J. Gen. Appl. Microbiol., **53**, 259–264 (2007)

Short Communication

Identification of Thai isolates assigned to the genus *Asaia* based on 16S rDNA restriction analysis

Vu Thi Lan Huong,[1,†] Taweesak Malimas,[1] Pattaraporn Yukphan,[1,*] Wanchern Potacharoen,[1] Somboon Tanasupawat,[2] Ly Thi Thanh Loan,[3] Morakot Tanticharoen,[1] and Yuzo Yamada[1,††]

[1] *BIOTEC Culture Collection (BCC), BIOTEC Central Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani 12120, Thailand*
[2] *Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*
[3] *Research Institute for Aquaculture Number 2, Ho Chi Minh City, Vietnam*

(Received February 5, 2007; Accepted May 25, 2007)

Key Words——acetic acid bacteria; *Asaia*; *Asaia bogorensis*; phylogeny; α-*Proteobacteria*; restriction analysis; 16S rDNA sequences; taxonomy

The genus *Asaia* Yamada et al. 2000 was introduced in acetic acid bacteria with a single species, *Asaia bogorensis* Yamada et al. 2000 (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 were described in total (Katsura et al., 2001; Yukphan et al., 2004). Strains of the three *Asaia* species show 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% acetic acid (v/v). The discrimination of the three *Asaia* species from one another is mainly based on phenotypic features, e.g., acid production from and growth on different carbon compounds, which sometimes give unreliable conclusions in their identification and classification.

We reported, as the second model, that the 16S-23S rDNA ITS restriction analysis using two restriction endonucleases *Taq*I and *Mva*I is applicable to species-level identification and classification of thirteen strains randomly selected from a large number of Thai isolates assigned to the genus *Asaia* (Yukphan et al., 2006a). 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 complicated clustering, which was composed of eight clusters.

In a subsequent study, the 16S rDNA restriction analysis using three restriction endonucleases *Sty*I, *Bsa*JI, and *Sna*BI gave four restriction groups in regard to the three *Asaia* species for the selected thirteen strains mentioned above (Yukphan et al., 2006b). In a

* Address reprint requests to: Dr. Pattaraporn Yukphan, BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Phaholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand.

E-mail: pattaraporn@biotec.or.th

† Present address: Microbiology Department, Faculty of Biology, University of Natural Sciences, Vietnam National University, Ho Chi Minh City, Vietnam.

†† JICA Senior Overseas Volunteer, Japan International Cooperation Agency (JICA), Shibuya-ku, Tokyo 151–8558, Japan; Professor Emeritus, Shizuoka University, Shizuoka 422–8529, Japan.

Abbreviations: ITS, internal transcribed spacer; BCC, BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani, Thailand.

phylogenetic tree based on 16S rDNA sequences, three clusters corresponding to the three *Asaia* species were recognized.

This paper is concerned with identification of a large number of Thai isolates mainly isolated from tropical flowers and phenotypically assigned to the genus *Asaia* based on 16S rDNA restriction analysis.

Eighty-seven strains were isolated mainly from flowers collected in Thailand by an enrichment culture approach using four different kinds of media, as described previously (Huong et al., 2007; Katsura et al., 2001; Yamada et al., 1976, 1999, 2000; Yukphan et al., 2004). These Thai isolates were phenotypically assigned to the genus *Asaia* by no or very weak oxidation of ethanol to acetic acid and no or very weak growth in the presence of 0.35% acetic acid (v/v) (Table 1). *Asaia bogorensis* BCC 12264^T, *A. siamensis* BCC 12268^T, and *A. krungthepensis* BCC 12978^T were used for reference strains.

PCR amplification of 16S rDNA was made (Yukphan et al., 2006b). The purified 16S rDNA PCR products were subjected to three different kinds of restriction analysis using three restriction endonucleases of *Sty*I (Fermentas, Hanover, Maryland, USA=*Eco*130I), *Bsa*JI (Fermentas=*Bse*DI), and *Sna*BI (Fermentas=*Eco*105I), as described previously (Yukphan et al., 2006b). Restriction products were analyzed by 2.5% agarose gel electrophoresis.

The eighty-seven Thai isolates phenotypically assigned to the genus *Asaia* tested were divided into the following five groups by the combination of the resulting restriction patterns in *Sty*I, *Bsa*JI, and *Sna*BI digestions (Table 1).

Group A was composed of Thai isolates that showed restriction patterns *a*, *c*, and *e*, which coincided with those of *A. bogorensis* BCC 12264^T, as found in isolate AF84 (Fig. 1). The isolates of Group A were forty-eight (data not shown except for isolate AF84) and amounted to 55.1%.

Group B was composed of Thai isolates that showed restriction patterns *b*, *d*, and *e*, which coincided with those of *A. siamensis* BCC 12268^T, as found in isolate AE97. The isolates of Group B were twenty-nine (data not shown except for isolate AE97) and amounted to 33.3%.

Group C was composed of Thai isolates that showed restriction patterns *b*, *c*, and *f*, which coincided with those of *A. krungthepensis* BCC 12978^T, as found in isolate AE73. The isolates of Group C were two (data not shown in isolate AE74) and amounted to 2.4%.

Group D was composed of Thai isolates that showed restriction patterns *b*, *c*, and *e*, which coincided with those of *A. bogorensis* BCC 15696 (Yukphan et al., 2006a), as found in isolate AG60. The isolates of Group D were three (data not shown except for isolate AG60) and amounted to 3.5%.

Group E was composed of Thai isolates that showed restriction patterns *a*, *c*, and *f*, as found in isolate AE68. The isolates of Group E were five (data not shown except for isolate AE68) and amounted to 5.7%.

In a previous study, a perfect correlation was found without any exception between the phylogenetic positions and the restriction groups in the species-level identification of the thirteen strains tested (Yukphan et al., 2006b). To confirm the results obtained above, isolates selected from each group containing a newly recognized group, Group E, were sequenced for 16S rDNA and analyzed phylogenetically, as described previously (Yukphan et al., 2006b). Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). The neighbor-joining method of Saitou and Nei (1987) was used for constructing a phylogenetic tree for 1,385 bases. Robustness for individual branches was estimated by bootstrapping with 1,000 replications (Felsenstein, 1985).

As shown in Fig. 2, isolates AF07 and AF84 of Group A formed a cluster together with the type strain of *A. bogorensis*. Isolate AE97 of Group B and isolate AE73 of Group C formed clusters respectively with the type strains of *A. siamensis* and *A. krungthepensis*. Isolate AG60 of Group D was included in the cluster of the type strain of *A. bogorensis*, as reported previously (Yukphan et al., 2006b). However, the clustering of *A. bogorensis* BCC 15696 of Group D and isolate AG60 was somewhat different. On the other hand, isolates AE68 and AE76 of Group E formed a small cluster, which was linked to the Group C cluster including the type strain of *A. krungthepensis*.

Isolates selected from the new restriction group, Group E were taxonomically examined for DNA-DNA hybridization. Bacterial chromosome DNAs were prepared, and DNA-DNA hybridization was made for isolates AE68 and AE76 by the photobiotin-labeling, microdilution-well method of Ezaki et al. (1989), as described previously (Yukphan et al., 2006a). The labeled DNA from *A. krungthepensis* BCC 12978^T gave

Table 1. Identification of Thai isolates assigned to the genus *Asaia* by 16S rDNA restriction analysis.

Restriction group, species and strain, and isolate	Restriction pattern with			Identified as
	*Sty*I	*Bsa*JI	*Sna*BI	
Group A				
A. bogorensis BCC 12264^T (=isolate 71^T)[a]	*a*	*c*	*e*	
AE61, AE62, AE78, AE79, AE80, AE81, AE82, AE84, AE85, AE86, AE87, AE94,	*a*	*c*	*e*	*A. bogorensis*
AE95, AE96, AF03, AF04, AF05, AF06, AF07, AF08, AF09, AF10, AF11, AF12,	*a*	*c*	*e*	*A. bogorensis*
AF13, AF14, AF15, AF23, AF24, AF25, AF26, AF27, AF28, AF29, AF30, AF35,	*a*	*c*	*e*	*A. bogorensis*
AF36, AF59, AF64, AF65, AF66, AF71, AF72, AF74, AF84, AG58, AH07, AH10	*a*	*c*	*e*	*A. bogorensis*
Group B				
A. siamensis BCC12268^T (=isolate S60-1^T)[a]	*b*	*d*	*e*	
AE63, AE70, AE71, AE72, AE97, AE99, AF01, AF02, AF32, AF33, AF34, AF39,	*b*	*d*	*e*	*A. siamensis*
AF63, AF73, AF75, AF76, AF77, AF78, AF79, AF80, AF81, AF82, AF83, AF85,	*b*	*d*	*e*	*A. siamensis*
AF86, AF87, AF88, AF89, AF90	*b*	*d*	*e*	*A. siamensis*
Group C				
A. krungthepensis BCC 12978^T (=isolate AA08^T)[a]	*b*	*c*	*f*	
AE73, AE74	*b*	*c*	*f*	*A. krungthepensis*
Group D				
A. bogorensis BCC 15696[b]	*b*	*c*	*e*	
AE64, AE69, AG60	*b*	*c*	*e*	*A. bogorensis*
Group E				
AE65, AE66, AE67, AE68, AE76	*a*	*c*	*f*	*A. krungthepensis*

Isolation sources and BCC numbers of isolates are not shown.

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

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

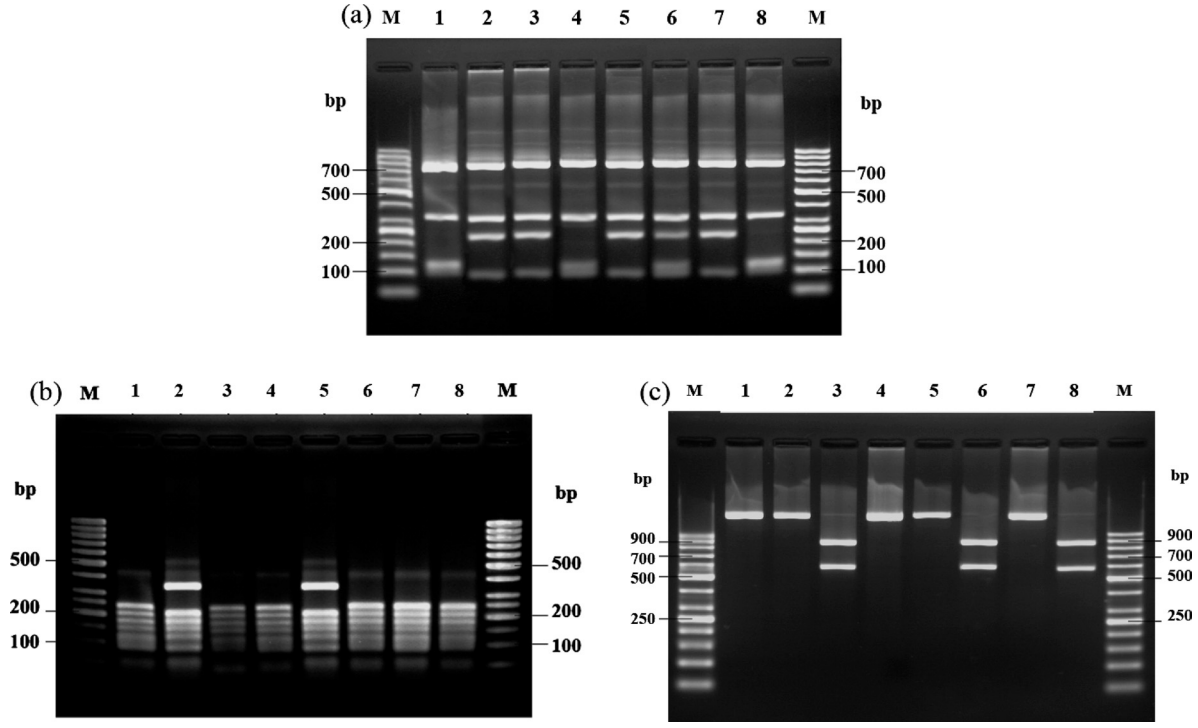

Fig. 1. Restriction analysis of 16S rDNA by digestion with *Sty*I, *Bsa*JI, and *Sna*BI for Thai isolates assigned to the genus *Asaia*.

Restriction of 16S rDNA PCR products was made with *Sty*I (a), *Bsa*JI (b), and *Sna*BI (c). For estimation of restriction fragments, 50-bp markers were used in gel elecrophoresis. Abbreviations: 1, *A. bogorensis* BCC 12264^T; 2, *A. siamensis* BCC 12268^T; 3, *A. krungthepensis* BCC 12978^T; 4, isolate AF84 (Group A); 5, isolate AE97 (Group B); 6, isolate AE73 (Group C); 7, isolate AG60 (Group D); 8, isolate AE68 (Group E); M, 50-bp marker.

DNA-DNA similarities respectively of 95 and 98% to isolates AE68 and AE76 of Group E. These results indicated that isolates of Group E were genetically identified as *A. krungthepensis*.

According to the phylogenetic and genetic data obtained above and previously (Yukphan et al., 2006a, b), the eighty-seven Thai isolates tested were identified as follows (Table 1).

Fifty-one isolates of Group A and Group D were identified as *A. bogorensis* and amounted to 58.6% of the total *Asaia* isolates. Twenty-nine isolates of Group B were identified as *A. siamensis* and amounted to 33.3%. The remaining seven isolates of Group C and E were identified as *A. krungthepensis* and amounted to 8.1%. And no possible new species was shown in the eighty-seven Thai isolates.

The 16S rDNA and/or 16S-23S rDNA ITS restriction analyses were reported for identifying acetic acid bacteria (Ruiz et al., 2000; Trček and Raspor, 1999; Trček and Teuber, 2002). However, there were no reports that a large number of *Asaia* strains were especially analyzed for species-level identification.

In the present study, five restriction groups were given for the eighty-seven isolates assigned to the genus *Asaia*. These numbers were quite few, when compared with those found in the 16S-23S rDNA ITS restriction analysis with *Taq*I and *Mva*I (Yukphan et al., 2006a), which gave twelve restriction groups for the only three *Asaia* species (data not shown). The results obtained above represented that the 16S rDNA restriction analysis is more convenient and useful than the 16S-23S rDNA ITS restriction analysis.

In a previous paper, we drew the following three conclusions from the species-level identification of the thirteen strains randomly selected from a large number of Thai *Asaia* isolates (Yukphan et al., 2006a): 1) *Asaia bogorensis* is the most popular in the genus *Asaia*. 2) Two strains were found in *A. krungthepensis* in addition to the three strains reported previously by Yukphan et al. (2004). 3) *Asaia siamensis* is a rather rare species in the genus *Asaia*, because identified were only two strains. In this study, however, our con-

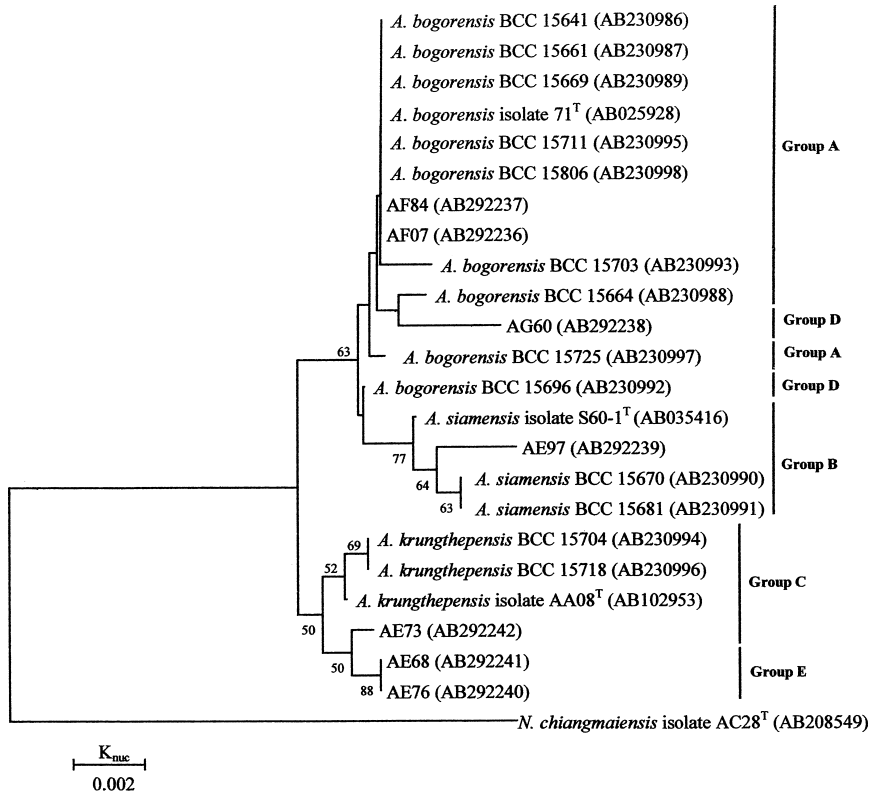

Fig. 2. A phylogenetic tree based on 16S rDNA sequences for Thai isolates assigned to the genus *Asaia*.

The phylogenetic tree was constructed by the neighbor-joining method. *Neoasaia chiangmaiensis* isolate AC28^T was used for an outgroup. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications.

clusions were slightly changed: 1) *Asaia siamensis* is not a rare species but not so popular, when compared with *A. bogorensis*. 2) *Asaia krungthepensis* is the most uncommon in the genus *Asaia*.

Acknowledgments

This study was supported in part by The HRD Program in Biotechnology for Neighboring Countries, National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, The Bioresources Research Network (BRN), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, and University of Natural Sciences, Vietnam National University, Ho Chi Minh City, Vietnam.

References

Ezaki, T., Hashimoto, Y., and Yabuuchi, E. (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int. J. Syst. Bacteriol.*, **39**, 224–229.

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783–791.

Huong, V. T. L., Malimas, T., Yukphan, P., Potacharoen, W., Tanasupawat, S., Loan, L. T. T., Tanticharoen, M., and Yamada, Y. (2007) Identification of Thai isolates assigned to the genus *Gluconobacter* based on 16S-23S rDNA ITS restriction analysis. *J. Gen. Appl. Microbiol.*, **53**, 133–142.

Katsura, K., Kawasaki, H., Potacharoen, W., Saono, S., Seki, T., Yamada, Y., Uchimura, T., and Komagata, K. (2001) *Asaia siamensis* sp. nov., an acetic acid bacterium in the α-*Proteobacteria*. *Int. J. Syst. Evol. Microbiol.*, **51**, 559–563.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**, 111–120.

Ruiz, A., Poblet, M., Mas, A., and Guillamón, J. M. (2000) Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. *Int. J. Syst. Evol. Microbiol.*, **50**, 1981–1987.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.

Trček, J. and Raspor, P. (1999) Molecular characterization of acetic acid bacteria isolated from spirit vinegar. *Food Technol. Biotechnol.*, **37**, 113–116.

Trček, J. and Teuber, M. (2002) Genetic and restriction analysis

of the 16S-23S rDNA internal transcribed spacer regions of the acetic acid bacteria. *FEMS Microbiol. Lett.*, **208**, 69–75.

Yamada, Y., Hosono, R., Lisdiyanti, P., Widyastuti, Y., Saono, S., Uchimura, T., and Komagata, K. (1999) Identification of acetic acid bacteria isolated from Indonesian sources, especially of isolates classified in the genus *Gluconobacter*. *J. Gen. Appl. Microbiol.*, **45**, 23–28.

Yamada, Y., Katsura, K., Kawasaki, H., Widyastuti, Y., Saono, S., Seki, T., Uchimura, T., and Komagata, K. (2000) *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α-*Proteobacteria*. *Int. J. Syst. Evol. Microbiol.*, **50**, 823–829.

Yamada, Y., Okada, Y., and Kondo, K. (1976) Isolation and characterization of "polarly flagellated intermediate strains" in acetic acid bacteria. *J. Gen. Appl. Microbiol.*, **22**, 237–245.

Yukphan, P., Malimas, T., Potacharoen, W., Tanasupawat, S., Tanticharoen, M., and Yamada, Y. (2006a) Identification of strains assigned to the genus *Asaia* Yamada et al. 2000 based on restriction analysis of 16S-23S rDNA internal transcribed spacer regions. *J. Gen. Appl. Microbiol.*, **52**, 55–62.

Yukphan, P., Malimas, T., Takahashi, M., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M., and Yamada, Y. (2006b) Identification of strains assigned to the genus *Asaia* Yamada et al. 2000 based on 16S rDNA restriction analysis. *J. Gen. Appl. Microbiol.*, **52**, 241–247.

Yukphan, P., Potacharoen, W., Tanasupawat, S., Tanticharoen, M., and Yamada, Y. (2004) *Asaia krungthepensis* sp. nov., an acetic acid bacterium in the α-*Proteobacteria*. *Int. J. Syst. Evol. Microbiol.*, **54**, 313–316.