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Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision

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The genetic diversity among strains in a worldwide collection of *Ralstonia solanacearum*, causal agent of bacterial wilt, was assessed by using three different molecular methods. PCR-RFLP analysis of the *hrp* gene region was extended from previous studies to include additional strains and showed that five amplicons were produced not only with all *R. solanacearum* strains but also with strains of the closely related bacteria *Pseudomonas syzygii* and the blood disease bacterium (BDB). However, the three bacterial taxa could be discriminated by specific restriction profiles. The PCR-RFLP clustering, which agreed with the biovar classification and the geographical origin of strains, was confirmed by AFLP. Moreover, AFLP permitted very fine discrimination between different isolates and was able to differentiate strains that were not distinguishable by PCR-RFLP. AFLP and PCR-RFLP analyses confirmed the results of previous investigations which split the species into two divisions, but revealed a further subdivision. This observation was further supported by 16S rRNA sequence data, which grouped biovar 1 strains originating from the southern part of Africa.

Keywords: bacterial wilt, PCR-RFLP, *hrp*, AFLP, 16S rRNA

INTRODUCTION

Ralstonia solanacearum causes bacterial wilt of a wide range of crops (potato, tomato, tobacco, banana, ginger, peanut, etc.) and is one of the most important plant diseases in tropical, subtropical and warm temperate regions of the world (Hayward, 1991). *R. solanacearum* shows great phenotypic and genotypic diversity, usually being divided into five races based on host range (Buddenhagen *et al*., 1962; He *et al*., 1983; Pegg & Moffett, 1971) and six biovars based on biochemical properties (Hayward, 1964; Hayward *et al*., 1990; He *et al*., 1983). The use of RFLP (Cook *et al*., 1989; Cook & Sequeira, 1994) allowed division of the species into two groups correlated with the geographical origin of strains: the 'Americanum' division contains biovar 1, 2 and N2 strains whereas the 'Asiaticum' division comprises biovars 3, 4 and 5 strains. Sequence analysis of the 16S rRNA gene (Li *et al*., 1993; Seal *et al*., 1993; Taghavi *et al*., 1996), the 16S–23S rRNA gene intergenic spacer region, the polygalacturonase gene and the endoglucanase gene (Fegan *et al*., 1998) have confirmed the two divisions and revealed a further subdivision including Indonesian isolates.

However, recent PCR-RFLP analysis of the *hrp* gene region (Poussier *et al*., 1999), including many African strains, which were rarely included in previous analyses of the genetic diversity of *R. solanacearum*, was not totally consistent with the above classification scheme since an African biovar 1 strains group belonged to the 'Asiaticum' division instead of the 'Americanum' division. Therefore, to clarify the relationships between these biovar 1 strains originating from the Southern part of Africa and other *R. solanacearum* isolates, three different approaches were compared and are presented

Abbreviations: AFLP, amplified fragment length polymorphism; BDB, blood disease bacterium; HCA, hierarchical cluster analysis; UPGMA, unweighted pair group method with arithmetic averages.

The GenBank accession numbers for the sequences determined in this work are AF207891–AF207897.

Table 1. *R. solanacearum*, *P. syzygii*, *R. pickettii* and the BDB strains used in this study

Strain*	Other designation†	Biovar or taxon	PCR-RFLP cluster and subcluster	AFLP cluster and profile‡	Geographical origin	Host
JS796	CFBP 1180	1	Ia	I 1	Puerto Rico	*Lycopersicon esculentum*
JS927	NCBBP 1225	1	Ia		Puerto Rico	*Lycopersicon esculentum*
JS833	UW30	1	Ia		Trinidad and Tobago	*Lycopersicon esculentum*
JR659	CFBP 2047	1	Ib	I 2	United States	*Lycopersicon esculentum*
JS783	CFBP 1036	1	Ib		United States	*Lycopersicon esculentum*
JT645	UW27	1	Ib		United States	*Nicotiana tabacum*
JS967	ICMP 7963	1	Ib	I 3	Kenya	*Solanum tuberosum*
JS831	UW26	1	Ic	I 4	United States	*Lycopersicon esculentum*
JS716	CFBP 705	1	IIa		Guyana	*Lycopersicon esculentum*
JS733	CFBP 2478	1	IIa	II 6	Guadeloupe	*Lycopersicon esculentum*
JS734	CFBP 2972	1	IIa	II 7	Martinique	*Solanum tuberosum*
JS768	CFBP 767	1	IIa		Guadeloupe	*Solanum tuberosum*
JS777	CFBP 770	1	IIa		Guadeloupe	*Lycopersicon esculentum*
JS784	CFBP 1036	1	IIa		Martinique	*Lycopersicon esculentum*
JS794	CFBP 1162	1	IIa		Guadeloupe	*Nicotiana tabacum*
JS837	UW90	1	IIa		Brazil	*Nicotiana tabacum*
JS838	UW275	1	IIa	II 8	Costa Rica	*Melampodium perfoliatum*
JS830	UW256	1	IIa		Costa Rica	*Solanum tuberosum*
JS779	CFBP 715	1	IIa	II 9	Burkina Faso	*Lycopersicon esculentum*
JS912	CFBP 3057	1	IIa		Burkina Faso	*Lycopersicon esculentum*
JS770	CFBP 712	1	IIa	II 10	Burkina Faso	*Solanum melongena*
JS845	CFBP 1175	1	IIa		Trinidad and Tobago	*Solanum melongena*
JS903	CFBP 3104	1	IIa		Peru	*Solanum tuberosum*
JT649	UW181	1	IIa	II 11	Venezuela	*Musa* sp. cv. plantain
JT652	UW469	1	IIb		Brazil	*Solanum tuberosum*
JS740	CFBP 1415	1	III	III 12	Colombia	*Solanum tuberosum*
JS788	CFBP 1412	1	III		Colombia	*Musa* sp. cv. plantain
JS847	CFBP 1419	1	III		Costa Rica	*Musa* sp.
JT648	UW162	1	III	III 13	Peru	*Musa* sp. cv. plantain
JT509		2	IVa	IV 16	Reunion Island	*Lycopersicon esculentum*
JT510		2	IVa	5	Reunion Island	*Solanum tuberosum*
JT511		2	IVa	IV 19	Reunion Island	*Solanum tuberosum*
JT512		2	IVa	IV 19	Reunion Island	*Lycopersicon esculentum*
JT513		2	IVa	IV 19	Reunion Island	*Solanum tuberosum*
JT514		2	IVa	IV 17	Reunion Island	*Solanum tuberosum*
JT515		2	IVa	IV 17	Reunion Island	*Lycopersicon esculentum*
JT516		2	IVa	IV 17	Reunion Island	*Solanum tuberosum*
JQ1102		2	IVa		Reunion Island	*Solanum tuberosum*
JQ1120		2	IVa		Reunion Island	*Solanum tuberosum*
JQ1084		2	IVa		Reunion Island	*Solanum tuberosum*
JQ1041		2	IVa		Reunion Island	*Solanum tuberosum*
JQ1051		2	IVa		Reunion Island	*Lycopersicon esculentum*
JQ1056		2	IVa	IV 27	Reunion Island	*Cyphomendra betacea*
JS780	CFBP 2148	2	IVa	IV 18	Reunion Island	*Solanum tuberosum*
JQ1144		2	IVa		Peru	*Solanum tuberosum*
JS738	CFBP 1413	2	IVa		Australia	*Solanum tuberosum*
JS737	CFBP 1417	2	IVa		Australia	*Solanum tuberosum*
JS758	CFBP 1420	2	IVa	IV 25	Colombia	*Solanum phureja*
JS792	CFBP 1810	2	IVa	IV 20	Haiti	*Solanum tuberosum*
JS774	CFBP 1414	2	IVa		Colombia	*Solanum tuberosum*

Table 1 (*cont.*)

Strain*	Other designation†	Biovar or taxon	PCR-RFLP cluster and subcluster	AFLP cluster and profile‡	Geographical origin	Host
JS887	CFBP 3785	2	IVa		Portugal	Unknown
JS895	CFBP 3673	2	IVa	IV 19	France	*Solanum tuberosum*
JS897	CFBP 3103	2	IVa		Peru	*Solanum tuberosum*
JS898	CFBP 3672	2	IVa		France	*Solanum tuberosum*
JS900	CFBP 3671	2	IVa		France	*Lycopersicon esculentum*
JS902	CFBP 3581	2	IVa		France	*Solanum tuberosum*
JS905	CFBP 3582	2	IVa	IV 19	Egypt	*Solanum tuberosum*
JS908	CFBP 3525	2	IVa	IV 19	Morocco	*Solanum tuberosum*
JS907	CFBP 3858	2	IVa	IV 19	The Netherlands	*Solanum tuberosum*
JS925	NCBBP 1323	2	IVa	IV 19	Sri Lanka	*Solanum tuberosum*
JS926	NCBBP 1331	2	IVa	IV 22	India	*Solanum tuberosum*
JS928	NCBBP 2797	2	IVa	IV 19	Sweden	*Solanum dulcamara*
JS929	NCBBP 2505	2	IVa		Sweden	*Solanum tuberosum*
JS930	NCBBP 1489	2	IVa	IV 21	Madeira	*Solanum tuberosum*
JS931	NCBBP 1049	2	IVa	IV 26	Kenya	*Lycopersicon esculentum*
JS932	NCBBP 1614	2	IVa	IV 23	Malaysia	*Solanum tuberosum*
JS935	NCBBP 339	2	IVa		Israel	Unknown
JS937	NCBBP 1789	2	IVa	IV 24	Greece	*Solanum tuberosum*
JS939	NCBBP 1824	2	IVa		Egypt	*Solanum tuberosum*
JS942	NCBBP 1019	2	IVa	IV 24	Portugal	*Lycopersicon esculentum*
JS943	NCBBP 613	2	IVa	IV 24	Brazil	*Solanum tuberosum*
JS948	NCBBP 2088	2	IVa	IV 24	Nigeria	*Solanum tuberosum*
JT572	NCBBP 752	2	IVa	IV 24	Zimbabwe	*Solanum tuberosum*
JT573	NCBBP 8088	2	IVa	IV 24	Rwanda	*Solanum tuberosum*
JT646	UW73	2	IVa		Sri Lanka	*Solanum tuberosum*
JT650	UW257	2	IVa		Costa Rica	*Solanum tuberosum*
JT653	UW470	N2	IVa		Brazil	*Solanum tuberosum*
JT658	R361	N2	IVa		Peru	*Solanum tuberosum*
JT660	R578	N2	IVa		Peru	*Solanum melongena*
JT683	R587	N2	IVa		Peru	*Solanum tuberosum*
JT662	R330	N2	IVa		Brazil	*Solanum tuberosum*
JT676	NCBBP 3990	N2	IVa		Peru	*Solanum tuberosum*
JT686	R572	N2	IVa	IV 29	Brazil	*Solanum american*
JT687	R564	N2	IVb		Brazil	*Solanum sysynbri*
JT677	NCBBP 3987	N2	IVb	IV 31	Brazil	*Solanum tuberosum*
JT689	R574	N2	IVc	IV 30	Peru	*Lycopersicon esculentum*
JT673	NCBBP 3985	N2	IVc		Peru	*Solanum melongena*
JT654	UW477	N2	IVd	IV 28	Peru	*Solanum tuberosum*
JS730	CFBP 1482	1	V		Panama	*Musa* sp.
JS775	CFBP 1409	1	V	III 14	Honduras	*Musa* sp.
JS793	CFBP 1183	1	V		Costa Rica	*Heliconia* sp.
JS791	CFBP 1416	1	V		Costa Rica	*Musa* sp. cv. plantain
JS748	CFBP 1416	1	V		Costa Rica	*Musa* sp. cv. plantain
JT644	UW9	1	V	III 15	Costa Rica	*Heliconia* sp.
JS781	CFBP 1185	3	Via	VI 43	Japan	*Lycopersicon esculentum*
JS836	UW8	3	Via	VI 44	Costa Rica	*Eupatorium odoratum*
JS842	UW119	3	Via		Costa Rica	*Solanum tuberosum*
JS940	NCBBP 500	3	Via	VI 45	Mauritius	*Vicia faba*
JS944	NCBBP 501	3	Via		Mauritius	*Brassica oleracea*
JS945	MAFF 301860	3	Via		Japan	*Capsicum annuum*
JS954	NCBBP 502	3	Via		Mauritius	*Casuarina equisetifolia*

Table 1 (*cont.*)

Strain*	Other designation†	Biovar or taxon	PCR-RFLP cluster and subcluster	AFLP cluster and profile‡	Geographical origin	Host
JS955	NCBBP 503	3	VIa		Mauritius	*Dahlia* sp.
JS743	CFBP 1418	3	VIa		Costa Rica	*Heliconia* sp.
JS763		3	VIa		Indonesia	Unknown
JS941§	NCBBP 3190	4	VIa	VI 46	Malaysia	*Lycopersicon esculentum*
JS832	UW378	4	VIa		China	*Olea* sp.
JS834	UW151	4	VIa	VI 56	Australia	*Zingiber officinale*
JS835	UW360	4	VIa		China	*Morus alba*
JS839	UW369	4	VIa		China	*Arachis hypogaea*
JT651	UW359	4	VIa		China	*Zingiber officinale*
JT517		3	VIb	VI 47	Reunion Island	*Pelargonium asperum*
JT518		3	VIb	VI 48	Reunion Island	*Solanum melongena*
JT519		3	VIb		Reunion Island	Unknown
JT520		3	VIb	VI 48	Reunion Island	*Pelargonium asperum*
JT521		3	VIb	VI 48	Reunion Island	*Lycopersicon esculentum*
JT522		3	VIb	VI 48	Reunion Island	*Lycopersicon esculentum*
JT523		3	VIb	VI 48	Reunion Island	*Solanum tuberosum*
JT524		3	VIb	VI 48	Reunion Island	*Lycopersicon esculentum*
JQ1060		3	VIb		Reunion Island	*Solanum tuberosum*
JQ1123		3	VIb		Reunion Island	*Solanum tuberosum*
JQ1090		3	VIb		Reunion Island	*Solanum tuberosum*
JQ1114		3	VIb		Reunion Island	*Solanum tuberosum*
JQ1072		3	VIb	VI 48	Reunion Island	*Allium cepa*
JQ1104		3	VIb		Reunion Island	*Solanum tuberosum*
JQ1138		3	VIb		Reunion Island	*Solanum tuberosum*
JQ1024		3	VIb		Reunion Island	*Lycopersicon esculentum*
JQ1129		3	VIb		Reunion Island	*Lycopersicon esculentum*
JQ1134		3	VIb	VI 49	Reunion Island	*Anthurium andreanum*
JS766	CFBP 726	3	VIb	VI 48	Reunion Island	*Solanum melongena*
JS778	CFBP 2041	3	VIb	VI 48	Reunion Island	*Solanum tuberosum*
JS747	CFBP 726	3	VIb		Reunion Island	*Solanum melongena*
JT571	ICMP 759	3	VIb		Kenya	*Solanum melongena*
JT674	NCBBP 1045	3	VIb	VI 48	Kenya	*Solanum melongena*
JT678	NCBBP 1486	3	VIb	VI 48	Uganda	*Arachis hypogaea*
JT675	NCBBP 1763	3	VIb	VI 48	Seychelles	*Lycopersicon esculentum*
JT574	NCBBP 7960	4	VIb		Kenya	*Capsicum annuum*
JS841	UW74	4	VIb	VII 58	Sri Lanka	*Solanum tuberosum*
JS933	MAFF 301418	4	VIb		Japan	*Lycopersicon esculentum*
JT690	MAFF 211266	1	VIb	40	Japan	*Lycopersicon esculentum*
JT691	MAFF 211267	1	VIb	40	Japan	*Lycopersicon esculentum*
JS722	CFBP 1813	3	VIc		Guyana	*Solanum melongena*
JS718	CFBP 2480	3	VIc		Guadeloupe	*Solanum melongena*
JS729	CFBP 2965	3	VIc	VI 50	Guadeloupe	*Solanum melongena*
JS719	CFBP 2970	3	VIc		Martinique	*Capsicum annuum*
JS715	CFBP 2976	3	VIc		Martinique	*Ensete ventricosum*
JS753	GMI1000	3	VIc	VI 52	Guyana	*Lycopersicon esculentum*
JS759	CFBP 1168	3	VIc	VI 50	Trinidad and Tobago	*Musa* sp.
JS772	CFBP 707	3	VIc	VI 53	Tahiti	*Lycopersicon esculentum*
JS773	CFBP 1960	3	VIc	VI 50	Algeria	*Capsicum annuum*
JS840	UW147	3	VIc	VI 54	Australia	*Nicotiana tabacum*
JS843	UW130	3	VIc	VI 51	Peru	*Lycopersicon esculentum*
JT647	UW152	3	VIc		Australia	*Solanum tuberosum*

Table 1 (*cont.*)

Strain*	Other designation†	Biovar or taxon	PCR-RFLP cluster and subcluster	AFLP cluster and profile‡	Geographical origin	Host
JS782	CFBP 1038	3	VIc		Guadeloupe	*Lycopersicon esculentum*
JS888	CFBP 3258	3	VIc		Unknown	Unknown
JS947	NCBBP 1123	4	VIc	VII 59	Papua New Guinea	*Lycopersicon esculentum*
JS953	MAFF 301552	3	VId	VI 55	Japan	*Lycopersicon esculentum*
JS936	NCBBP 3181	3	VIe	57	Gambia	*Solanum nicanum*
JS904	CFBP 3059	1	VIe	32	Burkina Faso	*Solanum melongena*
JT661	R292	5	VIf	VII 60	China	*Morus alba*
JT685	R322	5	VIf	VII 61	China	*Morus alba*
JS950	NCBBP 1018	1	VIIa	V 33	Angola	*Solanum tuberosum*
JT525		1	VIIb	V 34	Reunion Island	*Pelargonium asperum*
JT526		1	VIIb	V 34	Reunion Island	*Pelargonium asperum*
JT527		1	VIIb	V 34	Reunion Island	*Pelargonium asperum*
JT528		1	VIIb	V 34	Reunion Island	*Solanum tuberosum*
JT529		1	VIIb	V 34	Reunion Island	*Pelargonium asperum*
JT530		1	VIIb	V 34	Reunion Island	*Pelargonium asperum*
JT531		1	VIIb	V 34	Reunion Island	*Solanum tuberosum*
JQ1040		1	VIIb	V 34	Reunion Island	*Lycopersicon esculentum*
JT532		1	VIIb		Reunion Island	Unknown
JS756	CFBP 2146	1	VIIb	V 35	Reunion Island	*Pelargonium capitatum*
JS767	CFBP 734	1	VIIb	V 36	Madagascar	*Solanum tuberosum*
JS946	NCBBP 283	1	VIIb	V 37	Zimbabwe	*Solanum panduraforme*
JS949	NCBBP 332	1	VIIb	V 38	Zimbabwe	*Solanum tuberosum*
JS951	NCBBP 505	1	VIIb	V 39	Zimbabwe	*Symphytum* sp.
JS952	NCBBP 342	1	VIIb		Zimbabwe	*Nicotiana tabacum*
JS966	ICMP 748	1	VIIb		Zimbabwe	*Solanum tuberosum*
JS934§	MAFF 301558	N2	VIIc	62	Japan	*Solanum tuberosum*
JT656	R604	BDB	IXb	42	Indonesia	*Musa* sp.
JT657	R230	BDB	IXb		Indonesia	*Musa* sp.
JT680	NCBBP 3726	BDB	IXb		Indonesia	*Musa* sp.
	R006	*P. syzygii*	IXc		Indonesia	*Syzygium aromaticum*
JV1010	R024	*P. syzygii*	IXa		Indonesia	*Syzygium aromaticum*
JV1011	R028	*P. syzygii*	IXa		Indonesia	*Syzygium aromaticum*
JR660	CFBP 2459	*R. pickettii*		41	Unknown	Unknown

* Designation of strains of the Laboratoire de Phytopathologie, CIRAD-FLHOR, 97448 Saint-Pierre, La Réunion, France.

† CFBP , Collection Française de Bactéries Phytopathogènes, Angers, France; NCPPB , National Collection of Plant Pathogenic Bacteria, Harpenden, UK; ICMP , International Collection of Microorganisms from Plants, Auckland, New Zealand; UW, D. Cook and L. Sequeira, Department of Plant Pathology, University of Wisconsin-Madison, USA; GMI, M. Arlat and P. Barberis, CNRS-INRA, Auzeville, Castanet-Tolosan Cedex, France; MAFF , Ministry of Agriculture Forestry and Fisheries, National Institute of Agrobiological Resources, Japan; R, Institute of Arable Crops Research–Rothamsted, Harpenden, UK.

‡ Roman numerals indicate AFLP clusters and arabic numerals indicate AFLP profiles.

§ Strains described in our previous paper (Poussier *et al.*, 1999) and reclassified as the result of a new biovar determination.

in this paper. Firstly, 59 additional strains of *R. solanacearum*, including biovar N2 and 5 strains and new African strains, were analysed using PCR-RFLP. Two close relatives of *R. solanacearum*, *Pseudomonas syzygii* (causal agent of Sumatra disease of cloves) and the blood disease bacterium (BDB, causal agent of blood disease of bananas) (Eden-Green, 1994; Seal *et al.*, 1993; Taghavi *et al.*, 1996) were also analysed, permitting the specificity of our PCR-RFLP test and the phylogenetic relationships between these three bacteria to be assessed. Secondly, we have used the very powerful DNA fingerprinting technique AFLP (Vos *et al.*, 1995), which allows very fine whole genome analysis. AFLP methodology has already been used to study the diversity of race 3 isolates of *R. solanacearum* (Van der Wolf *et al.*, 1998) but has never been used to analyse a worldwide collection of *R. solanacearum* strains. Finally, we have determined nearly complete 16S rRNA gene sequences for seven (five African, one American and one Japanese) biovar 1 strains of *R. solanacearum*, and compared these

with 19 previously sequenced *R. solanacearum* 16S rRNA gene sequences (Taghavi *et al*., 1996).

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. Culture conditions and biovar determination of *R. solanacearum* isolates were as described previously (Poussier *et al*., 1999).

DNA isolation. Genomic DNA of strains was extracted using the hexadecyltrimethylammonium bromide method (Ausubel *et al*., 1991).

PCR-RFLP analysis. DNA amplification conditions and digestion with eight restriction endonucleases of the five PCR products were as previously described (Poussier *et al*., 1999). Each PCR-RFLP was duplicated to assure its reproducibility. Two different Hierarchical Cluster Analysis (HCA) methods were used to analyse the PCR-RFLP band data collected. Using STATLAB version 2.0 (SLP Statistiques, Monterey, CA, USA), clustering was based on the Euclidean distance between strains (Ward, 1963). The truncation level in the resulting dendrogram was thus determined to be that which provided the smallest number of clusters for which the variance within clusters was significantly ($P = 0{\cdot}05$) different from the variance between clusters. Using the PHYLIP software package (Felsenstein, 1995), a distance matrix was firstly constructed with the Nei (1973) genetic distance of the GENDIST program. A dendrogram was then constructed from genetic distance values by using the unweighted pair group method with arithmetic averages (UPGMA) (Sneath & Sokal, 1973) contained in the NEIGHBOR program. Finally, the strength of the tree topology was assessed by the bootstrap method (Felsenstein, 1985) of the SEQBOOT program.

AFLP analysis. Ninety-six *R. solanacearum* strains, one BDB strain and one *Ralstonia pickettii* strain were analysed using AFLP as described by Janssen *et al*. (1996) and Vos *et al*. (1995) with slight modifications. We used *Msp*I and *Sac*I to digest DNA instead of *Eco*RI and *Mse*I.

Genomic DNA (200 ng per sample) was digested for 2 h at 37 °C in 50 µl (final volume) containing 5 U *Msp*I, 5 U *Sac*I (Amersham Pharmacia Biotech), 0·125 µl BSA (10 µg µl^{-1}) and 2·5 µl 10× 'One Phor All' buffer (Amersham). Next, 50 pmol double-stranded *Msp*I-adapter (5′-GACGATGAGTCCTGAA-3′, 5′-CGTTCAGGACTCATC-3′) (50 pmol µl^{-1}), 5 pmol double-stranded *Sac*I-adapter (5′-CTCGTAGACTGCGTACAAGCT-3′, 5′-TGTACGCAGTCTAC-3′) (5 pmol µl^{-1}) (Genset), 1 µl ATP (10 mM), 1 U T4 DNA ligase (5 U µl^{-1}) (Appligene) and 2·5 µl 10× 'One Phor All' buffer were added to the digested DNA and the ligation reactions were performed for 3 h at 20 °C. Digested (D) and ligated (L) DNA were diluted (D) eightfold and the resulting DLD DNA was then stored at 4 °C until used.

Selective amplifications were done with two primers (*Msp*I-primer and *Sac*I-primer) (Genset) complementary to the adapter sequences, and the *Msp*I and *Sac*I restriction sites respectively, with additional selective nucleotides at their 3′ ends (cytosine for the *Sac*I-primer and cytosine plus guanine for the *Msp*I-primer). The *Sac*I-primer was labelled with γ-^{33}P [10 µCi (370 kBq) per DNA amplification; Nen Life Science Products] and T4 polynucleotide kinase (Gibco-BRL).

DNA amplifications were carried out in a 50 µl reaction mixture. DLD DNA (5 µl) was added to 45 µl mixture containing 5 µl 10× PCR buffer (Gibco-BRL), 1·5 µl labelled *Sac*I-primer (50 µg µl^{-1}), 2 µl unlabelled *Msp*I-primer (30 µg µl^{-1}), 1·5 µl $MgCl_2$ (50 mM), 8 µl of each dNTP (1·25 mM) and 0·6 µl *Taq* polymerase (5 U µl^{-1}; Gibco-BRL). Amplifications were performed with a thermocycler (Mastercycler gradient Eppendorf) by using the following protocol: 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min.

Amplified fragments were separated by electrophoresis on 5 % polyacrylamide gels and fingerprint patterns were visualized as described by Vos *et al*. (1995). The reproducibility of AFLP was assessed by comparing the DNA fingerprinting obtained from duplicate assays of 14 strains. Duplicate DNA fingerprints were produced using two aliquots from two different DNA amplifications that were run in different gels. AFLP data analysis was performed as described above for PCR-RFLP.

16S rRNA sequencing. Seven biovar 1 strains of *R. solanacearum* were used: CFBP 1036, CFBP 712, NCPPB 1018, CFBP 2146, CFBP 734, NCPPB 342 and MAFF 211266. 16S rRNA genes were amplified using the PCR as detailed by Taghavi *et al*. (1996) with slight modifications. PCR amplifications were carried out in a thermocycler (GeneAmp PCR system 9600; Perkin-Elmer) in a 50 µl (total volume) reaction mixture containing 10× buffer (200 mM Tris/HCl, 500 mM KCl, pH 8·4; Gibco-BRL), 1·5 mM $MgCl_2$ (Gibco-BRL) 200 µM of each dNTP (Boehringer Mannheim), 0·25 µM primer 27f, 0·25 µM primer 1525r (Genosys Biotechnologies) (Taghavi *et al*., 1996), 1 U *Taq* polymerase (Gibco-BRL) and 100 ng template DNA. The following PCR profile was used: initial denaturation at 96 °C for 2 min; 30 cycles consisting of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min; and final extension at 72 °C for 10 min.

PCR products were electrophoresed using 1 % agarose gels at 5 V cm^{-1} for 1 h and visualized with UV light after ethidium bromide staining. Amplification products were purified from the agarose gel slice by using the QIAquick purification kit PCR (Qiagen) according to the manufacturer's instructions.

PCR product sequences were determined by Cambridge Bioscience, Cambridge, UK. The GenBank accession numbers of the seven resulting 16S rRNA gene sequences are shown in Table 3. They were aligned using the 'gap proceed' program of the Genetics Computer Group software package (Genetics Computer Group, 1999) with the published 16S rRNA gene sequences of *R. solanacearum*, BDB and *P. syzygii* isolates studied by Taghavi *et al*. (1996). Data analysis was performed using the DNADIST (Jukes & Cantor, 1969) and NEIGHBOR (UPGMA method) programs of the PHYLIP software package, and using the STATLAB software.

RESULTS

PCR-RFLP analysis

Both of the bacteria closely related to *R. solanacearum* that were examined, *P. syzygii* and the BDB, which were not included in our previous study (Poussier *et al*., 1999), gave a single band of the expected size after amplification using each of the five pairs of primers. After suitable digestion of the amplicons, the BDB and *P. syzygii* strains could be separated from the *R. solanacearum* strains. Indeed, their resulting profiles displayed specific restriction patterns (Table 2). Pattern DHa7 (band sizes 608 and 281 bp) was common to the BDB and *P. syzygii* strains whereas APv5 (band size 1452 bp; no *Pvu*II restriction site within the amplified fragment) was specific to *P. syzygii* strains. EBs7 (band sizes visible on gel 1106, 125, 109 bp) was produced by only one *P. syzygii* strain, R006. In addition, two restriction

Table 2. Characterization of the eight new PCR-RFLP profiles identified within the 59 additional strains of *R. solanacearum*, three BDB strains and three *P. syzygii* strains

PCR-RFLP profile*	Subcluster	Organism
AAv2, APv1, BHi2, CSa1, DHa2, EBs5, ENo2, EPs3	IIb	*R. solanacearum*
AAv2, APv2, BHi2, CSa2, DHa2, EBs5, ENo2, EPs2	IVb	*R. solanacearum*
AAv2, APv2, BHi2, CSa2, DHa2, EBs5, ENo3, EPs2	IVc	*R. solanacearum*
AAv2, APv2, BHi2, CSa2, DHa2, EBs2, ENo3, EPs3	IVd	*R. solanacearum*
AAv2, APv1, BHi7, CSa1, DHa3, EBs3, ENo1, EPs1	VIf	*R. solanacearum*
AAv2, APv5, BHi5, CSa3, DHa7, EBs3, ENo1, EPs1	IXa	*P. syzygii*
AAv2, APv2, BHi5, CSa3, DHa7, EBs3, ENo1, EPs1	IXb	BDB
AAv2, APv5, BHi5, CSa3, DHa7, EBs7, ENo1, EPs1	IXc	*P. syzygii*

* A profile is the combination of eight restriction patterns generated through the digestion of the five amplified fragments by the designated enzymes. Each pattern was given a code containing three letters and one number; the first letter refers to the amplified fragment (A as delineated by primers RS20-RS201, B by RS30-RS31, C by RS50-RS501, D by RS600-R61and E by RS80-RS81); the following letters indicate the enzyme (*Ava*I, *Pvu*II, *Hind*II, *Sac*I, *Hae*II, *Bss*HII, *Not*I and *Pst*I); and the number refers to the pattern generated by the enzyme.

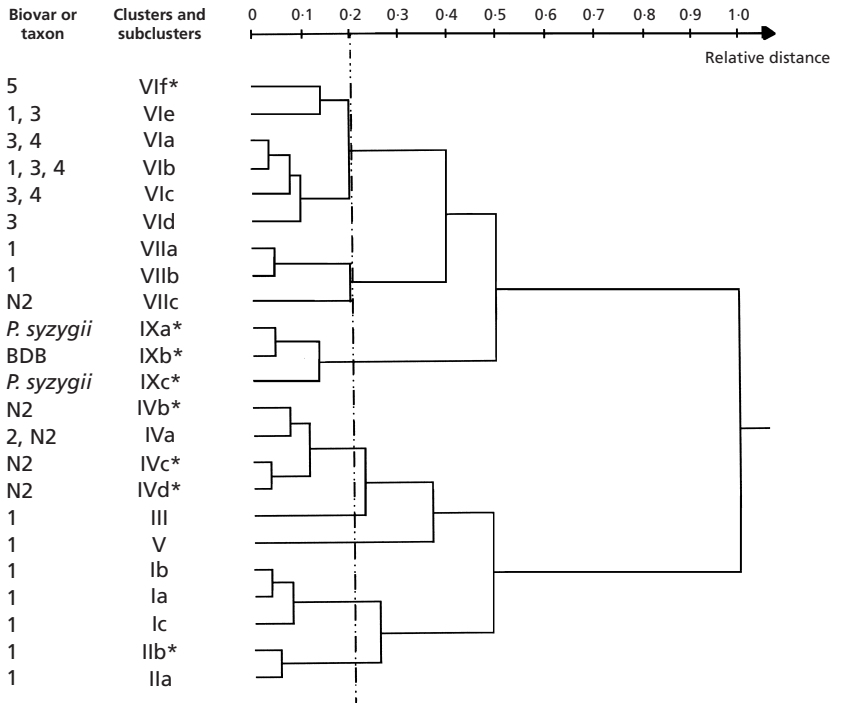

Fig. 1. Dendrogram resulting from a HCA (Ward, 1963) based on the restriction patterns of the five amplified fragments within the *hrp* gene region of the 184 strains of *R. solanacearum* and closely related bacteria (*P. syzygii* and the BDB). Asterisks indicate new subclusters as compared to those of Poussier *et al.* (1999). The relative distance between the farthest clusters is assumed to be 1. The biovar N2 strain MAFF 301558 was previously included in cluster VIII (Poussier *et al.*, 1999) but according to the new HCA, it was included in subcluster VIIc.

patterns, BHi5 and CSa3, produced by the BDB and *P. syzygii* strains were shared with the unique *R. solanacearum* strain MAFF 301558.

The dendrogram resulting from a HCA (Fig. 1), produced by STATLAB or PHYLIP software packages, showed that *R. solanacearum* could be separated into two divisions. The first division comprised about 64% of biovar 1 strains and all of the biovar 2 and N2 strains; the second division contained about 36% of biovar 1 strains, all of the biovar 3, 4 and 5 strains and one biovar N2 strain MAFF 301558. The BDB and *P. syzygii* isolates fell into the second division. No significant difference was observed between clusterings resulting from either of the two different software packages (results not shown). The dendrogram generated by the STATLAB software (Fig. 1) agreed with previous results (Poussier *et al.*, 1999). However, amongst the additional strains, five new profiles and one new restriction pattern were identified (Table 2). In particular, the biovar 1 strain UW469 gave a new profile (IIb), whilst the Japanese and Burkina Faso biovar 1 strains (MAFF 211266, MAFF 211267 and CFBP 3059) were grouped with biovar 3 strains into subclusters VIb and VIe. Three of the identified biovar 1/race 2 isolates, UW9, UW162 and UW181, were distributed into three different clusters: subcluster IIa, cluster III and cluster V. Five of the twelve biovar N2 strains used generated three new profiles

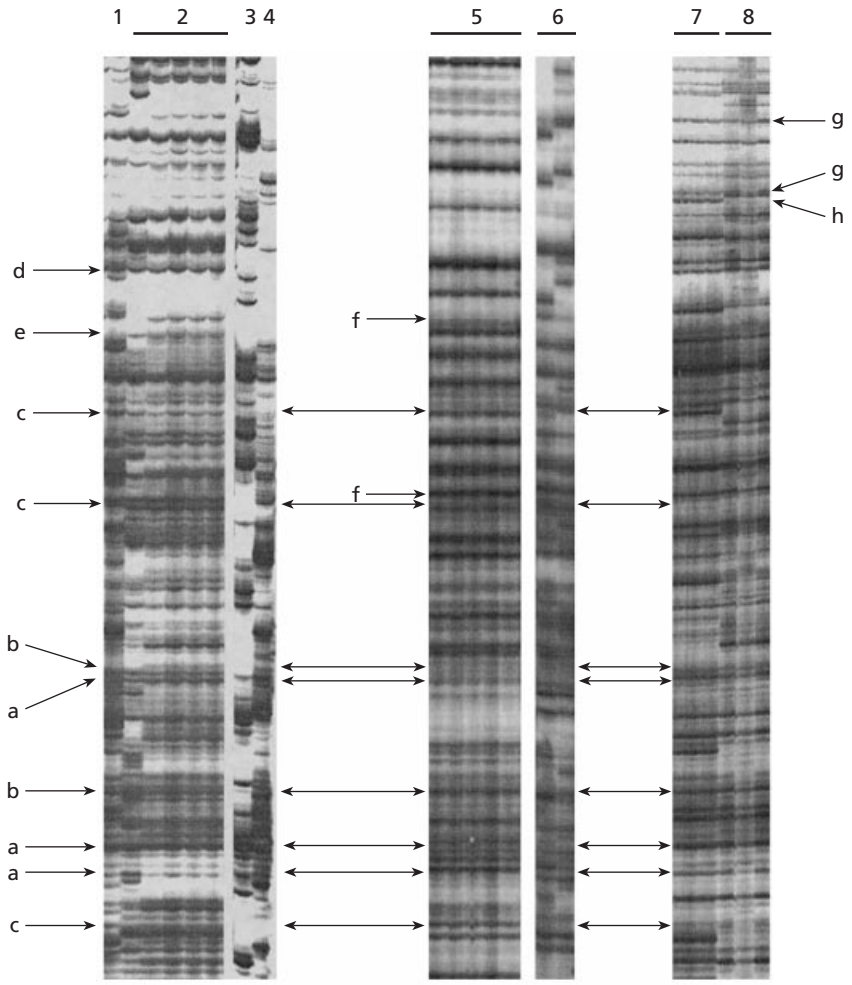

Fig. 2. A selection of AFLP fingerprints of *R. solanacearum* strains and related bacteria. Lanes: 1, Japanese biovar 1, MAFF 211267; 2, African biovar 1, NCPPB 1018, JT525, JT526, JT527, JT528; 3, *R. pickettii*, JR660; 4, BDB, JT657; 5, biovar 2, CFBP 3673, CFBP 3582, CFBP 3525, CFBP 3858, NCPPB 1323; 6, biovar N2, R572, R574; 7, African biovar 3, NCPPB 1045, NCPPB 1486, NCPPB 1763; 8, non-African biovar 3, CFBP 2965, GMI1000, CFBP 1168. Arrows indicate examples of polymorphic and specific fragments. Fragments are specific to (a) *R. solanacearum, R. pickettii* and the BDB; (b) *R. solanacearum* and the BDB; (c) *R. solanacearum;* (d) African and Japanese biovar 1; (e) African biovar 1; (f) biovar 2 and N2; (g) biovar 3, 4 and 5; (h) African biovar 3. Since five images derived from three different gels were associated to make this figure, exact alignment was not always possible (symbolized by double-headed arrows).

(IVb, IVc and IVd) and thus could be distinguished from biovar 2 strains whereas the seven remaining biovar N2 strains could not be separated (subcluster IVa). All of the new African biovar 3 strains fell into a single subcluster (VIb). Two strains belonging to biovar 5 were characterized by a new profile, VIf, and a new restriction pattern, BHi7 (band sizes 779, 745, 304 and 172 bp).

AFLP analysis

One hundred and fifty-nine DNA fragments which were reproducibly detected, ranging from 50 to 350 bp, were selected for the analysis of 96 *R. solanacearum* strains and two isolates belonging to two closely related taxa: the BDB and *R. pickettii*. Amongst these, 62 different DNA fingerprints were identified. Different DNA fingerprints were generated from strains belonging to the same biovar (for example 23, 12 and 13 from biovars 1, 2 and 3, respectively), but also originating from the same geographical area (for instance 6 from biovar 2 strains from Reunion Island). Identical DNA patterns could be produced by strains originating from different countries (for example IV 19 was found in strains from 7 countries).

Depending on the strain, DNA patterns contained 50–80 different DNA bands (Fig. 2). Comparison of AFLP fingerprints revealed that 95% of the fragments were polymorphic. Three fragments appeared to be specific to *R. solanacearum* species. Moreover, five AFLP fragments were common to all *R. solanacearum* strains: three of them were shared with the BDB and *R. pickettii* strains and two of them were shared with only the BDB strains. One fragment was specific to the BDB isolate. Many of the DNA fragments were useful in distinguishing subgroups within *R. solanacearum* species. For instance, one fragment was found only in all of the biovar 1, 2 and N2 strains (except for the biovar N2 strain MAFF 301558). Eight fragments were produced by all of the biovar 3, 4 and 5 strains (with one, two or three exceptions depending on the fragment). No DNA band appeared to be specific to all biovar 1 strains; however one fragment was common to all *R. solanacearum* strains other than biovar 1 strains. African and Japanese biovar 1 isolates were characterized by three and two fragments, respectively. Six particular DNA bands were specific to biovar 2 and N2 isolates, except for strains JT510 and MAFF 301558. Strain JT510 (biovar 2) was unusual since it shared less than 60% of the AFLP fragments with other biovar 2 isolates. One fragment was common to all biovar N2 strains, except

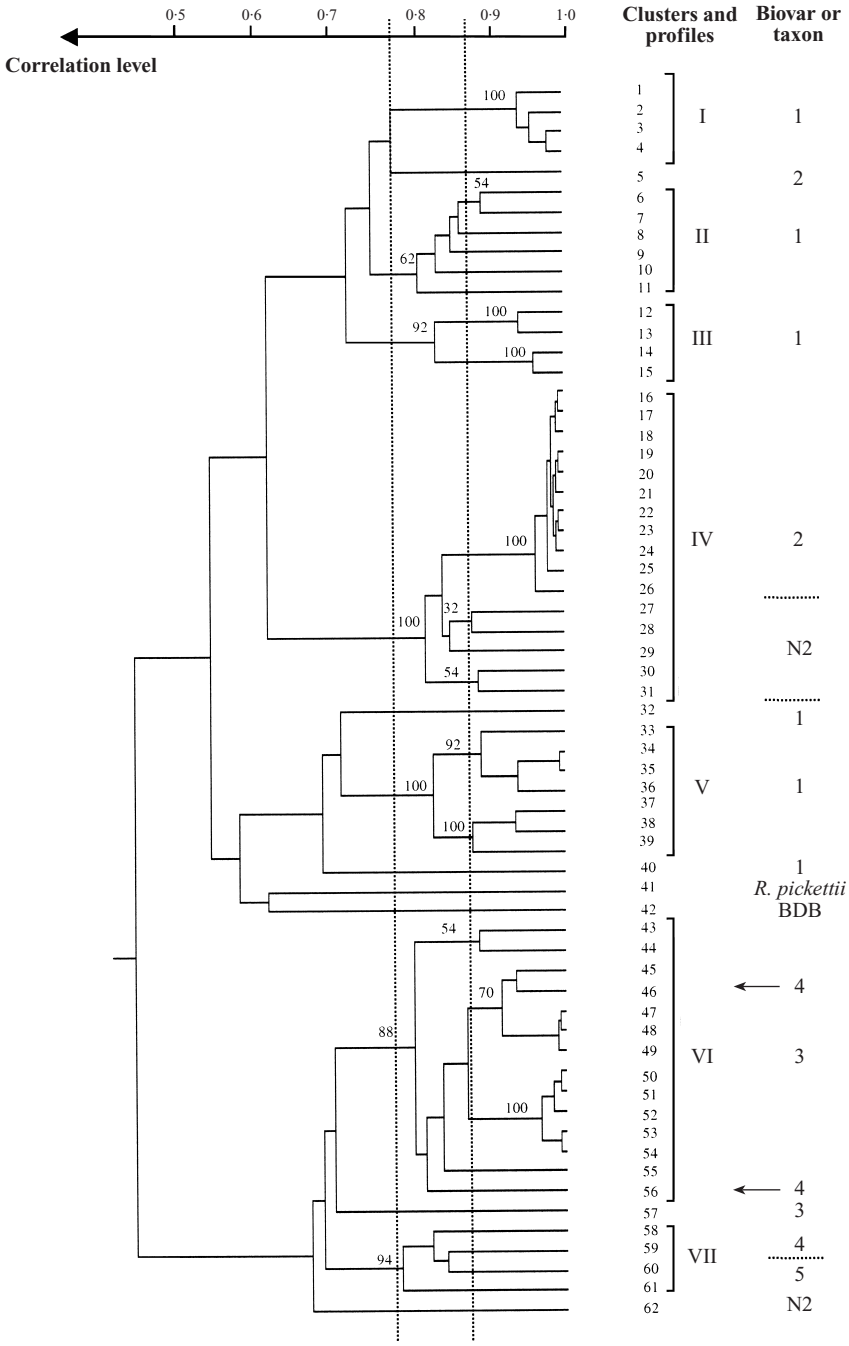

Fig. 3. Dendrogram constructed by using UPGMA showing correlation between AFLP fingerprints of 98 *R. solanacearum* strains and related bacteria (the BDB and *R. pickettii).* Roman numerals indicate clusters and arabic numerals indicate profile types. Numbers at the branch points are the percentages of bootstrap replicates in which the clusters were found.

for strains JT510, JQ1056 and MAFF 301558. Seventeen AFLP fragments could discriminate between the two similar biovars 2 and N2. One fragment was specific for biovar 3 strains (two exceptions) and one only for African biovar 3 strains. No fragments discriminating biovar 4 or 5 isolates were obtained, but these isolates were distinguished from biovar 3 strains by 14 DNA bands.

The dendrogram generated by the PHYLIP software package (Fig. 3) showed that *R. solanacearum* could be split into two divisions: the first division contained biovar 1, 2 and N2 isolates and the second division contained biovar 3, 4 and 5 isolates (except for one particular biovar N2 strain, MAFF 301558). The truncation level, at 0·78 correlation, allowed separation of seven AFLP clusters designated clusters I–VII. High bootstrap values indicated that this clustering was well supported and the tree was robust. The close relatives of *R. solanacearum*, *R. pickettii* and the BDB, were found to be in the first division and exhibited specific AFLP profiles. Clusters appeared to be grouped according to biovar, geographical origin or host origin. Clusters I, II and III included biovar 1 strains originating from the Americas (three coming from Northern Africa) whilst cluster V contained biovar 1 strains originating from the

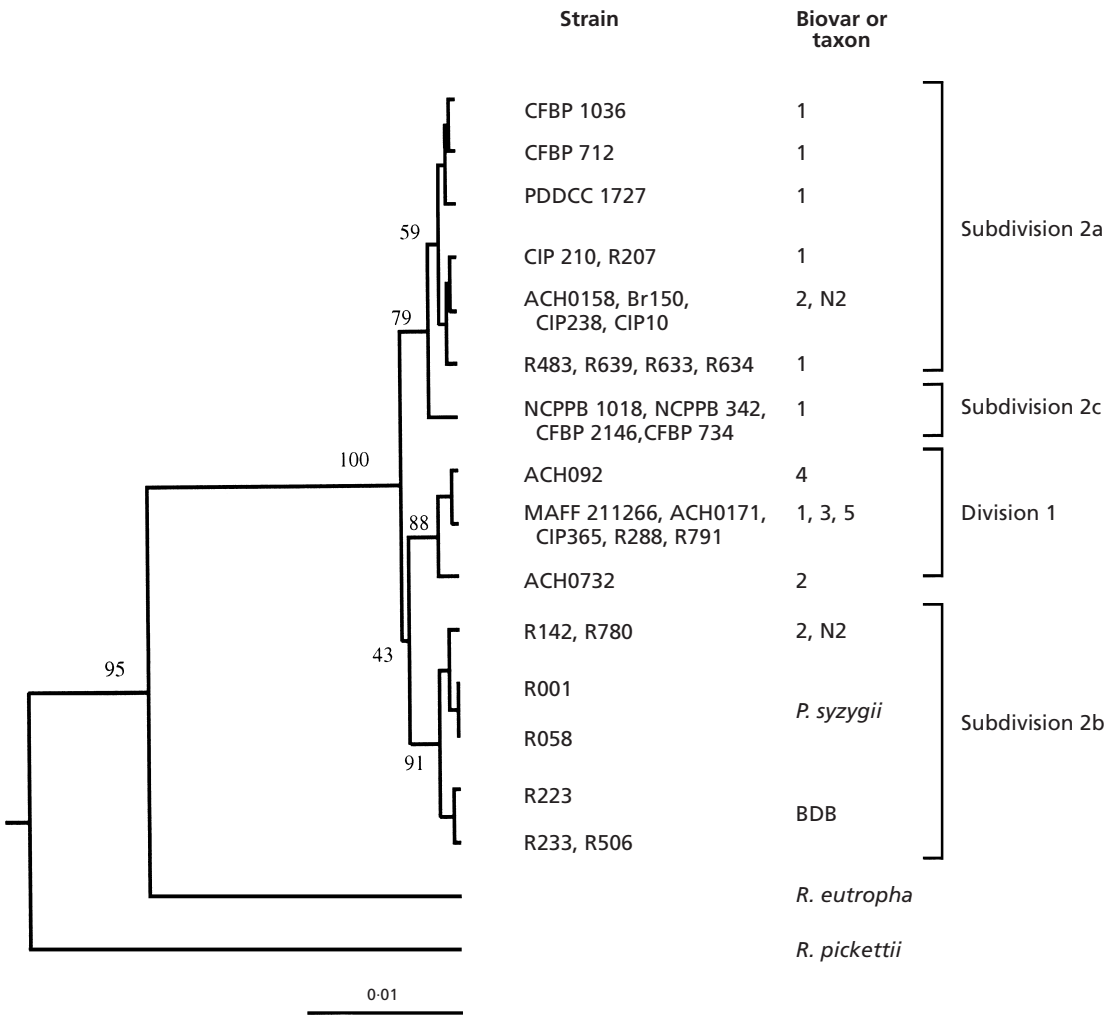

Fig. 4. Dendrogram constructed by using UPGMA showing the phylogenetic relationships of *R. solanacearum* strains and strains of closely related bacteria (*P. syzygii* and the BDB), based on 16S rDNA sequence comparisons. *Ralstonia eutropha* and *R. pickettii* were used as outgroups in this analysis. The numbers at the branch points are the percentages of bootstrap replicates in which the clusters were found. The GenBank accession numbers for the I6S rDNA sequences of the *R. eutropha* and *R. pickettii* strains are AF027407 and X67042, respectively.

Southern part of Africa. Strains belonging to cluster III were isolated from musaceous plants, except for one from potato. Cluster IV grouped biovar 2 and N2 isolates. Biovar 3 strains were assigned to cluster VI. Isolates belonging to biovars 4 and 5 were grouped together in cluster VII, except for two biovar 4 strains in cluster VI. Strains JT510, CFBP 3059, MAFF 211266, MAFF 211267, NCPPB 3181 and MAFF 301558 were unusual since they were members of unique branches. Moreover, a second truncation level (Fig. 3), at 0·87 correlation, could be used to make a more precise discrimination of strains, particularly between biovar 2 strains and biovar N2 strains, and between biovar 3 strains originating from Africa and biovar 3 strains from the other continents. This clustering was also well supported by high bootstrap values, except for some weaker values, 54% and 32%. The clustering obtained with the STATLAB software was approximately the same as that obtained with the PHYLIP software package except that certain strains which were not well separated from the other strains using STATLAB were separated using PHYLIP (result not shown).

16S rRNA sequence analysis

Nearly complete sequences of 16S rRNA genes were determined for 7 biovar 1 isolates of *R. solanacearum* and compared to 24 published sequences of *R. solanacearum* isolates and the closely related bacteria, *P. syzygii* and the BDB.

A phylogenetic tree was produced using the PHYLIP software package by comparing 1431 nucleotide positions, omitting all of the ambiguous nucleotides, and revealed two divisions, each of which was split into two subdivisions (Fig. 4). This tree was not completely consistent with that obtained by Taghavi *et al.* (1996) since the subdivision 2b appeared to be more closely related to division 1 than to subdivision 2a. However,

Table 3. The 16S rRNA sequence differentiation among 31 isolates of *R. solanacearum* and related bacteria

Organism	Strain*	Country	Host	Biovar	Division or subdivision	GenBank accession no.	Nucleotide(s) at position(s)†																					
							165	167	204	205	264	269	328	458–460	474	649	669	737	848	1208	1402	1424	1428	1441	1451	1456	1472
R. solanacearum	CFBP 1036	Martinique	Tomato	1	2a	AF207891	C	A	G	A	A	U	C	UUC	A	A	A	U	C	C	C	U	G	A	U	G	C
	CFBP 712	Burkina Faso	Eggplant	1	2a	AF207892	C	A	G	A	A	U	C	UUC	A	G	A	U	C	C	C	U	G	A	U	G	C
	NCPPB 1018	Angola	Potato	1	2c	AF207893	U	A	G	A	A	C	C	UCU	A	G	A	U	C	C	C	U	G	A	U	G	C
	CFBP 2146	Reunion Island	*Pelargonium capitatum*	1	2c	AF207894	U	A	G	A	A	C	C	UCU	A	G	A	U	C	C	C	U	G	A	U	G	C
	CFBP 734	Madagascar	Potato	1	2c	AF207895	U	A	G	A	A	C	C	UCU	A	G	A	U	C	C	C	U	G	A	U	G	C
	NCPPB 342	Zimbabwe	Tobacco	1	2c	AF207896	U	A	G	A	A	C	C	UCU	A	G	A	U	C	C	C	U	G	A	U	G	C
	MAFF 211266	Japan	Tomato	1	1	AF207897	C	A	G	A	A	C	C	ACU	U	G	A	U	C	C	C	C	A	A	C	A	U
	R288	China	Mulberry	5	1	U27984	C	A	G	A	A	C	C	ACU	U	G	A	U	C	C	C	C	A	A	C	A	U
	ACH092	Australia	Ginger	4	1	U27985	C	A	G	A	G	C	C	ACU	U	G	A	U	C	C	C	C	A	A	C	A	U
	ACH0171	Australia	Eggplant	3	1	U27986	C	A	G	A	A	C	C	ACU	U	G	A	U	C	C	C	C	A	A	C	A	U
	R791	Indonesia	Tomato	3	1	U27987	C	A	G	A	A	C	C	ACU	U	G	A	U	C	C	C	C	A	A	C	A	U
	CIP365	Philippines	Potato	5	1	U28220	C	A	G	A	A	C	C	ACU	U	G	A	U	C	C	C	C	A	A	C	A	U
	ACH0732	Australia	Tomato	2	1	U27983	C	A	G	A	A	U	C	UCU	G	G	A	U	C	C	C	C	A	A	C	A	U
	PDDCC 1727	United States	Tomato	1	2a	U28221	C	G	G	A	A	U	C	UUC	A	G	A	U	C	C	C	U	G	A	U	G	C
	CIP210	Brazil	Potato	1	2a	U28222	C	A	G	A	A	U	C	UUC	A	A	A	U	C	C	C	U	G	A	C	G	C
	R207	Belize	*Musa* AAB	1	2a	U28223	C	A	G	A	A	U	C	UUC	A	A	A	U	C	C	C	U	G	A	U	G	C
	ACH0158	Australia	Potato	2	2a	U28224	C	A	G	A	A	C	C	UUC	A	A	A	U	C	C	C	U	G	A	C	G	C
	Br150	United Kingdom	*Solanum dulcamara*	2	2a	U28225	C	A	G	A	A	C	C	UUC	A	A	A	U	C	C	C	U	G	A	C	G	C
	CIP238	Chile	Potato	2	2a	U28226	C	A	G	A	A	C	C	UUC	A	A	A	U	C	C	C	U	G	A	C	G	C
	CIP10	Peru	Potato	N2	2a	U28227	C	A	G	A	A	C	C	UUC	A	A	A	U	C	C	C	U	G	A	C	G	C
	R483	Philippines	Banana	1	2a	U28228	C	A	G	A	A	C	C	UUC	A	A	A	U	C	U	C	U	G	A	C	G	C
	R634	Philippines	Banana	1	2a	U28229	C	A	G	A	A	C	C	UUC	A	A	A	U	C	U	C	U	G	A	C	G	C
	R633	Philippines	Banana	1	2a	U28230	C	A	G	A	A	C	C	UUC	A	A	A	U	C	U	C	U	G	A	C	G	C
	R639	Philippines	Banana	1	2a	U28231	C	A	G	A	A	C	C	UUC	A	A	A	U	C	U	C	U	G	A	C	G	C
	R780	Indonesia	Potato	N2	2b	U28232	C	G	G	A	A	C	C	UUC	A	G	G	C	C	C	C	C	A	A	C	A	U
	R142	Indonesia	Clove	2	2b	U28233	C	G	G	A	A	C	C	UUC	A	G	G	C	C	C	C	C	A	A	C	A	U
BDB	R506	Indonesia	Banana		2b	U28234	C	G	G	A	A	C	C	UUC	A	G	G	C	U	C	C	C	A	G	C	A	U
	R233	Indonesia	Banana		2b	U28235	C	G	G	A	A	C	C	UUC	A	G	G	C	U	C	C	C	A	G	C	A	U
	R223	Indonesia	Banana		2b	U28236	C	G	G	A	A	C	C	UUC	A	G	G	C	U	C	A	C	A	G	C	A	U
P. syzygii	R001	Indonesia	Clove		2b	U28237	C	G	C	-	A	C	G	UUC	A	G	G	C	C	C	C	C	A	A	C	A	U
	R058	Indonesia	Clove		2b	U28238	C	G	N	-	A	C	G	UUC	A	G	G	C	C	C	C	C	A	A	C	A	U

*Except for the first seven strains, all of these data were described in the study of Taghavi *et al.* (1996). CIP, International Potato Center, Lima, Peru; ACH, A. C. Hayward, Department of Microbiology, Centre for Bacterial Diversity and Identification, University of Queensland, St Lucia, Australia; PDDCC, Culture Collection of Plant Diseases Division, DSIR, Auckland, New Zealand.

†*Escherichia coli* numbering (Woese *et al.*, 1983).

this result could be explained by the branch point separating division 1 and subdivision 2b, which was not as well supported (bootstrap value only 43%) as the branch point between subdivisions 2a and 2b (bootstrap value 59%) in the study of Taghavi *et al.* (1996). Moreover, the latter was supported by the dendrogram generated by the STATLAB software (result not shown). The four African biovar 1 strains originating from Angola, Madagascar, Reunion Island and Zimbabwe were included in a new subdivision, which was designated subdivision 2c. The branch point between subdivisions 2a and 2c was stable (bootstrap value 79%). The three other biovar 1 strains were distributed either into subdivision 2a (strains CFBP 712 and CFBP 1036), containing *R. solanacearum* biovar 1, 2 and N2 strains, or into division 1 (strain MAFF 211266), containing *R. solanacearum* biovars 3, 4 and 5 and a biovar 2 isolate.

As shown in Table 3, comparison of the differences between the seven analysed strains and with existing data gave useful new data. As expected (Taghavi *et al.*, 1996), the major sequence differences between the two divisions were in positions 458–460 (ACU for division 1 and UUC for division 2) and 474 (U for division 1 and A for division 2). However, four African strains were characterized at positions 458–460 by UCU and so differed from all other isolates, except for one biovar 2 strain, ACH0732. These four African isolates also differed from all other isolates at position 165 where U was substituted for C. The Burkina Faso strain, CFBP 712, was distinguished from the four other African strains particularly at positions 165, 269 and 458–460, and had the same nucleotide sequence as that of the American isolate, CFBP 1036, except at position 649. The sequence of the biovar 1 Japanese strain, MAFF 211266, was different from that of other biovar 1 isolates, especially at positions 458–460, 474, 1424, 1428, 1451, 1456 and 1472, but was not different from that of biovar 3, 4 and 5 strains.

DISCUSSION

Genomic diversity of *R. solanacearum* was evaluated by using different molecular approaches, extending the previous PCR-RFLP analysis of the *hrp* gene region (Poussier *et al.*, 1999). AFLP and PCR-RFLP analyses led

to comparable clustering but AFLP revealed more differences in the identification of clonal lines at the infrasubspecific level.

Clustering of the PCR-RFLP and AFLP profiles

Five PCR products were shown to be specific to *R. solanacearum*, *P. syzygii* and the BDB strains. This result is consistent with many other studies showing the close relationships between these three bacterial taxa (Eden-Green, 1994; Eden-Green & Sastraatmadja, 1990; Roberts *et al*., 1990; Seal *et al*., 1993; Taghavi *et al*., 1996). Nevertheless, *P. syzygii* as well as the BDB strains were distinguished from *R. solanacearum* strains since specific PCR-RFLP profiles, including specific restriction patterns, were obtained for these bacteria. To our knowledge, this is the first report of a simple and rapid method for discriminating *R. solanacearum* strains from *P. syzygii* and the BDB strains. New PCR-RFLP profiles were also obtained with new strains of *R. solanacearum*, particularly biovar N2 and 5 isolates. Biovar N2 strains generated five different subclusters demonstrating and confirming that biovar N2 is a genetically heterogeneous group of strains (Cook & Sequeira, 1994; Gillings & Fahy, 1994). Notably, the very similar biovars 2 and N2 (Hayward *et al*., 1990) showed a polymorphism within the RS80-RS81 amplified fragment spanning the *hrpB* regulatory gene (Genin *et al*., 1992). Since other groups of *R. solanacearum* strains are distinguishable by polymorphism in this gene (Poussier *et al*., 1999), the *hrpB* gene may be considered as a target for further phylogeny purposes, and for relating pathogenicity gene function with genetic variability. The PCR-RFLP and AFLP profiles were distributed into clusters which agreed well with biovar and geographical origin classifications, confirming the results obtained in the previous investigation (Poussier *et al*., 1999). In addition, this clustering was conserved using two different software packages which use different genetic distance methods, underlining its robustness. Biovar 1 and biovar 2 strains displayed the highest and the lowest diversity, respectively. Biovar 3 strains showed lower genetic diversity than biovar 1 strains. AFLP allowed very fine discrimination, close to the strain level, and reliable determination of genetic relationships between strains. This result is consistent with many previous investigations showing the usefulness of the AFLP procedure in strain identification, and for epidemiology and phylogeny purposes (Aarts *et al*., 1998; Arias *et al*., 1997; Blears *et al*., 1998; Clerc *et al*., 1998; Folkertsma *et al*., 1996; Hermans *et al*., 1995; Janssen *et al*., 1996, 1997; Keim *et al*., 1997; Lin *et al*., 1996; Restrepo *et al*., 1999). The BDB and *R. pickettii* strains, which showed specific AFLP profiles, could be distinguished from *R. solanacearum* strains. However, the overall level of polymorphism between these bacterial taxa was low, confirming their close relationship.

AFLP reveals a high level of polymorphism

The AFLP analysis revealed great variability within *R. solanacearum* since 60 different AFLP fingerprints were observed for the 96 strains. Thus, with 60 AFLP fingerprints (95% of fragments were polymorphic), AFLP has a higher resolution level for intraspecific differentiation of *R. solanacearum* strains than PCR-RFLP (20 profiles for 178 strains tested) and RFLP (46 profiles for 164 strains tested) (Cook *et al*., 1989, 1991; Cook & Sequeira, 1994). Several DNA fragments were common to all *R. solanacearum* species. Other DNA fragments could distinguish the divisions defined by Cook *et al*. (1989) within *R. solanacearum* species or differentiate strains according to their biovar or geographical origin and are therefore useful for the development of diagnostic tools and epidemiological studies. Although AFLP clustering was approximately the same as PCR-RFLP clustering, the AFLP procedure was more efficient for assessing intraspecific diversity since it permitted a clearer separation between biovar 2 and N2 and also between biovars 3, 4 and 5. AFLP and PCR-RFLP confirmed that biovar 2 strains are the least genetically diverse of all biovars (Cook *et al*., 1989; Cook & Sequeira, 1994; Smith *et al*., 1995; Van der Wolf *et al*., 1998); nevertheless AFLP, in contrast to PCR-RFLP, demonstrated genetic diversity among biovar 2 strains. In contrast, PCR-RFLP permitted strains isolated from musaceous plants to be grouped into two main clusters whereas AFLP grouped them into only one cluster.

New or unexpected groups

Trees resulting from PCR-RFLP analysis of the *hrp* gene region, AFLP and 16S rRNA sequencing showed the separation of *R. solanacearum* into two major groups, confirming and extending the conclusions of previous investigations using DNA–DNA hybridization (Palleroni & Doudoroff, 1971), and more recently of RFLP analyses (Cook *et al*., 1989, 1991; Cook & Sequeira, 1994), of PCR amplification with tRNA consensus (Seal *et al*., 1992), of 16S rRNA sequencing (Li *et al*., 1993; Taghavi *et al*., 1996), and of sequencing of the 16S–23S rRNA gene spacer region, the endoglucanase gene and the polygalacturonase gene (Fegan *et al*., 1998). The first division, named 'Americanum' by Cook *et al*. (1989), includes biovars 1, 2 and N2; and the second division named 'Asiaticum' contains biovars 3, 4 and 5.

However, our analyses revealed that there are numerous exceptions. Indeed, our extended PCR-RFLP analysis showed that an African biovar 1 strains group was associated with the 'Asiaticum' division rather than the 'Americanum' division, and so supported the conclusion of the first PCR-RFLP analysis (Poussier *et al*., 1999). The conclusions of AFLP and 16S rRNA sequencing were different to those of PCR-RFLP and appeared to be in complete agreement with the classification scheme proposed by Cook *et al*. (1989). Indeed, biovar 1 strains originating from the Southern part of Africa (Angola, Madagascar, Reunion Island, Zimbabwe) appeared to be more closely related to American strains even though they constituted a clearly separable group from American biovar 1 strains, and these were thus included in subdivision 2c, a new

subdivision compared to the work of Taghavi *et al*. (1996). The differentiation between American and African biovar 1 strains is more remarkable within the *hrp* gene region (PCR-RFLP analysis). To further clarify whether the discrimination between these two biovar 1 populations is clearer using regions of the genome involved in pathogenicity, other genes such as those encoding endoglucanase or polygalacturonase should be sequenced. The most probable explanation for the distinction between African and American biovar 1 isolates is separate evolution of the two populations due to geographical isolation. The two populations may have diverged under different natural selection pressures. The observation that other African isolates (three coming from Burkina Faso and one from Kenya) fell into clusters containing only American members may result from their introduction from the Americas through commercial exchanges.

The Japanese strains, MAFF 211266 and MAFF 211267, confirmed to be biovar 1 in our laboratory, were considered to be atypical. Both PCR-RFLP analysis and 16S rRNA sequence analysis indicated that these Japanese isolates appeared to be closely related to strains belonging to the 'Asiaticum' division, confirming the findings of Tsuchiya & Horita (1998). The peculiarity of these strains, which underlines the level of heterogeneity existing in the *R. solanacearum* species, may result from horizontal genetic transfers from biovar 3 or 4 members, which predominate in Japan. Moreover, another Japanese strain, MAFF 301558, was also unusual since PCR-RFLP and AFLP analyses showed that this biovar N2 strain was distantly related to all other biovar N2 strains and closely related to biovars 3, 4 and 5. This is not the first report of atypical isolates since Li & Hayward (1994) and Taghavi *et al*. (1996) also mentioned one atypical (ACH0732) biovar 2 strain. Furthermore, the two strains JT510 and JQ1056, which were identified as biovar 2 on several independent examinations, were unusual in AFLP grouping. It is possible that limited genomic rearrangements or genetic exchanges which do not modify the biovar typing occur. These considerations reinforce the need for a classification system that is based upon polymorphism between genes encoding pathogenicity functions. This would in turn permit more meaningful comparisons with specific phenotypic characteristics such as host specificity and survival in natural settings to be made.

Clearly the species *R. solanacearum* comprises two divisions, which may represent subspecies as suggested by Li *et al*. (1993). However, the 'Americanum' and 'Asiaticum' designations of these divisions proposed by Cook *et al*. (1989) in relation to the presumed geographical origin of strains could be reconsidered since our analyses reveal an African biovar 1 subdivision, which may have its own centre of genetic diversity, and thus likely evolutionary origin, in Africa.

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