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Genetic Diversity and Metabolites Production of Root-Nodule Bacteria Isolated from Medicinal Legumes *Indigofera tinctoria*, *Pueraria mirifica* and *Derris elliptica* Benth. Grown in Different Geographic Origins Across Thailand

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Abstract: A total of 215 root-nodule bacteria were isolated from 3 medicinal legumes including *Indigofera tinctoria*, *Pueraria mirifica* and *Derris elliptica* Benth. naturally grown in 16 provinces of Thailand. The isolates were evaluated for DNA polymorphism using randomly amplified polymorphic DNA (RAPD) analysis. Isolates generated 92 identical RAPD profiles, indicating highly significant genetic diversity among isolates from distinct geographic areas. The genetic diversity among root-nodule bacteria was affected slightly by the host plants rather than the geographic origins. The total 56 representative strains were characterized by small plasmid and megaplasmid profiles as well as production of acyl homoserine lactone (AHLs)-like quorum sensing molecules, indole-3-acetic acid (IAA) and melanin. Three *Rhizobium*-like strains nodulating *P. mirifica* and *D. elliptica* Benth. harbor the same profile with 2 small plasmids. One *Sinorhizobium*-like strain and 4 *Rhizobium*-like strains nodulating *I. tinctoria* and *D. elliptica* Benth. harbor the same profile with 4 megaplasmids. The strains varied in their ability to produce AHLs-like molecules and IAA. Melanin production was observed in the only one of *Pseudoalteromonas*-like strain.

Key words: RAPD analysis · Root-nodule bacteria · Acyl homoserine lactone (AHL) · Indole-3-acetic acid (IAA) · Melanin

INTRODUCTION

Diversity of root-nodule bacteria has been revealed by many studies and almost all of the data reported previously indicate that there is a high level of genetic diversity in these bacteria. An assessment of the genetic diversity and genetic relationships among strains could provide valuable information about bacterial genotypes that are well adapted to a certain environment [1]. The genetic diversity of root-nodule bacteria in relation to the host plant and geographic origin has been studied through the use of methodologies for phylogenetic analyses. However, the results obtained from the previous studies have been argumentative. Laguerre *et al.* [2] evaluated the genetic diversity of 44 rhizobial isolates from different host plants and different geographic locations and reported that rhizobial classification at the genus and probably also the species, level was independent of geographic origin and host plant affinity. Pongsilp *et al.* [3] reported a significant genetic diversity among bradyrhizobia that were isolated directly from different soil samples in Thailand. However, some of the isolates were closely related to each other. Lafay and Burdon [4] evaluated genetic structure of rhizobia nodulating *Acacia* spp. in southeastern Australia. The results showed that two genomospecies were both widespread and relatively abundant across the sampling site, whereas another genomospecies seemed to be restricted to the more temperate regions. A number of studies suggest that the host plant and the geographic origin are important factors affecting the genetic structure of rhizobial population. Andronov *et al.* [5] found that isolates from a particular site belonging to a limited range of chromosomal genotypes. Andronov *et al.* [6] found significant differences in the genetic structure between strains isolated from the soil under different legumes. Bromfield *et al.* [7] reported the difference in the distribution of genotypes of *Rhizobium meliloti* from nodules of different legumes. Carelli *et al.* [8] reported that the genetic structure of *Sinorhizobium*

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meliloti populations based mainly on differences among plants, while the effect of soil and cultivar were not significant.

Indigofera tinctoria (true indigo), *Pueraria mirifica* (white Kwao Krua) and *Derris elliptica* Benth. are medicinal legumes which are widely consumed in many applications of medicine. Phytochemical compounds from *I. tinctoria* have shown their several biological activities and pharmacological actions such as antioxidant activity [9,10] antidyslipidemic activity [11,12], hepatoprotective effect [13-15], protective effect against endotoxin [10] and liver injury [16], anticancer effect against chronic myelocytic leukemia [17] and inhibitory effect against cyclops, the carriers of dracunculiasis [18]. *P. mirifica*, an indigenous Thai medicinal plant, has been known as a source of phytoestrogens [19]. Therefore it has been used as a rejuvenating drug and a menopausal drug [20]. Phytochemical compounds from *D. elliptica* have been reported to possess antioxidant activity [21] and larvicidal activity [22]. Like other legumes, these plants can be nodulated by a specific group of bacteria. Since the genetic diversity of microsymbionts of these plants has not been studied so far, hence in this study we evaluated the genetic diversity of root-nodule bacteria isolated from these plants grown in different geographic origins based on RAPD analysis to determine whether host plant and geographic origin have influence on bacterial diversity. In addition, plasmid profiles and production of metabolites, including homoserine lactone-like quorum sensing molecules, indole-3-acetic acid (IAA) and melanin, were detected.

MATERIALS AND METHODS

Isolation of Bacteria and Culture Conditions: Root nodules were collected from 3 medicinal legumes including *Indigofera tinctoria*, *Pueraria mirifica* and *Derris elliptica* Benth. grown naturally in 16 provinces in Thailand. Root-nodule bacteria were isolated and the purity of the isolates was ensured as described previously [23]. Numbers of isolates and their geographic origins are presented in Table 1.

Nodulation Tests: A total of 215 isolates were examined for their nodulating ability with their original host plants. Seeds of *I. tinctoria* and seeds of *P. mirifica* were scarified and surface sterilized with 3% sodium hypochlorite. The seeds were laid on moistened cotton plates and incubated at 25°C in the dark for 1-2 days. Branches with adventitious roots of *D. elliptica* were surface sterilized with 95% alcohol. The germinated seeds and branches were planted in Leonard's jars and inoculated with bacterial suspensions as described by Somasegaran and Hoben [24]. After 3 weeks cultivation, plants were observed for the presence of nodules.

Random Amplified Polymorphic DNA (RAPD) Analysis: The total isolates of root-nodule bacteria were investigated by PCR-RAPD marker technique. Genomic DNAs were extracted from exponentially grown cultures by using Wizard^{R} genomic DNA purification kit (Promega, WI) and PCR reactions were carried out using an arbitary primer RAPD-2 (5' GTTTCGCTCC 3') as described

Table 1: The similarity percentage based on RAPD analysis of isolates from each host plant and each geographic origin

Host plant	Geographic origin	Number of isolates	Number of RAPD profiles	Similarity%
I. tinctoria	Chiang Mai (Lat 18° 46' N Long 98° 58' E)	51	20	25%
	Khon Kaen (Lat 16° 28' N Long 102° 46' E)	9	5	20%
	Lampang (Lat 18° 16' N Long 99° 28' E)	9	3	70%
	Ratchaburi (Lat 13° 31' N Long 99° 47' E)	7	2	80%
	Trad (Lat 12° 14' N Long 102° 30' E)	5	3	70%
	Chantaburi (Lat 12° 49' N Long 102° 10' E)	4	3	60%
	Chainat (Lat 15° 10' N Long 100° 7' E)	4	3	20%
	Nakhon Ratchasima (Lat 14° 58' N Long 102° 05' E)	4	2	70%
	Bangkok (Lat 13° 44' N Long 100° 30' E)	4	4	15%
	Leoi (Lat 17° 28' N Long 101° 43' E)	1	1	
P. mirifica	Chachoengsao (Lat 13° 42' N Long 101° 5' E)	14	6	30%
	Lampang	13	7	30%
	Pichit (Lat 16° 26' N Long 100° 22' E)	10	5	10%
	Kanchanaburi (Lat 14° 1' N Long 99° 31' E)	6	4	45%
	Burirum (Lat 15° 23' N Long 103° 25' E)	1	1	
D. elliptica	Lampang	44	12	25%
	Chonburi (Lat 13° 10' N Long 100° 55' E)	19	7	15%
	Saraburi (Lat 14° 31' N Long 100° 55' E)	10	6	10%

Lat: Latitude; Long: Logitude

elsewhere [23]. Negative controls (no DNA added) were included in all sets of reactions. PCR-RAPD amplifications were carried out in 25 µl of reaction mixture containing 2 µl of DNA solution (approx. 50 ng of DNA), 0.2 mM of each dNTPs, 4 mM of $MgCl_2$, 50 pmol of an arbitary primer and 1.25 units of *Taq* polymerase (Vivantis, Malaysia) with the PCR buffer A supplied with the enzyme. PCR conditions were as follows: 1 cycle at 94°C for 1 min., 45 cycles consisting of 94°C for 1 min., 36°C for 1 min., 72°C for 2 min. and 1 cycle at 72°C for 1 min. All PCR amplifications were performed in an Eppendorf Mastercycler (Brinkmann Instruments, Canada). The products of the reactions were separated on a 1% agarose gel electrophoresis. The sizes of the amplified fragments were determined by comparison with a 1-kb DNA ladder (Vivantis, Malaysia). Unweight pair groups using mathematical averages (UPGMA) dendrograms were performed using the Image Master ID Elite v 5.20 (GE Healthcare, UK).

Plasmid Profiles: The total 56 representative strains were examined for the presence of small plasmids and megaplasmids. Small plasmids were extracted from exponentially grown culture by using a GF-1 plasmid extraction kit (Vivantis, Malaysia) according to the manufacture's instruction. Megaplasmids were extracted from exponentially grown culture according to the following procedure. A 10-ml volume of culture was pelleted and cells were lysed by addition of 3.8 ml of lysozyme solution (50 mM glucose; 25 mM Tris, pH 8.0; 10 mM EDTA; lysozyme 2 mg/ml). Reaction was incubated at 25°C for 20 min. A 0.2-ml volume of 25% SDS and a 0.1-ml of RNase A (100 mg/ml) were added. The cell lysate was incubated at 37°C for 30 min., then 0.4 ml of pronase (2 mg/ml) was added. The suspension was incubated at 37°C for 2 h. About 4.5 ml of phenol was added and the suspension was mixed thoroughly by inversion. After centrifugation at 10,000 rpm for 20 min., the upper aqueous layer was transferred into a new tube. An equal volume of phenol : chloroform (1:1) was added into the aqueous solution. The solution was mixed thoroughly by inversion and the centrifugation was repeated. DNA was precipitated with ethanol, pelleted by centrifugation, air-dried and dissolved in a 1-ml of TE buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA). The DNA solution was subjected to gel electrophoresis for 6 h at 80 V. The presence of megaplasmid bands was observed under UV illumination. The reference plasmids of *Sinorhizobium fredii* (formerly *Rhizobium japonicum*) USDA 205 were used to estimate the sizes of megaplasmids [25].

Quantification of AHLs-like Molecules Production: The selected strains were grown in liquid Arabinose-Gluconate (AG) medium [26] for 1-2 days. Cells were removed and AHLs-Like molecules were extracted from 1 ml of supernatant by using ethyl acetate as described by Zhu *et al.* [27]. Ethyl-acetate-extracted AHLs were added to 5-ml of *Agrobacterium tumefaciens* NTL4 (pCF218) (pCF372) indicator strain, at 0.5 OD_{600} [28-30]. Cultures were incubated for 6-8 h, until reached OD_{600} = 1.0. Production of AHLs-like molecules was quantified by measuring β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate [31]. *Bradyrhizobium japonicum* USDA 290 producing large amount of AHL-like inducer activity was used as a positive strain and *Escherichia coli* HB101 producing very little AHL-like inducer activity was used as a negative strain [32].

Quantification of IAA Production: The selected strains were propagated in Tris-TMRT broth supplemented with L-tryptophan [33] at 28°C in the darks for 6 days. Cells were removed by centrifugation at 6,000 rpm for 10 min. The IAA production in the supernatant was determined by the colorimetric assay [34]. The concentration of IAA was determined by comparison of a standard curve.

Detection of Melanin Production: Melanin production was determined by the method described by del Papa *et al.* [35]. The presence of dark brown pigment was scored as positive for melanin production.

RESULTS AND DISCUSSION

Nodulation Tests and Random Amplified Polymorphic DNA (RAPD) Analysis: All 215 isolates were found to nodulate their original hosts. Genetic diversity amongst 215 isolates from root-nodules of 3 medicinal legumes grown naturally in different geographic origins across Thailand was assessed using RAPD. Some of the isolates generated specific patterns regarding to their particular genotypes. The highly significant genetic diversity among the isolates was found since 215 isolates generated 92 identical RAPD profiles. The RAPD profiles contained from 1 to 9 amplified bands, ranging from approximately 500 to 3,000 bp in size. The isolates of *I. tinctoria*, *P. mirifica* and *D. elliptica* Benth. presented 15% to 80%, 10% to 45% and 10% to 25% similarity, respectively, in the resulting dendrograms. Therefore, the highest genetic diversity was obtained from isolates nodulating *D. elliptica* Benth. Examples of RAPD profiles and the constructed dendrogram are shown in Fig. 1.

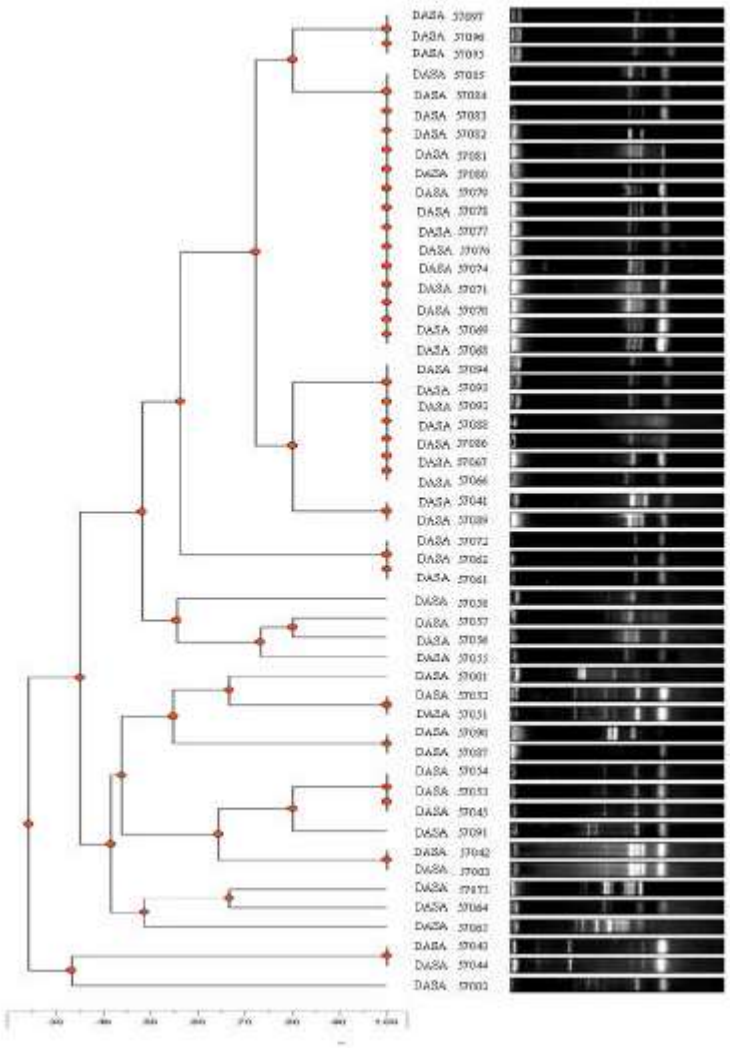

Fig. 1: Dendrogram generated from RAPD profiles of the isolates nodulating *I. tinctoria* grown in Chiang Mai province

The similarity percentage of isolates from each host plant and each geographic origin is presented in Table 1. The isolates from different host plants generated distinct RAPD profiles, even among isolates obtained from the same provinces. Whereas the identical RAPD profiles were found with isolates from the same host plants grown in different geographic origins. The *I. tinctoria* isolates DASA 57050 and DASA 57063 from Bangkok generated the identical RAPD profile with DASA 57060 from Ratchaburi. The *I. tinctoria* isolate DASA 57024 from Leoi also generated the identical RAPD profile with DASA 57017 and DASA 57018 from Nakhon Ratchasima. The *P. mirifica* isolate DASA 64040 from Burirum and DASA 64044 from Kanchanaburi generated the identical RAPD pattern. These results suggest that the genetic diversity among root-nodule bacteria was affected slightly by the host plants, while it was independent of geographic origins. This is in agreement with Carelli *et al.* [8] who reported that the genetic structure of *Sinorhizobium meliloti* populations based mainly on differences among plants, while the effect of soil and cultivar were not significant. Andronov *et al.* [6] and Bromfield *et al.* [7] also reported the effect of legumes on the genetic structure of rhizobial strains. On the contrary, Laguerre *et al.* [2] reported that rhizobial classification at the genus and probably also the species, level was independent of geographic origin and host plant affinity. This study also support that RAPD analysis provided a new tool for investigating genetic polymorphisms in many different organisms. de Bruijn [36] suggested that PCR with a suitable primer could be applied for molecular genetic characterization of rhizobia. Pongsilp and Nuntagij [23]

Table 2: Characteristics of the representative strains nodulating *I. tinctoria*, *P. mirifica* and *D. elliptica* Benth

			Plasmid profile		Production of metabolite		
Strain	Related genus group[a]	Geographic origin	No. of small plasmids (size in bp)	No. of megaplasmids (size in megadalton)	AHL-like inducer (unit of β-galacto-sidase activity)[c]	IAA (µg IAA equivalent/ ml of culture)[c]	Melanin
DASA 57053	*Rhizobium*	Chiang Mai	0	0	89.73±13.58	12.88±2.45	-
DASA 57065	*Rhizobium*	Chiang Mai	0	0	100.53±13.23	22.90±2.94	-
DASA 57076	*Rhizobium*	Chiang Mai	0	0	128.93±8.40	7.90±0.78	-
DASA 57010	*Rhizobium*	Ratchaburi	0	4 (57, 112, »195, >600)	138.00±9.46	19.70±2.63	-
DASA 57027	*Rhizobium*	Chainat	0	4 (57, 112, »195, >600)	57.07±15.16	24.09±1.28	-
DASA 57003	*Pseudoalteromonas*	Chiang Mai	0	0	66.27±9.26	21.92±1.67	+
DASA 57066	*Pseudoalteromonas*	Chiang Mai	0	0	109.87±10.23	16.35±0.59	-
DASA 57075	*Pseudoalteromonas*	Lampang	0	0	103.07±7.18	15.21±1.79	-
DASA 57004	*Pseudoalteromonas*	Chantaburi	0	0	39.73±13.93	18.88±2.30	-
DASA 57020	*Ralstonia/Cupriavidus*	Khon Kaen	0	0	94.67±12.10	23.54±2.54	-
DASA 57038	*Ralstonia/Cupriavidus*	Khon Kaen	0	0	126.27±19.09	8.17±1.01	-
DASA 57009	*Ralstonia/Cupriavidus*	Trad	0	0	149.43±4.79	7.35±1.08	-
DASA 57015	*Sinorhizobium*	Nahkon Ratchasima	0	0	110.67±12.81	34.76±0.18	n.d[d]
DASA 57019	*B. elkanii*	Khon Kaen	0	0	236.60±11.18	8.03±1.33	n.d[d]
DASA 57057	n.d.	Chiang Mai	0	0	60.80±18.85	27.89±1.35	-
DASA 57098	n.d.	Lampang	0	0	132.98±26.02	13.12±1.30	n.d[d]
DASA 57005	n.d.	Trad	0	0	133.47±4.84	32.50±1.48	-
DASA 57034	n.d.	Chantaburi	0	0	143.87±22.60	25.58±0.73	-
DASA 57050	n.d.	Bangkok	0	0	200.56±20.90	11.06±0.70	n.d[d]
DASA 57024	n.d.	Leoi	0	0	132.27±11.17	20.34±1.32	-
DASA 64006	*Rhizobium*	Chachoengsao	0	0	75.07±13.61	19.97±1.32	-
DASA 64021	*Rhizobium*	Chachoengsao	0	0	119.73±10.52	22.97±1.61	-
DASA 64038	*Rhizobium*	Lampang	0	0	115.73±14.37	8.20 ±1.18	-
DASA 64011	*Rhizobium*	Pichit	0	0	55.07±12.05	30.89±0.68	-
DASA 64012	*Rhizobium*	Pichit	0	0	198.73±19.59	23.58±2.28	-
DASA 64023	*Rhizobium*	Pichit	0	0	134.13±10.56	20.57±1.60	-
DASA 64016	*Rhizobium*	Kanchanaburi	2 (1,500; 900)	0	67.33±11.04	25.40±1.65	-
DASA 64040	*Rhizobium*	Burirum	0	0	82.00±10.67	4.31±0.67	-
DASA 64008	*Bradyrhizobium* sp.	Chachoengsao	0	0	63.33±13.98	6.73±1.57	-
DASA 64042	*Bradyrhizobium* sp.	Kanchanaburi	0	0	125.73±10.07	11.42±0.73	n.d[d]
DASA 64010	n.d.	Chachoengsao	0	0	91.33±25.44	9.10±1.00	-
DASA 64014	n.d.	Chachoengsao	0	0	84.40±7.34	21.08±1.42	-
DASA 64020	n.d.	Chachoengsao	0	0	107.32±10.36	13.23±0.91	-
DASA 64027	n.d.	Lampang	0	0	189.20±26.46	9.23±1.21	n.d[d]
DASA 64031	n.d.	Lampang	0	0	413.29±39.27	21.22±3.31	-
DASA 64034	n.d.	Lampang	0	0	75.20±8.38	21.45±1.80	-
DASA 64026	n.d.	Pichit	0	0	89.60±12.83	13.63±0.79	-
DASA 64022	n.d.	Kanchanaburi	0	0	61.20±11.93	4.87±0.85	-
DASA 68006	*Rhizobium*	Lampang	0	0	136.94±26.76	10.81±0.87	-
DASA 68020	*Rhizobium*	Lampang	2 (1,500; 900)	4 (57, 112, »195, >600)	59.60±13.40	18.25±1.54	-
DASA 68025	*Rhizobium*	Lampang	2 (1,500; 900)	4 (57, 112, »195, >600)	148.40±17.61	8.31±1.31	-
DASA 68066	*Rhizobium*	Lampang	0	0	100.00±22.98	12.06±1.56	-
DASA 68069	*Rhizobium*	Lampang	0	0	70.67±11.87	10.31±1.12	-
DASA 68070	*Rhizobium*	Lampang	0	0	141.16±21.58	12.14±1.60	-
DASA 68053	*Rhizobium*	Saraburi	0	0	138.80±50.72	7.35±0.48	n.d[d]
DASA 68061	*Rhizobium*	Saraburi	0	0	152.70±31.59	14.57±0.99	n.d[d]
DASA 68012	*Sinorhizobium*	Lampang	0	4 (57, 112, »195, >600)	83.87±7.77	23.53±2.26	-
DASA 68056	*B. elkanii*	Saraburi	0	0	299.20±28.74	9.38±0.68	n.d[d]
DASA 68003	n.d.	Lampang	0	0	155.07±8.71	8.79±0.54	-
DASA 68010	n.d.	Lampang	0	0	63.07±13.53	11.34±0.51	-
DASA 68030	n.d.	Lampang	0	0	110.53±24.83	5.52±0.44	-
DASA 68032	n.d.	Lampang	0	0	120.13±15.64	6.28±0.82	-
DASA 68071	n.d.	Lampang	0	0	108.93±9.08	11.36±2.06	n.d[d]
DASA 68055	n.d.	Saraburi	0	0	263.85±17.02	10.81±0.81	-
DASA 68058	n.d.	Saraburi	0	0	281.64±28.95	10.85±2.63	-
DASA 68062	n.d.	Saraburi	0	0	90.93±12.03	11.26±0.42	-
USDA 205	*S. fredii*	USDA collection[b]	n.d.	4 (57, 112, »195, >600)	n.d.	n.d.	n.d.
USDA 290	*B. japonicum*	USDA collection	n.d.	n.d.	588.87±50.89	n.d.	n.d.
HB 101	*E. coli*		n.d	n.d	37.05±12.64	n.d.	n.d.

n.d.: not determined

[a]Related genus group was based on 16S rRNA gene partial sequence (approx. 500 bp)

[b]USDA collection: USDA-ARS *Rhizobium* Culture Collection (Beltsville, MD)

[c]The values shown are the mean of 3 replicates±standard deviation

[d]n.d.: not determined due to the strains were sensitive to NaCl, $CuSO_4$ or L-tyrosine at the examining concentration

+, melanin production was observed; -, no melanin production was observed

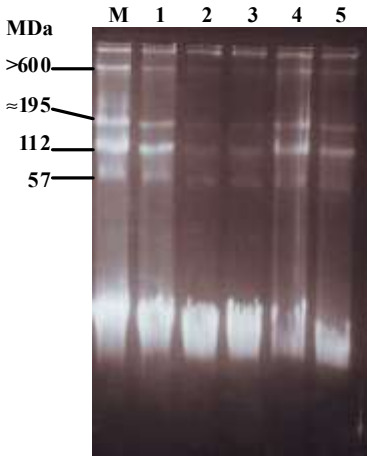

Fig. 2: Megaplasmid profiles of the selected strains. Lane M, USDA 205; 1, DASA 57010; 2, DASA 57027; 3, 68012; 4, DASA 68020; 5, DASA 68025.

examined a level of specificity among three arbitary primers and found that primer RAPD-2 could generate more discriminating patterns. Nimnoi and Pongsilp [37] examined the genetic relatedness amongst the IAA synthetic isolates employing RAPD and enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC PCR) and found that RAPD generated the slightly higher diversity than ERIC PCR. The results of Niemann *et al.* [1] also showed that RAPD discriminated slightly better among *Rhizobium meliloti* strains than ERIC PCR. The total 56 isolates belonging to distinct clusters of dendrograms were selected for further studies. These representative isolates generated the different RAPD patterns and therefore these isolates were identified as the individual strains. The representative strains and their geographical origins are listed in Table 2. The genera of 34 strains were determined by using partial sequencing of 16S rDNA. Among 34 strains, 4 strains nodultaing *I. tinctoria* showed 70% to 95% homology with *Pseudoalteromonas*. Partial *nif*H and *nod*C genes were amplified from a novel gammaproteobacterial symbiont *Pseudoalteromonas*-like strains (C. Leelahawong, A. Nuntagij, N. Teaumroong, N. Boonkerd and N. Pongsilp, unpublished). The further studies are required to identify the exact genus of these strains.

Plasmid Profiles: Among 56 representative strains, no small plasmid was found in 53 strains. The remaining 3 *Rhizobium*-like strains including DASA 64016 nodulating *P. mirifica* as well as DASA 68020 and DASA 68025 nodulating *D. elliptica* Benth. harbor the same profile with 2 small plasmids of 1,500 and 900 bp (Table 2). One *Sinorhizobium*-like strain DASA 68012 nodulating *D. elliptica* Benth. as well as 4 *Rhizobium*-like strains DASA 57010, DASA 57027, DASA 68020 and DASA 68025 nodulating *I. tinctoria* and *D. elliptica* Benth. harbor the same profile with 4 megaplasmids of 57, 112, ≈ 195 and > 600 MDa compared with the reference plasmids of *S. fredii* USDA 205 (Table 2). Mega plasmid profiles of these strains are shown in Fig 2.

Quantification of AHLs-like Molecules Production: The 56 representative strains were screened for their ability to produce AHLs-like molecules by using an *Agrobacterium tumefaciens* biosensor strain containing *tra*I-*lac*Z fusion. β-galactosidase activity were measured for the quantification of AHLs-like molecules. The strains produced different levels of AHLs-like molecules according to β-galactosidase activity. The class of the AHLs is the most common signaling compound (also referred to as autoinducer or quorum sensing molecule) which involves in cell-to-cell signaling within a microbial assemblage [38]. Quorum sensing has been shown to be important between root- and stem-nodule bacteria and their host plants and for the maintenance of viability, the synthesis of other AHLs and exopolysaccharides and plasmid transfer [29, 39-43]. The production of AHL-like inducers is widespread among root-nodule symbionts such as *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* [32], *Rhizobium* [29, 44], *Sinorhizobium* [45-46] and *Mesorhizobium* [47]. In this study, even though the highest activity was observed in 2 strains of *Bradyrhizobium elkanii*, the production of AHLs-like molecules was found to be varied from little to large amounts among strains even within strains belonging to the same related genus group and strains nodulating the same host plants (Table 2).

Quantification of IAA Production: The 56 representative strains varied in the ability to produce the plant hormone IAA ranging from 4.31±0.67 to 34.76±0.18 µg IAA equivalent/ml of culture (Table 2). Like AHLs-like molecules production, the IAA production was also found to be varied from little to large amounts among strains even within strains belonging to the same genus group and strains nodulating the same host plants.

Detection of Melanin Production: The dark brown pigment representing melanin production was observed in the only one strain DASA 57003 (Table 2 and Fig 3.). The strain DASA 57003 was related to *Pseudoalteromonas* based on 16S rRNA gene partial sequence (approx. 500 bp) (C. Leelahawong, A. Nuntagij, N. Teaumroong, N. Boonkerd and N. Pongsilp, unpublished). The further characterizations are required to identify the genus of this strain.

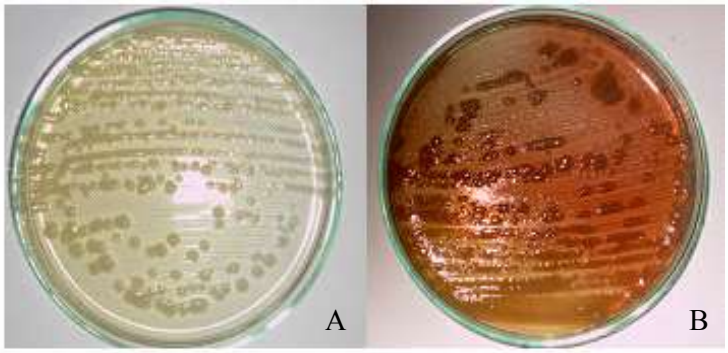

Fig. 3: Melanin production of *Pseudoalteromonas*-like strain DASA 57003. A, negative strain (DASA 57053); B, positive strain (DASA 57003).

The melanin production was found to be restricted to *Rhizobium leguminosarum* because none of 19 *Bradyrhizobium* strains could produce melanin [48]. The melanin production was also observed in *Rhizobium meliloti* [49] and some strains of alfafa-nodulating rhizobia [35]. The melanin production was not found in any of 21 *Rhizobium*-like strains, indicating that it does not commonly occur among strains of *Rhizobium* and may be restricted to the host plants.

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