Genetic Diversity of Japanese Strains of *Ralstonia solanacearum*

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ABSTRACT

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The genetic diversity of 74 Japanese strains of *Ralstonia solanacearum* was assessed by pathogenicity tests and the repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprint method. Based on their genomic fingerprints, biovar N2 strains were divided into two distinct groups, one consisting of potato isolates belonging to race 3, and the other consisting of tomato, eggplant, pepper, and tobacco isolates

belonging to race 1. Biovar 3 strains had low average similarity and were divided into five groups that differed in original host or pathogenicity. Biovar 4 strains consisted of only one group at the 80% similarity level. Comparative analysis of the rep-PCR fingerprints of 78 strains, including six biovars from Japan and various countries, revealed two main clusters. Cluster 1 comprised all biovar 3, 4, and 5 strains, biovar 1 strains from Reunion, and some biovar N2 strains from Japan. Cluster 2 included most of the biovar 1, 2, and N2 strains. The fingerprints showed low average similarity with biovar N2 strains from Japan and Brazil.

Ralstonia solanacearum (synonym *Pseudomonas*) (43,49) causes a lethal wilting disease on many important crops in tropical, subtropical, and warm regions in the world. The host range of the bacterium is exceptionally wide, and many economically important crops as well as many weed hosts have been recognized (16). *R. solanacearum* is a diverse species that differs in host range, geographical distribution, pathogenicity, epidemiological relationships, and physiological properties. To describe this intra-specific variability, binary classification systems are used. This pathogen is divided into five races (2,19,39) based on host range and six biovars (14,17,19) based on utilization of three disaccharides and three hexose alcohols. In Japan, bacterial wilt disease caused by *R. solanacearum* has been reported mainly for solanaceous crops including tomato (*Lycopersicon esculentum* Mill), potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), eggplant (*S. melongena* L.), and sweet pepper (*Capsicum annuum* L.). To date, more than 34 species in 18 families of plants have been reported as hosts in Japan (6,40). The classification of Japanese strains of *R. solanacearum* has been studied by several workers (24,33-35,37). However, the systematic relationship among strains is still poorly defined. Analysis of genetic diversity is important for understanding the distribution of strains worldwide and prevention of their further dissemination.

Recently, DNA-based analyses (restriction fragment length polymorphism [RFLP], 16S rDNA sequence, repetitive sequence-based polymerase chain reaction [rep-PCR], etc.) are effective in assessing the diversity and genetic relationships among *R. solanacearum* strains (3-5,9,11,23,26,41,42,44,47). The objectives of this study were to determine the pathogenic variation among Japanese strains of *R. solanacearum* and to compare them with strains of geographically diverse origin by the rep-PCR fingerprint method. The rep-PCR method uses primer sets based on conserved repetitive bacterial elements referred to as enterobacterial repetitive intergenic consensus (ERIC) (22), repetitive extragenic palindromic (REP) (12), and BOX (32). PCR

amplification by these primer sets can yield genomic fingerprints specific to pathovars or strains of phytopathogenic bacteria (9,23,27-29,44).

MATERIALS AND METHODS

Bacterial strains and growing conditions. Bacterial strains used in this study are listed in Table 1. Most strains were from the Ministry of Agriculture, Forestry, and Fisheries, Japan (MAFF) Genbank culture collection. Thirteen strains from outside Japan were derived from the National Center for Agricultural Research, Ghent, Belgium. 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 were revived by plating on tetrazolium chloride (TZC) medium (25). For tests, strains were grown at 30°C on TZC medium or casamino acids, peptone, and glucose (CPG) broth (20) on a rotary shaker at 150 rpm.

Pathogenicity, hypersensitivity tests, and biovar determination. For the pathogenicity test, tomato (cv. Houkin No. 2), eggplant (cv. Senryo No. 2), sweet pepper (cv. Wase Green), potato (cv. Danshaku), and tobacco (cv. Bright Yellow No. 4) were used. Seedlings were transplanted in plastic pots (8-cm diameter) containing a horticultural soil-vermiculite mixture, and grown in a greenhouse at 25 to 30°C. To prepare inoculum, bacteria were grown on TZC agar for 2 to 3 days at 30°C, suspended in sterile distilled water, and adjusted to 10⁹ CFU/ml. Plants at the fourth to fifth true-leaf stage were inoculated by puncturing the basal part of the stem with a needle dipped in inoculum (48). Two to four plants of each host were inoculated with each strain. Inoculated plants were placed in the greenhouse at 25 to 30°C under natural light. Severity of wilting was observed at weekly intervals on the following scale: 1 = no symptom, 2 = leaf above inoculation site wilted, 3 = two or three leaves wilted, 4 = four or more leaves wilted, and 5 = plant dead. The inoculation test was repeated two or more times on separate days. The hypersensitivity (HR) test was done as described by Lozano and Sequeira (30). Bacterial suspensions (≈10⁸ CFU/ml) were infiltrated into fully expanded tobacco leaves (cv. Bright Yellow No. 4). The reaction was recorded at 24, 48, and 72 h after infiltration. Biovar and the utilization of trehalose, *meso*-inositol,

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and D-ribose were determined by the methods of Hayward (14,15). Growth and acid production from carbohydrates were observed. Nitrite and gas production from nitrate were determined according to the method of Hayward et al. (18).

rep-PCR analysis. The rep-PCR method employed was adapted from Louws et al. (28). The primer sets: REP, REP1R-I (5'-III ICG ICG ICA TCI GGC-3') and REP2-I (5'-ICG ITT ATC IGG CCT AC-3'); ERIC, ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3'); and BOX, BOXA1R (5'-CTA CGG CAA GGC GAC

GCT GAC G-3') (28) were synthesized by Amersham Pharmacia Biotech (Tokyo, Japan). PCR amplifications were performed in a thermocycler (PC-800; Astec Inc., Tokyo, Japan) in 25- μ l reaction volumes of 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each dNTP, 50 pmol each primer, 2.5 unit of DNA polymerase Takara *Taq* (Takara Shuzo Co., Otsu, Japan), and 50 ng of template DNA overlaid with 25 μ l of mineral oil (Sigma Chemical Co., St. Louis). The PCR thermocycler was programmed as follows: an initial denaturation step of 95°C for 7 min, followed by 30 cycles of 94°C for 1 min, 44°C (REP) or

TABLE 1. Strains of *Ralstonia solanacearum* used in this study and classifications established according to race and biovar^a

Code no.	Strain	Host	Geographical origin	Race	Biovar	Source ^b
1	MAFF 211282	<i>Solanum melongena</i>	Kochi, Japan	1	3	MAFF
2	MAFF 301069	<i>Nicotiana tabacum</i>	Shizuoka, Japan	1	3	MAFF
3	MAFF 301070	<i>Lycopersicon esculentum</i>	Kochi, Japan	1	3	MAFF
4	MAFF 301522	<i>Lycopersicon esculentum</i>	Kagoshima, Japan	1	3	MAFF
5	MAFF 301560	<i>Strelitzia reginae</i>	Nagasaki, Japan	1	3	MAFF
6	MAFF 301561	<i>Strelitzia reginae</i>	Nagasaki, Japan	1	3	MAFF
7	MAFF 301841	<i>Solanum melongena</i>	Kanagawa, Japan	1	3	MAFF
8	MAFF 301849	<i>Capsicum annuum</i>	Tochigi, Japan	1	3	MAFF
9	MAFF 301860	<i>Capsicum annuum</i>	Ibaraki, Japan	1	3	MAFF
10	MAFF 302549	<i>Limonium</i> sp.	Kochi, Japan	1	3	MAFF
11	MAFF 302550	<i>Limonium</i> sp.	Kochi, Japan	1	3	MAFF
12	MAFF 302551	<i>Limonium</i> sp.	Kochi, Japan	1	3	MAFF
13	Ps29	<i>Nicotiana tabacum</i>	Okayama, Japan	1	3	H. Hara
14	U7	<i>Nicotiana tabacum</i>	Tochigi, Japan	1	3	H. Hara
15	U108	<i>Nicotiana tabacum</i>	Kagoshima, Japan	1	3	H. Hara
16	U137	<i>Nicotiana tabacum</i>	Kagoshima, Japan	1	3	H. Hara
17	U154	<i>Nicotiana tabacum</i>	Kochi, Japan	1	3	H. Hara
18	U158	<i>Nicotiana tabacum</i>	Yamagata Japan	1	3	H. Hara
19	U164	<i>Nicotiana tabacum</i>	Tochigi, Japan	1	3	H. Hara
20	U193	<i>Nicotiana tabacum</i>	Fukushima, Japan	1	3	H. Hara
21	U247	<i>Nicotiana tabacum</i>	Niigata, Japan	1	3	H. Hara
22	U248	<i>Nicotiana tabacum</i>	Yamagata Japan	1	3	H. Hara
23	K1	<i>Kalanchoe</i> sp.	Kagawa Japan	1	3	This study
24	K2	<i>Kalanchoe</i> sp.	Kagawa Japan	1	3	This study
25	MAFF 211266	<i>Lycopersicon esculentum</i>	Hiroshima, Japan	1	4*	MAFF
26	MAFF 211267	<i>Lycopersicon esculentum</i>	Shimane, Japan	1	4*	MAFF
27	MAFF 211268	<i>Solanum melongena</i>	Kochi, Japan	1	4	MAFF
28	MAFF 211269	<i>Solanum melongena</i>	Nara, Japan	1	4	MAFF
29	MAFF 211281	<i>Solanum melongena</i>	Kochi, Japan	1	4	MAFF
30	MAFF 301067	<i>Solanum melongena</i>	Kanagawa, Japan	1	4	MAFF
31	MAFF 301068	<i>Lycopersicon esculentum</i>	Shizuoka, Japan	1	4	MAFF
32	MAFF 301418	<i>Lycopersicon esculentum</i>	Fukui, Japan	1	4	MAFF
33	MAFF 301489	<i>Lycopersicon esculentum</i>	Oita, Japan	1	4	MAFF
34	MAFF 301491	<i>Lycopersicon esculentum</i>	Oita, Japan	1	4	MAFF
35	MAFF 301520	<i>Solanum melongena</i>	Saitama, Japan	1	4	MAFF
36	MAFF 301521	<i>Solanum melongena</i>	Nara, Japan	1	4	MAFF
37	MAFF 301523	<i>Solanum melongena</i>	Tokyo, Japan	1	4	MAFF
38	MAFF 301524	<i>Solanum tuberosum</i>	Nara, Japan	1	4	MAFF
39	MAFF 301526	<i>Lycopersicon esculentum</i>	Ibaraki, Japan	1	4	MAFF
40	MAFF 301528	<i>Lycopersicon esculentum</i>	Gunma, Japan	1	4	MAFF
41	MAFF 301556	<i>Solanum tuberosum</i>	Nagasaki, Japan	1	4	MAFF
42	MAFF 301557	<i>Solanum tuberosum</i>	Nagasaki, Japan	1	4	MAFF
43	MAFF 301819	<i>Solanum melongena</i>	Shizuoka, Japan	1	4	MAFF
44	MAFF 301823	<i>Capsicum annuum</i>	Kochi, Japan	1	4	MAFF
45	MAFF 301826	<i>Lycopersicon esculentum</i>	Hiroshima, Japan	1	4	MAFF
46	MAFF 301827	<i>Lycopersicon esculentum</i>	Hiroshima, Japan	1	4	MAFF
47	MAFF 301829	<i>Lycopersicon esculentum</i>	Gifu, Japan	1	4	MAFF
48	MAFF 301840	<i>Solanum melongena</i>	Kanagawa, Japan	1	4	MAFF
49	MAFF 301846	<i>Solanum tuberosum</i>	Hokkaido, Japan	1	4	MAFF
50	MAFF 301851	<i>Solanum melongena</i>	Shizuoka, Japan	1	4	MAFF
51	MAFF 301852	<i>Solanum melongena</i>	Shizuoka, Japan	1	4	MAFF
52	MAFF 301853	<i>Lycopersicon esculentum</i>	Shizuoka, Japan	1	4	MAFF
53	MAFF 301859	<i>Lycopersicon esculentum</i>	Niigata, Japan	1	4	MAFF
54	89C-9	<i>Cucurbita maxima</i> $\times$ <i>C. moschata</i>	Okayama, Japan	1	4	H. Date
55	89C-10	<i>Cucumis sativus</i>	Okayama, Japan	1	4	H. Date
56	MAFF 211280	<i>Solanum melongena</i>	Kochi, Japan	1	N2	MAFF
57	MAFF 301485	<i>Lycopersicon esculentum</i>	Oita, Japan	1	N2	MAFF

(continued on next page)

^a * Indicates reconfirmed in this study; nt = not tested.

^b MAFF = Ministry of Agriculture, Forestry, and Fisheries Genebank, National Institute of Agrobiological Resources, Tsukuba, Japan; ATCC = American Type Culture Collection, Rockville, MD; NCAR = National Center for Agricultural Research, Ghent, Belgium; and UPLB = University of the Philippines, Los Baños.

52°C (ERIC, BOX) for 1 min, and 65°C for 8 min with a final extension step of 65°C for 15 min. Amplified PCR products (4 to 8 µl) were separated by 1.5% agarose gel electrophoresis in 0.5× Tris-acetate-EDTA buffer (89 mM Tris-acetate, pH 8.0, and 2 mM EDTA) at 4 V/cm for 5 h, stained with ethidium bromide, and photographed under UV light. For the confirmation of banding patterns, rep-PCR experiments were repeated at least two times with independently prepared DNA stocks.

Data analysis. The rep-PCR fingerprint profiles were used to measure genetic similarity among strains. Each band with a dif-

ferent electrophoretic mobility was assigned a position number and scored as either 1 or 0 based on the presence or absence of the band, respectively, for this position. The similarity coefficients for all possible pairs of strains based on the fingerprint groups was estimated by the Dice method (7). Dendrograms were constructed from the similarity coefficient data by the unweighted pair group method with arithmetic averages clustering (45).

To evaluate the robustness of the groupings formed, the binary data set was subjected to bootstrapping. Using the WinBoot software program (50), the phenogram was reconstructed 2,000 times

TABLE 1. (continued from preceding page)

Code no.	Strain	Host	Geographical origin	Race	Biovar	Source ^b
58	MAFF 301486	<i>Lycopersicon esculentum</i>	Oita, Japan	1	N2	MAFF
59	MAFF 301487	<i>Lycopersicon esculentum</i>	Oita, Japan	1	N2	MAFF
60	MAFF 301488	<i>Lycopersicon esculentum</i>	Oita, Japan	1	N2	MAFF
61	MAFF 301492	<i>Solanum melongena</i>	Oita, Japan	1	N2	MAFF
62	MAFF 301527	<i>Capsicum annuum</i>	Oita, Japan	1	N2	MAFF
63	Ps27	<i>Nicotiana tabacum</i>	Okayama, Japan	1	N2	H. Hara
64	MAFF 211271	<i>Solanum tuberosum</i>	Shizuoka, Japan	3	N2	MAFF
65	MAFF 301558	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	MAFF
66	MAFF 301559	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	MAFF
67	SUPP 1917	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	Y. Takikawa
68	AA3122	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	Y. Suga
69	AA6002	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	Y. Suga
70	AA6004	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	Y. Suga
71	AA6009	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	Y. Suga
72	AA6011	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	Y. Suga
73	AA6012	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	Y. Suga
74	AA7001	<i>Solanum tuberosum</i>	Nagasaki, Japan	3	N2	Y. Suga
75	ATCC 11696 ^T	<i>Lycopersicon esculentum</i>	United States	1	1	ATCC
76	PD 1938	<i>Lycopersicon esculentum</i>	United States	1	1	NCAR
77	PD 1940	<i>Pelargonium capitatum</i>	Reunion	1	1	NCAR
78	2146c	<i>Pelargonium capitatum</i>	Reunion	1	1	S. Poussier
79	13	<i>Solanum tuberosum</i>	Brasilia, Brazil	1	1	N. Furuya
80	629	<i>Capsicum annuum</i>	Para, Brazil	1	1	N. Furuya
81	574	<i>Eucalyptus</i> sp.	Para, Brazil	1	1	N. Furuya
82	656	<i>Cucumis</i> sp.	Amazonas, Brazil	1	1	N. Furuya
83	PD 1445	<i>Musa</i> sp.	Panama	2	1	NCAR
84	PD 1446	<i>Heliconia</i> sp.	Costa Rica	2	1	NCAR
85	MoD3	<i>Musa</i> sp.	Philippines	2	1	UPLB
86	BuD4	<i>Musa</i> sp.	Philippines	2	1	UPLB
87	PD 1939	<i>Solanum tuberosum</i>	Israel	3	2	NCAR
88	PD 441	<i>Solanum tuberosum</i>	Sweden	3	2	NCAR
89	PD 1260	<i>Solanum tuberosum</i>	Reunion	3	2	NCAR
90	PD 1100	<i>Solanum tuberosum</i>	Egypt	3	2	NCAR
91	PD 1610	<i>Solanum tuberosum</i>	Belgium	3	2	NCAR
92	113	<i>Lycopersicon esculentum</i>	Parana, Brazil	3	2	N. Furuya
93	855	<i>Solanum tuberosum</i>	Brasilia, Brazil	3	2	N. Furuya
94	TK1-3-1	<i>Solanum tuberosum</i>	Thailand	3	2	This study
95	Garut 1	<i>Solanum tuberosum</i>	Indonesia	3	2	K. Katayama
96	Garut 2	<i>Solanum tuberosum</i>	Indonesia	3	2	K. Katayama
97	BBU1	<i>Solanum tuberosum</i>	Indonesia	3	2	K. Katayama
98	BBU2	<i>Solanum tuberosum</i>	Indonesia	3	2	K. Katayama
99	R568	<i>Solanum tuberosum</i>	Brazil	nt	N2	NCAR
100	61	<i>Solanum tuberosum</i>	Parana, Brazil	nt	N2	N. Furuya
101	66	<i>Solanum tuberosum</i>	Minas Gerais, Brazil	nt	N2	N. Furuya
102	67	<i>Solanum tuberosum</i>	Bahia, Brazil	nt	N2	N. Furuya
103	71	<i>Solanum tuberosum</i>	Brasilia, Brazil	nt	N2	N. Furuya
104	92	<i>Solanum tuberosum</i>	Brasilia, Brazil	nt	N2	N. Furuya
105	98	<i>Solanum tuberosum</i>	Santa Catarina, Brazil	nt	N2	N. Furuya
106	933	<i>Solanum tuberosum</i>	Brasilia, Brazil	nt	N2	N. Furuya
107	964	<i>Solanum tuberosum</i>	Santa Catarina, Brazil	nt	N2	N. Furuya
108	982	<i>Solanum tuberosum</i>	Rio de Janeiro, Brazil	nt	N2	N. Furuya
109	MAFF 211284	<i>Zingiber officinale</i>	Indonesia	1	3	MAFF
110	MAFF 302500	<i>Manihot esculenta</i>	Indonesia	1	3	MAFF
111	MAFF 302510	<i>Lycopersicon esculentum</i>	Indonesia	1	3	MAFF
112	MAFF 302514	<i>Croton hirtus</i>	Indonesia	nt	3	MAFF
113	LEV22	<i>Lycopersicon esculentum</i>	China	1	3	This study
114	L178	<i>Solanum tuberosum</i>	Nepal	1	3	NCAR
115	MAFF 302487	<i>Manihot esculenta</i>	Indonesia	1	4	MAFF
116	419-b-1-III	<i>Zingiber officinale</i>	Thailand	nt	4	T. Uematsu
117	412-c-1-I	<i>Zingiber officinale</i>	Thailand	nt	4	T. Uematsu
118	R277	<i>Zingiber officinale</i>	Australia	4	4	NCAR
119	R292	<i>Morus</i> sp.	China	5	5	NCAR

by repeated sampling with replacement, and the frequency with which a particular group was formed reflected the strength of the group.

RFLP analysis. Preparation of bacterial DNA was done according to the method of Cook et al. (3). Overnight cultures in CPG (3 ml) were used. Extracted DNA was digested with the restriction enzyme *EcoRI* (Toyobo Co., Osaka, Japan). Digested DNA samples (approximately 1 µg) were run on a 0.8% agarose (type II medium EEO; Sigma Chemical) gel in 0.5× Tris-borate-EDTA buffer (89 mM Tris-HCl, pH 8.0, 89 mM boric acid, and 2 mM EDTA) with electrophoresis at 6 V/cm for 5 h. Transfer of DNA fragments from agarose gel onto nylon membrane (Hybond-N+, Amersham International, Little Chalfont, England) and Southern hybridization using enhanced chemiluminescence labeling direct nucleic acid labeling and detection systems (Amersham International) were done according to the instructions of the manufacturer. After hybridization and stringency washes, signal was detected on a sheet of scientific imaging film (X-Omat; Eastman Kodak Co., Rochester, NY).

Two probes, *R. solanacearum* *hrpB* and *hrpO* genes controlling pathogenicity or hypersensitivity (10,13), were amplified by PCR method and used. Primers were synthesized by Amersham Pharmacia Biotech. Primers HBL1 (5'-TCG CCA AAA GCG AAA ACT-3') and HBR2 (5'-TCG AGT CAG ATG CAT GAT-3') amplified a nearly full-length *hrpB* gene from *R. solanacearum* MAFF 301520. Similarly, primers HOL1 (5'-AAC AGG AAC GAC GGA AAG-3') and HOR2 (5'-ATG GCC AAA AAG AAT GCA-3') amplified an internal region of the *hrpO* gene. The 100-µl reaction mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 µM each dNTP, 100 pmol primer, 2.5 unit of DNA polymerase Takara *Taq*, and 2 ng of template DNA) was overlaid with 90 µl of mineral oil (Sigma Chemical). Amplifications were performed on the thermocycler programmed as follows: an initial denaturation step of 94°C for 2 min, followed by 40 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min, with a final extension step of 72°C for 5 min. The amplified DNA was purified with an Easytrap kit (Takara Shuzo).

RESULTS

Pathogenicity and biovar determination. Seventy-four Japanese strains were tested for their pathogenicity and hypersensitivity, and were divided into four pathogenic groups (Table 2). Three groups (I to III) were pathogenic to tomato, eggplant,

sweet pepper, and potato, and mostly corresponded to race 1. Compatibility with tobacco (pathogenicity or hypersensitivity) differentiated group I from group II. Group III strains were characterized by weak pathogenicity to eggplant and potato. Group IV was pathogenic to potato, weakly pathogenic or nonpathogenic to tomato, and corresponded to race 3.

Race 1 strains were isolated from various plants that included cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita maxima* Duch. × *C. moschata* Duch.), kalanchoe (*Kalanchoe* sp.), statice (*Limonium* sp.), bird-of-paradise (*Strelitzia reginae* Ait.), and five solanaceous crops (tomato, eggplant, sweet pepper, tobacco, and potato) across Japan. But race 3 strains were only isolated from potato (Tables 1 and 3).

Of the 74 strains tested, 19 were biovar N2, 24 were biovar 3, and 31 were biovar 4, respectively. The 11 race 3 strains belonged to biovar N2, whereas race 1 strains were not directly related to specific biovars (Tables 1 and 2). All strains produced nitrite from nitrate. Race 1 strains, except for two isolates (MAFF 211268 and 211281), produced nitrogen gas profusely from nitrate, but this was not observed for any race 3 strains. Biovar N2 strains were isolated from five solanaceous crops, whereas biovar 3 strains were isolated from a range of species including kalanchoe, statice, bird-of-paradise, and five solanaceous crops. Biovar 4 strains were mainly isolated from tomato, eggplant, and potato (Table 1).

All strains of biovar 4 and some of biovar 3 induced tobacco HR within 24 h and were weakly pathogenic or nonpathogenic to tobacco, whereas some biovar 3 and N2 strains caused a slow or spreading necrosis and were all pathogenic to tobacco. On the other hand, biovar N2 strains corresponding to race 3 caused a slow collapse of infiltrated area of tobacco leaves, but were nonpathogenic to tobacco (Table 2).

Fingerprint analysis of Japanese strains based on the rep-PCR method. rep-PCR genomic fingerprints of 74 Japanese strains were generated (Fig. 1). Similarity coefficients were calculated based on the fingerprints and a dendrogram was constructed (Fig. 2). The three primer sets REP, ERIC, and BOX, after duplicate analyses, defined 35 fingerprint types at the 95% similarity level (greater than this could not discriminate each strain with confidence). Each strain that differed by race or biovar represented a distinct fingerprint type (Fig. 1). The strains tested were separated into two main groups; one with all race 1 strains, and the other with only race 3 strains. Average similarity within race 1 and race 3 strains was 53 and 93%, respectively, whereas

TABLE 2. Pathogenicity of Japanese strains of *Ralstonia solanacearum*

Code no.	Pathogenicity ^a					Tobacco HR ^b	Pathogenic group	Race	Biovar
	Tomato	Eggplant	Sweet pepper	Potato	Tobacco				
3, 7-12, 25-55	M-H	M-H	M-H	M-H	L-0	HR	I	1	3, 4
1, 2, 4, 13-24, 56-63	M-H	M-H	L-H	M-H	M-H	N	II	1	3, N2
5, 6	M-H	L	M-H	L	0	HR	III	1	3
64-74	L-0	0	0	M-H	0	C	IV	3	N2

^a Results based on average disease indices of two to four plants. Disease severity was observed 21 days after inoculation. H = high (4.1 to 5.0), M = medium (2.6 to 4.0), L = low (1.1 to 2.5), and 0 = no symptom (1.0).

^b HR = hypersensitive response within 24 h; N = slow or spreading necrosis on and after 48 h; and C = slow collapse of infiltrated area on and after 48 h.

TABLE 3. Grouping of Japanese strains of *Ralstonia solanacearum* on the basis of repetitive sequence-based polymerase chain reaction (rep-PCR) analysis

rep-PCR group	No. of strains	Geographical origin ^a	Race	Pathogenic group	Biovar	Original host
A	31	Hk, Ho, Sh, Ky	1	I	4	Potato, tomato, eggplant, sweet pepper, pumpkin, cucumber
B	9	Ho, Sh, Ky	1	II	3, N2	Tobacco, tomato, eggplant, sweet pepper
C	7	Ho, Sh	1	I	3	Tomato, eggplant, sweet pepper, statice
D	2	Ky	1	III	3	Bird-of-paradise
E	11	Ho, Sh	1	II	3	Tobacco, kalanchoe
F	3	Ho, Sh, Ky	1	II	3	Tobacco, tomato, eggplant
G	11	Ho, Ky	3	IV	N2	Potato

^a Hk = Hokkaido (North); Ho = Honshu (Middle East-Middle West); Sh = Shikoku (Middle West); and Ky = Kyushu (West-South West).

average similarity between race 1 and race 3 was 26%. Race 1 strains were further subdivided into six (A through F) groups at the 80% similarity level (Fig. 2). At this level, each of the groups (except group D) consisted of plural fingerprint types possessing many common bands with high reproducibility (Fig. 1) and in-

cluded strains that differed in biovar, pathogenic group, or original host (Table 3). In race 1, each of biovars N2 and 4 was included in a single group and showed high similarity within each biovar. On the other hand, biovar 3 strains were divided into five groups (B through F) and showed low average similarity (55%) among the strains.

Comparison of strains from Japan and other countries by the rep-PCR method. rep-PCR analysis was performed on 45 strains (representing six biovars and five races) from 15 countries (Table 1). The three primer sets defined 31 fingerprint types at the 95% similarity level and these types were compared with those of Japanese strains (Fig. 3). A dendrogram constructed based on rep-PCR data revealed two main clusters. Cluster 1 comprised biovar 3, 4, and 5 (race 1, 4, and 5) strains from Asia and Australia, and biovar 1 and N2 (race 1) strains from Reunion and Japan. Cluster 2 included biovar 1, 2, and N2 (race 1, 2, and 3) strains from 13 countries (Fig. 4). In cluster 2, average similarity within biovar 2 strains from eight countries (Belgium, Brazil, Egypt, Indonesia, Israel, Reunion, Sweden, and Thailand) and biovar N2 strains from Brazil were 94 and 65%, respectively, whereas average similarity between biovar N2 strains from Brazil and Japan was 21% (Table 4).

Comparison of biovar N2 and 2 strains from Japan and other countries by RFLP analysis. To confirm the genetic differences among the strains based on rep-PCR data, RFLP analysis was performed on 29 biovar N2 strains from Japan and Brazil and 12 biovar 2 strains from the eight countries. The RFLP patterns observed in Southern blots of *EcoRI*-digested DNA probed with

Fig. 1. Agarose gel showing representative patterns of Japanese strains generated by repetitive sequence-based polymerase chain reaction with **A**, BOX; **B**, enterobacterial repetitive intergenic consensus; and **C**, repetitive extragenic palindromic primers. Numbers above lanes indicate strain code number (Table 1) and group (Fig. 2). Lane m, DNA molecular size marker (kilobase DNA ladder, Stratagene, La Jolla, CA). Sizes are indicated on the left in kilobase pairs. Arrowheads identify similarities or differences among selected strains or groups.

Fig. 2. Genetic diversity of 74 strains of *Ralstonia solanacearum* isolated in Japan. The 74 strains were divided into 35 fingerprint types, and seven groups at the 80% similarity level on the basis of repetitive sequence-based polymerase chain reaction. Similarity coefficients were calculated based on fingerprints by the Dice method. A dendrogram was constructed by the unweighted pair group method with arithmetic average clustering. Values on the branches represent the results of bootstrap analysis with 2,000 iterations. Strain code number (Table 1), group, and biovar are indicated to the right.

Fig. 3. Agarose gel showing representative patterns of biovar N2 and 2 strains of *Ralstonia solanacearum* generated by repetitive sequence-based polymerase chain reaction with **A**, BOX; **B**, enterobacterial repetitive intergenic consensus; and **C**, repetitive extragenic palindromic primers. Numbers above lanes indicate strain code number (Table 1), race, biovar, and geographical origin. Lane m, DNA molecular size marker (kilobase DNA ladder, Stratagene, La Jolla, CA). Sizes are indicated on the left in kilobase pairs. Arrowheads identify similarities or differences among selected strains.

hrpO or *hrpB* are shown in Figure 5A and B. Biovar 2 strains showed only one RFLP pattern, whereas biovar N2 strains showed four RFLP patterns, which enabled Japanese strains to be distinguished from Brazilian strains.

DISCUSSION

There are several studies concerning the pathogenicity and biochemical properties of Japanese strains of *R. solanacearum* with four biovars (1, 2, 3, and 4) and two races (1 and 3) reported (24,33–37). The strains used in our experiments belong to biovars N2, 3, and 4 or races 1 and 3 (Tables 1 and 2). Among them, biovar N2 strains showed phenotypic and pathogenic variability and belonged to two distinct races. Biovar 2 comprises several phenotypes. Marin and El-Nashaar (31) and French et al. (8) reported biochemical and pathogenic differences between highland (Andes) and lowland biovar 2 strains. Highland biovar 2 mainly affects potato and tomato and are equivalent to race 3, whereas the lowland biovar 2 affected a wider range of plants (not equivalent to race 3) and was designated biovar N2 (new biovar 2) (11) or biovar 2T (tropical) (8). Biovar N2 is characterized by its use of trehalose, *meso*-inositol, and D-ribose, nitrite production from nitrate, and high pectolytic activity (17). The Japanese biovar 2 strains used in this study all utilized these three carbohydrates, produced nitrite from nitrate, and were almost identical to biovar N2. Katayama and Kimura (24) reported on the phenotypic char-

Fig. 4. Genetic diversity of 78 strains of *Ralstonia solanacearum* on the basis of repetitive sequence-based polymerase chain reaction. Similarity coefficient was calculated based on fingerprints by the Dice method. A dendrogram was constructed by the unweighted pair group method with arithmetic average clustering. Values on the branches represent the results of bootstrap analysis with 2,000 iterations. Strain code number (Table 1), group (in parenthesis), race, biovar, and geographical origin are indicated on the right.

acteristics of biovar 2 strains isolated from potato in Nagasaki prefecture, Japan, and their results were almost identical to ours. Our pathogenicity tests and theirs (24) showed that these potato strains only affect potato and belong to race 3 (Table 2).

Biovar N2 strains from tomato, eggplant, sweet pepper, and tobacco were pathogenic to five solanaceous crops and almost corresponded to race 1 (Table 2). Okabe and Goto (35) reported that Japanese strains that utilize lactose but not mannitol as a sole carbon source (resembling biovar N2 or 2) are strongly pathogenic to tomato, tobacco, eggplant, and peanut. Our results confirm this observation. These strains produced nitrogen gas from nitrate and were different from Japanese potato strains and biovar N2 and 2 strains from other countries.

rep-PCR analysis has been used to differentiate several species and strains of phytopathogenic bacteria (9,23,27–29,44). In this study, we used rep-PCR analysis to compare Japanese strains with other strains of *R. solanacearum*. Each primer set (REP, ERIC, or BOX) gave repeatable and distinct patterns (code no. 58 and 66 in Figs. 1 and 3). PCR-based techniques are usually subject to variations that reflect many factors, such as template and primer DNA concentrations and composition of the PCR buffer. We compared the band patterns that we observed (band sizes and intensities) with patterns previously observed by others and confirmed similarity for major bands. A dendrogram based on the rep-PCR fingerprints showed two main clusters; cluster 1 comprised all biovar 3, 4, and 5 strains, and cluster 2 included the majority of biovar 1, 2, and N2 strains (Fig. 4).

Cook et al. (3–5) assessed genetic diversity of *R. solanacearum* strains collected from many countries by the RFLP method and revealed two main divisions. Division I comprised all members of biovars 3, 4, and 5, and is thought to be of Asian origin. Division II contained all members of biovars 1, 2, and N2, and is thought to be of American origin. Taghavi et al. (47) analyzed 16S rRNA gene sequences of *R. solanacearum* and related species and identified two divisions that corresponded to the results obtained from RFLP analysis by Cook et al. (4).

Our rep-PCR results correspond closely with the divisions reported by Cook et al. (4). Our cluster 1 strains correspond to division I strains of Cook et al. (4), and our cluster 2 strains correspond to division II strains of Cook et al. (4).

R. solanacearum can be widely disseminated on vegetative propagating material (tomato, *Heliconia*, banana, and potato), and bacterial wilt of potato is spread locally and internationally by latently infected potato tubers (16). Buddenhagen (1) suggested that race 3 originated in the Andean region of South America where potato also originated and that the occurrence of race 3 outside South America was the result of human distribution of infected potato tubers. Several reports (3,5,11,44) using DNA-based techniques (RFLP, rep-PCR, PFGE, etc.) support this hypothesis.

In Japan, race 3 strains have been reported in some potato fields but not in others (24). Our data based on rep-PCR fingerprints showed low average similarity between race 3 biovar N2 from Japan and biovar N2 from Brazil or race 3 biovar 2 from eight different countries (Table 4). RFLP analysis using *hrpB* or *hrpO* as a probe also differentiated the Japanese strains from these other strains (Fig. 5).

TABLE 4. Average similarity coefficient within and between biovar N2 and biovar 2 strains of *Ralstonia solanacearum*^a

Biovar	Geographic origin (rep-PCR)	N2			
		Japan (B)	Japan (G)	Brazil	2
N2	Japan (B)	0.93	...	...	...
N2	Japan (G)	0.24	0.93	...	...
N2	Brazil	0.22	0.21	0.65	...
2	...	0.22	0.31	0.52	0.94

^a Similarity coefficient calculated by the Dice method (7).

Several biovar N2 strains have been reported in Indonesia and Africa (18,42,44,46). The genetic variability of Indonesian strains was shown by analyses of the rRNA gene (42,47). The 16S rRNA gene sequence from two race 3 biovar N2 Japanese strains was homologous to that of a race 1 biovar N2 strain (R780) from Indonesia (21). To decide whether the origin of Japanese race 3 is Indonesia or another area, further tests are needed.

Race 1 biovar N2 strains from Japan belong to cluster 1 (Fig. 4) with biovar 1 strains from Reunion (Africa), which showed a high similarity with biovar 3 and 4 strains in division I (41). Hayward et al. (18) reported that profuse gas production from nitrate is a valuable character for the identification of biovar 3, 4, and 5 strains. In cluster 1, all strains except two (code no. 116 and 117) provided profuse gas production from nitrate under anaerobic conditions (data not shown). Due to their nitrate metabolism and the rep-PCR data, race 1 biovar N2 strains from Japan might belong to the Asian origin group along with the biovar 3, 4, and 5 strains. However, to date, biovar N2 strains that atypically produce gas from nitrate have been reported only from Japan and a few African countries (18,46). The genetic relationship between the Japanese and the African biovar N2 strains is not clear.

Biovars 3 and 4 of *R. solanacearum* are homogeneous and distinct from other biovars of this species based on molecular analyses and physiological characteristics (3–5,26,38,47). However, analysis of Japanese strains based on rep-PCR fingerprint data showed that biovar 3 and 4 strains each belonged to distinct groups. Biovar 4 strains are homogeneous and belong to one group corresponding to one pathogenic group at the 80% similarity level. However, biovar 3 strains showed low average similarity among the strains and are divided into five groups that differ in pathogenic group or original host (Table 3; Fig. 2). Okabe and Goto (35) also reported that Japanese strains that utilize lactose and mannitol (probably corresponding to biovar 3) could be divided into 10 distinct groups that differ in pathogenicity and bacteriophage sensitivity. Phenotype and rep-PCR pattern may be correlated. Although our pathogenicity test results provide preliminary information concerning resistance/susceptibility levels for this wide range of strains, further large-scale tests are required for quantitative assessment of resistance/susceptibility.

Fig. 5. Southern blot analysis of total DNA from biovar N2 and 2 strains of *Ralstonia solanacearum*. The probe was A, *hrpO* or B, *hrpB*. Total DNA was digested with *EcoRI* and subjected to electrophoresis on a 0.8% agarose gel. Numbers above lanes indicate strain code number (Table 1), race, biovar, and geographical origin. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated on the left in kilobase pairs.

In the last 15 years, 13 new hosts (pumpkin, statice, kalanchoe, bird-of-paradise, etc.) of *R. solanacearum* have been reported in Japan (6,40). However, the origin of these new strains is unknown. The results presented here should help to clarify the genetic relationships among these strains and may contribute to the elucidation of their origin.

In a pumpkin field in Okayama prefecture, Japan, severely wilted plants were observed in close proximity to infected eggplant (6). rep-PCR results showed a high similarity between pumpkin and eggplant isolates (Table 3; Fig. 2), with a few of the eggplant isolates being pathogenic on pumpkin (data not shown). Our results also show that statice and kalanchoe isolates are closely related to tomato, eggplant, and sweet pepper isolates (group C) and tobacco isolates (group E), respectively. However, bird-of-paradise isolates consist of a single group (D) corresponding to one specific pathogenic group (III), and the relationship between these strains and other isolates is not clear.

In Japan, most strains from recently identified hosts are biovar 3 (6). This biovar has a wide host range (14,16) and has been isolated from various plants in Japan (6,40). Our results suggest that biovar 3 strains are phenotypically and genetically variable and have the potential to be compatible with more hosts than the other Japanese biovars. To confirm this hypothesis, further analysis with isolates from other hosts is under investigation.

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