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Short Communication

Asaia spathodeae sp. nov., an acetic acid bacterium in the α-*Proteobacteria*

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The genus *Asaia* was first described by Yamada et al. (2000) with a single species, *Asaia bogorensis*, the type strain of which was isolated from the flower of an orchid tree (*Bauhinia purpurea*). Subsequently, *Asaia siamensis*, *Asaia krungthepensis*, and *Asaia lannensis* were described (Katsura et al., 2001; Malimas et al., 2008a, b; Yukphan et al., 2004). The type strains of the three species were isolated respectively from flowers of the crown (*Calotropis gigantea*), heliconia (*Heliconia* sp.), and spider lily (*Hymenocallis speciosa*). Strains classified in the genus *Asaia* were characterized physiologically by no or weak oxidation of ethanol to acetic acid and by no or weak growth in the presence of 0.35% acetic acid (v/v) (Yamada et al., 2000).

Tanasupawat et al. (2009) found that the two isolates that were isolated from flowers of the African tulip (*Spathodea campanulata*) constitute a new species in the genus *Asaia* during the course of the systematic study of acetic acid bacteria isolated from the natural environment of Thailand.

In this paper, we propose the name of *Asaia spathodeae* sp. nov. to accommodate the two isolates as a fifth species of the genus *Asaia*.

Two isolates, designated as isolate GB23-2^T (= BCC 36458^T = NBRC 105894^T = PCU 307^T) and isolate GB23-3 (= BCC 31374 = NBRC 105182 = PCU 308), were isolated by an enrichment culture approach using a glucose/ethanol/acetic acid medium, which was composed of 1.5% D-glucose (w/v), 0.5% ethanol (v/v), 0.3% acetic acid (v/v), 0.8% peptone (w/v) and 0.5% yeast extract (w/v) and which was adjusted at pH 3.5

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Abbreviations: BCC, BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Pathumthani, Thailand; NBRC, NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Japan; PCU, Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

The DDBJ accession numbers for the 16S rRNA gene sequences of isolates GB23-2^T and GB23-3 are respectively AB511277 and AB469044.

(Katsura et al., 2001; Yamada et al., 1999, 2000; Yukphan et al., 2004). The isolates were maintained on agar slants comprised of 2.0% D-glucose (w/v), 0.5% ethanol (v/v), 0.3% peptone (w/v), 0.3% yeast extract (w/v), 0.7% $CaCO_3$ (w/v), and 1.5% agar (w/v). *Asaia bogorensis* BCC 12264^T (= NBRC 16594^T), *Asaia siamensis* BCC 12268^T (= NBRC 16457^T), *Asaia krungthepensis* BCC 12978^T (= NBRC 100057^T), *Asaia lannensis* BCC 15733^T (= NBRC 102526^T), and *Acetobacter aceti* NBRC 14818^T were used for reference strains.

Chromosomal DNA was prepared after Saito and Miura (1963). DNA base composition was determined by the method of Tamaoka and Komagata (1984). DNA-DNA hybridization was performed by use of the photobiotin-labeling method with microplate wells, as described by Ezaki et al. (1989). A single-stranded and labeled DNA was hybridized with DNAs from test strains in 2× SSC and 50% formamide at 48.0°C for 15 h. The levels of DNA-DNA relatedness (%) were determined colorimetrically (Verlander, 1992). The color intensity was measured at A_{450} on a model VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

The DNA G+C contents of isolates GB23-2^T and GB23-3 were respectively 59.7 and 59.8 mol% with the range of 0.1 mol% (Table 1). The data obtained were closely related to those of the type strains of *Asaia bogorensis*, *Asaia siamensis*, *Asaia krungthepensis*, and *Asaia lannensis* (Katsura et al., 2001; Malimas et al., 2008a; Yamada et al., 2000; Yukphan et al., 2004).

When single-stranded and labeled DNAs from isolates GB23-2^T and GB23-3 were hybridized with DNAs from tested strains, the levels of DNA-DNA relatedness were 100, 100, 27, 38, 43, 36, and 5% and 100, 100, 31, 40, 48, 40, and 6% respectively to isolates GB23-2^T and GB23-3 and the type strains of *Asaia bogorensis*, *Asaia siamensis*, *Asaia krungthepensis*, *Asaia lannensis*, and *Acetobacter aceti* (Table 1). The type strains of *Asaia bogorensis, Asaia siamensis, Asaia krungthepensis*, and *Asaia lannensis* gave the DNA-DNA relatedness levels of 21–53% to the two isolates. The data obtained indicated that the two isolates genetically constitute a new species in the genus *Asaia*.

Gene fragments specific for the 16S rRNA gene-encoding regions of isolates GB23-2^T and GB23-3 were amplified by PCR, as described previously (Tanasupawat et al., 2004; Yamada et al., 2000; Yukphan et al., 2004). Two primers used for PCR amplification were 20F (5′-GAGTTTGATCCTGGCTCAG-3′; positions 9–27 by the *Escherichia coli* numbering system, accession number V00348; Brosius et al., 1981) and 1500R (5′-GTTACCTTGTTACGACTT-3′; positions 1509–1492). Amplified 16S rRNA genes were directly sequenced with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems, Foster, CA, USA). For the direct sequencing, the following six primers were used: 20F, 1500R, 520F (5′-CAGCAGC CGCGGTAATAC-3′; positions 519–536), 520R (5′-GT ATTACCGCGGCTGCTG-3′; positions 536–519), 920F (5′-AAACTCAAATGAATTGACGG-3′; positions 907–926), and 920R (5′-CCGTCAATTCATTTGAGTTT-3′; positions 926–907). Multiple sequence alignments were performed for ca. 1,378 bases with the program CLUSTAL X (version 1.83, Thompson et al., 1997). Gaps and ambiguous bases were eliminated from calculation. Distance matrices for the aligned sequences were calculated by the two-parameter method of

Table 1. DNA base composition and DNA-DNA relatedness of *Asaia spathodeae* isolates GB23-2^T and GB23-3.

Labeled DNA from	DNA G+C (mol%)	DNA-DNA relatedness (%) of						
		1	2	3	4	5	6	7
A. spathodeae isolate GB23-2^T	59.7	100	100	27	38	43	36	5
A. spathodeae isolate GB23-3	59.8	100	100	31	40	48	40	6
A. bogorensis BCC 12264^T	60.2[a]	21	28	100	56	59	46	8
A. krungthepensis BCC 12978^T	60.3[a]	35	43	27	100	39	23	5
A. siamensis BCC 12268^T	59.3[a]	41	53	22	38	100	34	6
A. lannensis BCC 15733^T	60.8[a]	31	41	30	36	23	100	5

[a] Cited from Katsura et al. (2001), Malimas et al. (2008a), Yamada et al. (2000), and Yukphan et al. (2004).

Abbreviations: 1, *Asaia spathodeae* isolate GB23-2^T; 2, *Asaia spathodeae* isolate GB23-3; 3, *Asaia bogorensis* BCC 12264^T; 4, *Asaia krungthepensis* BCC 12978^T; 5, *Asaia siamensis* BCC 12268^T; 6, *Asaia lannensis* BCC 15733^T; 7, *Acetobacter aceti* NBRC 14818^T.

Kimura (1980). The phylogenetic trees based on 16S rRNA gene sequences were constructed by the neighbor-joining method (Saitou and Nei, 1987), the maximum-parsimony method (Felsenstein, 1983), and the maximum-likelihood method (Felsenstein, 1981) with the program MEGA (version 4.0; Tamura et al., 2007). The confidence values of individual branches in the phylogenetic trees were determined by using the bootstrap analysis of Felsenstein (1985) based on 1,000 replications. Pair-wise 16S rRNA gene sequence similarities were calculated for 1,414 bases between phylogenetically related strains.

The phylogenetic trees based on 16S rRNA gene sequences, which were constructed by the above-mentioned three methods, showed that isolates GB23-2^T and GB23-3 were located within the lineage of the genus *Asaia* and formed an independent cluster from the type strains of the four known *Asaia* species (Fig. 1). However, the two isolates were related phylogenetically to the type strain of *Asaia siamensis* with the bootstrap values respectively of 79, 77, and 83%. The calculated pair-wise sequence similarities of isolate GB23-2^T were 99.7, 99.9, 99.4, and 99.3% respectively to the type strains of *Asaia bogorensis*, *Asaia siamensis*, *Asaia krungthepensis*, and *Asaia lannensis*. The similarity between the two isolates was 100%. The data obtained suggested that isolates GB23-2^T and GB23-3 constitute a new taxon within the genus *Asaia*.

To discriminate isolates GB23-2^T and GB23-3 from the type strains of the four known *Asaia* species, a 16S rRNA gene restriction analysis was theoretically made by computer analysis using the program NEBcutter (version 2.0, New England BioLabs, Beverley, MA, USA) (Yukphan et al., 2006). In the computerized calculations, the two isolates produced restriction fragments comprised of: i) 790, 327, 214, and 83 bp in *Sty*I digestion; ii) 338, 205, 172, 148, 123, 91, 87, 87, 55, 29, 28, 24, 16, and 11 bp in *Bsa*JI digestion; iii) no restriction fragment in *Sna*BI digestion; iv) 445, 421, 216, 210, 58, 53, 15, and 11 bp in *Hpa*II digestion; v) 454, 350, 190, 196, 169, 152, and 99 bp in *Hpy*AV digestion. On the other hand, the type strains of the four *Asaia* species produced almost identical restriction fragments comprised of 454, 295, 190, 166, 151, 99, and 55 bp in *Hpy*AV digestion.

The 16S rRNA gene PCR products of the two isolates and the type strains of the four *Asaia* species were prepared and digested respectively with the five restriction endonucleases mentioned above. As shown in Fig. 2, isolates GB23-2^T and GB23-3 were completely distinguished by showing the *Asp* type of restriction patterns in *Hpy*AV digestion in spite of giving restriction patterns common to the type strains of some of the *Asaia* species examined in *Sty*I, *Bso*JI, *Sna*BI, and *Hpa*II digestions. In contrast, the type strains of the four *Asaia* species represented the *Ab* type of restriction patterns in *Hpy*AV digestion.

The cellular fatty acid composition of isolates GB23-2^T and GB23-3 as well as the type strains of the four *Asaia* species was determined in cells grown on NBRC 804 agar at 30°C for 24 h. Methyl esters of cellular fatty acids were prepared and identified according to the instructions of the Microbial Identification system (MIDI, Hewlett Packard, Palo Alto, CA, USA). The isolates contained unsaturated fatty acid of $C_{18:1}\omega7c$ (67.5−67.8%) and straight-chain fatty acid of $C_{16:0}$ (9.1−10.3%) as major components. The cellular fatty acids profiles determined were similar to those of the type strains of the *Asaia* species (Table 2).

Isolates GB23-2^T and GB23-3 were examined for morphological, physiological, biochemical, and chemotaxonomic characteristics (Asai et al., 1964; Hucker and Conn, 1923; Katsura et al., 2001; Tamaoka et al., 1983; Yamada et al., 1969, 1976, 2000; Yukphan et al., 2004). The phenotypic characterization was carried out by incubating the isolates and the test strains at 30°C for 2 days on glucose/glycerol/peptone/yeast extract agar or broth, which was composed of 1.0% D-glucose, 1.0% glycerol, 0.5% yeast extract and 1.0% peptone with or without 1.5% agar (all by w/v), unless otherwise mentioned.

Antibiotic susceptibility of isolates GB23-2^T and GB23-3 and the type strains of the four *Asaia* species was determined according to the conventional Kirby-Bauer method (Bauer et al., 1966). The two isolates and the type strains of the four *Asaia* species were susceptible to nalidixic acid (30 μg), novobiocin (5 μg), neomycin (30 μg), kanamycin (30 μg), gentamicin (10 μg), rifampicin (30 μg), tetracycline (30 μg), and streptomycin (10 μg) but resistant to erythromycin (15 μg), amoxycylin (10 μg), vancomycin (30 μg), ampicillin (10 μg), bacitracin (10 units), and chloramphenicol (30 μg).

The phenotypic and chemotaxonomic characteristics determined are given in the species description of *Asaia spathodeae* sp. nov.

Isolates GB23-2^T and GB23-3 were clearly distinguished from the type strains of the four known *Asaia*

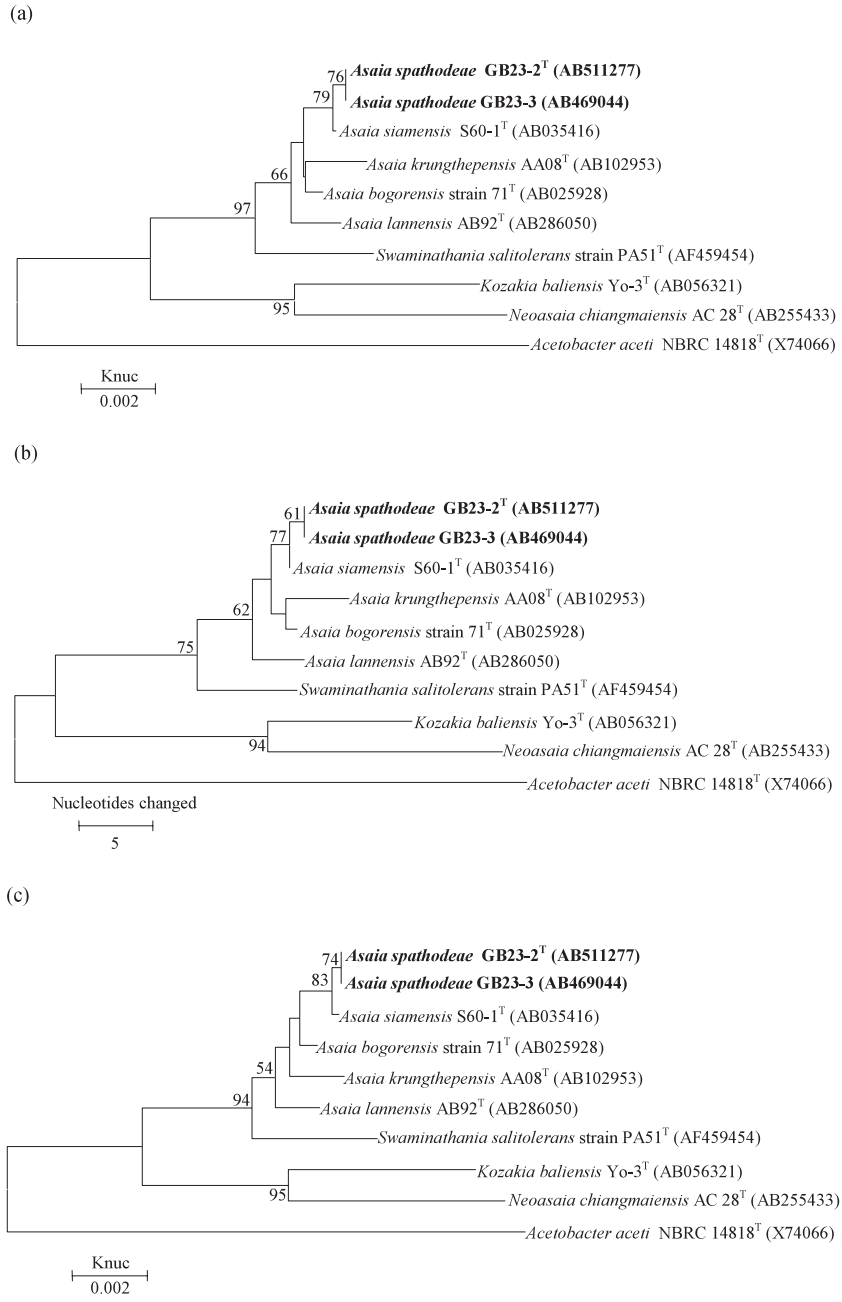

Fig. 1. Phylogenetic relationships of *Asaia spathodeae* isolates GB23-2^T and GB23-3 based on 16S rRNA gene sequences.

The phylogenetic trees were constructed by the neighbor-joining (a), maximum-parsimony (b), and maximum-likelihood (c) methods. Numbers at nodes indicate bootstrap percentages derived from 1,000 replications.

species, especially by the absence of acid production from L-rhamnose as well as by production of acid from ethanol, although the two isolates had common phenotypic characteristics such as peritrichous flagellation, weak oxidation of acetate and lactate, and growth on 30% D-glucose (w/v) in the genus *Asaia* (Table 3). In addition, the two isolates were molecular-phylogenetically differentiated by *Hpy*AV digestion.

From the results obtained above, a new species is therefore proposed in the genus *Asaia* with the name, *Asaia spathodeae* sp. nov.

Description of *Asaia spathodeae* sp. nov.

Asaia spathodeae [spa.tho'de.ae. L. gen. *spathode-*

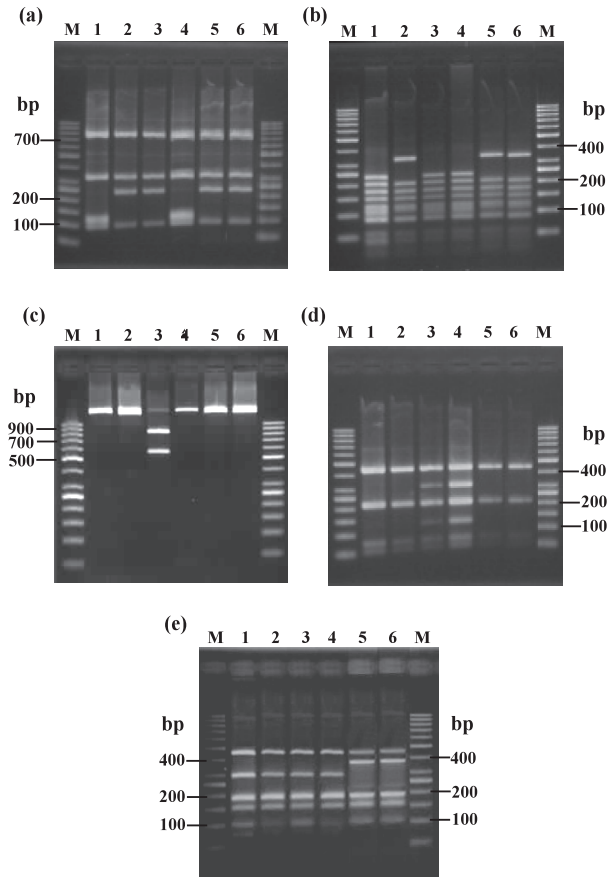

Fig. 2. Restriction of 16S rRNA gene PCR products of *Asaia spathodeae* isolates GB23-2^T and GB23-3 by digestion with five restriction endonucleases.

The restriction patterns were obtained by digestion with *Sty*I (a), *Bsa*JI (b), *Sna*BI (c), *Hpa*II (d), and *Hpy*AV (e). Lanes: 1, *Asaia bogorensis* BCC 12264^T; 2, *Asaia siamensis* BCC 12268^T; 3, *Asaia krungthepensis* BCC 12978^T; 4, *Asaia lannensis* BCC 15733^T; 5, *Asaia spathodeae* isolate GB23-2^T; 6, *Asaia spathodeae* isolate GB23-3; M, 50-bp DNA marker.

Table 2. Fatty acid profiles of *Asaia spathodeae* isolates GB23-2^T and GB23-3.

Fatty acid (%)	1	2	3	4	5	6
Straight-chain acid						
$C_{14:0}$	0.5	0.6	0.6	0.5	0.6	tr
$C_{16:0}$	10.3	9.1	11.1	9.6	8.5	6.3
$C_{17:0}$	tr		tr	tr	tr	
$C_{18:0}$	2.3	1.9	1.2	1.0	0.8	tr
Unsaturated acid						
$C_{13:1}$ AT 12−13			tr	0.6	tr	tr
$C_{17:1}$ ω6c	tr		tr	tr	tr	
$C_{18:1}$ ω7c	67.5	67.8	67.6	66.5	70.5	77.0
$C_{19:0}$ cyclo ω8c	tr		tr	0.7	0.5	1.7
Hydroxy acid						
$C_{14:0}$ 2-OH	3.2	2.7	3.6	4.2	3.0	3.3
$C_{16:0}$ 2-OH	6.6	5.4	5.8	7.0	5.9	4.4
$C_{16:0}$ 3-OH	2.5	1.9	2.4	3.1	2.2	2.3
$C_{18:0}$ 3-OH	1.0	0.9	0.8	1.2	0.8	0.5
$C_{18:1}$ 2-OH	3.1	7.8	3.1	2.2	4.0	0.6
Summed feature 2[a]	1.5	1.5	1.7	2.3	1.6	1.6

Less than 0.5% fatty acids were indicated as trace (tr). [a] Contained alde-$C_{12:0}$ and/or $C_{14:0}$ 3-OH and/or *iso* I-$C_{16:1}$.

Abbreviations: 1, *Asaia spathodeae* isolate GB23-2^T; 2, *Asaia spathodeae* isolate GB23-3; 3, *Asaia siamensis* BCC 12268^T; 4, *Asaia bogorensis* BCC 12264^T; 5, *Asaia krungthepensis* BCC 12978^T; 6, *Asaia lannensis* BCC 15733^T.

ae; N. L. fem. n. *Spathodea* the generic name of African tulip (*Spathodea campanulata*), the flower from which the type strain was isolated].

Cells are Gram-negative, aerobic, and rod-shaped, measuring 0.6−1.0×1.0−2.5 μm when cultured on glucose/ethanol/calcium carbonate agar. Motile with peritrichous flagella. Colonies are pink, shiny, smooth, and raised with an entire margin on glucose/ethanol/calcium carbonate agar. Grows at pH 3.0 and 3.5 at 30°C. Grows on 30% D-glucose (w/v). Growth is weak in the presence of 0.35% acetic acid (v/v). Methanol is not assimilated as a sole carbon source. Oxidizes acetate and lactate to carbon dioxide and water, but the activity is not intense. Grows on glutamate agar and mannitol agar. Acetic acid is produced on ethanol/calcium carbonate agar. Produces 2-keto-D-gluconate and 5-keto-D-gluconate, but not 2,5-diketo-D-gluconate from D-glucose. Produces dihydroxyacetone from glycerol, but the activity is not intense. Acid is produced from D-glucose, D-mannose, D-galactose, D-xylose, D-arabinose, L-arabinose, D-fructose, L-sorbose, D-mannitol (weakly positive), D-sorbitol (weakly positive), D-arabitol, L-arabitol, *meso*-ribitol, *meso*-erythritol, glycerol, melibiose, and ethanol. Acid is not produced from L-rhamnose, dulcitol, sucrose (weakly positive in isolate GB23-3), or raffinose. Grows on D-glucose, D-mannose, D-galactose, D-xylose, D-arabinose, L-arabinose (weakly positive), L-rhamnose (weakly positive), D-fructose, L-sorbose, D-mannitol (weakly positive in isolate GB23-2^T), D-sorbitol (weakly positive), D-arabitol, L-arabitol, *meso*-ribitol, *meso*-erythritol, glycerol, melibiose, sucrose, and ethanol but not on dulcitol, raffinose, maltose, or lactose. The major ubiquinone is Q-10. Major cellular fatty acids are $C_{18:1}$ω7c and $C_{16:0}$. The restriction analysis of 16S rRNA genes shows the *Asp* type of restriction patterns in *Hpy*AV digestion. DNA base composition is 59.7−59.8 mol% G+C with a range of 0.1 mol%.

Table 3. Differential characteristics of *Asaia spathodeae* isolates GB23-2^T and GB23-3 in the genus *Asaia*.

Characteristic	1	2	3	4	5	6
Oxidation of acetate and lactate	w	w	w	w	w	w
Growth on						
30% Glucose	+	+	+	+	+	+
0.35% Acetic acid	w	w	w	w	w	−
L-Rhamnose	w	w	+	w	w	+
D-Mannitol	w	+	+	+	w	+
D-Sorbitol	w	w	+	+	w	+
Dulcitol	−	−	w	−	w	+
Maltose	−	−	w	w	−	−
Lactose	−	−	−	−	−	−
Acid production from						
L-Rhamnose	−	−	+	w	+	+
D-Mannitol	w	w	+	+	w	w
D-Sorbitol	w	w	w	+	w	w
Dulcitol	−	−	w	−	w	w
Sucrose	−	w	−	−	−	−
Raffinose	−	−	w	−	−	−
Ethanol	+	+	−	w	−	w
16S rRNA gene restriction pattern type with[a]						
*Hpy*AV	*Asp*	*Asp*	*Ab*	*Ab*	*Ab*	*Ab*
DNA G+C content (mol%)[b]	59.7	59.8	60.2	59.3	60.3	60.8

+, positive; w, weakly positive; −, negative. [a] For detailed information on restriction fragments, see the references (Malimas et al., 2008a; Yukphan et al., 2006). [b] The data were cited from the references (Katsura et al., 2001; Malimas et al., 2008a; Yamada et al., 2000; Yukphan et al., 2004) except for the two isolates.

Abbreviations: 1, *Asaia spathodeae* isolate GB23-2^T; 2, *Asaia spathodeae* isolate GB23-3; 3, *Asaia bogorensis* BCC 12264^T; 4, *Asaia siamensis* BCC 12268^T; 5, *Asaia krungthepensis* BCC 12978^T; 6, *Asaia lannensis* BCC 15733^T.

The type strain is isolate GB23-2^T (= BCC 36458^T = NBRC 105894^T = PCU 307^T), which was isolated from a flower of the African tulip and has the DNA G+C content of 59.7 mol% and ubiquinone isoprenologs comprised of 85% Q-10, 12% Q-9, and 3% Q-8.

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