Phylogenetic analysis of strains originally assigned to *Bullera variabilis*: descriptions of *Bullera pseudohuiaensis* sp. nov., *Bullera komagatae* sp. nov. and *Bullera pseudoschimicola* sp. nov.

Feng-Yan Bai,¹ Masako Takashima² and Takashi Nakase²

¹ Systematic Mycology and Lichenology Laboratory, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China

² Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama, 351-0198, Japan

Author for correspondence: Feng-Yan Bai. Tel: +86 10 6255 5692. Fax: +86 10 6256 0912. e-mail: baify@sun.im.ac.cn

Twenty strains previously assigned to the species *Bullera variabilis* Nakase & Suzuki were reclassified using a molecular taxonomic approach. The strains were regrouped first by nucleotide sequence comparison of the rDNA internal transcribed spacer (ITS) regions, including the 5.8S gene. Phylogenetic positions of *B. variabilis* strains with different ITS region sequences were then analysed based on their 18S rDNA sequences. The taxonomic status of the original *Bullera variabilis* strains was clarified further by DNA–DNA hybridization experiments. Of the 20 strains studied, five remained in the species *B. variabilis*, six strains were reassigned to the species *Bullera mrakii* and three novel species were proposed for eight of the nine remaining strains, namely *Bullera pseudohuiaensis* sp. nov. (one strain; type strain JCM 5984^T = AS 2.2203^T), *Bullera komagatae* sp. nov. (one strain; type strain JCM 5983^T = AS 2.2202^T) and *Bullera pseudoschimicola* sp. nov. (six strains; type strain JCM 3915^T = AS 2.2201^T). The remaining strain, JCM 6140, was closely related to *B. pseudoschimicola*. However, differences in ITS region sequences between strain JCM 6140 and strains of *B. pseudoschimicola*, and the intermediate DNA–DNA relatedness to representative strains of *B. pseudoschimicola* did not allow a definite taxonomic decision to be made for strain JCM 6140.

Keywords: *Bullera variabilis*, *Bullera pseudohuiaensis* sp. nov., *Bullera komagatae* sp. nov., *Bullera pseudoschimicola* sp. nov., phylogeny

INTRODUCTION

Bullera variabilis Nakase & Suzuki (1987) was initially described to accommodate a group of yeast strains that formed ballistoconidia of various shapes. Heterogeneity of the species has been shown by markedly different electrophoretic enzyme patterns among strains studied. However, it has been difficult to separate clear-cut groups based on enzyme patterns or any other phenotypic characteristic.

The yeasts of this group are common inhabitants of plant materials. Since *B. variabilis* was established in

1987, a considerable number of ballistoconidium-forming yeast strains phenotypically similar or phylogenetically closely related to this species have been isolated from various countries, including China, Japan, New Zealand, Thailand and Vietnam (Nakase, 2000). Before novel strains can be accurately classified, it is necessary to redefine the species concept of *B. variabilis* and clarify the taxonomic status of the strains previously assigned to this species. Since it is difficult to reclassify *B. variabilis* strains by conventional yeast taxonomy, molecular phylogenetic approaches were used to resolve the problem.

METHODS

Yeast strains. The strains used are listed in Table 1. In addition to the 17 strains originally described as *B. variabilis* (Nakase & Suzuki, 1987), all other strains listed under this

Abbreviation: ITS, internal transcribed spacer.

The GenBank accession numbers for the ITS region and 18S rDNA sequences of the strains determined in the present study are AF314965–AF314989 and AF314990–AF314999, respectively.

Table 1. Selected physiological characters of the *Bullera variabilis* strains grouped by ITS region sequence comparison

$T_{\max}$, maximum growth temperature; +, positive; –, negative; L, latent; *l*, latent and weak; w, weakly positive.

Group	Strain	Source	Assimilation of:				Starch formation	$T_{\max}$ (°C)
			Lactose	Erythritol	Glucitol	Ethylamine		
I	JCM 5275 ^T	Dead leaf of <i>Oryza sativa</i> , Japan	–	–	+	–	+	29–30
	JCM 5276	Dead leaf of <i>Oryza sativa</i> , Japan	–	–	+	–	+	29–30
	JCM 5277	Dead leaf of <i>Oryza sativa</i> , Japan	–	–	+	–	+	29–30
	JCM 6137	Straw of <i>Oryza sativa</i> , Japan	–	–	+	–	+	30–31
	JCM 7267	Dead leaf of <i>Miscanthus sinensis</i> , Japan	–	–	+	–	+	27–28
IIa	JCM 5984 ^T	Dead leaf of <i>Sasa</i> sp., Japan	–	–	L	+	+	23–24
IIb	JCM 5986	Dead leaf of <i>Sasa</i> sp., Japan	<i>l</i>	L	–	–	–	27–28
	JCM 7265	Dead leaf of <i>Miscanthus sinensis</i> , Japan	L	L	–	–	–	27–28
	JCM 7266	Dead leaf of <i>Miscanthus sinensis</i> , Japan	L	L	–	–	–	27–28
	JCM 7491	Sooty moulds on <i>Pseudopanax arboreum</i> , New Zealand	<i>l</i>	+	<i>l</i>	–	–	28–29
	JCM 7479	Dead leaf of <i>Miscanthus sinensis</i> , Japan	<i>l</i>	<i>l</i>	–	–	–	28–29
IIIa	NB-94	Dead leaf of <i>Miscanthus sinensis</i> , Japan	<i>l</i>	<i>l</i>	–	–	–	27–28
	JCM 5983 ^T	Dead leaf of <i>Sasa</i> sp., Japan	–	–	+	–	–	24–25
	JCM 3914	Dead base of bamboo culm, Canada	–	<i>l</i>	L	–	–	23–24
	JCM 3915 ^T	Dead base of bamboo culm, Canada	–	w	+	–	–	23–24
	JCM 5985	Dead leaf of <i>Sasa</i> sp., Japan	–	w	+	–	–	26–27
IIIb	JCM 7486	Dead leaf of <i>Vitis ficifolia</i> var. <i>lobata</i> , Japan	–	–	–	–	–	26–27
	NB-143	Dead leaf of <i>Miscanthus sinensis</i> , Japan	–	–	–	–	–	26–27
	NB-239	Dead leaf of <i>Majanthemum bifolium</i> var. <i>dilatatum</i> , Japan	–	–	<i>l</i>	–	–	26–27
	JCM 6140	Dead leaf of <i>Magnolia grandiflora</i> , Japan	–	–	–	–	+-	29–30

name in the last edition of *JCM Catalogue of Strains* (Nakase, 1999) were included.

18S rDNA and internal transcribed spacer (ITS) region sequencing. Nucleotide sequences of small-subunit rDNA (18S rDNA) and internal transcribed spacer (ITS1 and ITS2) regions, including 5.8S rDNA, were determined according to Sugita & Nakase (1999) with the following modifications: cycle sequencing was performed using the ABI BigDye cycle sequencing kit and samples were electrophoresed on an ABI PRISM 310 Genetic Analyzer.

Molecular phylogenetic analysis. Sequences of the 18S rDNA and ITS regions determined in this study and reference sequences were aligned with the CLUSTAL W program (Thompson *et al.*, 1994) and adjusted manually. The reference sequences were obtained from DDBJ, EMBL and GenBank, where they had been deposited by other authors (Sugita *et al.*, 1999a; Suh & Sugiyama, 1993; Suh *et al.*, 1996a, b; Swann & Taylor, 1993; Takashima & Nakase, 1998, 1999; Van de Peer *et al.*, 1992). The phylogenetic tree was constructed from the evolutionary distance data calculated from Kimura's two-parameter model (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987). Sites where gaps existed in any of the aligned sequences were excluded. Bootstrap analyses (Felsenstein, 1985) were performed from 1000 random resamplings.

DNA–DNA reassociation. Crude DNA was extracted from ground freeze-dried cells using the methods of Raeder & Broda (1985). DNA purification, DNA base composition determination and DNA–DNA reassociation were performed according to Takashima & Nakase (2000).

RESULTS AND DISCUSSION

Grouping of the *B. variabilis* strains by ITS region sequence comparison

The complete ITS region (including 5.8S rDNA) sequences of all the *B. variabilis* strains used were determined first. A dendrogram was drawn by

Fig. 1. Dendrogram drawn from neighbour-joining analysis based on ITS region sequences showing the grouping of the *B. variabilis* strains.

neighbour-joining analysis based on alignment of the ITS region sequences. The 20 *B. variabilis* strains clearly separated into three groups corresponding to the three main clusters of the dendrogram (Fig. 1). Five strains, including *B. variabilis* JCM 5275^T, clustered into group I, seven into group II and the other eight were in group III. Groups II and III were further subdivided as depicted in Fig. 1.

The total lengths of ITS1–5.8S rDNA–ITS2 regions of the strains were as follows: group I, 389 nt; group IIa, 470 nt; group IIb, 488–490 nt; group IIIa, 493 nt; and group IIIb, 496–498 nt. The dramatic differences in ITS region length of group I from those of groups II and III indicated that the strains in group I were remarkably different from those in groups II and III. According to ITS region sequence similarity, group I

Fig. 2. Phylogenetic tree constructed from 18S rDNA sequence alignment depicting the positions of representative *B. variabilis* strains. The phylogram was constructed by neighbour-joining analysis. Bootstrap percentages over 50% from 1000 bootstrap replicates are shown. The GenBank/EMBL/DBJ accession numbers are indicated.

seemed to be homogeneous. Among the five strains in this group, four strains, including the type strain of *B. variabilis*, had identical ITS region sequences which differed from the fifth strain (JCM 5277) in only 1 nt. However, both groups II and III seemed to be heterogeneous. The two subgroups in each of these two groups differed from each other significantly in their ITS region sequences. Even within the same subgroups, especially in group IIIb, the ITS region sequences of the strains differed from each other to varying degrees, as indicated in Fig. 1.

Physiological profiles of the three groups recognized by ITS region sequence analysis were compared and differences were found in assimilation reactions of lactose, erythritol, glucitol and ethylamine, starch formation and maximum growth temperatures (Table 1). Strains in group I had almost identical assimilation patterns and similar maximum growth temperatures and could easily be distinguished from strains in the other two groups. In group II, strains in group IIb also had identical physiological characters, enabling them

to be differentiated from the strain in group IIa. Strains in group III could be distinguished from strains in the other two groups, but were less homogeneous in their physiological characters. Their erythritol and glucitol assimilation reactions and maximum growth temperatures were divergent. The two subgroups IIIa and IIIb were indistinguishable in their physiological characters.

Phylogenetic positions of the *B. variabilis* strains inferred from 18S rDNA sequencing

Heterogeneity of the *B. variabilis* strains was clearly demonstrated by their remarkable differences in ITS region sequences as mentioned above. To elucidate the phylogenetic relationships of the *B. variabilis* strains with one another and with other species in the genus *Bullera* and related xylose-containing basidiomycetous yeast taxa, 18S rDNA sequences of representative *B. variabilis* strains were determined, namely those belonging to groups II and III which differ from each other in more than one nucleotide in ITS region

Fig. 3. Phylogenetic tree drawn from neighbour-joining analysis based on ITS region (including 5-8S rDNA) sequence alignment showing the relationships of the *B. variabilis* strains with closely related *Bullera* species. Bootstrap percentages over 50% from 1000 bootstrap replicates are shown. The GenBank/EMBL/DBJ accession numbers are indicated.

sequences. A phylogenetic tree was constructed based on 18S rDNA sequence alignment using neighbour-joining analysis (Fig. 2).

The 18S rDNA sequence of the type strain of *B. variabilis* (JCM 5275^T), which represents group I, has already been determined (Suh & Nakase, 1995). The similarities in ITS region sequences and in physiological characters among the strains in group I as discussed above suggest that this group is homogeneous and can thus be assigned to the species *B. variabilis*. Therefore, no other strain was selected from group I for 18S rDNA sequencing.

The groupings of the *B. variabilis* strains based on ITS region sequences were correspondingly separated in the phylogenetic tree based on 18S rDNA sequences (Fig. 2), which shows the species of *Bullera* separated into two main clades as already indicated by Suh *et al.* (1996a) and Takashima & Nakase (1999). The representative strains of the three groups in this study are

limited to one of the clades, which coincides with the '*Cryptococcus luteolus* lineage' of Takashima & Nakase (1999). *B. variabilis* JCM 5275^T, representing group I, is basal to the clade and thus only distantly related to the others. Strains of group II clustered with *Bullera mrakii*, *Bullera boninensis*, *Bullera waltii* and *Bullera huiaensis*. The four strains of group IIb had identical 18S rDNA sequences and were closest to *B. mrakii*, whereas strain JCM 5984^T had a unique sequence. All strains in group III had identical 18S rDNA sequences and clustered with *Bullera schimicola*.

Phylogenetic relationships of the *B. variabilis* strains with closely related *Bullera* species demonstrated by ITS region sequence analysis

The ITS region sequences of the *Bullera* species in the clade containing the representative *B. variabilis* strains in the tree drawn from 18S rDNA sequences (Fig. 2) were then determined and aligned with those of all the

Table 2. DNA–DNA relatedness of selected group IIb strains of *B. variabilis* with the type strain of *Bullera mrakii*

Species/strain	G + C (mol %)	Relative binding (%) of DNA with JCM strain:				
		8934 ^T	7491	5986	7266	7265
<i>B. mrakii</i> JCM 8934 ^T	43.0	100	93	82	64	65
Strain JCM 7491	42.6	91	100	77	56	57
Strain JCM 5986	42.7	91	92	100	61	67
Strain JCM 7266	42.9	61	63	58	100	87
Strain JCM 7265	43.0	60	68	57	82	100

Table 3. DNA–DNA relatedness of strain JCM 5984^T with closely related species

Species/strain	G + C (mol %)	Relative binding (%) of DNA with JCM strain:				
		5984 ^T	8933 ^T	8934 ^T	10570 ^T	10575 ^T
<i>B. pseudohuiaensis</i> JCM 5984 ^T	43.9	100	18	17	17	17
<i>B. huiaensis</i> JCM 8933 ^T	44.5	31	100	ND	ND	ND
<i>B. mrakii</i> JCM 8934 ^T	43.0	23	ND	100	ND	ND
<i>B. boninensis</i> JCM 10570 ^T	43.3	22	ND	ND	100	ND
<i>B. waltii</i> JCM 10575 ^T	43.1	31	ND	ND	ND	100

ND, Not done.

B. variabilis strains used in the present study. A phylogenetic tree was drawn, with group I as the outgroup, according to the topology of the phylogenetic tree constructed from 18S rDNA sequences. Four main clades were recognized in the phylogenetic tree drawn from the ITS region sequences and the original *B. variabilis* strains were distributed in three of them (Fig. 3).

Taxonomic status of the strains in group II

The strains in group II were included in the clade represented by *B. mrakii* (Fig. 3). The single strain in group IIa, JCM 5984^T, clustered close to *B. huiaensis* and the five strains in group IIb were located together with *B. mrakii*. The numbers of nucleotide differences in ITS region sequences among all the strains in this clade were calculated by pairwise comparison. The strains in group IIb differed from each other in 1–6 nt and differed from the type strain of *B. mrakii* in 0–3 nt. Data predict that the group IIb strains belong to *B. mrakii*. Strain JCM 5984^T differed from the type strain of the closely related species *B. huiaensis* in 18 nt (3.8 %), implying that JCM 5984^T represents a distinct species.

DNA–DNA reassociation was performed between the type strain of *B. mrakii* and selected group IIb strains (Table 2). Strains JCM 5986 and JCM 7491 were clearly conspecific with *B. mrakii* because DNA homology values with the type strain of this species

were 77–93 %. The other two strains, JCM 7265 and JCM 7266, had 82–87 % DNA similarity, though they differed in 5 nt in ITS region sequences, but only had relatively low (56–68 %) DNA homology with the type strain of *B. mrakii*, JCM 5986 and JCM 7491. Data indicate that strains JCM 7265 and JCM 7266 represent a somewhat different population from that represented by the type strain of *B. mrakii*. Considering that the two strains differed from the type strain of *B. mrakii* in only 2–3 nt in the ITS region and that their DNA relatedness with *B. mrakii* was still within the range of the same species, it is thought that they should be assigned to the species *B. mrakii*. The morphological and physiological characters of the group IIb strains corresponded to those in the description of *B. mrakii* (Hamamoto & Nakase, 1996).

As well as marked ITS region sequence differences, DNA homology of strain JCM 5984^T with closely related species in the same clade was 17–31 % (Table 3), which suggested that this strain represents an undescribed species, for which the name *Bullera pseudohuiaensis* sp. nov. is proposed.

Taxonomic status of the strains in group III

The *B. variabilis* strains in group III were located in a clade containing only one recently described species, *B. schimicola* (Sugita *et al.*, 1999a) (Fig. 3). Group III strains differed from the type strain of *B. schimicola* in more than 18 nt (3.6 %) in the ITS region, implying

Table 4. DNA–DNA relatedness of representative group III strains of *B. variabilis* with closely related species

Species/strain	G + C (mol %)	Relative binding (%) of DNA with JCM strain:					
		3915 ^T	5985	7486	6140	10582 ^T	5983 ^T
<i>B. pseudoschimicola</i> JCM 3915 ^T	44.4	100	67	72	59	56	39
Strain JCM 5985	44.3	66	100	81	61	ND	38
Strain JCM 7486	43.9	67	81	100	60	48	37
<i>Bullera</i> sp. JCM 6140	44.7	54	52	62	100	50	35
<i>B. schimicola</i> JCM 10582 ^T	43.1	50		58	56	100	43
<i>B. komagatae</i> JCM 5983 ^T	44.5	35	39	46	45	29	100

ND, Not done.

that the former can be genetically differentiated from *B. schimicola*. Although the representative strains in group III all had identical 18S rDNA sequences, strain JCM 5983^T in group IIIa differed from the strains in group IIIb by 18–22 nt (3.6–4.4 %) in the ITS region, suggesting that it represents a separate species. The taxonomic relationships of the seven strains in group IIIb showed by ITS region sequencing are not clear. Strain JCM 6140 differed from the other strains in the same subgroup by 7–11 nt in the ITS region and was on a separate branch in the neighbour-joining tree drawn from ITS region sequences (Fig. 3). However, the separation was not clear-cut; excluding JCM 6140, the strains in group IIIb differed from each other in 0–7 nt in ITS region sequences.

DNA–DNA reassociation experiments were performed with strains having different ITS region sequences in group III and the type strain of *B. schimicola* (Table 4). Results confirmed that strain JCM 5983^T represented a distinct species: DNA homology values with strains in group IIIb and with the type strain of *B. schimicola* were 29–46 %. The name *Bullera komagatae* sp. nov. is therefore proposed for strain JCM 5983^T.

The strains in group IIIb (including JCM 6140) had intermediate DNA–DNA homology values (48–58 %) with the type strain of *B. schimicola* JCM 10582^T (Table 4). However, as mentioned above, the former had remarkable and clear-cut nucleotide divergence (3.6–4.4 %) with the latter in ITS region sequences. Moreover, in the phylogenetic tree drawn from 18S rDNA sequence comparison, the group IIIb strains were clearly separated from *B. schimicola* (Fig. 2). These sequence data suggest that the taxon or taxa represented by the group IIIb strains (including JCM 6140) are distinct from *B. schimicola* at species level.

Strains JCM 3915^T, JCM 5985 and JCM 7486 in group IIIb were shown to be conspecific by their 66–81 % DNA homology values. These three strains, together with JCM 3914, NB-143 and NB-239 (JCM 3914 and NB-143, and NB-239 have identical ITS region sequences with JCM 3915^T and JCM 7486, respectively), represent a novel *Bullera* species, for which the name *Bullera pseudoschimicola* sp. nov. is proposed.

The taxonomic status of the remaining strain in group IIIb, JCM 6140, is not easy to determine according to the data obtained so far. It does not seem reasonable to classify JCM 6140 as *B. pseudoschimicola* because this strain is located in a distinct branch, separated from the other strains of group IIIb by *B. schimicola* in the phylogenetic tree drawn from ITS region sequence comparison (Fig. 3). However, its intermediate DNA–DNA homology values (52–62 %) with, and its unclear ITS region sequence divergences from, other strains of group IIIb make it difficult to make a definite taxonomic decision for JCM 6140. Therefore, JCM 6140 should be treated as *Bullera* sp. at present.

The taxonomic significance of the ITS region sequences, electrophoretic patterns of enzymes and mycocins

Because of its relatively higher rate of divergence compared to small-subunit or large-subunit rDNA, ITS region sequences have been considered to be a useful tool in resolving relationships among closely related taxa (Berbee *et al.*, 1995; Fell *et al.*, 2000; James *et al.*, 1996; Oda *et al.*, 1997; Waalwijk *et al.*, 1996). Recent studies involving yeasts have found that ITS region sequence similarity is generally consistent with DNA–DNA relatedness and conspecific strains usually have fewer than 1 % nt differences in the ITS1 and ITS2 regions overall (Nagahama *et al.*, 1999; Sugita *et al.*, 1999a, b). The coincidence of the ITS region sequence similarity and DNA–DNA relatedness was observed among yeast strains used in the present study. However, up to approximately 2 % nt divergences in the ITS1 and ITS2 regions overall exist among some strains within each the species *B. mrakii* and *B. pseudoschimicola* sp. nov.

Boekhout *et al.* (1991) used six original *B. variabilis* strains in mating experiments. They found that mating only occurred between CBS 7347^T (= JCM 5275^T) and CBS 7367 (= JCM 7267). The former is the type strain of *B. variabilis* and the latter is retained in this species because of its identical ITS region sequence with the former. In the present study, the remaining four *B. variabilis* strains used by Boekhout *et al.* (1991) were

shown to belong to three other species: *B. pseudoschimicola* sp. nov. (CBS 7354 = JCM 3914), *B. pseudohuiaensis* sp. nov. (CBS 7364^T = JCM 5984^T) and *B. mrakii* (CBS 7365 = JCM 7265; CBS 7366 = JCM 7266).

As mentioned previously, the heterogeneity of *B. variabilis* was first observed in the remarkable differences in electrophoretic patterns of enzymes among the strains originally assigned to this species (Nakase & Suzuki, 1987). However, it is difficult to clearly correlate enzyme pattern similarities with ITS region sequence similarities among these strains. The present study showed that strains showing similarities below 30% in enzyme electrophoretic patterns may certainly be expected to represent different species. However, conspecific strains may have as low as 36% similarity, whereas strains belonging to different species may have as high as 69% similarity in enzyme patterns. A previous study (Bai *et al.*, 1999) also indicated that yeast strains with different enzyme patterns may have very high DNA–DNA complementarities.

Sensitivity to mycocins (killer toxins) has been considered to be a useful tool in yeast taxonomy (Golubev, 1998). Mycocinogeny studies (Golubev & Nakase, 1997, 1998) indicated that the killer-sensitive patterns of some strains originally classified as *B. variabilis* were different, implying that the species was heterogeneous. This observation has been confirmed here. However, the present study has shown that strains with different killer-sensitive patterns may be conspecific. For example, the two strains reclassified as *B. mrakii* in the present study, JCM 7479 and JCM 7491, have obviously different mycocin responses from each other and from the type strain of *B. mrakii* JCM 8934^T (Golubev & Nakase, 1998). The type strain of *B. variabilis*, JCM 5275^T, is insensitive to the *Bullera sinensis* mycocin, whereas strain JCM 6137, which has an ITS region sequence identical to that of JCM 5275^T, is weakly sensitive to this mycocin.

Latin diagnosis of *Bullera pseudohuiaensis* Bai, Takashima & Nakase sp. nov.

In YM (Difco) liquido post dies 5 ad 17 °C, cellulae vegetativae ovoideae, ellipsoideae et longi-ellipsoideae, 2–4 × 5–8 µm. Anulus et sedimentum formantur. Post unum mensem ad 17 °C, anulus, insulae et sedimentum formantur. In agaro YM post unum mensem ad 17 °C, cultura gilvo-flavae, rugosa, nitida aut non-nitida, butyracea, margine glabra. Mycelium et pseudomycelium formantur. Ballistoconidia napiformia, turbinatae vel subglobose, 3.5–4.5 × 4.0–5.0 µm. Fermentatio nulla. Glucosum, galactosum, saccharosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinose, melezitose, amyllum solubile, D-xylosum, L-arabinosum, D-arabinosum (lente), D-ribosum (lente et exigue), L-rhamnosum (lente), galactitolum (exigui), glucitolum (lente), methyl α-D-glucosidum (lente et exigui), salicinum (lente), glucono-δ-lactonum, acidum 2-keto-

Fig. 4. *B. pseudohuiaensis*. (left) Vegetative cells grown in YM broth for 3 d at 17 °C. (right) Ballistoconidia produced on corn meal agar after 7 d at 20 °C. Bars, 10 µm.

gluconicum, acidum 5-ketogluconicum, acidum succinicum (lente et exigue) et inositolum (lente et exigue) assimilantur at non L-sorbosum, lactosum, inulin, ethanolum, glycerolum, erythritolum, ribitolum, D-mannitolum, acidum DL-lacticum nec acidum citricum. Ammonium sulfatum, ethylaminum (lente) et L-lysinum assimilantur at non kalium nitricum, natrum nitrosum nec cadaverinum. Ad crescentiam thiaminum necessarium sunt. Maxima temperatura crescentiae: 23–24 °C. Materia amyloidea iodophila formantur. Urea finditur. Diazonium caeruleum B positivum. Proportio molaris G+C in acido deoxyribonucleico: 43.9 mol% (per HPLC). Ubiquinonum majus: Q-10. Xylosum in cellulis presens. Typus: isolatus ex folio Sasa sp. JCM 5984^T (originaliter ut NB-192^T) conservatur in collectionibus culturarum quas 'Japan Collection of Microorganisms', Wako, Saitama sustentat.

Description of *Bullera pseudohuiaensis* Bai, Takashima & Nakase sp. nov.

Bullera pseudohuiaensis (pseu.do.hui.a.en'sis. Gr. *pseudes* false; *pseudohuiaensis* referring to the close relationship of the species to *Bullera huiaensis*).

In YM broth, after 5 d at 17 °C, cells are oval, ellipsoidal or long ellipsoidal, 2–4 × 5–8 µm. They occur singly or in clusters (Fig. 4, left). True mycelia are also present. An incomplete ring and a sediment are formed. After 1 month at 17 °C, an incomplete ring, a fragile sediment and islets are present. On YM agar, after 1 month at 17 °C, the streak culture is light yellow, butyrous, wrinkled, semi-shining to dull and has an entire margin. In Dalmau plate culture on corn meal agar, pseudomycelia and true mycelia are produced. Ballistoconidia are produced on corn meal agar. Usually they are napiform, turbinate or subglobose, 3.5–4.5 × 4.0–5.0 µm (Fig. 4, right). Fermentation is absent. Glucose, galactose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose,

soluble starch, D-xylose, L-arabinose, D-arabinose (delayed), D-ribose (delayed and weak), L-rhamnose (delayed), galactitol (weak), glucitol (delayed), methyl α -D-glucoside (delayed and weak), salicin (delayed), glucono- δ -lactone, 2-ketogluconic acid, 5-ketogluconic acid, succinic acid (delayed and weak) and inositol (delayed and weak) are assimilated. L-sorbose, lactose, inulin, ethanol, glycerol, erythritol, ribitol, D-mannitol, DL-lactic acid and citric acid are not assimilated. Ammonium sulfate, ethylamine hydrochloride (delayed) and L-lysine are assimilated. Potassium nitrate, sodium nitrite and cadaverine dihydrochloride are not assimilated. Thiamin is required for growth. Maximum growth temperature is 23–24 °C. Starch-like substances are produced. Urease activity is positive. Diazonium blue B reaction is positive. The G + C content of nuclear DNA is 43.9 mol% as determined by HPLC. The major ubiquinone is Q-10. Xylose is present in the whole cell hydrolysate. The type strain, JCM 5984^T (originally NB-192^T), was isolated from a dead leaf of *Sasa* sp. collected from Suga-daira, Nagano Prefecture, Japan in May, 1985. This strain has also been deposited in the China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, as AS 2.2203^T.

As shown in Table 1, *B. pseudohuiaensis*, represented by strain JCM 5984^T, is physiologically distinct from the other taxa represented by the remaining strains originally assigned to the species *B. variabilis*. In practice, *Bullera pseudohuiaensis* can be distinguished from its closely related species *B. huiaensis* by its inability to assimilate mannitol and cadaverine.

Latin diagnosis of *Bullera komagatae* Bai, Takashima & Nakase sp. nov.

In YM (Difco) liquido post dies 5 ad 17 °C, cellulae vegetativae longi-ovoideae et longi-ellipsoideae, 2–4 × 4–8 µm. Anulus et sedimentum formantur. Post unum mensem ad 17 °C, anulus, insulae et sedimentum formantur. In agar YM post unum mensem ad 17 °C, cultura subaurantiaca, glabra, nitida, butyracea, margine glabra. Mycelium et pseudomycelium formantur. Ballistoconidia napiformia, turbinatae vel polygoniae, 3–4 × 4–6 µm. Fermentatio nulla. Glucosum, galactosum, saccharosum, maltosum, cellobiosum (lente), trehalosum, melibiosum, raffinose, melezitose, amyllum solubile, D-xylosum, L-arabinosum, D-arabinosum (lente), D-ribosum (lente), L-rhamnosum (lente), galactitolum, D-mannitolum (lente), glucitolum, methyl α -D-glucosidum (exigue), salicinum, glucono- δ -lactonum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum succinicum (lente), acidum citricum (lente et exigue) et inositolum (lente) assimilantur at non L-sorbosum, lactosum, inulin, ethanolum, glycerolum, erythritolum, ribitolum nec acidum DL-lacticum. Ammonium sulfatum et L-lysinum assimilantur at non kalium nitricum, natrum nitrosum, ethylaminum nec cadaverinum. Ad crescentiam thiaminum necessarium

Fig. 5. *B. komagatae*. (left) Vegetative cells grown in YM broth for 3 d at 17 °C. (right) Ballistoconidia produced on corn meal agar after 7 d at 20 °C. Bars, 10 µm.

sunt. Maxima temperatura crescentiae: 24–25 °C. Materia amyloidea iodophila non formantur. Urea finditur. Diazonium caeruleum B positivum. Proportio molaris G + C in acido deoxyribonucleico: 44.5 mol% (per HPLC). Ubiquinonum majus: Q-10. Xylosum in cellulis presens. Typus: isolatus ex folio *Sasa* sp., JCM 5983^T (originaliter ut NB-190^T) conservatur in collectionibus culturarum quas 'Japan Collection of Microorganisms', Wako, Saitama sustentat.

Description of *Bullera komagatae* Bai, Takashima & Nakase sp. nov.

Bullera komagatae (ko.ma.ga.tae. M.L. *komagatae* in honour of Professor K. Komagata, Japan).

In YM broth, after 5 d at 17 °C, cells are long oval to long ellipsoidal, 2–4 × 4–8 µm. They occur singly or in clusters (Fig. 5, left). True mycelia are also present. A ring and a sediment are formed. After 1 month at 17 °C, a ring, a fragile sediment and islets are present. On YM agar, after 1 month at 17 °C, the streak culture is orange-yellow, butyrous, smooth, semi-shining and has an entire margin. In Dalmau plate culture on corn meal agar, pseudomycelia and true mycelia are produced. Ballistoconidia are produced on corn meal agar. They are napiform, turbinate, trigonal or tetragonal, 3–4 × 4–6 µm (Fig. 5, right). Fermentation is absent. Glucose, galactose, sucrose, maltose, cellobiose (delayed), trehalose, melibiose, raffinose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose (delayed), D-ribose (delayed), L-rhamnose (delayed), galactitol, D-mannitol (delayed), glucitol, methyl α -D-glucoside (weak), salicin, glucono- δ -lactone, 2-ketogluconic acid, 5-ketogluconic acid, succinic acid (delayed), citric acid (delayed and weak) and inositol (delayed) are assimilated. L-Sorbose, lactose, inulin, ethanol, glycerol, erythritol, ribitol and DL-

lactic acid are not assimilated. Ammonium sulfate and L-lysine are assimilated. Potassium nitrate, sodium nitrite, ethylamine hydrochloride and cadaverine dihydrochloride are not assimilated. Thiamin is required for growth. Maximum growth temperature is 24–25 °C. Starch-like substances are not produced. Urease activity is positive. Diazonium blue B reaction is positive. The G + C content of nuclear DNA is 44.5 mol % as determined by HPLC. The major ubiquinone is Q-10. Xylose is present in the whole cell hydrolysate. The type strain, JCM 5983^T (originally NB-190^T), was isolated from a dead leaf of *Sasa* sp. collected from Suga-daira, Nagano Prefecture, Japan, in May 1985. This strain has also been deposited in the China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China as AS 2.2202^T.

Latin diagnosis of *Bullera pseudoschimicola* Bai, Takashima & Nakase sp. nov.

In YM (Difco) liquido post dies 5 ad 17 °C, cellulae vegetativae longi-ovoideae et longi-ellipsoideae, 2–4 × 5–13 µm. Anulus et sedimentum formantur. Post unum mensem ad 17 °C, anulus, insulae et sedimentum formantur. In agarō YM post unum mensem ad 17 °C, cultura glivo-flavae aut rubro-flavae, glabra aut rugosa, nitida aut non-nitida, butyracea, margine glabra. Mycelium et pseudomycelium formantur. Ballistoconidia napiformia, turbinatae vel polygoniae, 4.0–5.0 × 6.0–7.5 µm. Fermentatio nulla. Glucosum, galactosum, saccharosum (fortasse lente), maltosum (fortasse lente), cellobiosum (fortasse lente), trehalosum (fortasse lente), melibiosum, raffinose, melezitose, amyllum solubile, D-xylosum (fortasse lente), L-arabinosum (fortasse lente), D-arabinosum (fortasse lente), D-ribosum (lente), L-rhamnosum (fortasse lente), galactitolum (variabile), D-mannitolum (fortasse lente), glucitolum (variabile), methyl α-D-glucosidum (lente vel exigue), salicinum (fortasse lente vel exigue), glucono-δ-lactonum (fortasse lente), acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum succinicum (fortasse lente vel exigue), acidum citricum (lente et exigue) et inositolum (fortasse lente vel exigue) assimilantur at non L-sorbose, lactosum, inulin, ethanolum, glycerolum, erythritolum (vel exigue), ribitolum (vel exigue) nec acidum DL-lacticum (vel exigue). Ammonium sulfatum et L-lysinum assimilantur at non kalium nitricum, natrum nitrosus (vel lente et exigue), ethylaminum nec cadaverinum. Ad crescentiam thiaminum necessarium sunt. Maxima temperatura crescentiae: 23–27 °C. Materia amyloidea iodophila non formantur. Urea finditur. Diazonium caeruleum B positivum. Proportio molaris G + C in acido deoxyribonucleico: 43.9–44.4 mol % (per HPLC). Ubiquinonum majus: Q-10. Xylosum in cellulis presens. Typus: JCM 3915^T, conservatur in collectionibus culturalium quas 'Japan Collection of Microorganisms', Wako, Saitama sustentat.

Fig. 6. *B. pseudoschimicola* JCM 3915^T. (left) Vegetative cells grown in YM broth for 3 d at 17 °C. (right) Ballistoconidia produced on corn meal agar after 7 d at 20 °C. Bars, 10 µm.

Description of *Bullera pseudoschimicola* Bai, Takashima & Nakase sp. nov.

Bullera pseudoschimicola (pseu.do.schi.mi.co'la. Gr. *pseudes* false; *pseudoschimicola* referring to the close relationship of the species to *Bullera schimicola*).

In YM broth, after 5 d at 17 °C, cells are long oval, long ellipsoidal to club-shaped, 2–4 × 5–13 µm. They occur singly or in clusters (Fig. 6, left). True mycelia are also present. Usually a ring and a sediment are formed. After 1 month at 17 °C, a ring, a fragile sediment and islets are present. On YM agar, after 1 month at 17 °C, the streak culture is light yellow, orange-yellow or reddish-yellow, butyrous, smooth or wrinkled, shining or semi-shining and has an entire margin. In Dalmau plate culture on corn meal agar, pseudomycelia and true mycelia are produced. Ballistoconidia are produced on corn meal agar. Usually they are napiform or turbinate. Polygonal, including trigonal, tetragonal or pentagonal, ballistoconidia are also produced, 4.0–5.0 × 6.0–7.5 µm (Fig. 6, right). Fermentation is absent. Glucose, galactose, sucrose (or delayed), maltose (or delayed), cellobiose (or delayed), trehalose (or delayed), melibiose, raffinose, melezitose, soluble starch, D-xylose (or delayed), L-arabinose (or delayed), D-arabiose (or delayed), D-ribose (delayed), L-rhamnose (or delayed), galactitol (variable), D-mannitol (or delayed), glucitol (variable), methyl α-D-glucoside (delayed or weak), salicin (or delayed or weak), glucono-δ-lactone (or delayed), 2-ketogluconic acid, 5-ketogluconic acid, succinic acid (or delayed or weak), citric acid (delayed and weak) and inositol (or delayed or weak) are assimilated. L-Sorbose, lactose, inulin, ethanol, glycerol, erythritol (or weak), ribitol (or weak) and DL-lactic acid (or weak) are not assimilated. Ammonium sulfate and L-lysine are assimilated. Potassium nitrate, sodium ni-

trite (or delayed and weak), ethylamine hydrochloride and cadaverine dihydrochloride are not assimilated. Thiamin is required for growth. Maximum growth temperature is 23–27 °C. Starch-like substances are not produced. Urease activity is positive. Diazonium blue B reaction is positive. The G+C content of nuclear DNA is 43.9–44.4 mol% as determined by HPLC. The major ubiquinone is Q-10. Xylose is present in the whole cell hydrolysate. The type, JCM 3915^T, was isolated from the dead base of bamboo culm collected from Vancouver, Canada, in January, 1985. This strain has also been deposited in the China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China as AS 2.2201^T.

Phenotypically, *B. komagatae* and *B. pseudoschimicola*, originally *B. variabilis* strains in group III, are distinguishable from the species represented by the strains in groups I and II (Table 1). However, these two novel species are indistinguishable from each other by the morphological, physiological and biochemical characters examined in the present study. They differ slightly from their closely related species *B. schimicola* in L-sorbose assimilation reactions: the two novel species give negative results, whereas *B. schimicola* is weakly positive. In morphology, a considerable proportion of the ballistoconidia produced by strains of *B. komagatae* and *B. pseudoschimicola* were polygonal or irregularly shaped, whereas the ballistoconidia produced by *B. schimicola* were globose to napiform and polygonal or irregularly shaped ones were rarely observed.

Redefinition of *Bullera variabilis* Nakase & Suzuki

The original description of *B. variabilis* was based on the 17 strains studied by Nakase & Suzuki (1987). After reclassification of these strains in the present study, the demarcation of *B. variabilis* needs to be revised. According to the phenotypic characters of the five strains retained in *B. variabilis*, some physiological characters in the original description of this species are revised as follows: lactose and erythritol are not assimilated; galactitol and D-glucitol are assimilated; maximum growth temperature is 27–31 °C; and starch-like substances are produced. *B. variabilis* was also originally described as forming variously shaped ballistoconidia. However, the strains forming polygonal ballistoconidia were limited to group III of the present study and were assigned to the species *B. komagatae* and *B. pseudoschimicola*. The strains remaining in the species *B. variabilis* usually form napiform, turbinate to subglobose ballistoconidia as shown in the original figure of the type strain of this species (Nakase & Suzuki, 1987). The G+C contents of the type strain (JCM 5275^T) and two other strains (JCM 5276 and JCM 5277) of *B. variabilis* were reported to be 55.9–56.9 mol% (Nakase & Suzuki, 1987), which differ remarkably from those of the strains in groups II and III (42.7–44.7 mol%; Tables 2, 3 and 4) which have been reassigned to other species.

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