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Short Communication

Identification of strains assigned to the genus *Asaia* Yamada et al. 2000 based on 16S rDNA restriction analysis

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The genus *Asaia* Yamada et al. 2000 was introduced with a single species, *Asaia bogorensis* Yamada et al. 2000 in the family *Acetobacteriaceae* Gillis and De Ley 1980 (Yamada et al., 2000). Since the second and the third species were additionally described as *Asaia siamensis* Katsura et al. 2001 and *Asaia krungthepensis* Yukphan et al. 2004, three species have been reported in total (Katsura et al., 2001; Yamada et al., 2000; Yukphan et al., 2004c). Strains of the three *Asaia* species represent common phenotypic features such as no or very weak oxidation of ethanol to acetic acid and no or very weak growth in the presence of 0.35% (w/v) acetic acid. The discrimination of the three *Asaia* species from one another is mainly based on phenotypic characterization, e.g., acid production from and growth on different carbon compounds, which sometimes gives unreliable conclusion in their identification and classification and is different in this respect from DNA-DNA hybridization. However, the DNA-DNA hybridization is laborious and time-consuming, and it is therefore not interesting to construct databases with this technique. Sometimes, however, it is still the method that needs to be used to obtain a good identification, especially in the case of aberrant results.

In previous studies, we reported that 16S-23S rDNA ITS restriction analysis is applicable for species-level identification or classification of strains assigned to the

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Abbreviations: ITS, internal transcribed spacer; BCC, BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani, Thailand; NBRC, NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan; NRIC, NODAI Research Institute Culture Collection Center, Tokyo University of Agriculture, Tokyo, Japan; IFO, Institute for Fermentation, Osaka (IFO), Osaka, Japan; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland, UK; ATCC, American Type Culture Collection, Rockville, Maryland, USA; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.

genus *Gluconobacter* (Malimas et al., 2006; Yukphan et al., 2004a, b). On the basis of differences in restriction patterns, the new species and the revived name, *Gluconobacter albidus* (ex Kondo and Ameyama 1958) Yukphan et al. 2005 was described (Yukphan et. al., 2004d, 2005b). In a following study, thirteen strains randomly selected from a large number of Thai isolates assigned to the genus *Asaia* were examined for their 16S-23S rDNA ITS restriction analysis (Yukphan et al., 2006). However, the thirteen strains tested did not give three restriction groups, which corresponded to the above-mentioned three *Asaia* species, but six restriction groups were given. In addition, a phylogenetic tree based on 16S-23S rDNA ITS sequences represented very complicated clustering, which was composed of eight clusters.

This paper is concerned with the identification of the thirteen strains of the genus *Asaia* based on 16S rDNA restriction analysis, together with 16S rDNA sequence analysis.

Thirteen strains, which were isolated from flowers collected in Thailand and phenotypically assigned to the genus *Asaia*, were examined in this study (Table 1). *Asaia bogorensis* BCC 12264^T, *A. siamensis* BCC 12268^T, and *A. krungthepensis* BCC 12978^T were used as reference strains.

When the 16S rDNA sequences obtained from the DDBJ databases of the type strains of the three *Asaia* species, *A. bogorensis*, *A. siamensis*, and *A. krungthepensis*, were analyzed with the program NEBcutter (version 2.0), eight restriction endonucleases that discriminate one species from the other two were found (Table 2). A restriction endonuclease, *Sty*I (=*Eco*130I), discriminated the type strain of *A. bogorensis* from the type strains of *A. siamensis* and *A. krungthepensis*. Three restriction endonucleases, *Bsa*JI (=*Bse*DI), *Rsa*I, and *Tat*I, discriminated the type strain of *A. siamensis* from the type strains of *A. bogorensis* and *A. krungthepensis*. Four restriction endonucleases, *Sna*BI (=*Eco*105I), *Acc*I, *Afl*III, and *Hpy*8I, discriminated the

Table 1. Identification of the thirteen strains phenotypically assigned to the genus *Asaia* by restriction analysis of 16S rDNA.

Species and strain	Isolate and other designation[a]	Restriction group	Restriction pattern with			Cluster in 16S rDNA phylogenetic tree	Grouping by 16S-23S rDNA ITS restriction analysis[b]
			*Sty*I	*Bsa*JI	*Sna*BI		
A. bogorensis							
BCC 12264^T	=NBRC 16594^T, =NRIC 0311^T	A	*a*	*c*	*e*	*A. bogorensis*	I
A. siamensis							
BCC 12268^T	=NBRC 16457^T, =NRIC 0323^T	B	*b*	*d*	*e*	*A. siamensis*	II
A. krungthepensis							
BCC 12978^T	=NBRC 100057^T, =NRIC 0535^T	C	*b*	*c*	*f*	*A. krungthepensis*	III
A. bogorensis							
BCC 15641	=AA01	A	*a*	*c*	*e*	*A. bogorensis*	VI
BCC 15661	=AA35	A	*a*	*c*	*e*	*A. bogorensis*	I
BCC 15664	=AA38	A	*a*	*c*	*e*	*A. bogorensis*	IV
BCC 15669	=AA44	A	*a*	*c*	*e*	*A. bogorensis*	I
BCC 15696	=AB30	D	*b*	*c*	*e*	*A. bogorensis*	IV
BCC 15703	=AB40	A	*a*	*c*	*e*	*A. bogorensis*	VI
BCC 15711	=AB58	A	*a*	*c*	*e*	*A. bogorensis*	I
BCC 15725	=AB82	A	*a*	*c*	*e*	*A. bogorensis*	IV
BCC 15806	=AC73	A	*a*	*c*	*e*	*A. bogorensis*	I
A. siamensis							
BCC 15670	=AA49	B	*b*	*d*	*e*	*A. siamensis*	V
BCC 15681	=AA98	B	*b*	*d*	*e*	*A. siamensis*	V
A. krungthepensis							
BCC 15704	=AB41	C	*b*	*c*	*f*	*A. krungthepensis*	III
BCC 15713	=AB73	C	*b*	*c*	*f*	*A. krungthepensis*	III

[a] For the isolation sources of the representative strains, see a previous paper (Yukphan et al., 2006).

[b] Cited from a previous paper (Yukphan et al., 2006).

Table 2. Restriction endonucleases discriminating the three *Asaia* species, *A. bogorensis*, *A. siamensis*, and *A. krungthepensis*.

Restriction endonuclease	Number of restriction sites in 16S rDNA of			Molecular size of restriction fragments (bp) in		
	A. bogorensis BCC 12264^T	*A. siamensis* BCC 12268^T	*A. krungthepensis* BCC 12978^T	*A. bogorensis* BCC 12264^T	*A. siamensis* BCC 12268^T	*A. krungthepensis* BCC 12978^T
*Sty*I	4	3	3	790, 327, 123, 91, 80	790, 327, 214, 80	790, 327, 214, 80
*Bsa*JI	14	13	14	236, 205, 172, 148, 123,102, 91, 87, 87, 55, 29, 25, 24, 16, 11	338, 205, 172, 148, 123, 91, 87, 87, 55, 29, 25, 24, 16, 11	236, 205, 172, 148, 123, 102, 91, 87, 87, 55, 29, 25, 24, 16, 11
*Rsa*I	4	5	4	421, 405, 404, 98, 83	421, 405, 404, 98, 67, 16	421, 405, 404, 98, 83
*Tat*I	2	3	2	1228, 100, 83	1248, 118, 67, 16	1228, 100, 83
*Sna*BI	0	0	1	—	—	850, 561
*Acc*I	1	1	0	846, 565	846, 565	—
*Afl*III	2	2	1	588, 560, 263	588, 560, 263	1148, 263
*Hpy*8I	5	5	3	582, 508, 157, 97, 58, 9	582, 508, 157, 97, 58, 9	723, 582, 97, 9

—, no restriction fragment.

type strain of *A. krungthepensis* from the type strains of *A. bogorensis* and *A. siamensis*. For example, restriction endonuclease *Sty*I, which discriminated the type strain of *A. bogorensis*, cut the 16S rDNAs of the type strains of *A. bogorensis*, *A. siamensis*, and *A. krungthepensis* respectively at 4, 3, and 3 sites, and produced restriction fragments respectively composed of 790, 327, 123, 91, and 80 bp, 790, 327, 214, and 80 bp, and 790, 327, 214, and 80 bp. On the other hand, restriction endonuclease *Bsa*JI, which discriminated the type strain of *A. siamensis*, cut the 16S rDNAs of the type strains of the three *Asaia* species respectively at 14, 13, and 14 sites, and produced restriction fragments respectively composed of 236, 205, 172, 148, 123, 102, 91, 87, 87, 55, 29, 25, 24, 16, and 11 bp, 338, 205, 172, 148, 123, 91, 87, 87, 55, 29, 25, 24, 16, and 11 bp, and 236, 205, 172, 148, 123, 102, 91, 87, 87, 55, 29, 25, 24, 16, and 11 bp, and restriction endonuclease *Sna*BI, which discriminated the type strain of *A. krungthepensis*, cut respectively at 0, 0, and 1 sites, and produced restriction fragments composed of 850 and 561 bp only for the type strain of *A. krungthepensis*.

For preparation of 16S rDNA PCR products, two DNA primers, 20F (5′-GAG TTT GAT CCT GGC TCA G-3′; positions 9–27 on 16S rDNA by the *Escherichia coli* numbering system, Brosius et al., 1981) and 1500R (5′-GTT ACC TTG TTA CGA CTT-3′; positions 1509–1492 on 16S rDNA) were used as described previously (Yukphan et al., 2004c, d, 2005a). The purified 16S rDNA PCR products were subjected to three different kinds of restriction analysis, i.e., one with *Sty*I (Fermentas, Hanover, Maryland, USA, =*Eco*130I), one with *Bsa*JI (Fermentas, =*Bse*DI), and one with *Sna*BI (Fermentas, =*Eco*105I). The resulting reaction products were analyzed by 2.5% (w/v) agarose gel electrophoresis developed at 100 V for 40 min in 1× Tris-acetate running buffer.

As shown in Fig. 1, the sizes and numbers of the resulting restriction products coincided with those calculated theoretically (Table 2). The type strains of the three *Asaia* species, *A. bogorensis*, *A. siamensis*, and *A. krungthepensis* showed their own restriction patterns, when digested with restriction endonucleases *Sty*I, *Bsa*JI, and *Sna*BI, although certain restriction patterns were shared among the type strains tested (Table 1). The type strain of *A. bogorensis* respectively gave the type *a*, *c*, and *e* of restriction patterns, when digested with restriction endonucleases *Sty*I, *Bsa*JI, and *Sna*BI. The type strain of *A. siamensis* gave the type *b*, *d*, and *e* of restriction patterns, and the type strain of *A. krungthepensis* gave the type *b*, *c*, and *f* of restriction patterns. These data indicate that the type strains of the three *Asaia* species were completely discriminated from one another in combination of the resulting restriction patterns.

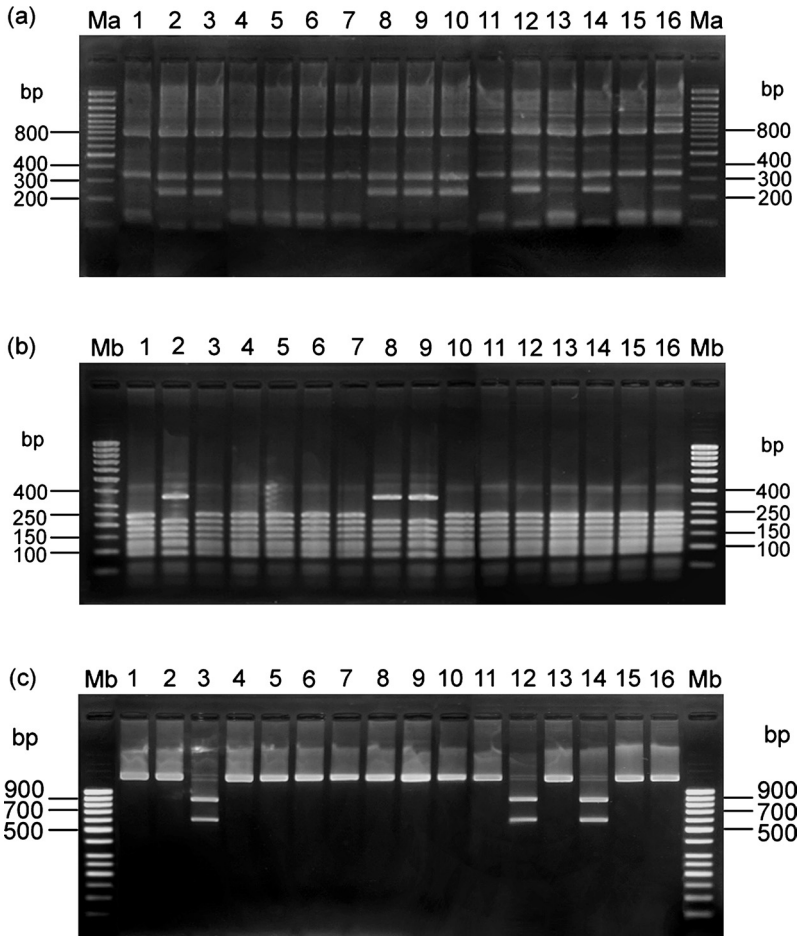

Fig. 1. Digestion of 16S rDNA PCR products of the type strains of the three *Asaia* species and the thirteen strains assigned to the genus *Asaia* with restriction endonucleases *Sty*I, *Bsa*JI, and *Sna*BI.

For estimation of digestion fragments, 100-bp DNA markers (Ma) and 50-bp DNA markers (Mb) were used in agarose gel electrophoresis. 1, *A. bogorensis* BCC 12264^T; 2, *A. siamensis* BCC 12268^T; 3, *A. krungthepensis* BCC 12978^T; 4, BCC 15641 (=AA01); 5, BCC 15661 (=AA35); 6, BCC 15664 (=AA38); 7, BCC 15669 (=AA44); 8, BCC 15670 (=AA49); 9, BCC 15681 (=AA98); 10, BCC 15696 (=AB30); 11, BCC 15703 (=AB40); 12, BCC 15704 (=AB41); 13, BCC 15711 (=AB58); 14, BCC 15713 (=AB73); 15, BCC 15725 (=AB82); 16, BCC 15806 (=AC73).

All the thirteen strains tested represented identical restriction patterns with the type strains of the three *Asaia* species except for one strain, BCC 15696 (= AB30), which presented a combination of restriction patterns (type *b*, *c* and *e*) that was not found in any of the type strains of the three *Asaia* species (Table 1).

The thirteen strains were sequenced for 16S rDNA and analyzed as described previously (Yukphan et al., 2004c, d, 2005a). The above-mentioned purified PCR products were directly sequenced on an ABI PRISM model 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) with the following six primers: 20F, 1500R, 520F (5′-CAG CAG CCG CGG TAA TAC-3′ positions 519–536), 520R (5′-GTA TTA CCG CGG CTG CTG-3′, positions 536–519), 920F (5′-AAA CTC AAA TGA ATT GAC GG-3′, positions 907–926), and 920R (5′-CCG TCA ATT CAT TTG AGT TT-3′ positions 926–907). Multiple sequence alignments were made with the program CLUSTAL X (version 1.8) (Thompson et al., 1997). Gaps and ambiguous bases were excluded. Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). Robustness for individual branches was estimated for bootstrapping with 1,000

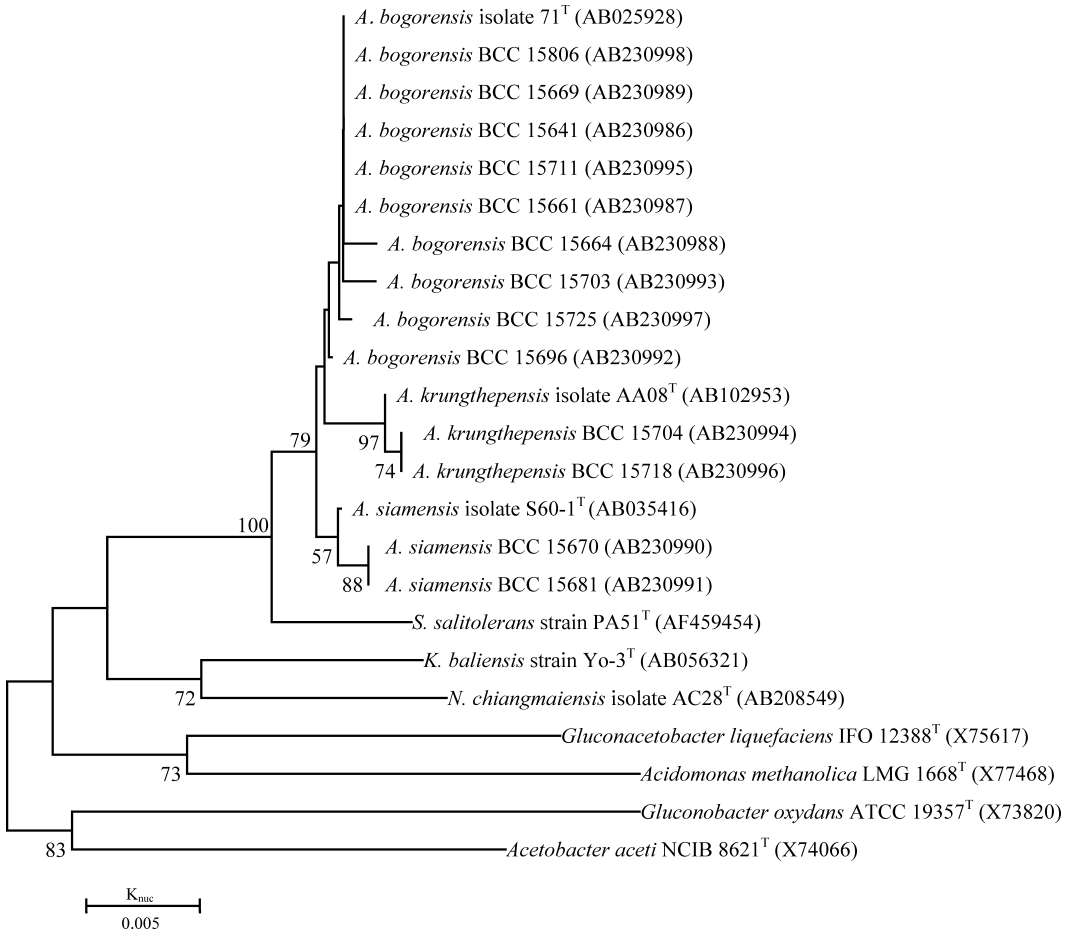

Fig. 2. A phylogenetic tree based on 16S rDNA sequences for the thirteen strains assigned to the genus *Asaia*.

The phylogenetic tree was constructed by the neighbor-joining method. *Swaminathania salitolerans* strain PA51^T, *Kozakia baliensis* strain Yo-3^T, *Neoasaia chiangmaiensis* isolate AC28^T, *Gluconacetobacter liquefaciens* IFO 12388^T, *Acidomonas methanolica* LMG 1668^T, *Gluconobacter oxydans* ATCC 19357^T, and *Acetobacter aceti* NCIB 8621^T were used as outgroups. Numerals at the nodes indicate bootstrap percentages. Abbreviations: *A., Asaia*; *K., Kozakia*; *S., Swaminathania*; *N., Neoasaia*.

replications (Felsenstein, 1985).

Pair-wise sequence similarities of 16S rDNA were calculated for 1,411 bases. The calculated sequence similarities were respectively 99.7, 99.5, and 99.5% between the type strains of *A. bogorensis* and *A. siamensis*, between the type strains of *A. bogorensis* and *A. krungthepensis*, and between the type strains of *A. siamensis* and *A. krungthepensis*. These numbers were very high, when compared with those of 16S-23S rDNA ITS sequences. The calculated numbers of the latter were respectively 94.5, 91.2, and 89.6% (Yukphan et al., 2006). This indicates that the 16S rDNA sequences were more highly conservative than the 16S-23S rDNA ITS sequences in the type strains of the three *Asaia* species.

As shown in Fig. 2, the type strains of the three *Asaia* species, *Asaia bogorensis*, *Asaia siamensis*, and *Asaia krungthepensis* constituted separate and independent clusters from one another in a phylogenetic tree based on 16S rDNA sequences for 1,387 bases. Strains BCC 15641, BCC 15661, BCC 15664, BCC 15669, BCC 15696, BCC 15703, BCC 15711, BCC 15725, and BCC 15806 were located in a cluster, along with the type strain of *A. bogorensis*. Strains BCC 15670 and BCC 15681 were included in a closely related cluster to the type strain of *A. siamensis*. Strains BCC 15704 and BCC 15718 were included in a cluster, which was closely related to the type strain of *A. krungthepensis*.

In combination of the resulting restriction patterns

formed by using the three restriction endonucleases, *Sty*I, *Bsa*JI and *Sna*BI, the type strains of the three *Asaia* species and the thirteen strains tested were grouped into the following four restriction groups (Table 1).

Group A was composed of the type strain of *A. bogorensis*, which showed the type *a*, *c*, and *e* of restriction patterns. Eight strains, BCC 15641, BCC 15661, BCC 15664, BCC 15669, BCC 15703, BCC 15711, BCC 15725, and BCC 15806 were included in Group A. These eight strains constituted the same cluster as the type strain of *A. bogorensis* in a phylogenetic tree based on 16S rDNA sequences (Fig. 2). In a previous study, these eight strains were accommodated to Group I, Group IV, and Group VI in the 16S-23S rDNA ITS restriction analysis using two restriction endonucleases, *Taq*I and *Mva*I, and showed high DNA-DNA similarities (96–90%) to the type strain of *A. bogorensis* (Yukphan et al., 2006). The present study confirmed the eight strains of Group A to be members of *A. bogorensis*.

Group B was composed of the type strain of *A. siamensis*, which showed the type *b*, *d*, and *e* of restriction patterns. Two strains, BCC 15670 and BCC 15681 were included in Group B. These two strains constituted the same cluster, along with the type strain of *A. siamensis* (Fig. 2). In a previous study, the two strains were accommodated to Group V, and showed high DNA-DNA similarities (95–92%) to the type strain of *A. siamensis* (Yukphan et al., 2006). The present study confirmed the two strains of Group B to be members of *A. siamensis*.

Group C was composed of the type strain of *A. krungthepensis*, which showed the type *b*, *c*, and *f* of restriction patterns. Two strains, BCC 15704 and BCC 15718 were included in Group C. These two strains were distributed in the same cluster as *A. krungthepensis* (Fig. 2). In a previous study, the two strains were accommodated to Group III, and showed high DNA-DNA similarities of 97% and 97% to the type strain of *A. krungthepensis* (Yukphan et al., 2006). The present study confirmed the two strains of Group C to be members of *A. krungthepensis*.

Group D was composed of only one strain, BCC 15696 (=AB30), which showed the type *b* of restriction patterns, when digested with restriction endonuclease *Sty*I, which was not identical with that of *A. bogorensis* but with those of *A. siamensis* and *A. krungthepensis*. The phylogenetic analysis located the strain in the same cluster as the type strain of *A. bogorensis*, but the strain was somewhat far from the type strain of *A. bogorensis* (Fig. 2). In a previous study, the strain was accommodated to Group IV, along with strains BCC 15664 and BCC 15725, which were grouped into Group A in this study, and showed 91% DNA-DNA similarities to the type strain of *A. bogorensis* (Yukphan et al., 2006). This strain can be identified as a member of *A. bogorensis*.

It is of interest that strain BCC 15696 (=AB30) of Group D represented the same restriction pattern, i.e., the type *b*, when digested with restriction endonuclease *Sty*I, as the type strains of *A. siamensis* and *A. krungthepensis*. The restriction pattern obtained above suggests the presence of the same recognition sequence (5′-CCA CGG-3′) as the type strain of *A. siamensis* and *A. krungthepensis*, which was found at position 135–140 by the *Escherichia coli* numbering system (Brosius et al., 1981). The only base substitution by transition of T to C was found at position 138, as reported in the 16S-23S rDNA ITS restriction analysis of *Gluconobacter frateurii* NBRC 3251 and *Gluconobacter cerinus* NBRC 3274 in the genus *Gluconobacter* (Yukphan et al., 2004a, b).

As described above, one strain (BCC 15696, =AB30), which was grouped into Group D, was exceptionally found in the 16S rDNA restriction analysis by use of three restriction endonucleases. This indicates that base substitution may occur in the so-called conservative 16S rDNA sequences. The possibility that base substitution occurs was only one thirty-ninth or 2.6% among the thirty-nine digestions concerned. When this value was compared with that of the 16S-23S rDNA ITS restriction analysis reported previously, the possibility was greatly reduced (Yukphan et al., 2006).

The exceptional one strain (BCC 15696, =AB30) grouped into Group D and identified as *A. bogorensis* was located slightly far from the type strain of *A. bogorensis* in a phylogenetic tree based on 16S rDNA sequences (Fig. 2). Phylogenetically, the presence of such an exceptional strain was not meaningless but reasonable in the grouping mentioned above. The 16S rDNA restriction analysis using three restriction endonucleases, *Sty*I, *Bsa*JI, and *Sna*BI is utilized for species identification and classification as well.

In a previous paper, we reported the presence of six restriction groups by use of only two restriction endonucleases, *Taq*I and *Mva*I, in the 16S-23S rDNA ITS

restriction analysis (Yukphan et al., 2006). These numbers in grouping were so high to accommodate the test strains to the three species of the genus *Asaia*. However, the restriction analysis was useful, since the only species was recognized in each of the six restriction groups.

We described in that paper that the strain was to be examined for a new taxon, if a restriction pattern was additionally found in a certain strain (Yukphan et al., 2006). We are screening a large number of isolates phenotypically assigned to the genus *Asaia* by the 16S-23S rDNA ITS restriction analysis using two restriction endonucleases in pursuit of the strain to be classified into a new taxon. When the presence of a different kind of restriction patterns is observed in a certain isolate during the course of this screening, the additional 16S rDNA restriction analysis using three restriction endonucleases described above will give more information that the isolate is possible to constitute a new taxon or not. Then, the interesting strains will be continued to make DNA-DNA hybridization to get the final decision.

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