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Genetic Diversity of Zingiberaceae Plant Isolates of *Ralstonia solanacearum* in the Asia-Pacific Region

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Abstract

The genetic diversity of *Ralstonia solanacearum* strains isolated from Zingiberaceae plants in the Asia-Pacific region was assessed by examining their biochemical properties, discriminating the phylogeny by polymerase chain reaction (PCR), and analyzing the *egl* and *mutS* gene sequences. These data were compared with those of reference strains covering the known diversity within the *R. solanacearum* species complex. Fifty-two of the Zingiberaceae plant isolates belong to either biovar 3 or biovar 4. Multiplex PCR analyses indicated that these strains belong to phylotype I. Phylogenetic analyses revealed that the investigated strains could be further divided into five or more groups and three major groups, based on the *egl* and *mutS* gene sequences, respectively. These groups were closely correlated with the host species and/or geographical origin. Our findings suggest that *R. solanacearum* strains affecting Zingiberaceae plants have multiple origins from within the Asia-Pacific region, and may have been disseminated with seed rhizomes.

Discipline: Plant disease
Additional key words: biovar, dissemination, endoglucanase, ginger, phylotype

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (Smith)[45] is among the most devastating bacterial diseases. *R. solanacearum* is widely distributed in tropical, subtropical, and warm temperate regions of the world, and can infect more than 200 plant species and 50 plant families[12]. This bacterial pathogen can cause serious economic damage to various crops[7], and the disease is very difficult to control.

R. solanacearum is a genetically and physiologically diverse bacterial pathogen. It has been divided into five races as based on host range differences, and six biovars as based on biochemical properties[6,10,14]. Strains that affect ginger (*Zingiber officinale* Rosc.) have been distinguished from other plant isolates and designated as race 4[6]; most of these strains have been classified as biovar 3 or biovar 4[13].

Recent DNA-based analyses have revealed that *R. solanacearum* is a species complex that can be divided into four subgroups (phylotypes), each corresponding to a separate species or subspecies[9]. Strains in phylotype I originate in Asia, whereas strains in phylotype II are predominantly from America. Phylotype III comprises strains from Africa and nearby islands, while phylotype IV includes strains from some Asian countries and Australia. Each phylotype is related to specific races and biovars. Phylotype II contains race 2 (affecting triploid banana and *Heliconia*)/biovar 1 and race 3 (primarily affecting potato)/biovar 2 strains, while phylotype I includes race 5/biovar 5 (affecting mulberry) strains.

R. solanacearum is known as a soilborne plant pathogen, but can also be transported over long distances on vegetative propagating material[12]. For example, the race 3/biovar 2 strain that primarily originates in South America (phylotype II) has been spread worldwide on latently infected potato tubers and/or cut flower seedlings[7,12,18,43]. Such long-range dispersal of the pathogen is likely to exacerbate a severe disease problem.

In Africa, Asia, and the Americas, Zingiberaceae plants (including ginger) are traditionally cultivated from rhizomes and used as condiments, for medicinal and ornamental purposes, and in the preparation of food. Bacterial wilt of

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Zingiberaceae plants has occurred in several countries or regions (e.g., Hawaii, Mauritius, some Asian countries, Australia)[11,13,15,21,25,27,33,36,37,39,46,48]. The situation is made worse by the use of infected rhizomes as planting material[12,21,25,34,38].

To avoid further damage to crop production and prevent the dissemination of strains, it is necessary to identify the origin of occurrence, clarify the pathogenic and epidemiological characteristics of isolates, and develop diagnostic methods effective against pathogens for plant quarantine and seed rhizome management.

Recently, sequence information on the *R. solanacearum* genome (e.g., rRNA, ITS, *hrpB*, *egl*, *mutS*, *gyrB*) has been rapidly accumulated, thereby becoming an efficient tool for the identification and discrimination of individual strains[2,8,23,24,30,31,32,40]. Phylogenetic analyses based on the sequence data of a specific gene (*egl*) have facilitated the division of worldwide strains into more than 50 groups (sequevars)[3,9,22,26,41,42,44]. However, to date, only limited analytical data are available on Zingiberaceae plant isolates of *R. solanacearum* because the bacterial wilt of specific host plants is considered an endemic problem, and given the limited collection of strains. In the present study, we assessed the genetic diversity and relations among *R. solanacearum* strains isolated from Zingiberaceae plants in the Asia-Pacific region, by analyzing *egl* (an encoding endoglucanase protein and one of the pathogenicity-related genes located in megaplasmid)[9] and *mutS* (an encoding methy-directed DNA mismatch repair protein and one of the housekeeping genes located in chromosome)[26,32], and thus showed the diversity of sequences types (haplotypes) within the same species[42]. We will discuss the implications of our data for plant protection.

Materials and methods

1. Bacterial strains, media, and culture conditions

Table 1 lists the *R. solanacearum* strains used in this study. Fifteen Japanese strains were from the culture collection of the National Institute of Agrobiological Sciences (NIAS) Genebank. Most strains from outside Japan were derived from the Research Institute of Spices and Medical Crops, Bogor, Indonesia, and the Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. All strains were maintained in long-term storage as suspensions in sterile distilled water or by freezing in a medium containing 10% skimmed milk supplemented with 1% sodium glutamate at -30°C, and then revived by plating on a triphenyltetrazolium chloride (TTC) medium[19] at 28°C. Typical bacterial colonies were grown in casamino acids-peptone-glucose broth[16] on a rotary shaker at 30°C for 24–48 h.

2. Biovar determination

R. solanacearum strains were classified into biovars based on their ability to oxidize or utilize a range of carbon sources (e.g., maltose, lactose, cellobiose, mannitol, sorbitol, dulcitol, trehalose), according to the method proposed by Hayward[10,14].

3. Pathogenicity test

Ginger plants (cv. Sanshu) were used for the pathogenicity test. Rhizomes were transplanted in plastic pots (9 cm in diameter) containing horticultural soil, and then grown in a greenhouse. To prepare inoculum, bacteria were grown on TTC agar plates for three days at 30°C, suspended in sterile distilled water, and then adjusted to ca. 10^8 cfu/ml. Plants at the seventh to ninth leaf stage were inoculated by puncturing the basal part of the stem with a needle dipped in inoculum. Symptoms of disease were then observed for three weeks after inoculation at 28°C.

4. DNA extraction

The genomic DNA of the *R. solanacearum* strain was extracted using the following protocol. Bacterial broth (1.5 mL) was centrifuged at 12,000 × *g* for 5 min., and the precipitate was re-suspended in 1 mL of sterilized Milli-Q water. The resulting suspension was heated at 100°C for 5 min., and then stored at -30°C until further use.

5. Multiplex PCR for strain identification

Multiplex polymerase chain reaction (PCR) was used to determine the phylotype of each investigated strain. Phylotype-specific primers (Nmult21:1F, Nmult21:2F, Nmult23:AF, Nmult22:InF, and Nmult21:RR) and *R. solanacearum* species universal primers (759 and 760) were used, according to the method proposed by Fegan & Prior[9]. The bands were amplified by PCR in a 25-μL reaction mixture containing 2.5 μL of 10× reaction buffer (supplied), 2 μL of dNTP mixture (200 μM/L each), 240 nmol/L of each primer, 0.5 U of Takara *Taq* DNA Polymerase (Takara Bio, Otsu, Japan), and 1 μL of extracted DNA. Amplifications were performed in an automated thermocycler (model 9700, Applied Biosystems, Foster City, CA, USA) with initial denaturation at 95°C for 5 min., followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 1 min. Final extension was performed at 72°C for 10 min. The amplified DNA was separated by 1.5% agarose gel electrophoresis in 0.5× Tris-acetate-EDTA (TAE) buffer. The gel was stained in ethidium bromide solution and photographed under UV light.

Strains were further tested using two PCR primer sets (AKIF-AKIR and 21F-21R), each specially amplifying a 165-bp and 125-bp band from race 4 strains representing type I or type II DNA fingerprints, respectively[17]. These

Table 1. *Ralstonia solanacearum* strains used in this study

Strain	Host	Origin	Year	Biovar	Phylotype	DNA type[1]	Sequevar	Accession number (reference)	
								egl	*mutS*
MAFF107639	*Zingiber officinale*	Tochigi, Japan	2009	3	I	I	n.i.[2]	AB620014 (TS)[3]	AB621641 (TS)
MAFF107640	*Z. officinale*	Tochigi, Japan	2009	3	I	I	n.i.	AB620015 (TS)	AB621642 (TS)
MAFF107641	*Z. officinale*	Tochigi, Japan	2009	3	I	I	n.i.	AB620016 (TS)	AB621643 (TS)
MAFF107642	*Z. officinale*	Tochigi, Japan	2009	3	I	I	n.i.	AB620017 (TS)	AB621644 (TS)
MAFF107643	*Z. officinale*	Tochigi, Japan	2009	3	I	I	n.i.	AB620018 (TS)	AB621645 (TS)
TG8-2	*Z. officinale*	Tochigi, Japan	2011	4	I	II	14	AB732947 (TS)	AB732952 (TS)
TG9-1	*Z. officinale*	Tochigi, Japan	2011	4	I	II	14	AB732948 (TS)	AB732953 (TS)
MAFF241651	*Z. officinale*	Kochi, Japan	2004	4	I	II	14	AB732950 (TS)	AB732954 (TS)
MAFF211471	*Z. officinale*	Kochi, Japan	1997	4	I	II	16	AY464998 (40)	AB621651 (TS)
MAFF211472	*Z. officinale*	Kochi, Japan	1997	4	I	II	16	AB678435 (TS)	AB678437 (TS)
MAFF211474	*Z. officinale*	Kochi, Japan	1997	4	I	II	16	AB678436 (TS)	AB732951 (TS)
MAFF211272	*Curcuma alismatifolia*	Kochi, Japan	1995	4	I	I	n.i.	AB508611 (24)	AB621652 (TS)
MAFF211479	*Z. officinale*	Kochi, Japan	1997	4	I	I	n.i.	AY464997 (40)	AB621653 (TS)
MAFF211490	*Z. mioga*	Kochi, Japan	2000	4	I	I	n.i.	AY465012 (40)	AB621650 (TS)
MAFF211493	*Z. mioga*	Kochi, Japan	2000	4	I	I	n.i.	AY465013 (40)	AB621654 (TS)
32	*Z. officinale*	Thailand	unknown	4	I	I	n.i.	AB621655 (TS)	AB621674 (TS)
1052	*Z. officinale*	Thailand	unknown	4	I	n.a.[4]	47	AB621656 (TS)	AB621675 (TS)
1294	*Z. officinale*	Thailand	unknown	4	I	I	n.i.	AB621657 (TS)	AB621676 (TS)
1445	*Curcuma* sp.	Thailand	unknown	3	I	I	n.i.	AB621659 (TS)	AB621678 (TS)
1446	*Curcuma* sp.	Thailand	unknown	3	I	I	n.i.	AB621660 (TS)	AB621679 (TS)
1447	*Curcuma* sp.	Thailand	unknown	3	I	I	n.i.	AB621661 (TS)	AB621680 (TS)
1448	*Curcuma* sp.	Thailand	unknown	3	I	I	n.i.	AB621662 (TS)	AB621681 (TS)
1478	*Z. officinale*	Thailand	unknown	3	I	I	n.i.	AB621658 (TS)	AB621677 (TS)
419B-1-I	*Z. officinale*	Thailand	1982	4	I	I	n.i.	AB621636 (TS)	AB621682 (TS)
419B-1-III	*Z. officinale*	Thailand	1982	4	I	I	n.i.	AB621637 (TS)	AB621683 (TS)
412C-1-I	*Z. officinale*	Thailand	1982	4	I	I	n.i.	AB621638 (TS)	AB621684 (TS)
Z8a	*Z. officinale*	China	unknown	4	I	II	16	AB621639 (TS)	AB621646 (TS)
Z8b	*Z. officinale*	China	unknown	4	I	II	16	AY465010 (40)	AB621647 (TS)
R277	*Z. officinale*	Australia	1965	4	I	II	16	AY465011 (40)	AB621648 (TS)
T447	*C. mangga*	Indonesia	1988	3	I	n.a.	17	AB678480 (TS)	AB678517 (TS)
T454-B	*C. domestica*	Indonesia	1988	3	I	n.a.	17	AB678481 (TS)	AB678518 (TS)
T585-98	*Z. officinale*	Indonesia	1992	3	I	n.a.	14	AB678485 (TS)	AB678522 (TS)
T625	*Z. officinale*	Indonesia	1992	3	I	n.a.	17	AB678486 (TS)	AB678523 (TS)
T625-98	*Z. officinale*	Indonesia	1992	3	I	n.a.	17	AB621663 (TS)	AB621685 (TS)
T736	*Z. officinale*	Indonesia	1993	3	I	n.a.	14	AB621664 (TS)	AB621686 (TS)
T740	*Z. officinale*	Indonesia	1993	3	I	n.a.	14	AB621665 (TS)	AB621687 (TS)
T741	*Z. officinale*	Indonesia	1993	3	I	n.a.	14	AB678487 (TS)	AB678524 (TS)
T748	*Z. officinale*	Indonesia	1993	3	I	n.a.	17	AB621666 (TS)	AB621688 (TS)
T749	*Z. officinale*	Indonesia	1993	3	I	n.a.	17	AB621667 (TS)	AB621689 (TS)
T871	*Z. arobaticum*	Indonesia	1996	3	I	n.a.	17	AB621668 (TS)	AB621690 (TS)
T874	*Z. cassumunar*	Indonesia	1996	3	I	n.a.	17	AB678488 (TS)	AB678525 (TS)
T874-98	*Z. cassumunar*	Indonesia	1996	3	I	n.a.	17	AB621669 (TS)	AB621691 (TS)
T917	*Z. officinale*	Indonesia	1996	3	I	n.a.	17	AB621670 (TS)	AB621692 (TS)
T924-2	*Z. officinale*	Indonesia	1996	3	I	n.a.	17	AB678489 (TS)	AB678526 (TS)
T925-2	*Z. officinale*	Indonesia	1998	4	I	n.a.	14	AB621671 (TS)	AB621693 (TS)
T948	*Z. officinale*	Indonesia	1998	3	I	n.a.	17	AB621672 (TS)	AB621694 (TS)
T952-B	*Z. officinale*	Indonesia	1999	3	I	n.a.	14	AB678491 (TS)	AB678528 (TS)
T963	*Z. officinale*	Indonesia	2000	3	I	n.a.	17	AB678490 (TS)	AB678527 (TS)
T967	*C. xanthorrhiza*	Indonesia	2000	3	I	n.a.	17	AB678482 (TS)	AB678519 (TS)
T968	*C. aeruginosa*	Indonesia	2000	3	I	n.a.	17	AB621673 (TS)	AB621695 (TS)
Psg-8	*Z. officinale*	Indonesia	1990	3	I	n.a.	17	AB678484 (TS)	AB678521 (TS)
Ps6-3-1	*Z. officinale*	Indonesia	1990	3	I	n.a.	17	AY465009 (40)	AB621649 (TS)
MAFF301070	*Solanum lycopersicum*	Kochi, Japan	1966	3	I	n.a.	14	AB508612 (24)	AB732956 (TS)
MAFF302549	*Limonium* sp.	Kochi, Japan	1987	3	I	n.a.	14	AB732949 (TS)	AB732955 (TS)

[1] Based on the method proposed by Horita et al.[17]

[2] n.i. = not identical to any previously designated sequevar

[3] TS = this study

[4] n.a. = no bands amplified

bands were amplified by PCR in 25-μL reaction volume containing 2.5 μL of 10× reaction buffer (supplied), 2 μL of dNTP mixture (200 μM/L each), 2 μM/L of each primer, 0.5 U of Takara *Taq* DNA Polymerase (Takara Bio), and 1 μL of extracted DNA. The amplifications were performed in an automated thermocycler (model 9700, Applied Biosystems) with an initial denaturation at 95°C for 5 min., followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 1 min., and then final extension at 72°C for 10 min. The amplified DNA was separated by 2% agarose gel electrophoresis in 0.5× TAE buffer.

6. Sequencing of *egl* and *mutS* genes

The *egl* gene was amplified by PCR in a 25-μL reaction mixture containing 2.5 μL of 10× reaction buffer (supplied), 2 μL of dNTP mixture (200 μM/L each), 1 mM/L $MgSO_4$, 800 nmol/L of each primer (endo-F: 5′-ATGCATGCCGCTGGTCGCCGC-3′; endo-R: 5′-GCGTTGCCCGGCACGAACACC-3′)[8], 0.5 U of KOD-Plus DNA Polymerase (Toyobo Co., Osaka, Japan), and 1 μL of extracted DNA. Amplifications were performed in an automated thermocycler (model 9700, Applied Biosystems) with initial denaturation at 95°C for 5 min., followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 1 min., and then extension at 72°C for 1 min. Final extension was performed at 72°C for 10 min. The *mutS* gene was amplified by PCR in a 25-μL reaction mixture containing 12.5 μL of 2× reaction buffer (supplied), 4 μL of dNTP mixture (400 μM/L each), 750 nmol/L of each primer (mutS-RsF.1570: 5′-ACAGCGCCTTGAGCCGGTACA-3′; mutS-RsR.1926: 5′-GCTGATCACCGGCCCGAACAT-3′)[32], 1.0 μL of DMSO, 0.5 U of KOD FX or KOD FX neo (Toyobo Co.), and 1 μL of extracted DNA. Amplifications were performed in an automated thermocycler (model 9700, Applied Biosystems) with initial denaturation at 94°C for 2 min., followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 70°C for 30 s, and then extension at 68°C for 1 min.

The amplified DNA was separated by 1.5% agarose gel electrophoresis in 0.5× TAE buffer and recovered from the gel using the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. PCR products were sent to Fasmac Co. (Kanagawa, Japan) for sequencing. The *egl* and *mutS* DNA sequences of both strands were determined using the above-mentioned PCR primers.

7. Data analysis

The derived DNA sequences were deposited in the DDBJ/EMBL/GenBank database systems under accession numbers AB620014–AB620018, AB621636–AB621695, AB678435–AB678437, AB678480–AB678491, AB678517–AB678528 and AB732947–AB732956. Forty-six reference strain sequences were also selected and used in the analysis (Table 2).

The sequences were aligned using DNASIS pro (Hitachi, Tokyo, Japan). The genetic distances between the sequences were calculated using the Kimura-2-parameter method[20]. A dendrogram was constructed from the genetic distance data, using the neighbor-joining method in the Clustal W program[35]. The strength of tree branches was tested using 1,000 bootstrap trials.

Results

1. Biovar of Zingiberaceae plant isolates

Fifty-two *R. solanacearum* strains isolated from various Zingiberaceae plants (ginger, mioga, two other *Zingiber* spp., and five *Curcuma* spp.) in Japan, Thailand, Indonesia, China, and Australia, and two additional strains isolated from other kind of crops (tomato and *Limonium* sp. grown near the ginger fields in Kochi Prefecture, Japan) were used for analysis (Table 1). These strains belonged to two biovars—biovar 3 and biovar 4. Of the 52 Zingiberaceae strains studied, 32 were classified as biovar 3, and 20 as biovar 4. The biovar 4 strains were found distributed in all five countries, while the biovar 3 strains were isolated only from Japan, Thailand, and Indonesia. The two other crop isolates belonged to biovar 3.

2. PCR-based discrimination of *R. solanacearum* strains

PCR-based phylotype discrimination analysis was conducted. The *R. solanacearum* species complex universal band (281 bp) and the phylotype I-specific band (144 bp) were derived from all tested strains (Table 1). PCR analysis for DNA type discrimination was further demonstrated. The 52 Zingiberaceae plant isolates included 19 isolates of type I and nine of type II. The specific DNA band for type I or type II was not amplified from 24 other strains. Ten strains from Thailand and nine from Japan (i.e., the prefectures of Tochigi and Kochi) were included in type I. All strains from China and Australia, and six strains from Japan were included in type II. All strains from Indonesia and one (1052) from Thailand were not included in either DNA type (Table 1). No bands were amplified from two other crop isolates.

3. *egl* gene analysis

The *egl* gene of 45 strains isolated from Zingiberaceae plants and one other strain were sequenced. A dendrogram was generated by comparing the 666 nucleotide positions of the 97 strains including 43 reference strains [*R. solanacearum*, *R. syzygii*, and blood disease bacterium of banana (BDB)] that cover the known diversity within the *R. sola-*

Table 2. Reference strains used for sequence analysis

Strain	Host	Origin	Biovar	Phylotype	Sequevar	Accession number (reference)	
						egl	*mutS*
Ralstonia solanacearum							
Z1	*Zingiber officinale*	China	4	I	16	FJ561138 (44)	-
Z2	*Z. officinale*	China	4	I	16	FJ561163 (44)	-
Z3	*Z. officinale*	China	4	I	16	FJ561164 (44)	-
Z6	*Z. officinale*	China	4	I	14	FJ561141 (44)	-
Z7	*Z. officinale*	China	4	I	16	FJ561142 (44)	-
Z14	*Z. officinale*	China	4	I	18	FJ561139 (44)	-
Z78	*Z. officinale*	China	4	I	14	FJ561125 (44)	-
Zo4	*Z. officinale*	Philippines	4	I	14	FJ561156 (44)	-
ACH92	*Z. officinale*	Australia	4	I	16	AF295254 (30)	AY756746 (32)
CIP365	*Solanum tuberosum*	Philippines	3	I	45	GQ907151 (22)	AY756787 (32)
GMI1000	*S. lycopersicum*	Guyana	3	I	18	AF295251 (30)	AY756804 (32)
MAFF211266	*S. lycopersicum*	Japan	4	I	15	AF295250 (30)	AY756791 (32)
R288	*Morus alba*	China	5	I	12	GQ907153 (22)	AY756797 (32)
R292	*M. alba*	China	5	I	12	AF295255 (30)	AY756801 (32)
M2	*M. alba*	China	5	I	48	FJ561067 (44)	JF702707 (42)
P11	*Arachis hypogaea*	China	3	I	17	FJ561068 (44)	JF702705 (42)
O3	*Olea europae*	China	3	I	44	FJ561069 (44)	JF702706 (42)
PSS4	*S. lycopersicum*	Taiwan		I	15	EU407264 (42)	JF702698 (42)
PSS81	*S. lycopersicum*	Taiwan	3	I	14	FJ561066 (44)	JF702701 (42)
PSS219	*S. lycopersicum*	Taiwan	3	I	34	FJ561167 (44)	JF702700 (42)
PSS358	*S. lycopersicum*	Taiwan	3	I	15	EU407298 (42)	JF702699 (42)
PSS366	*S. lycopersicum*	Taiwan		I	15	EU407299 (42)	JF702696 (42)
CFBP7058	*S. scabrum*	Cameroon		I	13	EF439740 (26)	EF439794 (26)
MAD17	*Capsicum annuum*	Madagascar		I	46	GU295040 (3)	JF702703 (42)
JT519	*Pelargonium* sp.	Reunion		I	31	GU295032 (3)	JF702713 (42)
GMI8254	*S. lycopersicum*	Indonesia		I	47	GU295014 (3)	JF702719 (42)
UW551	*Pelargonium* sp.	Kenya		II	1	DQ657596 (2)	JF702736 (42)
ICMP7963	*S. tuberosum*	Kenya		II	7	AF295263 (30)	AY766776 (32)
CMR87	*S. lycopersicum*	Cameroon		II	35	EF439727 (26)	EF439805 (26)
CMR39	*S. lycopersicum*	Cameroon		II	41	EF439726 (26)	EF439803 (26)
CMR121	*S. lycopersicum*	Cameroon		II	52	EF439725 (26)	EF439800 (26)
MOLK2	*Musa* sp.	Philippines	1	II	3	EF371841 (26)	AY756813 (32)
UW162	*Musa* sp..	Peru	1	II	4	AF295256 (30)	AY756795 (32)
CFBP2972	*S. tuberosum*	Martinique	1	II	35	EF371809 (26)	EF371847 (26)
JT525	*P. asperum*	Reunion	1	III	19	AF295272 (30)	AY756786 (32)
NCPPB332	*S. tuberosum*	Zimbabwe	1	III	22	DQ657649 (2)	AY756760 (32)
CFBP3059	*S. melongena*	Burkina Faso	1	III	23	AF295270 (30)	AY756766 (32)
CMR66	*S. scabrum*	Cameroon		III	49	EF439729 (26)	EF439783 (26)
MAFF301558	*S. tuberosum*	Japan	N2	IV	8	AY465002 (40)	AY756812 (32)
PSI07	*S. lycopersicum*	Indonesia	2	IV	10	EF371804 (26)	AY756752 (32)
ACH732	*S. lycopersicum*	Australia	2	IV	11	GQ907150 (22)	AY756743 (32)
Blood disease bacterium of banana (BDB)							
R230	*Musa* sp.	Indonesia		IV	10	AF295280 (30)	AY756788 (32)
R. syzygii							
R1	*Syzygium aromaticum*	Indonesia		IV	9	JF702320 (42)	JF702734 (42)
R. insidiosa							
LMG21421	*Homo sapiens*	United States				-	AY756777 (32)
R. mannitolilytica							
LMG6866	*H. sapiens*	United Kingdom				-	JF702732 (42)
R. pickettii							
LMG5942	*H. sapiens*	United States				-	JF702733 (42)

nacearum species complex[9,32,42,44] (Tables 1 and 2). The strains were divided into four major clusters (Fig. 1). Each cluster corresponded to a specific phylotype (phylotype I, phylotype II, phylotype III, or phylotype IV). All 61 strains from Zingiberaceae plant isolates were classified into a single cluster that contained all phylotype I strains, and revealed 12 haplotypes and six sequevars (Table 1).

Zingiberaceae plant isolates in the phylotype I cluster were further divided into five major groups (A, B, CI, CII, and D) showing significant (>50%) bootstrap values, and

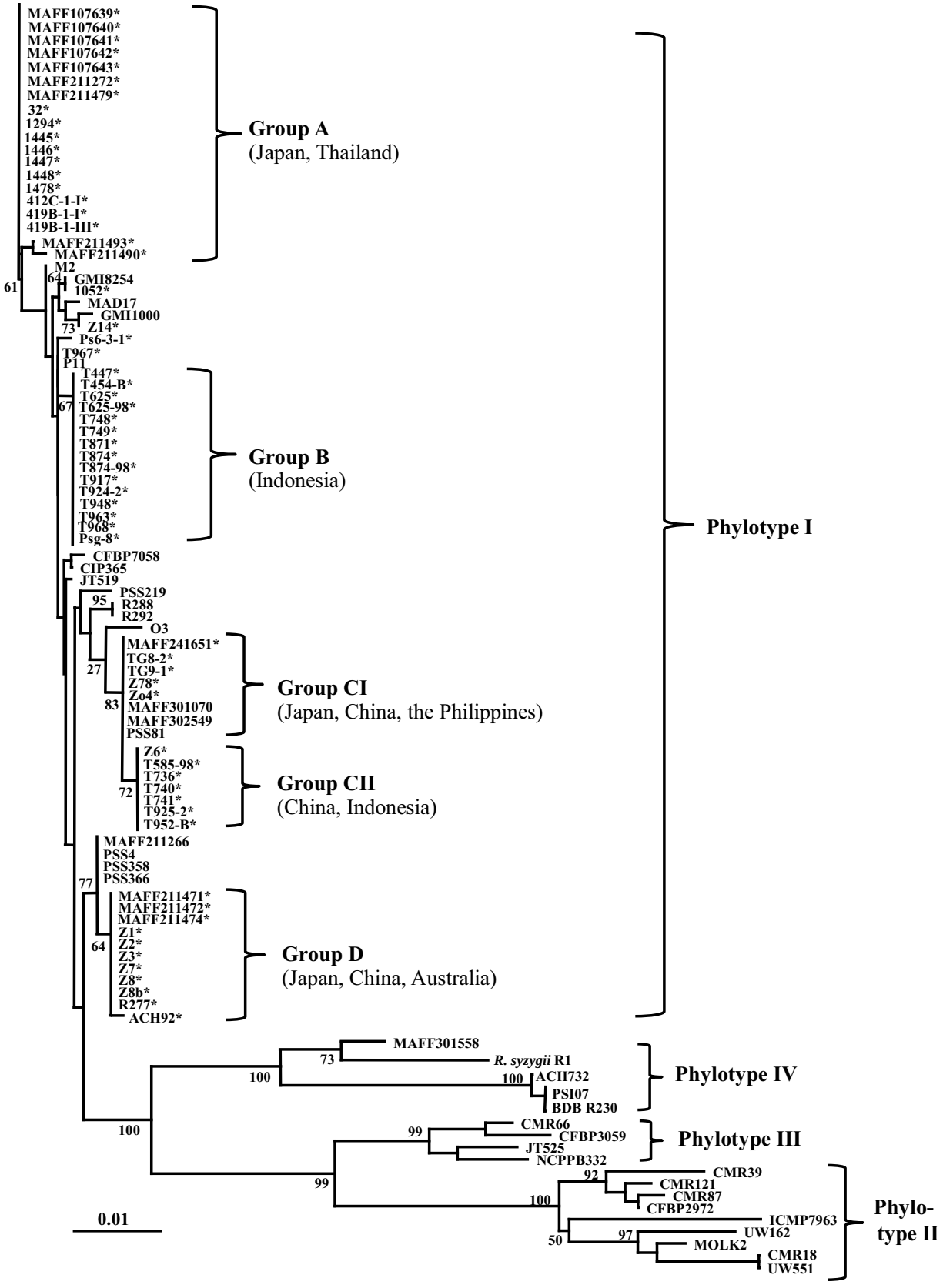

Fig. 1. Phylogenetic neighbor-joining tree based on partial sequences of the endoglucanase (*egl*) gene from *Ralstonia solanacearum*, *R. syzygii*, and the blood disease bacterium of banana (BDB) strains

Asterisks near the strain names signify isolates from Zingiberaceae plants. Values at the branches indicate percentage bootstrap support for 1000 resamplings. The scale bar represents one nucleotide substitution per 100 nucleotides.

with each corresponding closely to a single sequevar (in which partial sequences differ by less than 1%)[9,42] (Fig. 1). Group A (not identical to any previously designated sequevar) included nine strains from Japan and ten from Thailand; group B (corresponding to sequevar 17) comprised 15 strains—all from Indonesia; group CI (corresponding to sequevar 14) consisted of three strains from Japan, one strain from China, and one from the Philippines; group CII (corresponding to sequevar 14) consisted of one strain from China and six strains from Indonesia; group D (corresponding to sequevar 16) contained three strains from Japan, six from China, and two from Australia. Though strains in groups CI and CII were included in sequevar 14, bootstrap trials indicated each as an independent group with high confidence. Two Zingiberaceae strains from Indonesia (Ps6-3-1 and T967) were homogeneous to the P11 strain (representing sequevar 17)[42,44], and included in sequevar 17 as well as the group B strains. However, bootstrap trials showed that these strains do not completely consist of the same cluster as the group B strains. Two additional ginger strains (1052 and Z14 from Thailand and China, respectively) were homogeneous to the strains in sequevar 47 (GMI8254) and sequevar 18 (GMI1000), respectively. Two other crop isolates from Japan (MAFF301070 and MAFF302549) belonged to group CI, along with several ginger strains and the PSS81 strain (representing sequevar 14)[42,44].

4. *mutS* gene analysis

The *mutS* gene of 52 strains isolated from Zingiberaceae plants and two other strains was sequenced. The dendrogram was generated by comparing the 651 nucleotide positions of the 92 strains (Tables 1 and 2). The tree was rooted based on the outgroups (*R. pickettii*, *R. insidiosa*, and *R. mannitolylitica*). The 89 strains, including *R. solanacearum*, *R. syzygii*, and BDB, were divided into four major clusters (Fig. 2). Fifty-three strains from Zingiberaceae plants (15 from Japan, 11 from Thailand, 23 from Indonesia, two from China, and two from Australia) were classified into a single cluster that contained all phylotype I strains, and revealed six haplotypes.

Zingiberaceae plant isolates were further divided into three major groups. Group 1 included six strains from Indonesia [all belonging to group CII (sequevar 14) in Fig. 1]; group 2 consisted of two strains from China, two from Australia, and three from Japan [corresponding to group D (sequevar 16)]; group 3 comprised 12 strains from Japan, 10 from Thailand, and 16 from Indonesia [including most strains in group A (not identical to any previously designated sequevar) and group B (sequevar 17), and two independent strains (Ps6-3-1 and 1052) in sequevars 17 and 47, respectively]. Two Zingiberaceae plant isolates (T967 and 412C-1-I, from Indonesia and Thailand, respectively) were not included in any of the three groups. Two other crop isolates belonged to group 3 with many Zingiberaceae plant isolates and eight reference strains[9,32,42].

5. Pathogenicity of Zingiberaceae plant isolates to ginger

We checked the pathogenicity of 14 strains that differed in terms of host, country, biovar, DNA group (shown in Figs. 1 and 2), DNA type and/or sequevar (Table 1) relative to ginger (Table 3). All Zingiberaceae plant isolates tested were strongly pathogenic to ginger (causing wilting or death of the inoculated plants). However, two other crop isolates were not pathogenic to ginger.

Discussion

R. solanacearum strains affecting Zingiberaceae plants have been isolated from different areas of the Asia-Pacific region, where the climate varies from tropical and subtropical to warm temperate[7,12,13,15,25,27,36,37,38,39,44,46,48] (Tables 1 and 2). Therefore, these strains could possibly affect host plants regardless of the cultivation conditions (e.g., race 3/biovar 2 strains belonging to phylotype II/sequevar 1 that affect

Table 3. Pathogenic and genetic characters of Zingiberaceae and other plant isolates of *Ralstonia solanacearum*

Host	Strain	Country	Biovar	Group (*egl*/*mutS*)[1]	DNA type	Sequevar	Pathogenicity test[2]
Zingiberaceae	MAFF107639, MAFF211272, MAFF211490, 1445	Japan, Thailand	3, 4	A/3	I	n.i.	+
	T447, T454-B, T874	Indonesia	3	B/3	n.a.	17	
Ginger	TG8-2, MAFF241651	Japan	4	CI/3	II	14	
	T585-98	Indonesia	3	CII/1	n.a.		
	MAFF211474, Z8a	Japan, China	4	D/2	II	16	
Others	MAFF301070, MAFF302549	Japan	3	CI/3	n.a.	14	-

[1] Grouping based on *egl* and *mutS* gene sequence analyses (Figs. 1 and 2).

[2] Ginger plants (cv. Sanshu) at the 7th to 9th leaf stage (n=3) were inoculated by pucturing the basl part of the stem with a needle dipped in inoculum (ca. 10^8cfu/ml). Symptoms were checked for three weeks after inoculation at 28°C. (+ = wilted or died; - = no symptom)

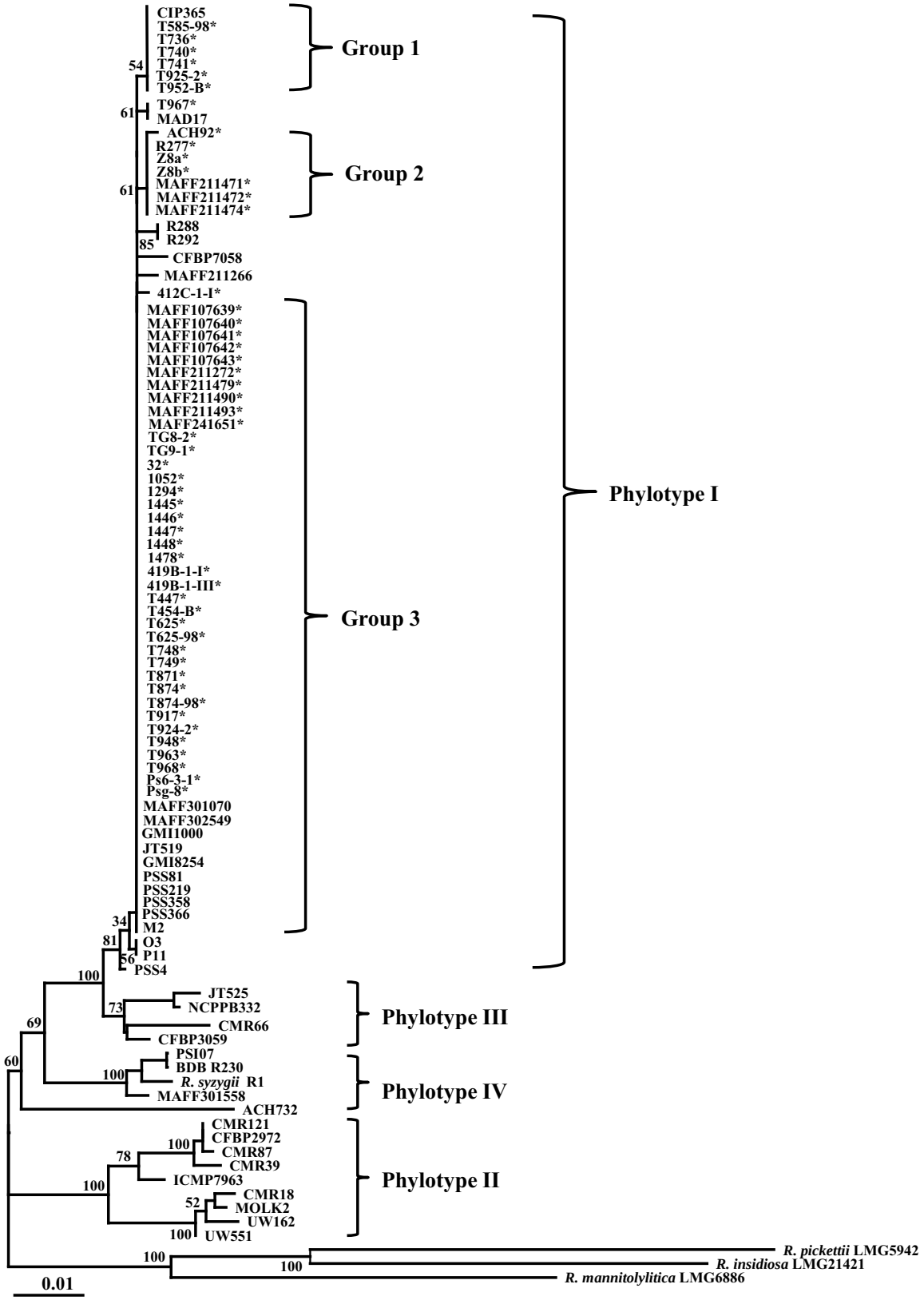

Fig. 2. Phylogenetic neighbor-joining tree based on partial sequences of the methy-directed DNA repair (*mutS*) gene from *Ralstonia solanacearum, R. syzygii*, and the blood disease bacterium of banana (BDB) strains

The tree was rooted based on the outgroups *R. pickettii*, *R. insidiosa*, and *R. mannitolylitica*. Asterisks near the strain names signify isolates from Zingiberaceae plants. Values at the branches indicate percentage bootstrap support for 1000 resamplings. The scale bar represents one nucleotide substitution per 100 nucleotides.

potato cultivation in wide-ranging areas)[7,9,12,26,43,44]. Conversely, diverse types of the strains, each adapting to different climatic conditions, may exist. The DNA-based analyses used in the present study showed that these strains are genetically divergent.

Zingiberaceae plant isolates used were classified into six sequevars (sequevars 14, 16, 17, 18, 47, and one new additional sequevar corresponding to group A in Fig. 1), in which sequevars 14, 17, 18, and 47 comprised various plants isolates as well as Zingiberaceae plant isolates from Japan and worldwide[3,24,26,30,42,44] (Tables 1 and 2). The other two (sequevar 16 and the new one) only consisted of Zingiberaceae plant isolates, and were closely related to specific host plants.

In this study, these Zingiberaceae strains can be divided into five or more groups (each almost corresponding to a separate sequevar) based on *egl* sequence analysis (Fig. 1), and three major groups based on *mutS* sequence analysis (Fig. 2).

Strains in each group were closely correlated with a host species and/or its geographic origin (country), especially in *egl* sequence analysis. Group A (presumed to be a new sequevar) included ginger, mioga, and *Curcuma* spp. isolates from Thailand and Japan. Group B (corresponding to sequevar 17) consisted of various Zingiberaceae plant isolates from Indonesia (three *Zingiber* spp. and three *Curcuma* spp. isolates). The remaining three groups comprised ginger isolates from several countries; group CI (sequevar 14) included isolates from Japan, China, and the Philippines, group CII (sequevar 14) consisted of isolates from China and Indonesia, while group D (sequevar 16) was composed of isolates from China, Australia, and Japan.

Groups A, B, CII, and D consisted solely of Zingiberaceae plant isolates, whereas group CI included other plant isolates (PSS81 from tomato, representing sequevar 14, MAFF301070 from tomato, and MAFF302549 from *Limonium* sp.). However, PCR-based DNA typing[17] discriminated ginger isolates from other plant isolates within group CI (Tables 1 and 3). Conversely, *mutS* gene analysis revealed that the Zingiberaceae plant isolates in group CII and group D comprised an independent group (group 1 and group 2, respectively) (Fig. 2).

Overall, our results indicate that strains affecting Zingiberaceae plants are genetically distinct from other plant isolates, have five or more origins, and are distributed throughout host cultivation fields in the Asia-Pacific region. Moreover, some of these strains may have previously been disseminated locally and globally.

Severe outbreaks of bacterial wilt of Zingiberaceae crops have been reported in several Asian countries (e.g., Indonesia, Thailand, Malaysia, India, the Philippines)[7,12,21,25,34]. Some of these outbreaks were caused by the use of infected rhizomes as planting material. Thus, the disease may spread both within a country[7,21,25] and globally[12].

In Australia, outbreaks of bacterial wilt of ginger occurred in the 1960s. The pathogen was believed to have been disseminated from seed rhizomes imported from China[29]. Restriction fragment length polymorphism analysis was used to clarify the relations among *R. solanacearum* strains, and confirmed that the strains from Australia were closely related to those from China[4,5]. In the present study, we revealed that strains from Australia and China belonged to group D (Fig. 1) and group 2 (Fig. 2). Thus, our results are in accordance with the previous findings.

In Japan, bacterial wilt of Zingiberaceae plants was first reported in cultivation fields containing *Curcuma alismatifolia* (introduced from Thailand as a planting material for cut flowers in 1989) located in Kochi Prefecture in 1995[27]. Since 1997, outbreaks of the disease have occurred in ginger and mioga cultivation fields throughout neighboring areas of the same prefecture[37,46]. More recently, the disease has spread to ginger fields outside Kochi (such as Tochigi and several other prefectures in 2009). In most cases, the introduction of ginger rhizomes originating from Kochi Prefecture or some Asian countries as planting material was immediately followed by an outbreak of disease. In the present study, we demonstrated that Zingiberaceae plant isolates of *R. solanacearum* from Japan were genetically homogeneous to those from Thailand (group A in Fig. 1), China (groups CI and D), and the Philippines (group CI) based on *egl* analysis. Our findings indicate that the pathogen originated in other Asian countries, and was disseminated with infested seed rhizomes, thereby dispersing bacterial wilt in Japan.

Pathogenic characteristics of some Japanese strains of *R. solanacearum* isolated from Zingiberaceae plants were previously assessed by using root-injuring inoculation methods[38,47]. Strains belonging to group A (Fig. 1) were shown to be strongly pathogenic to ginger, mioga, and *C. alismatifolia*. In contrast, strains in group D were found to be strongly pathogenic to ginger, but weakly pathogenic or nonpathogenic to mioga and *C. alismatifolia*. In the present study, all strains in group D were isolated only from ginger. In contrast, group A included strains isolated from Zingiberaceae plants other than ginger. Taken together, these findings may indicate differences between group A and group D strains in terms of host range.

The previous and current cross-inoculation testing of Japanese strains of *R. solanacearum* have revealed that isolates other than Zingiberaceae plant isolates were not pathogenic to ginger[47] (Table 3). Additionally, the Zingiberaceae plant isolates in group A were strongly pathogenic to potato (causing wilting or death), but weakly pathogenic or nonpathogenic to other Solanaceae crops (e.g., tomato, tobacco, eggplant, pepper) and peanuts. Some Thai ginger strains of *R. solanacearum* in group A showed similar pathogenic

characteristics[36,39]. In contrast, the ginger strains in group D were strongly pathogenic to most Solanaceae crops, but weakly pathogenic or nonpathogenic to tobacco and peanuts. On the basis of previous reports[11,15,29], we conclude that the pathogenic characteristics of Chinese (Z2 and Z3 in group D) and Australian (also belonging to group D) ginger strains towards Solanaceae crops and peanuts are almost identical to those of the Japanese strains in group D. Taken together, these findings suggest that DNA-based groupings of the strains are closely correlated with the pathogenic characteristics. Quinon et al[33]. and Lum[25] reported that the cross-inoculation of Hawaiian and Malaysian ginger strains of *R. solanacearum* failed to wilt tomato, tobacco, and peanuts, and that the characteristics of these strains were almost identical to those of group A strains. Further DNA-based comparison of these strains is thus required.

In the present study, group B strains (all from Indonesia) were isolated from various Zingiberaceae plants, whereas groups CI and CII strains (from Japan, China, the Philippines, and Indonesia) were isolated only from ginger. Zehr[48] reported that ginger isolates of *R. solanacearum* showed strong virulence only to ginger in the Philippines, and exhibited unique pathogenic characteristics towards other crops. Further pathogenicity tests are now being conducted on the strains in groups B, CI, and CII as isolated in the present study.

Biovar classification is a classic and simple means of categorizing *R. solanacearum* strains[6,10]. Biovars of Zingiberaceae plant isolates from several countries were previously assessed in terms of their pathogenic characteristics, with most being classified as biovar 3 or biovar 4[13]. The Zingiberaceae strains used in the present study also belonged to either biovar 3 or biovar 4. Strains in biovar 3 were distributed among three distinct groups in *egl* analysis (groups A, B, and CII in Fig. 1), and two groups in *mutS* analysis (group 1 and group 3 in Fig. 2). Biovar 4 strains were divided into four (A, CI, CII, and D) or more groups in *egl* analysis, and three (1–3) groups in *mutS* analysis. We detected no clear relation between biovar and DNA-based grouping.

In the present study, strains in biovar 3 were isolated from various Zingiberaceae plants (including ginger), whereas most strains in biovar 4 were isolated from ginger (Table 1). Hayward et al[11]. reported that biovar 4 strains were more virulent to ginger than biovar 3 strains. The association of virulence with a specific biovar thus requires further investigation.

Our present findings indicate that the bacterial wilt pathogen affecting Zingiberaceae plants in the Asia-Pacific region can be divided into several genetically distinct groups. These groups may differ in terms of geographic origin, pathogenicity (host range, virulence to specific plants), biovar, etc. Moreover, some strains may have been previously disseminated locally and globally via transplanting material.

Efficient countermeasures must therefore be independently considered and implemented according to the local situation (i.e., whether the pathogen is established, economic importance and degree of damage to the host crop, pathogenic characteristics of the pathogen). Newly introduced (or imported) seed rhizomes should be subject to a quarantine check, and the host cultivation history of the rhizome-producing area (i.e., whether bacterial wilt disease has previously been recorded) must be investigated. Recently, immunodiagnostic and DNA-based detection assays for screening ginger rhizomes for specific pathogens have been developed[1,34].

Once a pathogen has been established and spreads in a cultivation area, a range of control methods (e.g., restricted seed rhizome management, soil fumigation, crop rotation with non-hosts, fallow) should be considered. In Hawaii, bacterial wilt-free ginger seed rhizomes have been produced using a tissue culture method. And the use of plant essential oils as biofumigants in integrated disease management systems has also been investigated[28]. Kumar et al[21]. reported that rhizome solarization and microwave treatment were effective in sterilizing infected pathogens. In the present study, each of the Zingiberaceae plant isolates showed a distinct host range or pathogenicity to various plants, including Zingiberaceae, Solanaceae, and Fabaceae. Therefore, according to the invasive strains, different types of plants (crops) may be useful for rotation, to prevent disease, and/or reduce the pathogen population in the field. Pegg and Moffett[29] reported that *Alpina caerulea* (wild ginger) and some specific weeds are resistant to bacterial wilt in Australia. Moreover, the pathogen (belonging to group D in Fig.1) was not isolated from plants grown in an infested field. Such plants would be useful for eradicating the pathogen, which may have already been disseminated to other countries. Therefore, further research on this strategy is now be considered.

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