 2000; Scorzetti *et al.*, 2002). This clade is closely related to taxa in the Luteolus clade of the Tremellales (Scorzetti *et al.*, 2002). Interestingly, the *B. mrakii* clade is composed exclusively of species belonging to the single genus *Bullera*, although these species are only distantly related to the type species *Bullera alba* (anamorph of *Bulleromyces albus*), as showed in Fig. 1. This could imply that species in the *B. mrakii* clade should be moved to a different genus. The precedent is that the genus *Dioszegia* was reinstated and redefined by rDNA sequence analysis to accommodate both ballistoconidium-forming and other yeast species in a sister clade of the *B. mrakii* clade (Takashima *et al.*, 2001). In addition to the signature sequences in the 18S rDNA, *Dioszegia* species form orange-coloured colonies (Bai *et al.*, 2002b; Takashima *et al.*, 2001; Wang *et al.*, 2003). Though a sequence signature (AAGCTC) was found near the 3' end of the ITS2 regions of all the species in the *B. mrakii* clade (data not shown), no particular morphological or physiological characteristics could be found to discriminate species in this clade from other *Bullera* species. Therefore, it is thought to be premature to propose a new genus for this clade based only on rDNA sequence data at present.

Physiological differences between species in the *B. mrakii* clade are shown in Table 1. *B. cylindrica* sp. nov. can be distinguished from closely related species by its ability to assimilate nitrate and to grow in vitamin-free medium. *B. hubeiensis* sp. nov. is distinct in its inability to assimilate D-arabinose, D-ribose, L-rhamnose, salicin and succinate. *B. nakasei* sp. nov. can be differentiated by the combination of assimilation reactions of L-sorbose, ribitol, nitrite and ethylamine and the formation of starch-like substances.

Morphologically, *B. cylindrica* sp. nov. and *B. nakasei* sp. nov. are also distinguishable from other species in the *B. mrakii* clade. The former forms characteristic cylindrical vegetative cells and the latter forms relatively large, somewhat apiculate vegetative cells.

Latin diagnosis of *Bullera cylindrica* Bai, Wang et Takashima sp. nov.

In YM (Difco) liquido post dies 5 ad 20 °C, cellulae vegetativae ovoideae, ellipsoideae aut cylindratae, 2.5–4.5 × 5.5–7.5 µm, singulae aut binae. Annulus et sedimentum formantur. In agar YM post unum mensem ad 20 °C, cultura flavae-cremea, glabra vel rugosa, non-nitida, butyracea, margine glabra. Pseudomycelium formantur. Ballistoconidia napiformia vel subglobosa, 3.5–5.5 × 3.0–5.0 µm. Fermentatio nulla. Glucosum, galactosum, L-sorbosum (lente et exigue), saccharosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinose, melezitosum, amyllum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum, D-mannitolium (lente et exigue), galactitolium (lente et exigue), glucitolium, methyl α-D-glucosidum,

Table 1. Salient physiological characteristics of species in the *Bullera mrakii* clade

Species: 1, *B. cylindrica* sp. nov.; 2, *B. hubeiensis* sp. nov.; 3, *B. nakasei* sp. nov.; 4, *B. boninensis*; 5, *B. huiensis*; 6, *B. komagatae*; 7, *B. mrakii*; 8, *B. schimicola*; 9, *B. pseudohuiaensis*; 10, *B. pseudo-schimicola*; 11, *B. waltii*. +, Positive; –, negative; D, delayed; V, variable; W, weak; DW, delayed and weak.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Assimilation of:											
L-Sorbose	DW	–	+	–	–	–	D, –	W	–	–	D
Soluble starch	+	–	W	+	W	+	V	+	+	+	+
D-Arabinose	+	–	+	+	+	D	+	+	D	+	+
D-Ribose	+	–	+	+	D	D	+	+	DW	D	+
L-Rhamnose	+	–	+	+	+	D	+	+	D	+	+
Erythritol	–	–	DW	DW	–	–	+	DW	–	–	D
Ribitol	–	–	–	+	D	–	–	+	–	–	DW
D-Mannitol	DW	W	DW	+	+	D	+	+	–	+	+
Salicin	+	–	+	+	D	+	D	+	D	+	+
DL-Lactate	–	–	–	D	D	–	V	DW	–	–	+
Succinate	+	–	+	+	+	D	+	+	DW	+	+
Nitrate	D	–	–	–	–	–	–	–	–	–	–
Nitrite	+	–	–	+	–	–	+	–	–	–	+
Ethylamine	+	–	+	–	+	–	–	–	D	–	+
Cadaverine	+	–	–	–	W	–	–	–	–	–	+
Growth without vitamins	+	–	–	–	–	–	–	–	–	–	–
Starch formation	+	–	–	–	+	–	+	–	+	–	+

salicinum, acidum succinicum et inositolum assimilantur, at non lactosum, inulinum, methanolum, ethanolum, glycerolum, erythritolum, ribitolium, acidum DL-lacticum, acidum citricum nec hexadecanum. Ammonium sulfatum, kalium nitricum, natrum nitrosum, L-lysinum, ethylaminum et cadaverinum assimilantur. Ad crescentiam vitaminum non necessarium est. Maxima temperatura crescentiae: 27 °C. Materia amyloidea iodophila formantur. Urea finditur. Diazonium caeruleum B positivum. Ubiquinonum majus: Q-10. Typus: CB 169^T (= AS 2.2308^T = CBS 9744^T), isolatus ex folio *Brachybotrys paridiformis* Maxim.

Description of *Bullera cylindrica* Bai, Wang & Takashima sp. nov.

Bullera cylindrica (cy.lin'dri.ca. L. nom. fem. adj. *cylindrica* referring to the cylindrical shaped vegetative cells of the species).

In YM broth, after 5 days at 20 °C, cells are ovoid, ellipsoidal or cylindrical, 2.5–4.5 × 5.5–7.5 µm, and occur singly or in pairs (Fig. 2a). Budding is polar. A sediment and a ring are formed. On YM agar, after 1 month at 20 °C, the streak culture is yellowish cream, butyrous, dull, smooth or somewhat wrinkled. The margin is entire. In Dalmau plate culture on cornmeal agar, pseudohyphae are formed. Ballistoconidia are produced on cornmeal agar and are

napiform or subglobose, $3.5\text{--}5.5 \times 3.0\text{--}5.0\text{ }\mu\text{m}$ (Fig. 2b). Fermentation of glucose is negative. Glucose, galactose, L-sorbose (delayed and weak), sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, galactitol (delayed and weak), D-mannitol (delayed and weak), D-glucitol, methyl α -D-glucoside, salicin, succinic acid and inositol are assimilated. Lactose, inulin, methanol, ethanol, glycerol, erythritol, ribitol, DL-lactic acid, citric acid and hexadecane are not assimilated. Ammonium sulfate, potassium nitrate (delayed), sodium nitrite, L-lysine, ethylamine hydrochloride and cadaverine dihydrochloride are assimilated. Maximum growth temperature is 27°C . Growth in vitamin-free medium is positive. Starch-like substances are produced. Growth on 50 % (w/w) glucose/yeast extract agar is negative. Urease activity is positive. Diazonium blue B reaction is positive. The major ubiquinone is Q-10. The type strain, CB 169^T (=AS 2.2308^T=CBS 9744^T), was isolated from a wilting leaf of *Brachybotrys paridiformis* Maxim. collected in Changbai Mountain, northeast China in October, 1998.

Latin diagnosis of *Bullera hubeiensis* Bai, Wang et Takashima sp. nov.

In YM (Difco) liquido post dies 5 ad 20°C , cellulae vegetativae ovoideae aut ellipsoideae, $2.5\text{--}4.5 \times 5.0\text{--}7.5\text{ }\mu\text{m}$, singulae, binae aut adhaerentes. Annulus et sedimentum formantur. In agar YM post unum mensem ad 20°C , cultura crenea aut gilvo-flavae, non-nitida, glabra vel rugosa, butyracea, margine glabra aut erosa. Pseudomycelium formantur. Ballistoconidia napiformia vel subglobosa, $3.7\text{--}5.0 \times 4.9\text{--}6.2\text{ }\mu\text{m}$. Fermentatio nulla. Glucosum, galactosum, saccharosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinsum, melezitosum, D-xylosum, L-arabiosum, galactitolum (exigue), D-mannitolum (exigue), glucitolum, methyl α -D-glucosidum (exigue) et inositolum (exigue) assimilantur, at non L-sorbosum, lactosum, inulinum, amyllum solubile, D-arabiosum, D-ribosum, L-rhamnosum, D-glucosaminum, methanolum, ethanolum, glycerolum, erythritolum, ribitolum, salicinum, acidum DL-lacticum, acidum succinicum, acidum citricum nec hexadecanum. Ammonium sulfatum et L-lysinum assimilantur, at non kalium nitricum, natrum nitrosolum, ethylaminum nec cadaverinum. Vitaminarum externarum ad crescentiam necessaria sunt. Maxima temperatura crescentiae: 22°C . Materia amyloidea iodophila non formantur. Urea finditur. Diazonium caeruleum B positivum. Ubiquinonum majus: Q-10. Typus: HX 19.3^T (=AS 2.2466^T=CBS 9747^T), isolatus ex folio *Tilia* sp.

Description of *Bullera hubeiensis* Bai, Wang & Takashima sp. nov.

Bullera hubeiensis (hu.be.i.en'sis. L. nom. masc. adj. *hubeiensis* referring to Hubei Province, China, the geographical origin of the species).

In YM broth, after 5 days at 20°C , cells are ellipsoidal or

ovoid, $2.5\text{--}4.5 \times 5.0\text{--}7.5\text{ }\mu\text{m}$, and occur singly, in pairs or in clusters (Fig. 3a). Budding is multilateral. A ring and a sediment are formed. On YM agar, after 1 month at 20°C , the streak culture is cream to light yellow, butyrous, dull, smooth and somewhat wrinkled. The margin is entire or eroded. In Dalmau plate culture on cornmeal agar, pseudohyphae are formed. Ballistoconidia are produced on cornmeal agar and are napiform or subglobose, $3.7\text{--}5.0 \times 4.9\text{--}6.2\text{ }\mu\text{m}$ (Fig. 3b). Fermentation of glucose is negative. Glucose, galactose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, D-xylose, L-arabinose, galactitol (weak), D-mannitol (weak), D-glucitol, methyl α -D-glucoside (weak) and inositol (weak) are assimilated. L-Sorbose, lactose, inulin, soluble starch, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, methanol, ethanol, glycerol, erythritol, ribitol, salicin, DL-lactic acid, succinic acid, citric acid and hexadecane are not assimilated. Ammonium sulfate and L-lysine are assimilated. Potassium nitrate, sodium nitrite, ethylamine hydrochloride and cadaverine dihydrochloride are not assimilated. Maximum growth temperature is 22°C . Growth in vitamin-free medium is negative. Starch-like substances are not produced. Growth on 50 % (w/w) glucose/yeast extract agar is negative. Urease activity is positive. Diazonium blue B reaction is positive. The major ubiquinone is Q-10. The type strain, HX 19.3^T (=AS 2.2466^T=CBS 9747^T), was isolated from a wilting leaf of *Tilia* sp. collected in Xingshan County, Hubei Province, China in October, 2002.

Latin diagnosis of *Bullera nakasei* Bai, Wang et Takashima sp. nov.

In YM (Difco) liquido post dies 5 ad 20°C , cellulae vegetativae ovoideae, ellipsoideae aut apiculatae, $2.0\text{--}6.0 \times 6.2\text{--}14.5\text{ }\mu\text{m}$, singulae aut binae. Annulus et sedimentum formantur. In agar YM post unum mensem ad 20°C , cultura crenea aut gilvo-flavae, nitida aut non-nitida, glabra vel rugosa, butyracea, margine glabra aut erosa. Mycelium formantur. Ballistoconidia napiformia vel subglobosa, $3.2\text{--}7.4 \times 4.5\text{--}8.7\text{ }\mu\text{m}$. Fermentatio nulla. Glucosum, galactosum, L-sorbosum, saccharosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinsum, melezitosum, amyllum solubile (exigue), D-xylosum, L-arabiosum, D-arabiosum, D-ribosum, L-rhamnosum, erythritolum (lente et exigue), D-mannitolum (lente et exigue), glucitolum, methyl α -D-glucosidum, salicinum, acidum succinicum et inositolum assimilantur, at non lactosum, inulinum, D-glucosaminum, methanolum, ethanolum, glycerolum, ribitolum, galactitolum, acidum DL-lacticum, acidum citricum nec hexadecanum. Ammonium sulfatum, L-lysinum et ethylaminum assimilantur, at non kalium nitricum, natrum nitrosolum nec cadaverinum. Vitaminarum externarum ad crescentiam necessaria sunt. Maxima temperatura crescentiae: 27°C . Materia amyloidea iodophila non formantur. Urea finditur. Diazonium caeruleum B positivum. Ubiquinonum majus: Q-10. Typus: HX 15.5^T (=AS 2.2435^T=CBS 9746^T), isolatus ex folio *Litsea* sp.

Fig. 2. *Bullera cylindrica* sp. nov. CB 169^T vegetative cells grown in YM broth for 5 days at 20 °C (a) and ballistoconidia produced on cornmeal agar after 5 days at 20 °C (b). Bars, 10 µm.

Fig. 3. *Bullera hubeiensis* sp. nov. HX 19.3^T vegetative cells grown in YM broth for 5 days at 20 °C (a) and ballistoconidia produced on cornmeal agar after 5 days at 20 °C (b). Bars, 10 µm.

Fig. 4. *Bullera nakasei* sp. nov. HX 15.5^T vegetative cells grown in YM broth for 5 days at 20 °C (a) and ballistoconidia produced on cornmeal agar after 5 days at 20 °C (b). Bars, 10 µm.

Description of *Bullera nakasei* Bai, Wang & Takashima sp. nov.

Bullera nakasei (na.ka.se.i. L. gen. masc. n. *nakasei* in honour of Dr Takashi Nakase, Japan, for his outstanding contributions to the progress of systematics of ballistoconidium-forming yeasts).

In YM broth, after 5 days at 20 °C, cells are ovoid, ellipsoidal or apiculate, 2.0–6.0 × 6.2–14.5 µm, and occur singly or in pairs (Fig. 4a). Budding is multilateral. Sediment and a ring are formed. On YM agar, after 1 month at 20 °C, the streak culture is cream to light yellow, butyrous, semi-shiny to dull, smooth and somewhat wrinkled. The margin is entire or eroded. In Dalmau plate culture on cornmeal agar, true hyphae are formed. Ballistoconidia are produced on cornmeal agar and are napiform, subglobose or somewhat trigonal, 3.2–7.4 × 4.5–8.7 µm (Fig. 4b). Fermentation of glucose is negative. Glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, soluble starch (weak), D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, erythritol (delayed and weak), D-mannitol (delayed and weak), D-glucitol, methyl α-D-glucoside, salicin, succinic acid and inositol are assimilated. Lactose, inulin, D-glucosamine, methanol, ethanol, glycerol, ribitol, galactitol, DL-lactic acid, citric acid and hexadecane are not assimilated. Ammonium sulfate, L-lysine and ethylamine hydrochloride are assimilated. Potassium nitrate, sodium nitrite and cadaverine dihydrochloride are not assimilated. Maximum growth temperature is 27 °C. Growth in vitamin-free medium is negative. Starch-like substances are not produced. Growth on 50 % (w/w) glucose/yeast extract agar is negative. Urease activity is positive. Diazonium blue B reaction is positive. The major ubiquinone is Q-10. The type strain, HX 15.5^T (= AS 2.2435^T = CBS 9746^T), was isolated from a wilting leaf of *Litsea* sp. collected in Xingshan County, Hubei Province, China in October, 2002.

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