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Differentiation of "*Candidatus* Liberibacter asiaticus" Isolates by Variable-Number Tandem-Repeat Analysis▽

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Four highly polymorphic simple sequence repeat (SSR) loci were selected and used to differentiate 84 Japanese isolates of "*Candidatus* Liberibacter asiaticus." The Nei's measure of genetic diversity values for these four SSRs ranged from 0.60 to 0.86. The four SSR loci were also highly polymorphic in four isolates from Taiwan and 12 isolates from Indonesia.

Citrus greening (Huanglongbing) is one of the most devastating citrus diseases prevalent in many parts of the world. This disease is a major cause of yield and tree losses in Asia and Africa (8). This disease was first noted in southern China at the end of the 19th century, and it was known as yellow shoot disease in this region (35). By the 1920s, diseases similar to yellow shoot disease were recorded in Taiwan (likubin or drooping disease) (25) and India (citrus dieback) (4). The disease was first recorded in Indonesia in the 1940s and was described as vein phloem degeneration (31). The causal agents, which are phloem-limited and Gram-negative bacteria, belong to the genus "*Candidatus* Liberibacter." Thus far, three species of this organism have been identified: "*Ca.* Liberibacter africanus" is found mainly in African countries, "*Ca.* Liberibacter americanus" is found in Brazil (3), and "*Ca.* Liberibacter asiaticus" is widely found in Asian countries as well as in Sao Paulo (Brazil) and Florida (United States). The pathogens are transmitted mainly by the psyllids *Trioza erytreae* in Africa (2) and *Diaphorina citri* in Asia, Florida, and Sao Paulo (12). Contaminated plant materials used for the propagation of nursery plants also transmit these pathogens.

In Japan, this disease was first found in 1988 on Iriomote Island, the southernmost island in the chain of the Ryukyu Islands that stretch near Taiwan in the subtropical East China Sea (21) (Fig. 1). The disease apparently moved northward through the Ryukyu Islands, being recognized on Okinawa Main Island in 1994 (16), Yoron Island in 2002 (13), and Okinoerabu, Tokunoshima, and Kikai Islands in 2003 (28) (Fig. 1). Extensive field surveys by local governments revealed about 1,200 infected trees around these areas (22, 28). The transmission vector *D. citri* was distributed throughout the Ryukyu Islands, and "*Ca*. Liberibacter asiaticus"-positive psyllids were found on most of these islands (24). Kyusyu, which is the main citrus production area, is located at the north of these subtropical islands. Northbound dispersion of the disease poses a large threat to citrus cultivation in this region.

Methods for distinguishing bacterial isolates are important for epidemiological analysis and understanding the genetic structure of microbial populations. Simple sequence repeat (SSR) markers, also known as microsatellites, are tandem repetitive DNA sequences with repeat motif lengths of 2 to 6 bp or more (33). The variability of the repeats is believed to be caused by slipped-strand mispairing (29), the genetic instability of polynucleotide tracts, especially poly(G-T) (14), and DNA recombination between homologous repeat sequences (33). SSRs with a potential variable number of tandem repeats (VNTR) in bacterial DNA have been used as markers for differentiating and subtyping strains of several bacterial species, including *Yersinia pestis* (1), *Haemophilus influenzae* (15), *Mycobacterium tuberculosis* (11, 18), *Mycobacterium africanum* (34), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (20), *Bacillus anthracis* (17), and *Xylella fastidiosa* (7, 19). Strains of *X. fastidiosa* cause serious diseases, such as Pierce's disease of grapevine and variegated chlorosis of citrus (5). The multilocus SSR primers, distributed across the *X. fastidiosa* genome, clearly differentiated and clustered *X. fastidiosa* strains collected from grape, almond, citrus, and oleander (19).

Recently, Chen et al. applied a similar strategy to characterize the variation in "*Ca*. Liberibacter asiaticus" strains from Guangdong, China, and Florida by using one repeat unit (AGACACA) (6). However, VNTR analysis using only one SSR locus is apparently insufficient to reveal the precise genetic diversity of "*Ca*. Liberibacter asiaticus," especially in newly invaded areas, such as Japan and the United States, where less genetic variation is expected. The complete genomic sequence of the pathogenic "*Ca*. Liberibacter asiaticus" psy62 strain (1.23 Mb) (9) was determined, and this allowed the analysis of SSRs in the entire genome. The objectives of this study were to identify SSR loci with VNTR within Japanese, Taiwanese, and Indonesian isolates and to determine genetic

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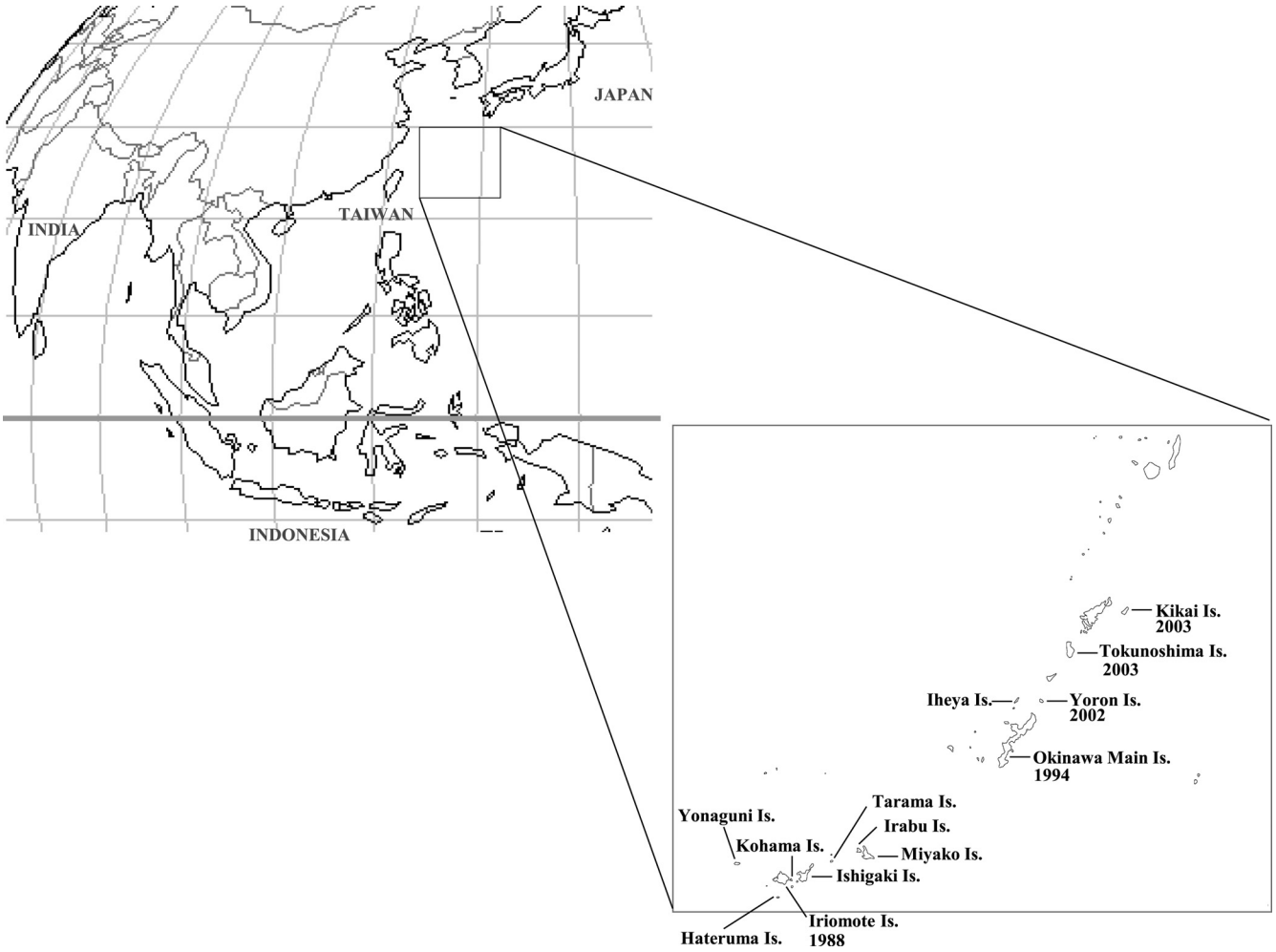

FIG. 1. Spread of citrus greening disease in Japan. Maps of the Ryukyu Islands and the world were downloaded from free map websites (http://www.freemap.jp/japan/ja_island1.html and http://www.craftmap.box-i.net/map.php, respectively). The numbers under the island names indicate the year when citrus greening disease was found on each island.

diversity among approximately 100 isolates of "*Ca*. Liberibacter asiaticus," collected from a total of about 1,200 trees found in major infested sites in the Ryukyu Islands (22, 28), by using several SSR regions. Comparison was also made with isolates from Taiwan and Indonesia. We investigated the relationship between genetic diversity and the geographic origin of the isolates on the Ryukyu Islands.

A genome-wide search was performed on the complete sequence of "*Ca*. Liberibacter asiaticus" to identify SSR loci by using the Tandem Repeats Finder software, version 2.0 (7), which is available from the Tandem Repeats Finder website (http://tandem.bu.edu/trf/trf.html). The complete genomic sequence (1.23 Mb) of the pathogenic "*Ca*. Liberibacter asiaticus" strain psy62 (accession number CP001677) was obtained from the GenBank DNA database. Samples were collected from "*Ca*. Liberibacter asiaticus"-infected citrus trees in different groves in Japan, Taiwan, and Indonesia (Fig. 1 and Table 1). Total DNA was extracted from the leaf midrib tissue from the infected citrus tree using the DNeasy plant minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions with minor modifications, which was that ~0.2 g of the leaf midrib was placed in 400 μl of AP1 buffer (in kit) in a mortar and ground with a pestle until the leaf midrib became a fine green liquid.

All primers in Table 2 were selected and designed from the sequences of surrounding SSRs found in the complete sequence of the pathogenic "*Ca*. Liberibacter asiaticus" psy62 (1.23 Mb) strain by using a program available on the Primer3 website (http://frodo.wi.mit.edu/primer3/). PCR was performed using GeneAmp PCR system 9700 (Applied Biosystem, Foster City, CA) in 20-μl reaction mixture volumes containing 1 μl of DNA template, 0.1 μM each primer, 200 μM deoxynucleoside triphosphate (dNTP) mixture, 1× PCR buffer, and 2.5 units of *Ex Taq* DNA polymerase, Hot Start version (TaKaRa, Shiga, Japan). The thermal cycling conditions were as follows: initial denaturation at 92°C for 2 min and 35 cycles of denaturing at 92°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min.

Amplified PCR products were separated by electrophoresis in a 1.5% (wt/vol) agarose gel in Tris-boric acid EDTA buffer. The PCR products were extracted from the gel slice by using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

TABLE 1. Isolates of "*Candidatus* Liberibacter asiaticus" used in this study and comparison of the repeat numbers at respective SSR regions

Island or country	Isolate	Code	Location	Yr of collection	VNTR			
					001	002	005	077
Kikai Island	Kikai-130	Hm1	Osato, Kikai, Kagoshima	2006	15	7	11	9
	Kikai-145	Hm2	Osato, Kikai, Kagoshima	2006	15	7	11	9
	Kikai-147	Hm3	Osato, Kikai, Kagoshima	2006	15	7	11	9
	Kikai-269	Hm4	Osato, Kikai, Kagoshima	2007	15	7	11	9
	Kikai-301	Hm5	Osato, Kikai, Kagoshima	2007	15	7	11	9
	Kikai-318	Hm6	Osato, Kikai, Kagoshima	2007	15	7	11	9
	Kikai-323	Hm7	Osato, Kikai, Kagoshima	2007	15	7	11	9
Tokunoshima Island	Toku-225	Hm8	Kinen, Isen, Tokunoshima, Kagoshima	2006	8	7	5	9
	Toku-228	Hm9	Kinen, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-229	Hm10	Kinen, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-230	Hm11	Kinen, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-231	Hm12	Kinen, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-232	Hm13	Kinen, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-233	Hm14	Kinen, Isen, Tokunoshima, Kagoshima	2006	14	7	5	9
	Toku-234	Hm15	Nishi-metegu, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-235	Hm16	Higashi-metegu, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-236	Hm17	Higashi-metegu, Isen, Tokunoshima, Kagoshima	2006	12	7	5	8
	Toku-237	Hm18	Higashi-metegu, Isen, Tokunoshima, Kagoshima	2006	12	7	5	8
	Toku-238	Hm19	Higashi-metegu, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-239	Hm20	Higashi-metegu, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-240	Hm21	Saben, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-241	Hm22	Saben, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
	Toku-244	Hm23	Saben, Isen, Tokunoshima, Kagoshima	2006	12	7	5	9
Yoron Island	Yoron-57	H1	Yoron, Kagoshima	2002	12	8	10	9
	Yoron-83	H2	Yoron, Kagoshima	2002	16	7	15	9
	Yoron-121	H3	Yoron, Kagoshima	2002	16	7	11	9
	Yoron-127	H4	Yoron, Kagoshima	2002	15	7	9	9
Iheya Island	Iheya-2	K13	Iheya, Okinawa	2007	18	7	11	10
Okinawa Main Island	OgimiA-3	K20	Ogimi, Okinawa	2007	12	7	12	9
	Nakijin-5	K18	Nakijin, Okinawa	2007	14	6	12	9
	MotobuB-1	K16	Motobu, Okinawa	2007	15	7	11	9
	HigashiA-3	K19	Higashi, Okinawa	2007	11	7	13	9
	Nago-Nc-1	K14	Nago, Okinawa	2007	15	7	12	9
	Nago-4	K15	Nago, Okinawa	2007	15	7	12	9
	Kin2-1	K17	Kin, Okinawa	2007	14	6	12	10
	KIN-3	Ns1	Kin, Okinawa	2007	15	7	13	8
	KIN-1	Iw2	Kin, Okinawa	1994	14	7	11	9
	Uruma1-1	K21	Gushikawa, Uruma, Okinawa	2007	13	7	8	9
	UrunaKA-5	K22	Katsuren, Uruma, Okinawa	2007	15	7	11	9
	Ishi-2	Ns2	Uruma, Okinawa	2007	15	7	15	9
	A-17	K23	Okinawa, Okinawa	2007	12	7	12	9
	A2-12	K24	Okinawa, Okinawa	2007	16	7	13	9
	B-8	K25	Okinawa, Okinawa	2007	7	8	8	8
	A-11	K26	Tomigusuku, Okinawa	2007	13	7	13	9
	C-3	K27	Itoman, Okinawa	2007	18	6	6	9
	A-3	K28	Naha, Okinawa	2007	12	7	6	9
	Hae-5	K29	Haebaru, Okinawa	2007	17	7	11	9
	Ishi-4	Iw5	Okinawa, Okinawa	2005	14	7	9	9
	KO-7	K30	Yaese, Okinawa	2007	15	7	11	9
Miyako Island	08GA-5	08M1	Jobe, Miyakojima, Okinawa	2008	15	11	10	8
	08G-3	08M2	Irie, Miyakojima, Okinawa	2008	15	10	9	11
	08U-1	08M3	Ueno, Miyakojima, Okinawa	2008	14	10	11	8
	08U-2	08M4	Nohara, Miyakojima, Okinawa	2008	15	10	9	8
	08U-3	08M5	Ueno, Miyakojima, Okinawa	2008	17	8	5	7
	08GB-3	08M6	Jobe, Miyakojima, Okinawa	2008	14	8	4	7
	06S-2-2	06M3	Shimoji, Miyakojima, Okinawa	2006	15	10	9	7
	06S-2-3	06M4	Shimoji, Miyakojima, Okinawa	2006	18	11	9	7
	06S-2-5	06M5	Shimoji, Miyakojima, Okinawa	2006	16	11	9	7
	06G-3	06M10	Jobe, Miyakojima, Okinawa	2006	12	8	6	7
	06G-4	06M11	Jobe, Miyakojima, Okinawa	2006	15	11	12	7
	S-2-4	K1	Shimoji, Miyakojima, Okinawa	2006	19	10	9	8
	H-3	K3	Hiraya, Miyakojima, Okinawa	2006	14	8	11	8
	U-4	K4	Ueno, Miyakojima, Okinawa	2006	15	10	4	8
Irabu Island	06I-5	06M9	Irabu, Miyakojima, Okinawa	2006	16	10	6	7
	I-1	K2	Irabu, Miyakojima, Okinawa	2006	14	11	10	8
Tarama Island	Tarama-12	K12	Trama, Okinawa	2006	16	8	6	7
	MT-3	Mt3	Trama, Okinawa	2008	15	9	4	9
	MT-4	Mt4	Trama, Okinawa	2008	8	7	4	8
	MT-6	Mt6	Trama, Okinawa	2008	9	6	4	8
	MT-7	Mt7	Trama, Okinawa	2008	9	6	4	8
	MT-8	Mt8	Trama, Okinawa	2008	9	6	4	8

Continued on following page

TABLE 1—*Continued*

Island or country	Isolate	Code	Location	Yr of collection	VNTR			
					001	002	005	077
	MT-9	Mt9	Trama, Okinawa	2008	8	6	4	8
	MT-10	Mt10	Trama, Okinawa	2008	16	8	6	7
	MT-11	Mt11	Trama, Okinawa	2008	23	10	6	7
	MT-12	Mt12	Trama, Okinawa	2008	9	6	4	7
Ishigaki Island	Ishi-1	Iw3	Ishigaki, Okinawa	2005	14	7	8	9
	Hirakubo-5	K5	Hirakubo, Ishigaki, Okinawa	2007	19	9	6	9
	Hirano-4	K6	Hirano, Ishigaki, Okinawa	2007	14	7	12	10
	Kawahara-4	K7	Kawahara, Ishigaki, Okinawa	2007	17	8	5	8
	Hirae-1	K8	Hirae, Ishigaki, Okinawa	2007	22	7	5	9
Iriomote Island	OK-901	Iw1	Iriomote, Taketomi, Okinawa	1988	14	7	11	9
Kohama Island	Kohama-4	K10	Kohama, Taketomi, Okinawa	2007	13	10	17	8
Yonaguni Island	Higawa-1	K11	Higawa, Yonaguni, Okinawa	2007	16	11	10	8
Hateruma Island	Hateruma-1	K9	Hateruma, Taketomi, Okinawa	2007	13	10	12	11
Taiwan		II-2	Pingting	2006	11	10	7	9
		II-5	Douliu	2006	25	11	5	13
		II-6[a]	Pingting	2006	9, 22, 26, 27, 28	9	6	13
		II-7	Hualian	2006	11	10	21	8
Indonesia	1	Pum 12	Magetan	2007	23	8	9	10
	9	Pum 3	Magetan	2007	18	7	6	10
	11	EJ5-1	Magetan	2007	25	7	2	9
	17	Pum 8	Magetan	2007	29	8	11	10
	4	Pu1	Purworejo	2007	23	10	8	9
	5	ND5	Purworejo	2007	29	7	9	10
	7	Pu3	Purworejo	2007	29	7	9	10
	16	Pu2	Purworejo	2007	11	8	7	11
	18	ND3	Purworejo	2007	23	7	8	6
	25	P1-9-4	Purworejo	2007	23	7	2	10
	12	KIT-3	Kintamani	2007	24	8	8	10
	13	B3T3	Buleleng	2007	14	9	7	11

[a] Doublet bands were observed when the 001 primer set was used.

The nucleotide sequence of the DNA fragment was obtained by directly sequencing both strands of the purified PCR products by using the dideoxynucleotide triphosphate (ddNTP) termination method (26). DNA sequences were aligned using the ClustalW program (30), and homology analysis was performed following instructions from the website of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/Welcome-j.html). The number of repetitions in each SSR was manually counted from the aligned sequence data.

No one- or two-base SSRs were found, but there were 27 perfect SSRs with four to 63 nucleotides per unit (Table 2), including a previously reported repeat motif (AGACACA) (6). Typically, four-nucleotide SSRs were present at six loci with copy numbers varying from three to eight copies per repeat.

For DNA polymorphism analysis of the SSR regions, we designed primers on each side of these 27 SSRs (Table 2). First, amplification using all SSR primers was performed in nine "*Ca*. Liberibacter asiaticus" isolates collected from Miyako Island because we obtained many isolates from this island, which also has a long history of invasion by "*Ca*. Liberibacter asiaticus" (22). Attempts to amplify SSR regions using three SSR primer sets (081, 083, and 091) failed, although several different amplification programs and reaction mixtures were utilized. The 078 primers generated the same PCR products for the nine domestic isolates, even though these repeat numbers were different from American psy62. On the other hand, the repeat sequences generated by 093 primers were $(TCGTTACGCT)_3$ (psy62) and $(ACGCTTCATC)_3$ (Japanese isolates) (subscript 3 indicates the number of repetitions for each motif in the genome). Although the 006, 007, 010, 013, 014, 022, 024, 080, 082, 084, 085, 086, 087, 089, 090, and 092 primers generated the same PCR products for the nine isolates from Japan, five pairs of primers (001, 002, 005, 077, and 088) generated different results for the nine isolates and thus appear to represent genuine VNTRs. When 088 primers were used, polymorphic PCR products were generated for Japanese isolates. However, we did not consider 088 as a VNTR because the motif was imperfect and appeared only a few times. Therefore, we investigated the diversity of "*Ca*. Liberibacter asiaticus" within a set of 84 isolates in the Ryukyu Islands, Japan, as well as four and 12 isolates from Taiwan and Indonesia, respectively, using four pair of primers (001, 002, 005, and 077).

Table 1 shows the variable numbers of tandem repeats in the four SSR loci. SSR loci amplified by four pairs of primers (001, 002, 005, and 077) had different repeat numbers within a set of 84 isolates in the Ryukyu Islands, Japan, as well as four and 12 isolates from Taiwan and Indonesia, respectively. Doublet bands were consistently observed when the 001 primer set was used with Taiwanese source II-6. The PCR product was then subcloned into the plasmid vector pCR4-TOPO (Invitrogen, Tokyo, Japan), and sequencing of the inserts from multiple clones revealed several lengths of SSRs (Table 1). This indicates the presence of five alleles for the same SSR locus, presumably due to mixed infection with five isolates. In particular, 12 alleles in VNTR locus 001, six alleles in VNTR locus 002, nine alleles in VNTR locus 005, and five alleles in VNTR locus 077 were confirmed among isolates spread in the south-

TABLE 2. Characteristics of SSR primer sequences produced and used to study "*Ca.* Liberibacter asiaticus" bacteria[a]

SSR primer	Forward primer sequence	Reverse primer sequence	Type of repeat motif[b]	ORF definition[c]	Locus location in genome
001	TGAAGTAGCTCTGCAATATCTGA	GGTGAATTAGGATGGAAATGC	$(TACAGAA)_8$	Noncoding	255591–255646
002	TTGATAATATAGAAAGAGGCGAAGC	TCCATACCCAAAAGAAAAGCA	$(CAGT)_8$	Noncoding	537729–537760
005	ATTGAAGGACGAAACCGATG	TCCCAAGGTTTTCAAATTGC	$(AGACACA)_5$	Bacteriophage repressor protein C1	354493–354527
006	TCATGTTGATCAGACGCTTTTT	CACTTAATAACGCCCCGAAA	$(TCTTTACA)_3$	Noncoding	684193–684216
007	TGGATAGCATGCTCATTTGAA	AAGGCAAATTTCCCCATACG	$(TCAGTA)_3$	Cell division protein	670617–670634
010	CGTCAGAATAATCAGCGCATA	TGGATTCGAAAG AACCGTCT	$(CAAT)_3$	Noncoding	950213–950224
013	AGATTGATGGGCGATAGCTG	TGTCGCATTGTAGACCCTGA	$(TAACTTG)_2$	Noncoding	747800–747813
014	AATCCCTTGCTCGTA GGTGA	AAAGATAAGCGACCCGGATT	$(TAAAGAG)_2$	Aminodeoxychorismate lyase	748463–748476
022	AATCCCTTGCTCGTA GGTGA	ATTTGAGCCGTGAAACTTCG	$(AAAC)_3$	Conserved hypothetical protein	2063–2074
024	GTGGGGAGAGAAGTCGGTTT	ACCGTACCGCTCCAATATGA	$(TTGG)_3$	SNF2	1193755–1193766
077	TGACTGATGGCAAAAGATGG	AGACACGCCAAACAAGGAAT	$(TTTG)_{14}$	Noncoding	655277–655332
078	CCCCCAGAACTTCATTTTTC	GAGGCAATACGTCCATCGTT	$(TTTTAA)_3$	Noncoding	360549–360566
079	GGCGCACTCAGCATCTAAA	TCGCCTTTCGCAATACTTCT	$(ATTG)_3$	Predicted GTPase	405894–405905
080	TCCGATGCGTCTAGTTGTTG	GCCGGATTCATAATGACCTT	$(TTTTTA)_2$	Predicted membrane protein	444036–444047
081	TGAGAAAATTTCGCGATAAAAA	TGCTTTCGCATAACATTAGCA	$(TTTTTA)_3$	Noncoding	444507–444524
082	GGATTATAGCGACGCTGGTT	GAAGCAGCTGGAGAAGTCGT	$(TTAAT)_5$	Noncoding	535168–535192
083	AATCCTGCCAAGGTTGATTG	TACGCCTGTATCGCATGGTA	$(TCAGTCTTGTGCGTTCAATGT)_2$	Conserved hypothetical protein	576776–576817
084	GGAAGAACGTTTCCAAGCTG	AATTGTGTCGCGAGTCTGTG	$(TGTCCATATGATCTTCGATAATGCGAG)_2$	Noncoding	698567–698620
085	TTTCAGGGCAAGATAGCACA	ATGCTTCGAAGAGCACATTG	(TCTTTTTGCTATTTTTAGTAAATAAAGCGTTTAGAT ATTTATTAAAAAGTTGATGTTACCAAG)$_2$	Noncoding	803665–803790
086	TGGTTTGTGATGGCGATAAA	ATGAGGTCGAAATCCATCCA	$(TTTTATCGGTCCA)_2$	Noncoding	974568–974593
087	TTGCTTTCGCATCATACAGG	CTGTTGGTGGAAGTGGAGGT	$(ATTTATTTTTT)_2$	Noncoding	982762–982783
088	CGTTGGGATATCTGACCACA	TGCTAGCAGGCTATCTTTGGA	(ATCAGGCAGGTTTTCTATTGCAATATCGATCTCAC TAGCTTGATGGTTTGATTTCAA)$_2$	Conserved hypothetical protein	1009710–1009823
089	AGGGGTGTTTCTGTCGTTTTT	CAGAGGCCATGAGAACGATT	$(TGTTACATTC)_2$	Noncoding	1188378–1188397
090	TTGAGGCAAGCCATACAAAA	GGTTGATGGCTTCACTGCTT	$(TCGCAAATGTACGTATAGAAG)_2$	Guanylate kinase	1196101–1196142
091	TCACGTAGATTGGCACTTCG	AAACGGAAGATGTTGGTCGT	$(CTCTAGTGTCATCAA)_2$	Conserved hypothetical protein	1197512–1197541
092	AAGGGAGCCCTAAACCAAAA	GGGGGATAAGTCGGATGAGT	(TCCTTATCCGCTTTCTCTCTGTCGGCTTTTTCTTTA GCT)$_2$	Methyl-accepting chemotaxis protein	1208243–1208320
093	GCCACTTTGGGGTAGCAGTA	AGAAAAGCCCCAAAAAGACC	$(TCGTTACGCT)_2$	Noncoding	1219080–1219099

[a] All data are based on the genome sequence of "*Ca.* Liberibacter asiaticus" strain psy62. The accession number is CP001677 (9). The primer sets that amplify the four most variable SSR loci (001, 002, 005, and 077) listed in Table 1 are underlined.

[b] Numerical subscripts indicate the number of repetitions for each motif in the genome of psy62.

[c] The open reading frame (ORF) definition refers to the gene that is adjacent to or contains the SSR locus.

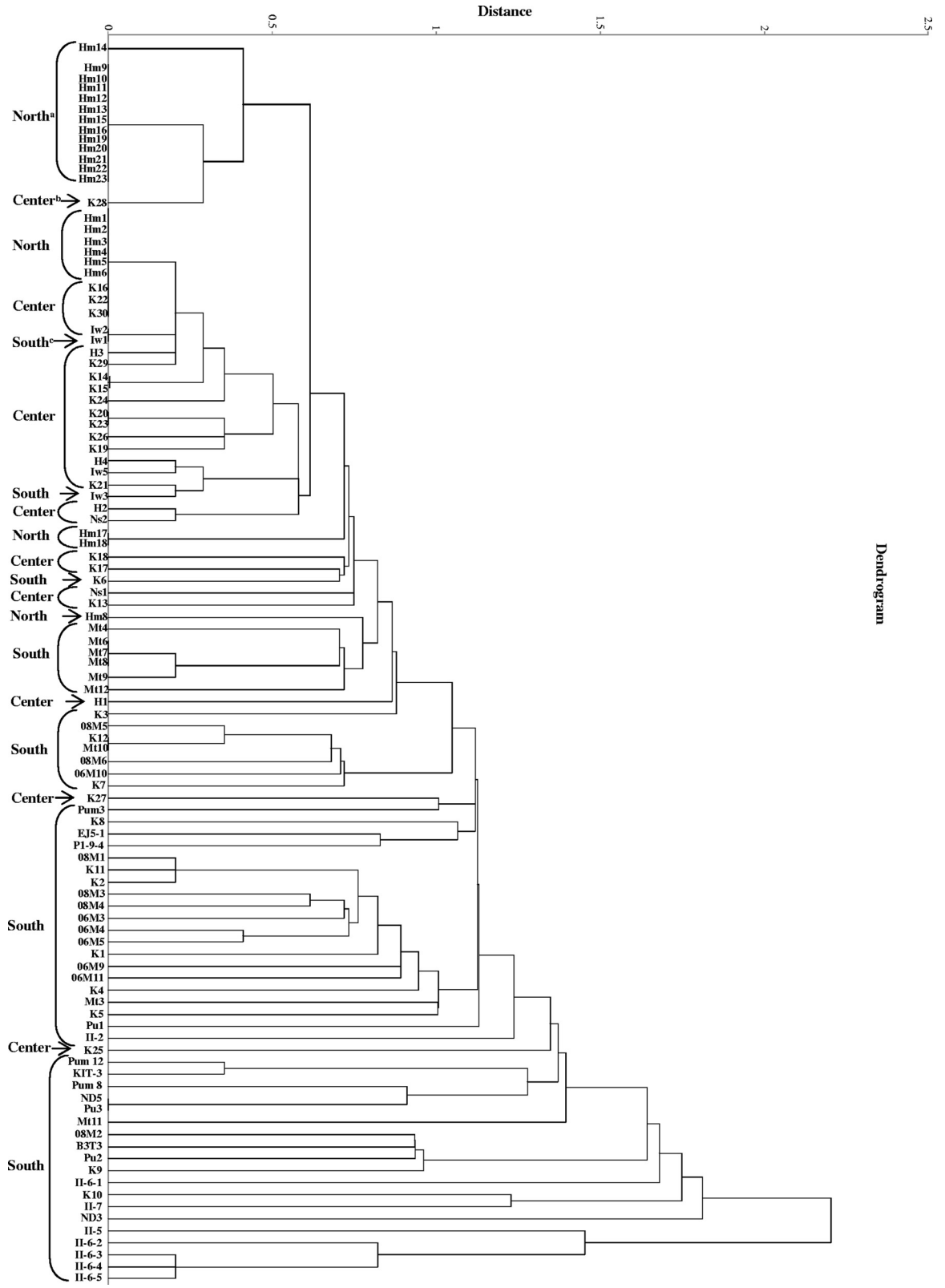

FIG. 2. Dendrogram of genetic similarity among 104 "*Ca*. Liberibacter asiaticus" isolates based on the unweighted paired-group method using arithmetic averages cluster analysis of data from four VNTR loci (001, 002, 005, and 077). Superscript letters: a, isolates originated from Kikai and Tokunoshima Islands; b, isolates originated from Yoron, Iheya, and Okinawa Main Islands; c, isolates originated from Miyako, Irabu, Tarama, Ishigaki, Kohama, Iriomote, Hateruma, and Yonaguni Islands and isolates originated from Taiwan and Indonesia.

ern parts of the Ryukyu Islands (Miyako Island, Irabu Island, Tarama Island, Ishigaki Island, Kohama Island, Iriomote Island, Hateruma Island, and Yonaguni Island) near Taiwan (Table 1); these findings suggested that the four VNTR loci are diverse among these isolates.

Tomimura et al. estimated the genetic diversity among "*Ca*. Liberibacter asiaticus" isolates by sequencing a bacteriophage-type DNA polymerase region (32). The 3,610-nucleotide sequence of the bacteriophage-type DNA polymerase region was analyzed for 27 isolates (32). Among 27 isolates, 86 single nucleotide polymorphisms (SNPs) were found (32). In contrast, among approximately 100 isolates used in this study, no nucleotide differences were observed in the genomic region surrounding four VNTRs (001, 002, 005, and 077) (data not shown), suggesting that VNTR could differentiate isolates of "*Ca*. Liberibacter asiaticus" more precisely than SNPs.

The unweighted paired-group method using arithmetic averages cluster analysis was performed with AEW3220DA (Nihon NAG, Tokyo, Japan) by using SSR numbers of the four VNTR loci, 001, 002, 005, and 077. Since Taiwanese source II-6 had five alleles at locus 001 and one allele at 002, 005, and 077, it was treated as five isolates in the dendrogram analysis. The resulting clusters were expressed as a dendrogram. Cluster analysis of genetic distance divided the 104 isolates into 10 major clusters (Fig. 2). These clusters were correlated with geographical origins of the isolates (Fig. 2).

Twenty-one isolates from Okinawa Main Island had nine alleles in VNTR locus 001, three alleles in VNTR locus 002, seven alleles in VNTR locus 005, and three alleles in VNTR locus 077. On the other hand, for all seven isolates from Kikai Island, which is located on the northern border of the Ryukyu Islands, none of the four loci showed polymorphism (Table 1), suggesting that these seven isolates are highly homologous. Kikai Island is located on the northern border of the Ryukyu Islands and is also the last island involved in the recent outbreak of "*Ca*. Liberibacter asiaticus" in Japan (28). The homogeneity of "*Ca*. Liberibacter asiaticus" in Kikai Island is in accordance with the apparent short incubation period of the bacterium on this island. Isolates with the same repeat numbers as the four VNTR loci were not found in the neighboring islands of Kikai Island. The three isolates (K16, K22, and K30) collected from Okinawa Main Island had the same number of tandem repeats in each of the four loci as the seven isolates collected from Kikai Island (Table 1), which indicated that the isolates from these two islands share the same origin. Okinawa Main Island and Kikai Island are separated by approximately 270 km and several islands. It is more likely that "*Ca*. Liberibacter asiaticus" was introduced into Kikai Island by contaminated budwood rather than by dispersion of "*Ca*. Liberibacter asiaticus"-positive psyllids.

Nei's measure (*H*) is useful to compare genetic diversity among biological populations, and it is frequently applied for VNTR loci of bacteria (1, 7, 17). The value was calculated as $H = 1 - \Sigma pi^2$, where *pi* is the frequency of allele *i* at the locus (23). VNTR typing of *B. anthracis*, *Y. pestis*, and *X. fastidiosa* has been shown to produce the highest *H* values, of 0.80, 0.82, and 0.83, respectively (1, 7, 17). The *H* value of VNTR locus 005 within 84 Japanese isolates from the Ryukyu Islands was 0.86 (Table 3), which was the highest among the four VNTR loci, closely followed by the *H* value of VNTR locus 001 (Table 3). All four VNTR loci (001, 002, 005, and 077) were also highly variable within four and 12 isolates from Taiwan and Indonesia, respectively (Table 1). In the analysis of Japanese "*Ca*. Liberibacter asiaticus" isolates, the population in the southern area had higher *H* values than those from the central and northern areas of Ryukyu Islands (Table 3). These results showed that the genetic diversity was higher in southern areas than in any other areas of the Ryukyu Islands, which suggested that Japanese "*Ca*. Liberibacter asiaticus" isolates were primarily introduced in the southern area, most probably from Taiwan. It is also surmised that the spread of the pathogen in the northern border region took place recently.

TABLE 3. Values of Nei's genetic diversity (*H*) for the variable-number tandem-repeat (VNTR) loci in 84 Japanese isolates of "*Ca.* Liberibacter asiaticus" bacteria from different areas

Location	*H* values for VNTR locus:			
	001	002	005	077
Northern area[a]	0.53	0.00	0.42	0.16
Central area[b]	0.83	0.33	0.83	0.27
Southern area[c]	0.87	0.80	0.84	0.67
Whole area[d]	0.84	0.62	0.86	0.60

[a] *H* values for the SSR loci in 23 isolates originated from Kikai and Tokunoshima Islands.
[b] *H* values for the SSR loci in 26 isolates originated from Yoron, Iheya, and Okinawa Main Islands.
[c] *H* values for the SSR loci in 35 isolates originated from Miyako, Irabu, Tarama, Ishigaki, Kohama, Iriomote, Hateruma, and Yonaguni Islands.
[d] *H* values for the SSR loci in all 84 Japanese isolates of "*Ca.* Liberibacter asiaticus."

On the basis of the four VNTR markers found in this study (001, 002, 005, and 077) the 21 isolates from Okinawa Main Island were differentiated into 17 genetic groups (Table 1), whereas on the basis of a single VNTR marker that was previously reported (6), the isolates were divided into only seven genetic genotypes. The results suggested that the analysis using several VNTR loci, rather than a single VNTR locus, reveals genetic diversity more precisely. We have reported for the first time that several SSR regions in the genome of "*Ca*. Liberibacter asiaticus" are genuine VNTR loci, and these VNTR markers could be used to estimate the genetic diversity and population structures of "*Ca*. Liberibacter asiaticus" in Japan, Taiwan, and Indonesia.

The growing numbers of prokaryotic DNA sequences, including those from plant pathogens in databases and computer programs available for the detection of SSR loci, have facilitated the evaluation of SSR within DNA sequences. SSR markers are useful not only for their hypervariability and reproducibility, but "*Ca*. Liberibacter asiaticus"-specific primers allow *in situ* analysis of a known gene without bacterial isolation. This approach could greatly facilitate epidemiological, genetic, and ecological studies of fastidious bacteria, such as "*Ca*. Liberibacter asiaticus," which are difficult to isolate and grow stably despite the recent advances in cultivation (10, 27).

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