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16S rRNA gene sequences analysis of acetic acid bacteria isolated from Thailand

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We determined the 16S rRNA gene sequences of 302 strains of acetic acid bacteria isolated from Thailand. The isolates were divided into 35 sequence groups based on differences in 16S rRNA gene sequences. A phylogenetic tree constructed from these sequences showed that 5 strains should be classified into new genera in the family *Acetobacteraceae*. The other 297 strains were assigned to 33 sequence groups of 4 known genera *Acetobacter*, *Asaia*, *Gluconacetobacter*, and *Gluconobacter*. Seventeen strains belonging to *Acetobacter* were divided into 8 sequence groups (AB1-AB8). Seven groups except for AB2 were closely related to known *Acetobacter* species. AB2 was remote from known *Acetobacter* species. We sorted 150 strains of *Asaia* into 11 sequence groups (AS1-AS11). AS11 was distinct from all known *Asaia* species. Nine strains assigned to *Gluconacetobacter* showed 100% sequence similarity to *Gluconacetobacter liquefaciens* NBRC 12388^T. Further, 121 strains of *Gluconobacter* were divided into 13 sequence groups (GB1-GB13). Of these, GB1 and GB6 seemed to constitute 2 distinct lineages in the genus. Based on these results, 155 Thai strains were deposited from the BIOTEC culture collection (BCC) in the NITE Biological Resource Center (NBRC) for public use.

Keywords: acetic acid bacteria, 16S rRNA gene sequence, Thailand

INTRODUCTION

In accordance with the Convention on Biological Diversity (CBD), the National Institute of Technology and Evaluation in Japan and the National Center for Genetic Engineering and Biotechnology in Thailand completed the Memorandum of Understanding for conducting cooperative research to promote the conservation and sustainable use of biological resources in Japan and Thailand for academic, industrial, and other purposes. It is expected that their utilization and commercialization will provide benefits to both countries. The NITE Biological Resource Center (NBRC) and the BIOTEC Culture Collection (BCC) started joint research projects on bacteria, yeast, and fungi in 2005 to increase microbiological resources in both collections.

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Acetic acid bacteria are important organisms in food and beverage industries etc. It is known that they adapt well to sugary and alcoholized fluid and are isolated from vinegar, fruit juice, sap water, alcoholic beverages and flowers. A common feature of this group is the aerobic oxidation of ethanol to acetic acid. The family *Acetobacteraceae* in the *Alphaproteobacteria* currently accommodates 10 genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Kozakia*, *Neoasaia*, *Saccharibacter*, and *Swaminathania*. They have been isolated in various countries in East and Southeast Asia, such as Japan, Indonesia, Thailand, and the Philippines since 1996 (Kersters, *et al*., 2006; Sievers & Swings, 2005; Uchimura, 2007; Yamada & Yukphan, 2008). More than 300 strains of acetic acid bacteria were isolated from various sources collected from various areas in Thailand and maintained at the BCC (Yukphan *et al*., 2004c) to be identified at a later date. To publicize these strains in our strain catalogues and to opti-

mize their use, we determined their 16S rRNA gene sequences to understand their taxonomic positions. It was also expected that some new taxa would be found in these strains because many new acetic acid bacteria have been isolated from tropical zones.

MATERIAL AND METHODS

Bacterial strains and cultivation conditions

We isolated 302 strains from various samples, including fruit, fermented fruit, flowers, seeds, and mushrooms collected in various regions of Thailand, such as Bangkok, Pak Kret, Ratchaburi, Suratthani, Samutsakorn, Ayutthaya, Chiang Mai, Sakaerat, and Tong Pha Phum (Kanchanaburi). A glucose/ethanol/acetic acid medium, a sorbitol medium, a dulcitol medium, and/or a sucrose medium were used for enrichment (Huong *et al*., 2007; Lisdiyanti *et al*., 2002; Yamada *et al*., 1976, 1999, 2000; Yukphan *et al*., 2004c, 2005). To obtain cell mass for DNA extraction, the strains were cultivated at 30℃ in 5 m*l* of NBRC 804 broth (Polypepton 5 g (Wako Pure Chemical Ind. Ltd., Osaka, Japan), yeast extract 5 g, glucose 5 g, $MgSO_4 \cdot 7H_2O$ 1 g, distilled water 1 *l*, pH 7.0) or NBRC 350 broth (Polypepton 5 g, yeast extract 5 g, glucose 5 g, mannitol 5 g, ethanol 5 m*l*, $MgSO_4 \cdot 7H_2O$ 1 g, and distilled water 1 *l*; pH 7.0).

PCR amplification, sequencing, and phylogenetic analysis of 16S rRNA genes

Genomic DNA extraction and 16S rRNA sequencing were performed as previously described (Takahashi *et al*., 2006). Sequence data obtained were aligned with those of representative members of *Alphaproteobacteria* by using ClustalX (Thompson *et al*., 1997) and then modified manually by referring to the 16S rRNA secondary structure of *Escherichia coli* (Gutell *et al*., 1994), and using the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic trees were constructed by the neighbor-joining method (Saitou & Nei, 1987), with calculated K_{nuc} values (Kimura, 1980). The topology of the trees was evaluated using Felsenstein's (1985) bootstrap method with 1000 replicates. Nearly complete sequences of the 16S rRNA genes from positions 28 through 1494 according to the *Escherichia coli* numbering system (Brosius *et al*., 1978) were determined for all strains. The 16S rRNA gene sequences of type strains were obtained from Japan's DNA databank.

RESULTS AND DISCUSSION

The analysis of the phylogenetic tree based on the 16S rRNA gene sequences indicated that 297 strains belonged to the genera *Acetobacter*, *Asaia*, *Gluconacetobacter*, and *Gluconobacter*. The other 5 strains (16S rRNA gene sequence groups GA1 and GA2) were not closely related to any known species and occupied an independent position in the family *Acetobacteraceae* (Fig. 1). Their 16S rRNA gene sequence similarities to all known species were lower than 96.5%. We propose that the group GA1, including the three strains BCC 15772 (=NBRC 103193), BCC 15773 (=NBRC 103194), and BCC 15774 (=NBRC 103195), be classified in a new genus *Tanticharoenia sakaeratensis* (Yukphan *et al*., 2008). We will publish a new taxon elsewhere for the group GA2, consisting of the 2 strains BCC 15744 (=NBRC 103196) and BCC 15745 (=NBRC 103197). The 302 isolates were divided into 35 groups based on differences in the 16S rRNA gene sequence. Their closely related species are summarized in Table 1.

Acetobacter

The genus *Acetobacter* currently accommodates 18 species. Based on the 16S rRNA gene sequence analysis, 17 strains were classified in the genus *Acetobacter* and divided into 8 sequence groups (AB1-AB8) (Fig. 2). AB1 clustered with *A. cerevisiae* LMG 1625^T, *A. malorum* LMG 1746^T, and *A. orleanensis* NBRC 13752^T , and was closely related to *A. cerevisiae*. AB2 was relatively remote from all known species. AB3, AB4, AB5, and AB6 were closely related to *A. indosinensis* NRIC 0313^T, *A. lovaniensis* NBRC 13753^T, *A. orientalis*, and *A. peroxydans* NBRC 13755^T, respectively. AB7 clustered with *A. ghanaensis* 430A^T, *A. syzygii* 9H-2^T, and *A. lovaniensis*. AB8 was closely related to *A. tropicalis* NIRC 0312^T. We are investigating their taxonomic characteristics to reveal whether they constitute a new species in the genus *Acetobacter*.

Asaia

In the genus *Asaia*, we grouped 150 strains, and they were divided into 11 sequence groups (AS1-AS11) (Fig. 3). We found that AS4, including BCC 15733^T (=NBRC 102526) and BCC 15734 (=NBRC 102527) constituted an independent species. We proposed to classify them as *Asaia lannensis* (Malimas *et al*., 2008). Other sequence groups (AS1-AS3 and AS5-AS11) showed high 16S rRNA gene sequence

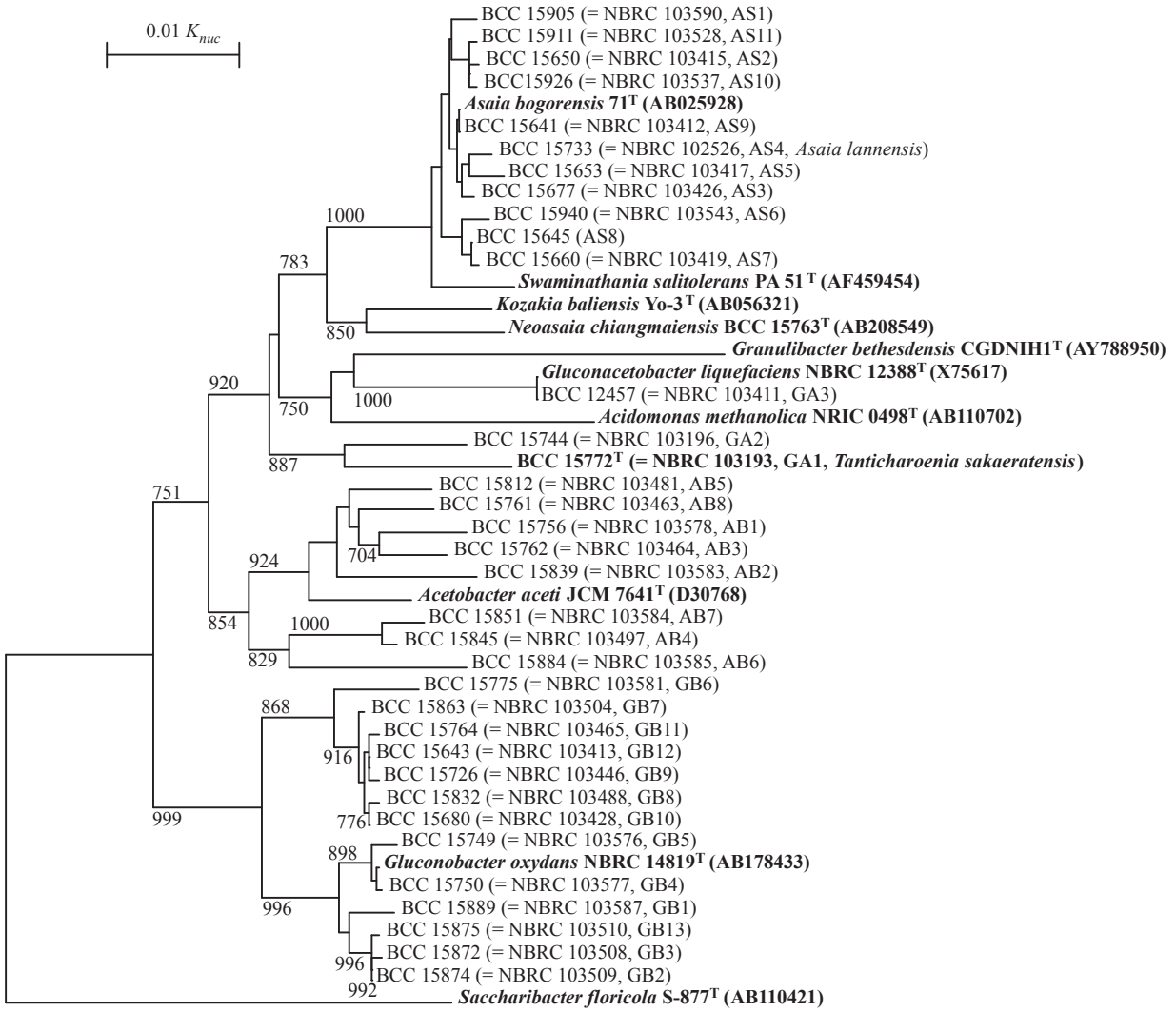

Fig. 1 A neighbor-joining tree showing the phylogenetic positions of the family *Acetobacteraceae* based on the 16S rRNA gene sequences. Bar, 0.01 K_{nuc}. Bootstrap values greater than 700 are shown in 1000 replicates. Type species are highlighted in bold.

similarities to known *Asaia* species. Because differences in the 16S rRNA gene sequences are extremely small in the genus *Asaia*, as shown previously (Yamada & Yukphan, 2008), we could not obtain reliable phylogenetic relationships based on the low bootstrap values. For example, the number of nucleotide differences between *A. bogorensis* (AB025928) and *A. siamensis* (AB035416) is merely 2 bases. However, it was reported that they can be distinguished from each other by DNA-DNA relatedness and several phenotypes (Katsura *et al.* 2001).

Gluconacetobacter

Nine strains (sequence group GA3) belonging to the genus *Gluconacetobacter* showed an identical 16S rRNA gene sequence with the type strain of *Gluconacetobacter liquefaciens* NBRC 12388^T (Fig. 1).

Gluconobacter

We divided 121 strains into 13 sequence groups (GB1-GB13) under the genus *Gluconobacter* (Fig. 4). Three sequence groups, namely GB2, GB3 and GB13, formed a cluster with *G. albidus* and *G. kondonii*. GB1 showed the highest 16S rRNA gene sequence similarity with *G. albidus*. GB4 and GB5 were closely related to *G. oxydans* NBRC 14819^T. GB6 was relatively remote from all known species. GB7-11 formed a cluster with *G. frateurii* NBRC 3264^T or

Table 1 List of sequence groups of Thai isolates

Phylogenetic group		Number of strains	Closest species based on 16S rRNA sequence similarities[a]
New genus	GA1	3	Proposed as *Tanticharoenia sakaeratensis*
	GA2	2	*Tanticharoenia sakaeratensis* (97.8)
Acetobacter	AB1	1	*A. cerevisiae* (99.6)
	AB2	1	*A. orientalis* (97.9)
	AB3	1	*A. indonesiensis* (99.3)
	AB4	6	*A. lovaniensis* (99.4)
	AB5	5	*A. orientalis* (99.9)
	AB6	1	*A. peroxydans* (99.4)
	AB7	1	*A. syzygii* (99.7)
	AB8	1	*A. tropicalis* (99.8)
Asaia	AS1	1	*A. bogorensis* (99.7)
	AS2	2	*A. siamensis* (99.9)
	AS3	6	*A. lannensis* (99.5)
	AS4	7	Proposed as *Asaia lannensis*
	AS5	12	*A. lannensis* (99.5)
	AS6	1	*A. krungthepensis* (99.5)
	AS7	13	*A. krungthepensis* (99.7)
	AS8	3	*A. krungthepensis* (99.9)
	AS9	95	*A. bogorensis* (100)
	AS10	6	*A. krungthepensis* (99.5)
	AS11	4	*A. bogorensis* (99.6)
Gluconacetobacter	GA3	9	*G. liquefaciens* (100)
Gluconobacter	GB1	2	*G. albidus* (99.5)
	GB2	6	*G. albidus* (100)
	GB3	2	*G. albidus* (99.9)
	GB4	1	*G. oxydans* (99.9)
	GB5	3	*G. oxydans* (99.7)
	GB6	2	*G. frateurii* (99.1)
	GB7	1	*G. frateurii* (99.8)
	GB8	1	*G. frateurii* (99.9)
	GB9	1	*G. frateurii* (99.9)
	GB10	41	*G. frateurii* (99.9)
	GB11	20	*G. frateurii* (99.9)
	GB12	39	*G. frateurii* (100)
	GB13	2	*G. kondonii* (100)

[a] 16S rRNA sequence similarities (%) are shown in parenthesis.

G. japonicus NBRC 3271^T (Malimas *et al*., 2009). Intrageneric relationships of the genus *Gluconobacter* based on 16S rRNA gene sequences were not reliable because the bootstrap values were not high, as shown in Fig. 4. It was reported that the analysis of the 16S-23S ITS sequence analysis was useful for the identification of *Gluconobacter* species (Takahashi *et al*., 2006; Yukphan *et al*., 2004a, 2004b). Restriction analysis of 16S-23S ITS using 6 restriction endonucleases was used to identify *Gluconobacter* species (Huong *et al*., 2007; Malimas *et al*., 2006). More information through several analyses, such as restriction analysis of 16S-23S ITS and phenotypic characteristics, is required to identify isolates of the genus *Gluconobacter*.

CONCLUSION

As described above, since the 302 isolates from

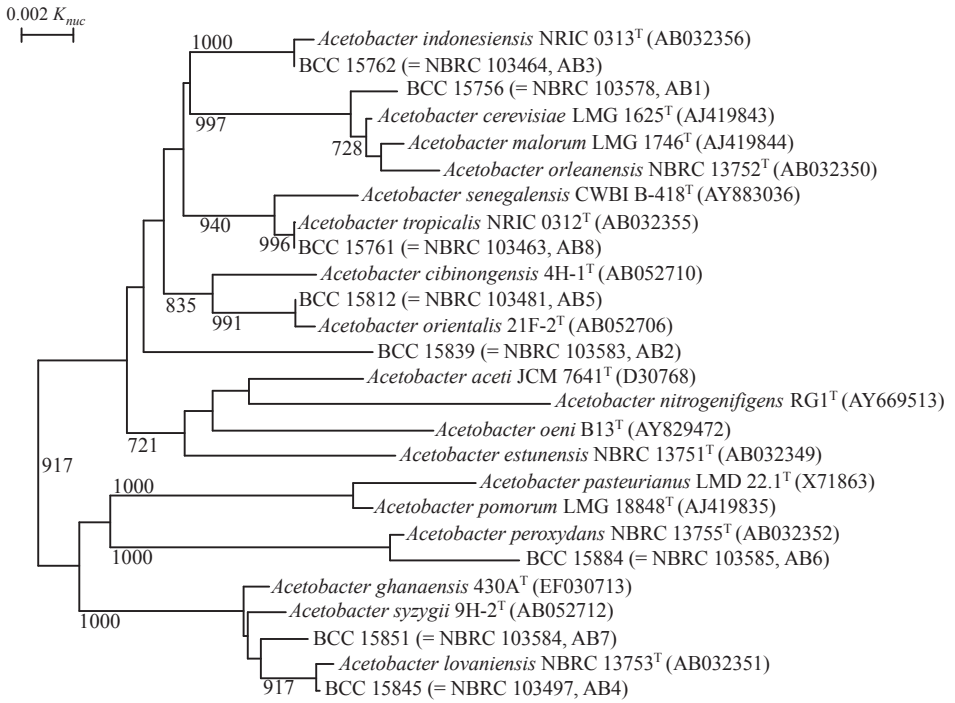

Fig. 2 A neighbor-joining tree showing the phylogenetic positions of the genus *Acetobacter* based on the 16S rRNA gene sequences. Bar, 0.002 K_{nuc}. Bootstrap values greater than 700 are shown in 1000 replicates.

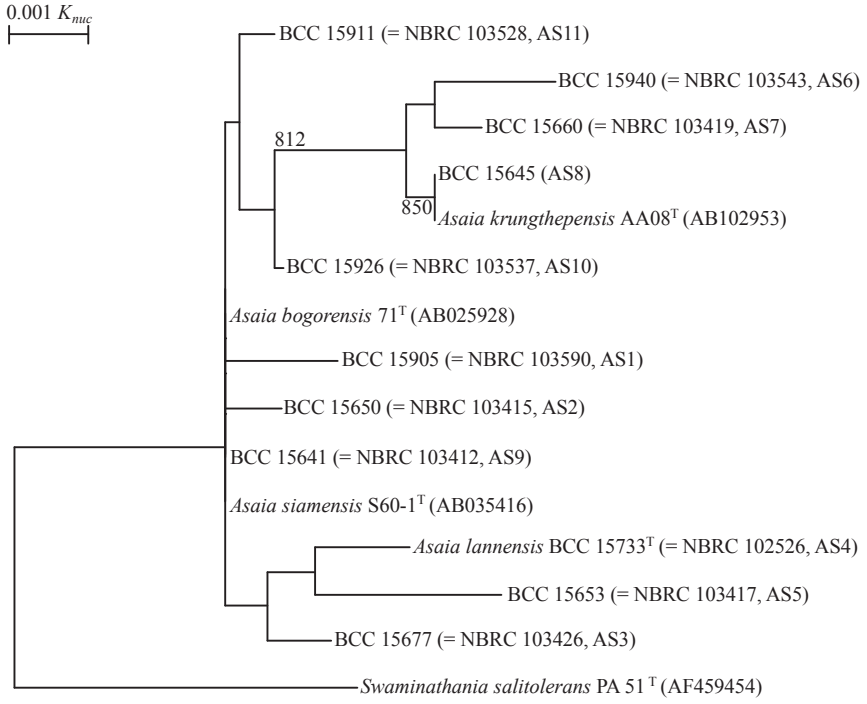

Fig. 3 A neighbor-joining tree showing the phylogenetic positions of the genus *Asaia* based on the 16S rRNA gene sequences. Bar, 0.001 K_{nuc}. Bootstrap values greater than 700 are shown in 1000 replicates.

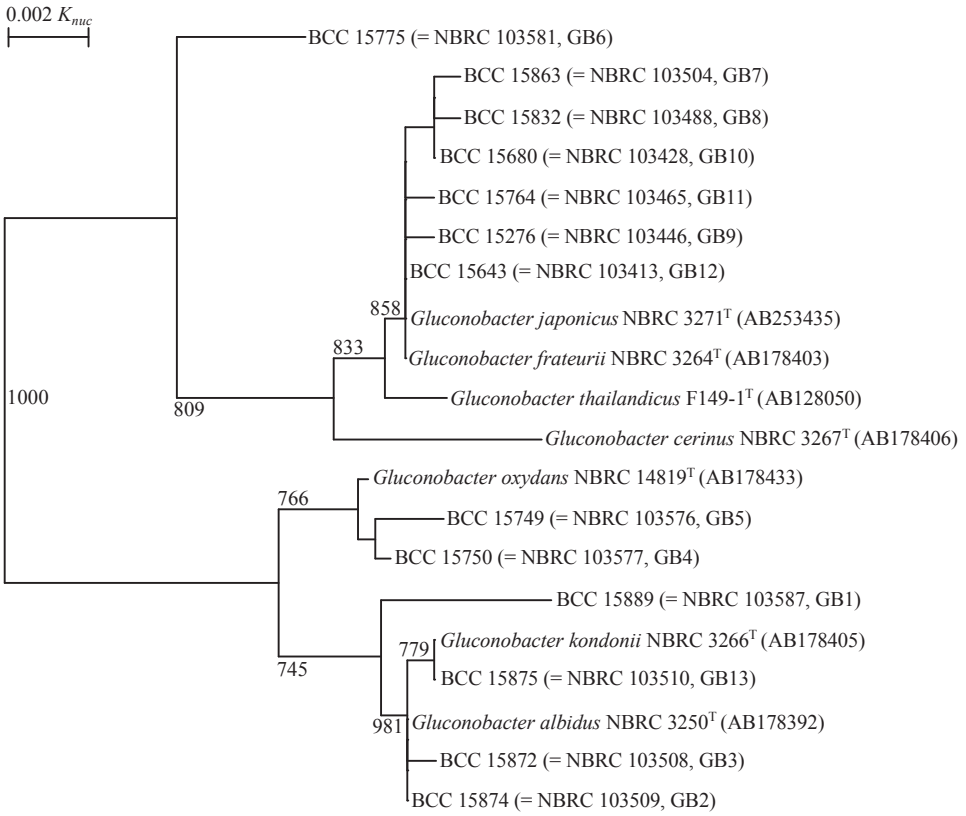

Fig. 4 A neighbor-joining tree showing the phylogenetic positions of the genus *Gluconobacter* based on the 16S rRNA gene sequences. Bar, 0.002 K_{nuc}. Bootstrap values greater than 700 are shown in 1000 replicates.

Thailand constituted a variety of a phylogenetic group in the genus *Acetobacter*, *Asaia*, and *Gluconobacter*, it is expected that they will possess some new and/or useful properties. In addition, we found several new sequence groups that will be classified into new species or genera. We are investigating their taxonomic characteristics to confirm whether they constitute new taxa, and the results will be published elsewhere. After the CBD is validated, access to biological resources in foreign countries becomes difficult. Establishment of techniques for classification and identification of microorganisms has become important for performing microbiological studies in compliance with the CBD. This study resulted in depositing 155 Thai strains from the BCC into the NBRC and making them available to the public. Accordingly, cooperative research through culture collections in different countries, such as those from the NBRC and the BCC in this study, should be useful to promote the conservation and sustainable use of biological resources under the CBD. We will maintain and, in fact, strengthen this partnership to explore and use microbiological resources both in Japan and Thailand.

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タイ原産酢酸菌の 16S rRNA 遺伝子解析

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タイ国で分離された酢酸菌 302 株の 16S rRNA 遺伝子塩基配列を決定し，系統解析を行った．5 株は *Acetobacteraceae* 科の新属に分類すべきと考えられ，残りの 297 株は *Acetobacter*，*Asaia*，*Gluconacetobacter* あるいは *Gluconobacter* 属のいずれかに含まれた．*Acetobacter* 属には 17 株が含まれ，8 グループ（AB1-AB8）に分かれた．グループ AB2 以外の 7 グループは *Acetobacter* 属の既知種に近縁であった．*Asaia* 属には 150 株が属し，11 グループ（AS1-AS11）に分かれた．グループ AS11 は *Asaia* 属の既知種から独立していた．*Gluconacetobacter* 属に含まれた 9 株は，*Gluconacetobacter liquefaciens* NBRC 12388^T と 100% の 16S rRNA 塩基配列相同性を示した．残りの 121 株は *Gluconobacter* 属に含まれ，13 グループ（GB1-GB13）に分かれた．2 つのグループ GB1 と GB6 は *Gluconobacter* 属の既知種から独立していた．

新種あるいは新属に分類すべきと考えられたいくつかのグループが見つかり，タイの酢酸菌は系統的に多様であると考えられた．日本国内のユーザーが利用できるよう，今回研究した株のうち 155 株を BIOTEC Culture Collection (BCC) から NITE Biological Resource Center (NBRC) に寄託して公開した．

（担当編集委員：河村好章）