Mycoscience 40: 491–501, 1999

Three new species of *Bullera* isolated from leaves in the Ogasawara Islands

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Accepted for publication 20 September 1999

Thirteen strains of ballistoconidium-forming yeasts were isolated from leaves collected in the Ogasawara Islands, Japan. They represent three different species in the genus *Bullera* on the basis of morphological, physiological, and biochemical characteristics, analyses of the sequences of internal transcribed spacer regions and small subunit ribosomal DNA, and a nuclear DNA-DNA hybridization study. Three new species, *Bullera boninensis* (five strains), *B. waltii* (seven strains), and *B. schimicola* (one strain), are proposed for these 13 strains.

Key Words——ballistoconidium-forming yeast; *Bullera boninensis*; *Bullera schimicola*; *Bullera waltii*

In a survey of ballistoconidium-forming yeasts from the leaves of plants collected in the Ogasawara Islands, Japan, we isolated 13 strains of hitherto undescribed yeasts. These isolates appear to belong to the genus *Bullera*, because they have Q-10 as the major ubiquinone, contain xylose in the cells, and proliferate through budding cells and ballistoconidia. Three new species of *Bullera* are proposed for these 13 isolates.

Materials and Methods

Strains used The strains studied and their sources are shown in Table 1. They were isolated using the ballistoconidium-fall method using YM agar (Difco Lab, MI, U.S.A.) plates as reported by Nakase and Takashima (1993). *Bullera huiaensis* Hamamoto et Nakase and *B. mrakii* Hamamoto et Nakase were used for comparative study (Hamamoto and Nakase, 1996).

Traditional taxonomic criteria Most of the methods used to examine the morphological, physiological, and biochemical characteristics were described by van der Walt and Yarrow (1984). The assimilation of nitrogen compounds was investigated on solid media with starved inoculum as described by Nakase and Suzuki (1986). Vitamin requirements were investigated following the methods of Komagata and Nakase (1967). The maximum growth temperature was determined in YM broth, using metal block baths.

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Investigation of chemotaxonomic criteria Extraction, purification, and identification of ubiquinone were carried out according to Nakase and Suzuki (1986). Xylose in the cells was analyzed as described by Suzuki and Nakase (1988). Isolation and purification of DNA was carried out according to Nakase and Suzuki (1986). The DNA base composition (mol% G+C) was determined by HPLC after enzymatic digestion of DNA into deoxyribonucleosides. The DNA-GC Kit (Yamasa Shouyu, Chiba, Japan) was used according to the manufacturer's instructions. DNA-DNA hybridization was performed using the membrane-filter method according to Nakase and Suzuki (1985).

Nucleotide sequences of small subunit ribosomal DNA and internal transcribed spacer regions The nucleotide sequences of small subunit ribosomal DNA (SSU rDNA) and the internal transcribed spacer (ITS 1 and ITS 2) regions including the 5.8S rDNA were directly determined using PCR products according to the method of Sugita and Nakase (1999), and Sugita et al. (1999). The sequences were aligned using the Clustal W computer program (Thompson et al., 1994). For phylogenetic analysis, the SSU rDNA sequences of related yeasts shown in Fig. 1 were obtained from international DNA database. For the Neighbor-Joining analysis (Saitou and Nei, 1987), the distances between the sequences were calculated using Kimura's two-parameter model (Kimura, 1980). Sites where gaps existed in any of the sequences were excluded. Bootstrap analysis (Felsenstein, 1985) was performed with 1,000 repetitions.

Results and Discussion

Bullera boninensis Sugita, Cañete-Gibas, Takashima et Nakase, sp. nov. Fig. 1

In liquido YM post 5 dies ad 17°C: cellulae ovoideae,

Table 1. Strains used in this study.

Species	Strain	Isolation number	Source	Locality
Bullera boninensis	JCM 10570^T	OK-3	*Osteomeles lanata*	Chichijima
	JCM 10571	OK-11	*Schima mertensiana*	Chichijima
	JCM 10572	OK-37	*Schima mertensiana*	Chichijima
	JCM 10573	OK-40	*Schima mertensiana*	Chichijima
	JCM 10574	OK-49	*Schima mertensiana*	Chichijima
Bullera waltii	JCM 10575^T	OK-9	*Schima mertensiana*	Chichijima
	JCM 10576	OK-79	*Schima mertensiana*	Chichijima
	JCM 10577	OK-130	*Machilus kobu*	Anijima
	JCM 10578	OK-206	*Syzygium buxifolium*	Hahajima
	JCM 10579	OK-215	*Syzygium buxifolium*	Hahajima
	JCM 10580	OK-229	*Callicarpa subpubescens*	Hahajima
	JCM 10581	OK-233	*Callicarpa subpubescens*	Hahajima
Bullera schimicola	JCM 10582^T	OK-34	*Schima mertensiana*	Chichijima
Reference strains				
Bullera huiaensis	JCM 8933^T		*Pseudopanax arboreus*	New Zealand
Bullera mrakii	JCM 8934^T		*Nothofagus fusca*	New Zealand
	JCM 8935		*Pseudowintera colonata*	New Zealand
	JCM 8936		*Coprosma tenuifolia*	New Zealand

T, type strain.

ellipsoidales, elongatae, 2.1–4.2 × 2.2–5.3 μm, singulae aut binae; sedimentum formatur; post unum mensem ad 17°C pellicula fragilis et sedimentum compactum formantur. Coloniae in agaro YM post unum mensem ad 17°C gilvo-flavae, glabrae, butyraceae, margine glabrae. In agaro farinae zeae pseudomycelium non formatur; ballistoconidia globosa, subglobosa vel napiformia, 5.3–5.8 × 7.4–7.6 μm. Fermentatio nulla. Glucosum, galactosum, saccharosum, maltosum, cellobiosum (lente), trehalosum, lactosum (lente vel lente et infirme), melibiosum, raffinosum, melezitosum, amylum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum (lente et infirme), *N*-acetyl-D-glucosaminum, erythritolum (lente et infirme), ribitolum (lente et infirme), galactitolum (varie), mannitolum (lente), glucitolum (lente), α-methyl-D-glucosidum, salicinum, glucono-δ-lactonum, acidum D-gluconicum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum (lente vel lente et infirme), acidum succinicum, acidum citricum (lente vel lente et infirme), inositolum (lente), acidum saccharicum (lente vel lente et infirme) xylitolum, L-arabinitolum (infirme), acidum D-glucronicum et acidum galacturonicum assimilantur, autem L-sorbosum, inulinum, methanolum, ethanolum, glycerolum, hexadecanum, 1,2-propanediolum et 2,3-butanediolum non assimilantur. Ammonium sulfatum, natrium nitrosum et L-lysinum assimilantur, autem kalium nitricum, ethylaminum et cadaverinum non assimilantur. Maxima temperatura crescentiae 28–31°C est. Ad crescentiam thiaminum necessarium est. Commutatio coloris per diazonium caeruleum B positiva est. Proportio molaris guanini+cytosini in acido deoxyribonucleico 42.2–43.1 mol% (per HPLC) est. Ubiquinonum majus Q-10 adest. Xylosum in cellulis praesens. Holotypus: Colonia in cultura a T. Nakase isolata (originaliter ut OK-3) ex folio *Osteomelis lanatae*, Chichijima, Ins. Ogasawara, Japonia, Nov. 1994, T. Sato leg. et in Collectione Culturarum Japonensium qua 'Japan Collection of Microorganisms,' Wako, Saitama sustentat conservata (JCM 10570).

Etymology: Latin, *boninensis*, pertaining to the Bonin Islands (Ogasawara Islands) where the isolation source was collected.

Growth in YM broth: After 5 d at 17°C, cells are ovoidal, ellipsoidal, elongate, 2.1–4.2 × 2.2–5.3 μm, single or in pairs (Fig. 1A). A sediment is formed. After one mo at 17°C, a fragile pellicle and a compact sediment are present.

Growth on YM agar: After one mo at 17°C, the streak culture is light yellow, smooth, butyrous, and has an entire margin.

Dalmau plate culture on corn meal agar: No pseudomycelia are produced.

Production of ballistoconidia: Ballistoconidia are produced on corn meal agar. They are globose, subglobose to napiform, 5.3–5.8 × 7.4–7.6 μm (Fig. 1B).

Fermentation: Absent.

Assimilation of carbon compounds:

glucose	+	erythritol	+ (latent and weak)
galactose	+	ribitol	+ or + (latent and weak)

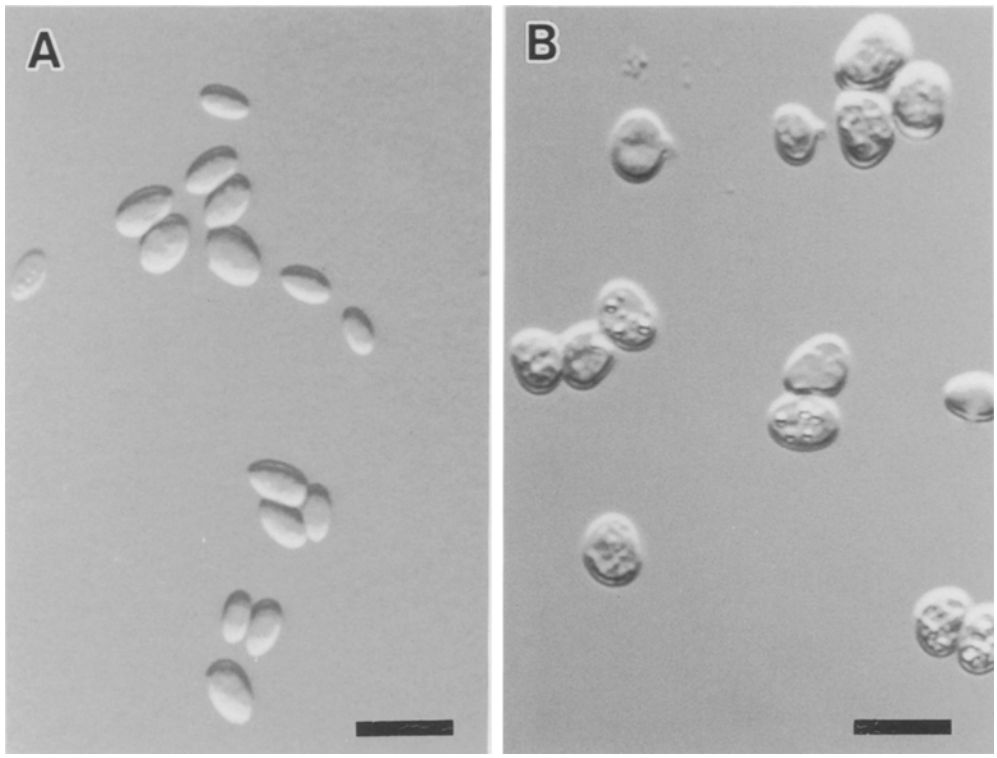

Fig. 1. *Bullera boninensis*. (A) Vegetative cells grown in YM broth for 5 d at 17°C. (B) Ballistoconidia produced on corn meal agar after 5 d at 17°C. Scale bars indicate 10 μm.

L-sorbose	−	galactitol	+ or + (latent or latent and weak) or −
sucrose	+	mannitol	+ or + (latent)
maltose	+	glucitol	+ or + (latent)
cellobiose	+ or + (latent and weak)	α-methyl-D-glucoside	+
treharose	+	*salicin*	+
lactose	+ or + (latent or latent and weak)	glucono-δ-lactone	+
melibiose	+	D-gluconic acid	+
raffinose	+	2-ketogluconic acid	+
melezitose	+	5-ketogluconic acid	+
inulin	−	DL-lactic acid	+ (latent or latent and weak)
soluble starch	+	succinic acid	+
D-xylose	+	citric acid	+ (latent or latent and weak)
L-arabinose	+	inositol	+ or + (latent)
D-arabinose	+	n-hexadecane	−
D-ribose	+	saccharic acid	+ (latent or latent and weak)
L-rhamnose	+	xylitol	+
D-glucosamine	+ (latent and weak)	L-arabinitol	+ or + (weak)
N-acetyl-D-glucosamine	+	propane 1, 2 diol	−
methanol	−	butane 2, 3 diol	−
ethanol	−	D-glucronic acid	+
glycerol	−	D-galacturonic acid	+
Assimilation of nitrogen compounds:			
ammonium sulfate	+	ethylamine hydrochloride	−
potassium nitrate	−	L-lysine hydrochloride	+
sodium nitrite	+	cadaverine dihydrochloride	−

Maximum growth temperature: 28–31°C.
Vitamin required: Thiamine.
Production of starch-like substances: Negative.
Growth on 50% (w/w) glucose-yeast extract agar: Negative.
Urease: Positive.
Liquefaction of gelatin: Negative.
Hydrolysis of fat: Negative.

Diazonium blue B reaction: Positive.

G+C content of nuclear DNA: 42.2–43.1 mol% (by HPLC).

Major ubiquinone: Q-10.

Xylose in the cells: Present.

Type strain: OK-3, isolated by T. Nakase from a leaf of *Osteomeles lanata* Nakai collected by T. Sato in Nov. 1994, Chichijima, Ogasawara Islands, Japan. This strain has been deposited in the Japan Collection of Microorganisms, Wako, Saitama, as JCM 10570. The other strains, OK-11, OK-37, OK-40, and OK-49, have also been deposited in the Japan Collection of Microorganisms, as JCM 10571, 10572, 10573, and 10574, respectively.

Bullera waltii Sugita, Cañete-Gibas, Takashima et Nakase, sp. nov. Fig. 2

In liquido YM post 5 dies ad 17°C: cellulae ovoideae, ellipsoidales, elongatae, 3.2–6.3×4.2–7.4 μm, singulae aut binae; sedimentum formatur; post unum mensem ad 17°C pellicula fragilis et sedimentum fluitans formantur. Coloniae in agaro YM post unum mensem ad 17°C griseo-flavae, glabrae aut rugulosae, butyraceae, nitidae, margine integrae aut lobulatae. In agaro farinae zeae pseudomycelium non formatur; ballistoconidia globosa, subglobosa vel napiformia, 5.8–6.3×6.8–7.4 μm. Fermentatio nulla. Glucosum (lente), galactosum (lente), L-sorbosum (lente vel lente et infirme), saccharosum (lente vel infirme), maltosum (lente), cellobiosum (lente), trehalosum (lente), melibiosum (lente), raffinosum (lente), melezitosum (lente), amylum solubile, D-xylosum (lente), L-arabinosum (lente), D-arabinosum (lente vel infirme), D-ribosum (lente), L-rhamnosum (lente vel lente et infirme), D-glucosaminum (exiguum vel lente et infirme), *N*-acetyl-D-glucosaminum (vel lente et infirme), erythritolum (lente vel lente et infirme), ribitolum (lente, infirme vel lente et infirme), galactitolum (lente), mannitolum (lente), glucitolum (lente vel lente et infirme), α-methyl-D-glucosidum (lente), salicinum (lente vel lente et infirme), glucono-δ-lactonum (lente), acidum gluconicum (lente), acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum (lente vel lente et infirme), acidum succinicum (lente), acidum citricum (lente et infirme), inositolum (lente), acidum saccharicum (lente vel lente et infirme), xylitolum (lente), L-arabinitolum (lente vel lente et infirme), acidum D-glucronicum (lente) et acidum D-galacturonicum (lente) assimilantur, autem lactosum, inulinum, methanolum, ethanolum, glycerolum, hexadecanum, 1, 2-propanediolum et 2,3-butanediolum non assimilantur. Ammonium sulfatum, natrium nitrosum, ethylaminum, L-lysinum et cadaverinum assimilantur, autem kalium nitricum non assimilatur. Maxima temperatura crescentiae 28–29°C est. Ad crescentiam thiaminum necessarium est. Commutatio coloris per diazonium caeruleum B positiva est. Proportio molaris guanini +cytosini in acido deoxyribonucleico 42.8 mol% (per HPLC) est. Ubiquinonum majus Q-10 adest. Xylosum in cellulis praesens. Holotypus: Colonia in cultuva a T. Nakase isolata (originaliter ut OK-9) ex folio *Schimae mertensianae*, Chichijima, Ins. Ogasawara, Japonia, Nov. 1994, T. Sato leg. et in Collectione Culturarum qua 'Japan Collection of Microorganisms,' Wako, Saitama sustentat conservata (JCM 10575).

Etymology: The specific epithet, *waltii*, was given in honor of Dr. J. P. van der Walt of South Africa.

Growth in YM broth: After 5 d at 17°C, cells are ovoidal, ellipsoidal, elongate, 3.2–6.3×4.2–7.4 μm, single or in pairs (Fig. 2A). A sediment is formed. After one mo at 17°C, a fragile pellicle and a flocculent sediment are present.

Growth on YM agar: After one mo at 17°C, the streak culture is greyish yellow, smooth or delicately wrinkled, butyrous, shining and has an entire to slightly lobate margin.

Dalmau plate culture on corn meal agar: No pseudomycelia are produced.

Production of ballistoconidia: Ballistoconidia are produced on corn meal agar. They are globose, subglobose to napiform, 5.8–6.3×6.8–7.4 μm (Fig. 2B).

Fermentation: Absent.

Assimilation of carbon compounds:

glucose	+ or + (latent)	erythritol	+ (latent or latent and weak)
galactose	+ or + (latent)	ribitol	+ (latent or weak or latent and weak)
L-sorbose	+ (latent or latent and weak)	galactitol	+ or + (latent)
sucrose	+ or + (latent or weak)	mannitol	+ or + (latent)
maltose	+ or + (latent)	glucitol	+ (latent or latent and weak)
cellobiose	+ or + (latent)	α-methyl-D-glucoside	+ or + (latent)
treharose	+ or + (latent)	salicin	+ or + (latent or latent and weak)
lactose	−	glucono-δ-lactone	+ or + (latent)
melibiose	+ or + (latent)	D-gluconic acid	+ or + (latent)
raffinose	+ or + (latent)	2-ketogluconic acid	+
melezitose	+ or + (latent)	5-ketogluconic acid	+
inulin	−	DL-lactic acid	+ or + (latent or latent and weak)
soluble starch	+	succinic acid	+ or + (latent)
D-xylose	+ or + (latent)	citric acid	+ (latent and weak)

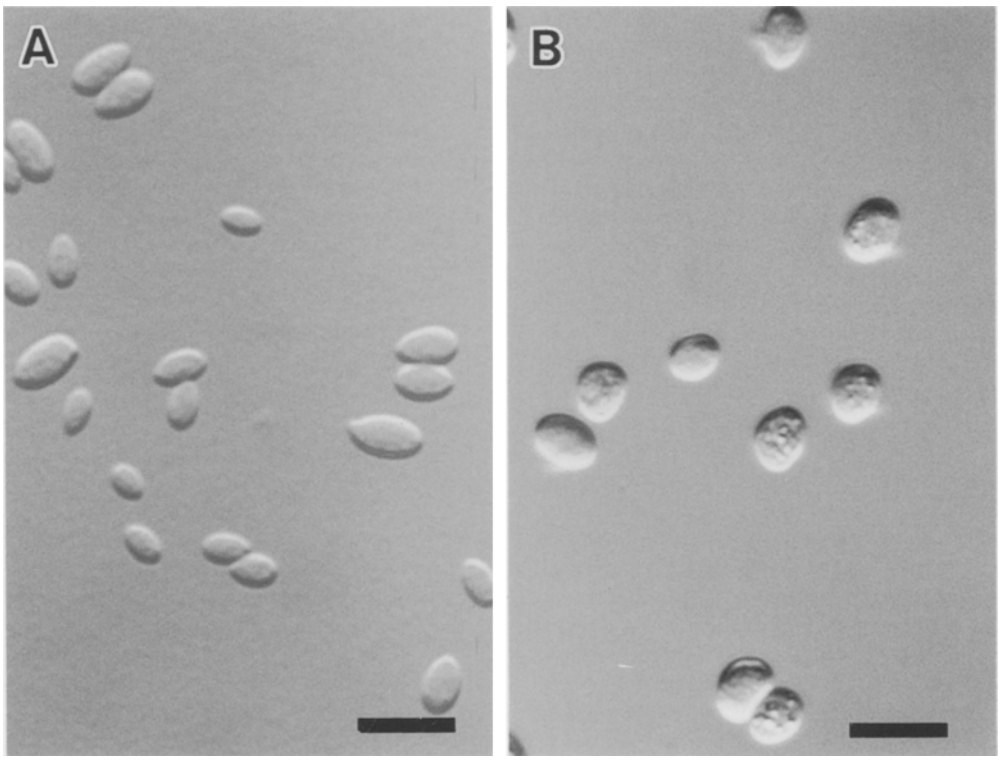

Fig. 2. *Bullera waltii*. (A) Vegetative cells grown in YM broth for 5 d at 17°C. (B) Ballistoconidia produced on corn meal agar after 5 d at 17°C. Scale bars indicate 10 μm.

L-arabinose	+ or + (latent)	inositol	+ or + (latent)
D-arabinose	+ or + (latent or weak)	n-hexadecane	−
D-ribose	+ or + (latent)	saccharic acid	+ (latent or latent and weak)
L-rhamnose	+ or + (latent or latent and weak)	xylitol	+ or + (latent)
D-glucosamine	+ or + (weak or latent and weak)	L-arabinitol	+ or + (latent or latent and weak)
N-acetyl-D-glucosamine	+ or + (latent and weak)	propane 1, 2 diol	−
methanol	−	butane 2, 3 diol	−
ethanol	−	D-glucronic acid	+ or + (latent)
glycerol	−	D-galacturonic acid	+ or + (latent)

Assimilation of nitrogen compounds:

ammonium sulfate	+	ethylamine hydrochloride	+
potassium nitrate	−	L-lysine hydrochloride	+
sodium nitrite	+	cadaverine dihydrochloride	+

Maximum growth temperature: 28–29°C.

Vitamin required: Thiamine.

Production of starch-like substances: Positive.

Growth on 50% (w/w) glucose-yeast extract agar: Negative.

Urease: Positive.

Liquefaction of gelatin: Negative.

Hydrolysis of fat: Negative.

Diazonium blue B reaction: Positive.

G+C content of nuclear DNA: 42.8 mol% (by HPLC).

Major ubiquinone: Q-10.

Xylose in the cells: Present.

Type strain: OK-9, isolated by T. Nakase from a leaf of *Schima mertensiana* (Sieb. et Zucc.) Koidz. collected by T. Sato in Nov. 1994, Chichijima, Ogasawara Islands, Japan. This strain has been deposited in the Japan Collection of Microorganisms, Wako, Saitama, as JCM 10575. The other strains, OK-79, OK-130, OK-206, OK-215, OK-229 and OK-233, have also been deposited in the Japan Collection of Microorganisms, as JCM 10576, 10577, 10578, 10579, 10580 and 10581, respectively.

Bullera schimicola Sugita, Cañete-Gibas, Takashima et Nakase, sp. nov. Fig. 3

In liquido YM post 5 dies ad 17°C: cellulae ovoideae, ellipsoidales, elongatae, 6.8–9.5 × 7.4–

14.7 μm, singulae aut binae; sedimentum formatur; post unum mensem ad 17°C pellicula fragilis et sedimentum compactum formantur. Coloniae in agaro YM post unum mensem ad 17°C griseo-aurantiacae glabrae aut rugulosae, margine serrulatae. In agaro farinae zeae pseudomycelium non formatur; ballistoconidia globosa, subglobosa vel napiformia, 5.3–5.8 × 7.4–7.6 μm. Fermentatio nulla. Glucosum, galactosum, L-sorbosum (infirme), saccharosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinosum, melezitosum, amylum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum (infirme), *N*-acetyl-D-glucosaminum, erythritolum (lente et infirme), ribitolum, galactitolum, mannitolum, glucitolum, α-methyl-D-glucosidum, salicinum, glucono-δ-lactonum, acidum D-gluconicum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum (lente et infirme), acidum succinicum, acidum citricum (lente et infirme), inositolum, acidum saccharicum (lente et infirme), xylitolum, L-arabinitolum (infirme), acidum D-glucronicum et acidum D-galacturonicum assimilantur, autem lactosum, inulinum, methanolum, ethanolum, glycerolum, hexadecanum, 1,2-propanediolum et 2,3-propanediolum non assimilantur. Ammonium sulfatum et L-lysinum assimilantur, autem kalium nitricum, natrium nitrosum, ethylaminum et cadaverinum non assimilantur. Maxima temperatura crescentiae 28–29°C est. Ad crescentiam thiaminum necessarium est. Commutatio colori per diazonium caeruleum B positiva est. Proportio molaris guanini+cytosini in acido deoxyribonucleico 42.9 mol% (per HPLC) est. Ubiquinonum majus Q-10 adest. Xylosum in cellulis praesens. Holotypus: Colonia in cultura a T. Nakase isolata (originaliter ut OK-34) ex folio *Schimae mertensianae*, Chichijima Ins. Ogasawara, Japonia, Nov. 1994, T. Sato, leg. et in Collectionibus Culturarum Japononsium qua 'Japan Collection of Microorganisms,' Wako, Saitama conservata (JCM 10582).

Etymology: The specific ephithet, *schimicola*= dwelling on *Schima*, referring to its habitat.

Growth in YM broth: After 5 d at 17°C, cells are ovoidal, ellipsoidal, elongate, 6.8–9.5 × 7.4–14.7 μm, single or in pairs (Fig. 3A). A sediment is formed. After one mo at 17°C, a fragile pellicle and a compact sediment are present.

Growth on YM agar: After one mo at 17°C, the streak culture is orange grey, delicately wrinkled and has a slightly serrated margin.

Dalmau plate culture on corn meal agar: No pseudomycelia are produced.

Production of ballistoconidia: Ballistoconidia are produced on corn meal agar. They are globose, subglobose to napiform, 5.3–5.8 × 7.4–7.6 μm (Fig. 3B).

Fermentation: Absent.

Assimilation of carbon compounds:

glucose	+	erythritol	+ (latent and weak)

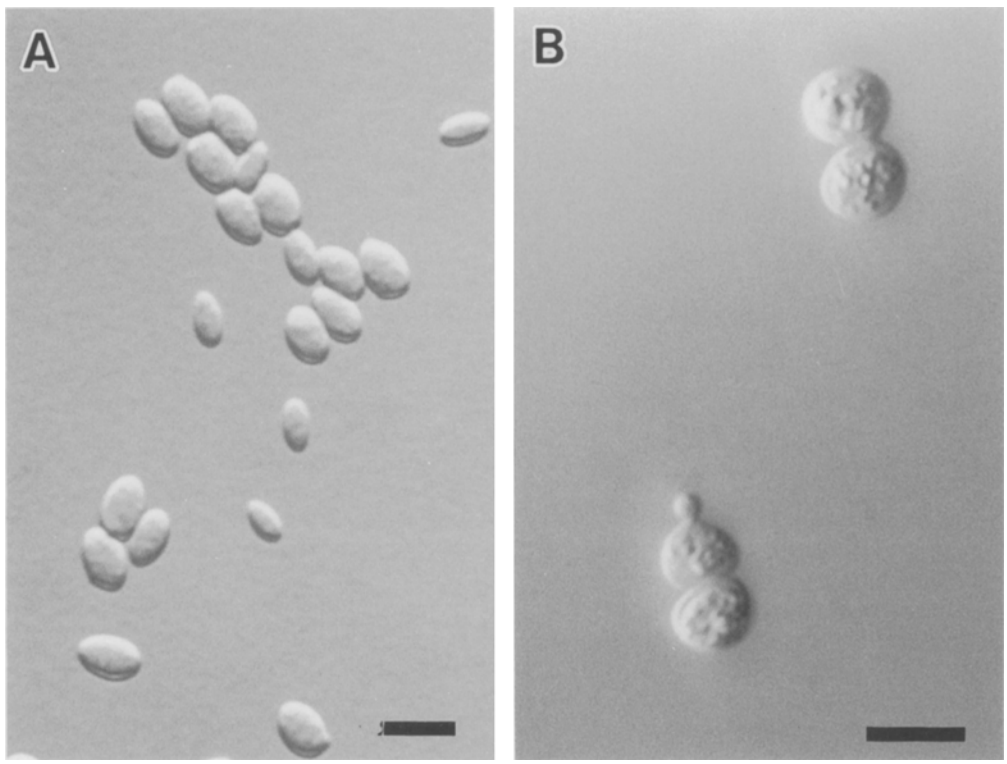

Fig. 3. *Bullera schimicola*. (A) Vegetative cells grown in YM broth for 5 d at 17°C. (B) Ballistoconidia produced on corn meal agar after 5 d at 17°C. Scale bars indicate 10 μm.

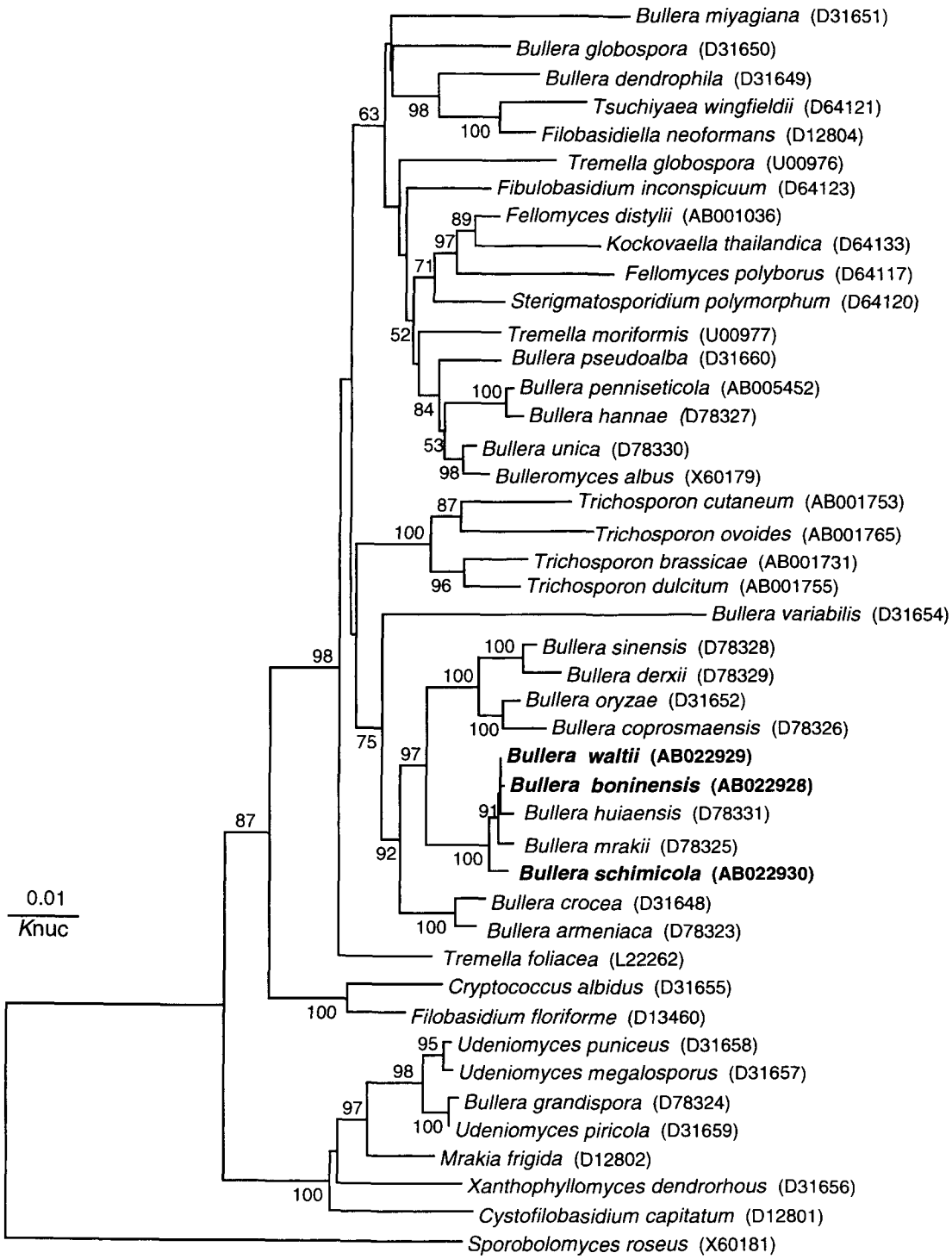

Fig. 4. Molecular phylogenetic tree of three new species of *Bullera* and related species based on the SSU rDNA sequences. The numerals represent the percentages from 1,000 replicate bootstrap samplings (frequencies of less than 50% are not indicated). Sequences were retrieved from the GenBank database under the accession numbers indicated.

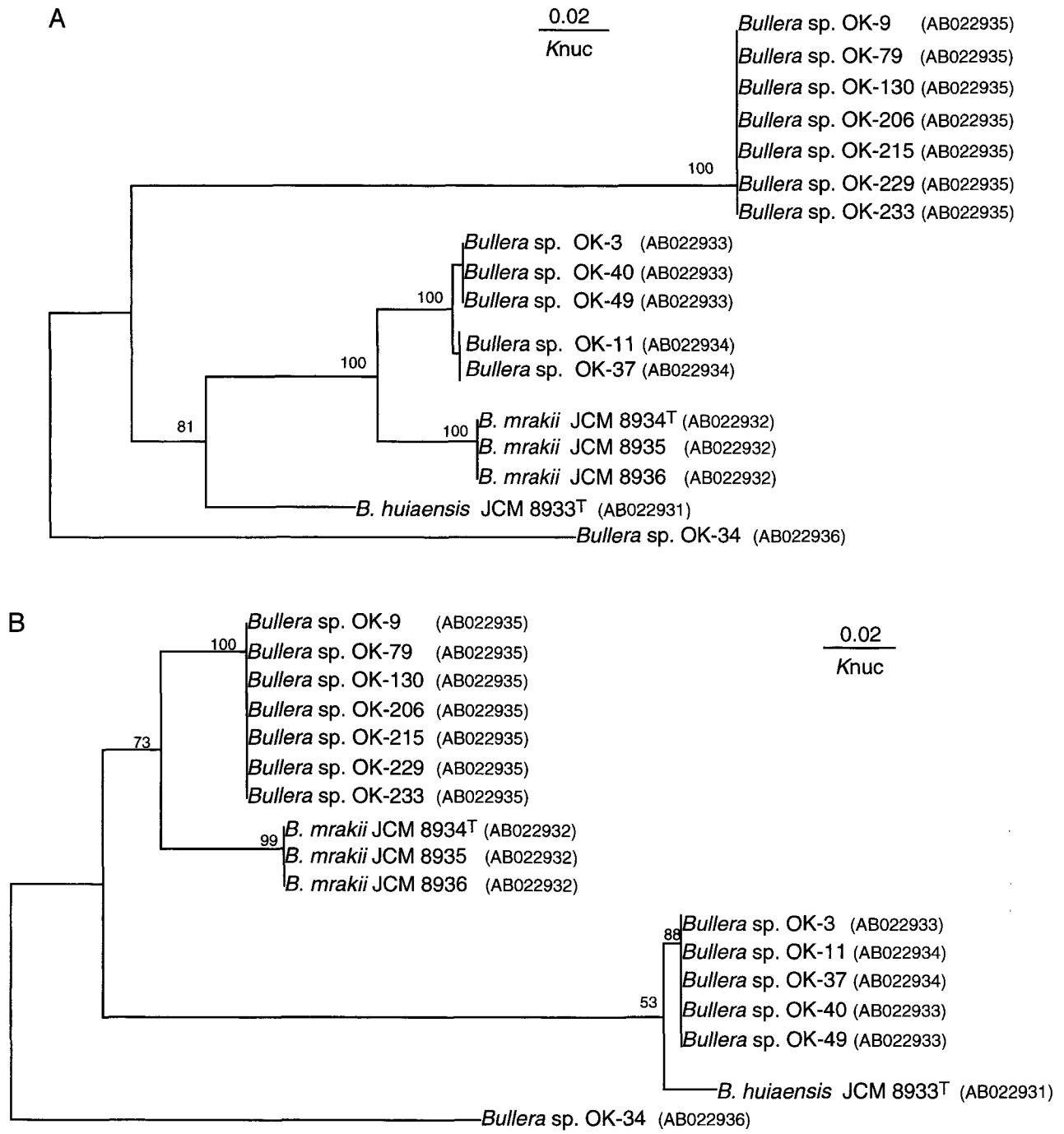

Fig. 5. Molecular phylogenetic tree of *Bullera* isolates, *B. huiaensis,* and *B. mrakii* based on the sequences of the ITS 1 (Fig. 5A) and 2 (Fig. 5B) regions. The numerals represent the persentages from 1,000 replicate bootstrap samplings (frequencies of less than 50% are not indicated). ITS1 is 161 to 181 bp long, and ITS2 is 149 to 155 bp long.

Table 2. Matrix of ITS 1 and 2 similarity (%) for 13 *Bullera* isolates.

	Bullera sp. OK-3 *Bullera* sp. OK-40 *Bullera* sp. OK-49	*Bullera* sp. OK-11 *Bullera* sp. OK-37	*Bullera* sp. OK-9 *Bullera* sp. OK-79 *Bullera* sp. OK-130 *Bullera* sp. OK-206 *Bullera* sp. OK-215 *Bullera* sp. OK-229 *Bullera* sp. OK-233	*Bullera* sp. OK-34	*Bullera huiaensis* JCM 8933	*Bullera mrakii* JCM 8937 *Bullera mrakii* JCM 8935 *Bullera mrakii* JCM 8936
Bullera sp. OK-3 *Bullera* sp. OK-40 *Bullera* sp. OK-49	—	99.4	85.6	74.6	89.0	93.8
Bullera sp. OK-11 *Bullera* sp. OK-37	100.0	—	86.5	74.7	89.0	93.8
Bullera sp. OK-9 *Bullera* sp. OK-79 *Bullera* sp. OK-130 *Bullera* sp. OK-206 *Bullera* sp. OK-215 *Bullera* sp. OK-229 *Bullera* sp. OK-233	94.2	94.2	—	73.7	77.8	86.0
Bullera sp. OK-34	86.2	86.2	80.9	—	75.7	68.3
Bullera huiaensis JCM 8933	97.3	97.3	96.3	86.2	—	89.0
Bullera mrakii JCM 8937 *Bullera mrakii* JCM 8935 *Bullera mrakii* JCM 8936	94.0	94.0	96.3	83.5	94.7	—

Data in the upper right portion of the table refer to ITS 1 similarity, and data in the lower left portion to ITS 2 similarity.

galactose	+	ribitol	+	ethanol	−
L-sorbose	+ (weak)	galactitol	+	glycerol	−
sucrose	+	mannitol	+		
maltose	+	glucitol	+		
cellobiose	+	α-methyl-D-glucoside	+		
treharose	+	salicin	+		
lactose	−	glucono-δ-lactone	+		
melibiose	+	D-gluconic acid	+		
raffinose	+	2-ketogluconic acid	+		
melezitose	+	5-ketogluconic acid	+		
inulin	−	DL-lactic acid	+ (latent and weak)		
soluble starch	+	succinic acid	+		
D-xylose	+	citric acid	+ (latent and weak)		
L-arabinose	+	inositol	+		
D-arabinose	+	n-hexadecane	−		
D-ribose	+	saccharic acid	+ (latent and weak)		
L-rhamnose	+	xylitol	+		
D-glucosamine	+ (weak)	L-arabinitol	+ (weak)		
N-acetyl-D-glucosamine	+	propane 1,2 diol	−		
methanol	−	butane 2, 3 diol	−		

D-glucronic acid	+		
D-galacturonic acid	+		
Assimilation of nitrogen compounds:			
ammonium sulfate	+	ethylamine hydrochloride	−
potassium nitrate	−	L-lysine hydrochloride	+
sodium nitrite	−	cadaverine dihydrochloride	−

Maximum growth temperature: 28–29°C.
Vitamin required: Thiamine.
Production of starch-like substances: Negative.
Growth on 50% (w/w) glucose-yeast extract agar: Negative.
Urease: Positive.
Liquefaction of gelatin: Negative.
Hydrolysis of fat: Negative.
Diazonium blue B reaction: Positive.
G+C content of nuclear DNA: 42.9 mol% (by HPLC).
Major ubiquinone: Q-10.
Xylose in the cells: Present.
Type strain: OK-34, isolated by T. Nakase from a leaf of *Schima mertensiana* (Sieb. et Zucc.) Koidz. collected by T. Sato in Nov. 1994, Chichijima, Ogasawara Islands, Japan. This strain has been deposited in the Japan Collection of Microorganisms, Wako, Saitama, as JCM

Table 3. DNA relatedness among *Bullera* sp. OK isolates and phylogenetically closely related species.

Species	Strain	Mol% G+C	% relative binding of DNA with								
			OK3	OK11	OK37	OK40	OK49	OK9	OK34	JCM8933	JCM8934
Bullera sp.	OK3	42.2	100	86	75	75	79	19	13	16	21
Bullera sp.	OK11	43.1	76	100							
Bullera sp.	OK37	42.4	84		100						
Bullera sp.	OK40	42.6	71			100					
Bullera sp.	OK49	42.6	93				100				
Bullera sp.	OK9	42.8	25					100	28	29	34
Bullera sp.	OK34	42.9	31					22	100	7	16
B. huiaensis	JCM 8933^T	44.3[a]	19					13	21	100	29[a]
B. mrakii	JCM 8934^T	43.7[a]	25					20	18	24[a]	100

a, Hamamoto and Nakase (1996).
T, type strain.

Table 4. Differential characteristics of three new *Bullera* species and phylogenetically closely related species.

species	Assimilation of:						Production of starch-like substrates	Maximum growth temperature (°C)
	Ribitol	DL-Lactate	Citrate	Nitrite	Etylamine	Cadaverine		
B. boninensis	+/L	L/LW	LW	+	−	−	−	28–31
B. waltii	L/W/L	+/L/LW	LW	+	+	+	+	28–29
B. schimicola	+	LW	LW	−	−	−	−	28–29
B. huiaensis	LW	−	−	−	+	+	W	21–22
B. mrakii	−	W/LW	LW	−	−	−	−	28–29

+, positive; L, latent; W, weak; LW, latent and weak; −, negative.

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Thirteen undescribed yeasts were taxonomically investigated. These isolates were divided into three groups on the basis of their physiological characteristics. SSU rDNA of a representative isolate of each group OK-3, OK-9 and OK-34, was sequenced. The three isolates were phylogenetically closely related to each other and constituted a cluster with *B. huiaensis* and *B. mrakii* (Fig. 4). The sequences of the ITS 1 and ITS 2 regions were then determined for all of the isolates. Since the ITS regions have a higher rate of divergence than the SSU or LSU rRNA genes, their sequence analysis is generally considered to be a useful identification tool. Figures 5A and 5B show the molecular phylogenetic trees based on the sequences of the ITS 1 and ITS 2 regions, respectively, of the 13 isolates. There are some minor incongruencies in the ITS 1 and ITS 2, and 18S rDNA data. These are thought to be due to differences in the rate of divergence of each molecule. Table 2 also shows the matrix of ITS 1 and ITS 2 similarity for the 13 isolates and four strains of *B. huiaensis* and B. *mrakii*. Isolates OK-3, 40, and 49 showed 99.4% and 100% similarities in ITS 1 and ITS 2 sequences, respectively, to the isolates OK-11 and 37. The ITS 1 and ITS 2 sequences of OK-9 were identical to those of the isolates OK-79, 130, 206, 215, 229, and 233. The OK-3 group, the OK-9 group, and OK-34 showed 68.3–93.8% and 83.5–97.3% similarities in ITS 1 and ITS 2 sequences, respectively, to the phylogenetically closely related species *B. huiaensis* and *B. mrakii*. Recently, Sugita et al. (1999) found that conspecific strains differ by fewer than 1% of nucleotides in the ITS 1 and ITS 2 regions overall. According to this species concept, it is postulated that the 13 isolates represent three new species. This was confirmed by a nuclear DNA-DNA hybridization experiment (Table 3). The isolates of the OK-3 group showed 71 to 93% DNA relatedness values to each other. The DNA-DNA hybridization experiment was not performed for the OK-9 group, as the ITS sequences of the isolates in that group were completely identical. The three new *Bullera* specie were named as follows: *Bullera boninensis* for OK-3, 11, 37, 40, and 49; *Bullera waltii* for OK-9, 79, 130, 206, 215, 229, and 233; and *Bullera schimicola* for OK-34. The new species can be distinguished from both *B. huiaensis* and *B. mrakii* by their assimilation of ribitol, DL-lactate, citrate, nitrite, ethylamine and cadaverine. The production of a starch-like substance and maximum growth temperature can also be used to distinguish these new species (Table 4).

Acknowledgements——We thank Ms. Paipan Budhaka, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand, for her help in several experiments. We also thank Dr. Toyozo Sato, Shikoku National Agricultural Experiment Station, Japan, for kindly supplying plant materials collected in the Ogasawara Islands. This study was supported in part by special coordination funds for promoting science and technology of the Science and Technology Agency of the Japanese Government.

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