Genetic Diversity of African and Worldwide Strains of *Ralstonia solanacearum* as Determined by PCR-Restriction Fragment Length Polymorphism Analysis of the *hrp* Gene Region

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The genetic diversity among a worldwide collection of 120 strains of *Ralstonia solanacearum* was assessed by restriction fragment length polymorphism (RFLP) analysis of amplified fragments from the *hrp* gene region. Five amplified fragments appeared to be specific to *R. solanacearum*. Fifteen different profiles were identified among the 120 bacterial strains, and a hierarchical cluster analysis distributed them into eight clusters. Each cluster included strains belonging to a single biovar, except for strains of biovars 3 and 4, which could not be separated. However, the biovar 1 strains showed rather extensive diversity since they were distributed into five clusters whereas the biovar 2 and the biovar 3 and 4 strains were gathered into one and two clusters, respectively. PCR-RFLP analysis of the *hrp* gene region confirmed the results of previous studies which split the species into an "Americanum" division including biovar 1 and 2 strains and an "Asiaticum" division including biovar 3 and 4 strains. However, the present study showed that most of the biovar 1 strains, originating from African countries (Reunion Island, Madagascar, Zimbabwe, and Angola) and being included in a separate cluster, belong to the "Asiaticum" rather than to the "Americanum" division. These African strains could thus have evolved separately from other biovar 1 strains originating from the Americas.

Ralstonia (formerly *Pseudomonas*) *solanacearum* (E. F. Smith) Yabuuchi et al. (47) is the causal agent of bacterial wilt, a severe and devastating plant disease in most tropical and subtropical and some warm temperate areas (22). Moreover, it can also occur in cool temperate areas (9, 33). Many economically important food crops such as potatoes, tomatoes, and bananas are affected. The disease was recorded on several hundred plant species distributed in more than 50 families (23). The species *R. solanacearum* is a complex taxonomic unit in which strains display an important diversity at different levels (physiological, serological, genetic characteristics, and host range). In order to describe this intraspecific variability, several systems of classification have been proposed. Thus, the species was subdivided into five races according to its host range (7, 25, 35) and into six biovars based on the utilization of three disaccharides and three hexose alcohols (21, 24, 25). Fatty acid analysis (26, 42) and protein profiling (15) were also performed but did not further clarify the relationships among *R. solanacearum* strains. Restriction fragment length polymorphism (RFLP) analysis (involving nine probes, seven of which encode information required for virulence and the hypersensitive response) (12–14) has provided a new classification scheme dividing the species into 46 groups in relation to geographic origin of strains and sometimes host range. The species was then separated into two major groups, the "Asiaticum" and the "Americanum" divisions, which regrouped strains from Asia and America, respectively. Further investigations comparing sequences of 16S rRNA (30, 40, 43) or using PCR amplification with tRNA consensus primers (39) supported the separation according to geographic origin.

Only a few strains originating from Africa, and only one from Reunion Island (21), were included in these previous studies. However, strains related to the three major biovars (1, 2, and 3) were isolated from various crops in Reunion (17). The aim of our study was to assess the genetic diversity within the local populations of *R. solanacearum*. Since we also wanted to develop molecular tools for the identification and detection of *R. solanacearum* biovars, we used the PCR-RFLP procedure to analyze the diversity. Recently, several authors have successfully performed PCR-RFLP analysis to assess genetic diversity among bacterial species (27, 28, 31, 44, 45). The *hrp* (hypersensitive reaction and pathogenicity) gene region, which is required by many phytopathogenic bacteria to produce symptoms on susceptible hosts and a hypersensitive reaction on resistant hosts or on nonhosts (1, 3, 4, 6, 18, 29), was explored for studying the variability within a collection of 120 strains isolated from different hosts over the five continents and belonging to biovars 1, 2, 3, and 4.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains studied (Table 1) included diverse strains of *R. solanacearum* with special attention to those isolated from Africa (51 strains including 28 from Reunion Island) and strains belonging to more or less closely related species (*Ralstonia pickettii, Ralstonia eutropha, Burkholderia cepacia, Pseudomonas* spp., *Xanthomonas* spp., *Erwinia chrysanthemi,* and *Clavibacter michiganensis* subsp. *michiganensis*). Identification of *R. solanacearum* strains at the biovar level was performed by using a modification of Hayward's method (21). All strains were stored on beads in cryovials at −80°C (Microbank Pro-Lab Diagnostics). Nutrient broth cultures were grown for 24 h on a rotary shaker (150 rpm) at 28°C. Bacteria were cultivated either (*R. solanacearum*) on a modified Granada and Sequeira medium (19) (tryptone, 1 g/liter; peptone, 10 g/liter; agar, 18 g/liter; glycerine, 6.3 ml/liter; crystal violet, 0.002 g/liter; polymyxin sulfate, 0.01 g/liter; tyrothricin, 0.02 g/liter; chloramphenicol, 0.005 g/liter; triphenyltetrazolium chloride, 0.025 g/liter; propiconazole, 0.4 ml/liter; penicillin, 20 U/liter; pH 7.2) or (other species) on YPGA medium (yeast extract, 7 g/liter; peptone, 7 g/liter; glucose, 7 g/liter; agar, 15 g/liter; pH 7.2) and incubated for 3 days at 28°C.

DNA extraction. DNA was extracted from *R. solanacearum* cells grown overnight at 28°C in 30 ml of YP (yeast extract, 7 g/liter; peptone, 7 g/liter) by the hexadecyltrimethylammonium bromide method (2). DNA concentration was estimated by fluorometry (TKO 100 minifluorometer; Hoefer Scientific Instruments, San Francisco, Calif.).

DNA amplification. Pairs of primers from the nucleotide sequence of the *hrp* gene region of strain GMI1000 of *R. solanacearum* (accession no. Z14056 for

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TABLE 1. Strains of *R. solanacearum* used in this study

Strain	Other designation	Source[a]	Geographic origin	Host	Biovar	PCR-RFLP cluster and subcluster[b]
JS796	CFBP1180	B	Puerto Rico	*Lycopersicon esculentum*	1	Ia
JS927	NCPPB1225	C	Puerto Rico	*Lycopersicon esculentum*	1	
JS833	UW30	E	Trinidad and Tobago	*Lycopersicon esculentum*	1	
JR659	CFBP2047[c]	B	United States	*Lycopersicon esculentum*	1	Ib
JS783	CFBP1036	B	United States	*Lycopersicon esculentum*	1	
JS967	ICMP7963	D	Kenya	*Solanum tuberosum*	1	
JS831	UW26	E	United States	*Lycopersicon esculentum*	1	Ic
JS768	CFBP767	B	Guadeloupe	*Solanum tuberosum*	1	II
JS794	CFBP1162	B	Guadeloupe	*Nicotiana tabacum*	1	
JS777	CFBP770	B	Guadeloupe	*Lycopersicon esculentum*	1	
JS733	CFBP2478	B	Guadeloupe	*Lycopersicon esculentum*	1	
JS784	CFBP1036	B	Martinique	*Lycopersicon esculentum*	1	
JS734	CFBP2972	B	Martinique	*Solanum tuberosum*	1	
JS716	CFBP705	B	Guyana	*Lycopersicon esculentum*	1	
JS837	UW90	E	Brazil	*Nicotiana tabacum*	1	
JS838	UW275	E	Costa Rica	*Melampodium perfoliatum*	1	
JS830	UW256	E	Costa Rica	*Solanum tuberosum*	1	
JS770	CFBP712	B	Burkina Faso	*Solanum melongena*	1	
JS779	CFBP715	B	Burkina Faso	*Lycopersicon esculentum*	1	
JS912	CFBP3057	B	Burkina Faso	*Lycopersicon esculentum*	1	
JS740	CFBP1415	B	Colombia	*Solanum tuberosum*	1	III
JS788	CFBP1412	B	Columbia	*Musa* sp. cv. *plantain*	1	
JS847	CFBP1419	B	Costa Rica	*Musa* sp.	1	
JT509		A	Reunion Island	*Lycopersicon esculentum*	2	IV
JT515		A	Reunion Island	*Lycopersicon esculentum*	2	
JT512		A	Reunion Island	*Lycopersicon esculentum*	2	
JT510		A	Reunion Island	*Solanum tuberosum*	2	
JT511		A	Reunion Island	*Solanum tuberosum*	2	
JT513		A	Reunion Island	*Solanum tuberosum*	2	
JT514		A	Reunion Island	*Solanum tuberosum*	2	
JT516		A	Reunion Island	*Solanum tuberosum*	2	
JS780	CFBP2148	B	Reunion Island	*Solanum tuberosum*	2	
JS931	NCPPB1049	C	Kenya	*Lycopersicon esculentum*	2	
JS948	NCPPB2088	C	Nigeria	*Solanum tuberosum*	2	
JS780	CFBP2148	B	Reunion Island	*Solanum tuberosum*	2	
JS931	NCPPB1049	C	Kenya	*Lycopersicon esculentum*	2	
JS948	NCPPB2088	C	Nigeria	*Solanum tuberosum*	2	
JS905	CFBP3582	B	Egypt	*Solanum tuberosum*	2	
JS939	NCPPB1824	C	Egypt	*Solanum tuberosum*	2	
JS908	CFBP3525	B	Morocco	*Solanum tuberosum*	2	
JS902	CFBP3581	B	France	*Solanum tuberosum*	2	
JS900	CFBP3671	B	France	*Lycopersicon esculentum*	2	
JS898	CFBP3672	B	France	*Solanum tuberosum*	2	
JS895	CFBP3673	B	France	*Solanum tuberosum*	2	
JS942	NCPPB1019	C	Portugal	*Lycopersicon esculentum*	2	
JS887	CFBP3785	B	Portugal	Unknown	2	
JS930	NCPPB1489	C	Madeira	*Solanum tuberosum*	2	
JS937	NCPPB1789	C	Greece	*Solanum tuberosum*	2	
JS935	NCPPB339	C	Israel	Unknown	2	
JS907	CFBP3858	B	The Netherlands	*Solanum tuberosum*	2	
JS928	NCPPB2797	C	Sweden	*Solanum dulcamara*	2	
JS929	NCPPB2505	C	Sweden	*Solanum tuberosum*	2	
JS792	CFBP1810	B	Haiti	*Solanum tuberosum*	2	
JS943	NCPPB613	C	Brazil	*Solanum tuberosum*	2	
JS758	CFBP1420	B	Colombia	*Solanum phureja*	2	
JS774	CFBP1414	B	Colombia	*Solanum tuberosum*	2	
JS897	CFBP3103	B	Peru	*Solanum tuberosum*	2	
JS926	NCPPB1331	C	India	*Solanum tuberosum*	2	
JS925	NCPPB1323	C	Sri Lanka	*Solanum tuberosum*	2	
JS932	NCPPB1614	C	Malaysia	*Solanum tuberosum*	2	
JS738	CFBP1413	B	Australia	*Solanum tuberosum*	2	
JS737	CFBP1417	B	Australia	*Solanum tuberosum*	2	

Continued on following page

TABLE 1—*Continued*

Strain	Other designation	Source[a]	Geographic origin	Host	Biovar	PCR-RFLP cluster and subcluster[b]
JS775	CFBP1409	B	Honduras	*Musa* sp.	1	V
JS730	CFBP1482	B	Panama	*Musa* sp.	1	
JS791	CFBP1416	B	Costa Rica	*Musa* sp. cv. *plantain*	1	
JS793	CFBP1183	B	Costa Rica	*Heliconia* sp.	1	
JS781	CFBP1185	B	Japan	*Lycopersicon esculentum*	3	VIa
JS945	MAFF301860	G	Japan	*Capsicum annuum*	3	
JS941	NCPPB3190	C	Malaysia	*Lycopersicon esculentum*	3	
JS836	UW8	E	Costa Rica	*Eupatorium odoratum*	3	
JS842	UW119	E	Costa Rica	*Solanum tuberosum*	3	
JS940	NCPPB500	C	Mauritius	*Vicia faba*	3	
JS944	NCPPB501	C	Mauritius	*Brassica oleracea*	3	
JS954	NCPPB502	C	Mauritius	*Casuarina equisetifolia*	3	
JS955	NCPPB503	C	Mauritius	*Dahlia* sp.	3	
JS834	UW151	E	Australia	*Zingiber officinale*	4	
JS835	UW360	E	China	*Morus alba*	4	
JS839	UW369	E	China	*Arachis hypogaea*	4	
JS832	UW378	E	China	*Olea* sp.	4	
JT517		A	Reunion Island	*Pelargonium asperum*	3	VIb
JT520		A	Reunion Island	*Pelargonium asperum*	3	
JT519		A	Reunion Island	*Pelargonium asperum*	3	
JS766	CFBP726	B	Reunion Island	*Solanum melongena*	3	
JT518		A	Reunion Island	*Solanum melongena*	3	
JT523		A	Reunion Island	*Solanum tuberosum*	3	
JS778	CFBP2041	B	Reunion Island	*Solanum tuberosum*	3	
JT521		A	Reunion Island	*Lycopersicon esculentum*	3	
JT522		A	Reunion Island	*Lycopersicon esculentum*	3	
JT524		A	Reunion Island	*Lycopersicon esculentum*	3	
JS841	MAFF301418	G	Japan	*Lycopersicon esculentum*	4	
JS933	UW74	E	Sri Lanka	*Solanum tuberosum*	4	
JS719	CFBP2970	B	Martinique	*Capsicum annuum*	3	VIc
JS715	CFBP2976	B	Martinique	*Ensete ventricosum*	3	
JS718	CFBP2480	B	Guadeloupe	*Solanum melongena*	3	
JS729	CFBP2965	B	Guadeloupe	*Solanum melongena*	3	
JS722	CFBP1813	B	Guyana	*Solanum melongena*	3	
JS753	GMI1000	F	Guyana	*Lycopersicon esculentum*	3	
JS764	GMI1336	F	*hrp* mutant of GMI1000	*Lycopersicon esculentum*	3	
JS843	UW130	E	Peru	*Lycopersicon esculentum*	3	
JS772	CFBP707	B	Tahiti	*Lycopersicon esculentum*	3	
JS773	CFBP1960	B	Algeria	*Capsicum annuum*	3	
JS759	CFBP1168	B	Trinidad and Tobago	*Musa* sp.	3	
JS840	UW147	E	Australia	*Nicotiana tabacum*	3	
JS947	NCPPB1123	C	Papua New Guinea	*Lycopersicon esculentum*	4	
JS953	MAFF301552	G	Japan	*Lycopersicon esculentum*	3	VId
JS936	NCPPB3181	C	Gambia	*Solanum nicanum*	3	VIe
JS950	NCPPB1018	C	Angola	*Solanum tuberosum*	1	VIIa
JT526		A	Reunion Island	*Pelargonium asperum*	1	VIIb
JT527		A	Reunion Island	*Pelargonium asperum*	1	
JT529		A	Reunion Island	*Pelargonium asperum*	1	
JT530		A	Reunion Island	*Pelargonium asperum*	1	
JT528		A	Reunion Island	*Solanum tuberosum*	1	
JT531		A	Reunion Island	*Solanum tuberosum*	1	
JS756	CFBP2146	B	Reunion Island	*Pelargonium capitatum*	1	
JT525		A	Reunion Island	*Pelargonium asperum*	1	
JT532		A	Reunion Island	Unknown	1	
JS767	CFBP734	B	Madagascar	*Solanum tuberosum*	1	
JS949	NCPPB332	C	Zimbabwe	*Solanum tuberosum*	1	
JS966	ICMP748	D	Zimbabwe	*Solanum tuberosum*	1	
JS946	NCPPB283	C	Zimbabwe	*Solanum panduraforme*	1	
JS951	NCPPB505	C	Zimbabwe	*Symphytum* sp.	1	
JS952	NCPPB342	C	Zimbabwe	*Nicotiana tabacum*	1	

TABLE 1—*Continued*

Strain	Other designation	Source[a]	Geographic origin	Host	Biovar	PCR-RFLP cluster and subcluster[b]
JS934	MAFF301558	G	Japan	*Solanum tuberosum*	3	VIII

[a] Strains were contributed as follows: A, this study; B, Collection Française de Bactéries Phytopathogènes, Angers, France; C, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; D, International Collection of Microorganisms from Plants, Auckland, New Zealand; E. D. Cook and L. Sequeira, Department of Plant Pathology, University of Wisconsin—Madison, Madison; F, M. Arlat and P. Barberis, CNRS-INRA, Auzeville, Castanet-Tolosan Cedex, France; G, K. Tsuchiya, Ministry of Agriculture, Forestry and Fisheries, National Institute of Agrobiological Resources, Tokyo, Japan.

[b] PCR-RFLP clusters and subclusters were defined by this study.

[c] Type strain.

EMBL-GenBank-DDBJ databases) were designed with Oligo 5.0 software (32). Eleven pairs were selected in order to explore the whole region. They delineated fragments with sizes ranging from 213 to 2,456 bp. Primers were synthesized by Genosys Biotechnologies, Cambridge, England.

PCRs were carried out in a total volume of 50 µl and performed in a thermocycler (GeneAmp PCR system 9600; Perkin-Elmer Corporation, Norwalk, Conn.). Two kinds of enzymes were used for PCR amplifications, either *Taq* DNA polymerase (GIBCO BRL Life Technologies, Cergy Pontoise, France) used with the 10× buffer (200 mM Tris-HCl, 500 mM KCl; pH 8.4) for an expected fragment length smaller than 1,000 bp, or a mix containing *Taq* and *Pwo* DNA polymerases used with buffer 3 (Expand Long Template PCR system; Boehringer Mannheim, Meylan, France) for a length over 1,000 bp. DNA, selected primers, MgCl$_2$ (GIBCO BRL), dATP, dCTP, dGTP, dTTP (Boehringer Mannheim), and water (high-pressure liquid chromatography grade; Sigma-Aldrich, Steinheim, Germany) were added to the reaction mixture. Optimal conditions of amplification were determined by using the Taguchi methods as modified by Cobb and Clarkson (11).

PCR products were electrophoresed onto agarose gels and visualized with UV light after ethidium bromide staining (37).

Restriction fragment analysis. The amplified DNA fragments considered to be specific to *R. solanacearum* were digested with restriction endonucleases according to the manufacturer's directions (GIBCO BRL; Boehringer Mannheim). Enzymes were chosen on the basis of the nucleotide sequence of the *hrp* gene region of strain GMI1000 by using Oligo 5.0 software (32). Among all considered enzymes, only 13 available in the laboratory were retained: *Ava*I, *Bgl*II, *Bss*HII, *Ecl*XI, *Eco*RI, *Hae*II, *Hind*II, *Not*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, and *Sma*I. Restriction fragments were separated by electrophoresis and visualized as described previously (37).

Data analysis. Data derived from the different RFLP patterns exhibited by the tested strains (presence or absence of bands) were used for a hierarchical cluster analysis (HCA). With Statlab software (41), clustering was based on the Euclidean distance between strains (Ward's method [46]). 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 ($P = 0.05$) different from the variance between clusters.

RESULTS

Specificity of primers to the *hrp* gene region of *R. solanacearum*. A suitable amplification pattern was obtained with only 6 of the 11 pairs of primers, giving fragments ranging from 213 to 1,993 bp (according to the sequence of the reference strain GMI1000). The amplified fragments were distributed along the *hrp* gene region and cover a variable number of genes (two to four) which have been previously defined: RS20-RS201, 1,452 bp over *hrpV-hrpU-hrpT-hrpQ*; RS30-RS31, 1,993 bp over *hrpO-hrpN*; RS50-RS501, 1,200 bp over *hrpN-hrpK-hrpJ-hrpI*; RS600-RS61, 905 bp, and RS80-RS81, 1,537 bp, both over *hrpC-hrpB* (Fig. 1); and RS90-RS91, 213 bp over *hrpB-hrpA*.

For each of the six pairs of primers which led to a suitable amplification and for all of the tested strains which belonged to *R. solanacearum*, a single band with the expected size was observed. However, the density of the band appeared to be variable depending upon the pair of primers and the bacterial DNA. In contrast, no amplification could be obtained for strains belonging to another bacterial species even for such closely related species as *R. eutropha* or *R. pickettii* (data not shown).

Restriction endonuclease analysis of specific amplified *hrp* sequences. The six amplified DNA fragments, which were considered to be specific to *R. solanacearum*, were digested with the 13 selected restriction endonucleases. Different restriction patterns among the 120 strains of *R. solanacearum* were observed with different restriction endonucleases: *Ava*I (four patterns) and *Pvu*II (four patterns) for the RS20-RS201 sequence; *Hind*II (six patterns) for the RS30-RS31 sequence; *Sac*I (three patterns) for the RS50-RS501 sequence; *Hae*II (six patterns) for the RS600-RS61 sequence; *Bss*HII (six patterns), *Not*I (three patterns), and *Pst*I (four patterns) for the RS80-RS81 sequence (Fig. 2).

Clustering of the PCR-RFLP profiles. Among the 120 strains of *R. solanacearum*, 15 different profiles could be distinguished (Table 2). A profile was the result of the combination of the RFLP patterns given by the eight restriction endonucleases which generated polymorphism and which were selected for the data analysis. The HCA resulted in a dendrogram showing the genetic relatedness between strains (Fig. 3). The truncation level allowed separation of eight PCR-RFLP clusters designated clusters I to VIII. While five clusters (II, III, IV, V, and VIII) contained a unique profile, the three remaining contained two (cluster VII), three (cluster I), and five (cluster VI) different profiles. The number of strains in each cluster ranged from 1 to 40, but each cluster contained strains belonging to the same biovar, with the exception of cluster VI, which included strains of biovars 3 and 4. Biovar 1 strains were distributed over five clusters (I, II, III, V, and VII), biovar 3 was grouped into both cluster VI and cluster VIII (only one strain within the latter), and the 36 strains belonging to biovar 2 were grouped together in cluster IV.

Most restriction patterns were common to different clusters. However, the restriction patterns AAv1, BHi1, DHa1 (one exception), and EBs1 appeared to be specific to cluster VII whereas BHi4 and DHa4 characterized cluster I (pattern designations are explained in Table 2). In addition, BHi5 and CSa3 were characteristic of the unique strain within cluster VIII, and DHa6, EBs6, ENo3, and EPs4 seemed to be specific to cluster V. Similarly, when the distribution of the restriction patterns within the biovars was analyzed DHa2 was found only in biovar 2 strains and BHi3, DHa3, and EBs3 were found only in biovars 3 and 4.

The restriction patterns generated by *Hae*II (DHa1 to DHa6) and *Bss*HII (EBs1 to EBs6) appeared to be the most useful for separating the eight clusters and distinguishing the three biovars (Table 3). Strains of biovars 2 and 3 or 4 had a unique distinctive profile, DHa2-EBs2 and DHa3-EBs3, respectively, and were classified either in cluster IV or in clusters VI and VIII, while the biovar 1 strains displayed five different profiles which characterized the five remaining clusters.

In addition, the dendrogram obtained suggests that these *R. solanacearum* strains can be separated into two distinct groups, namely, clusters I to V (all biovar 2 strains and approximately 64% of biovar 1 strains) and clusters VI to VIII (all biovar 3
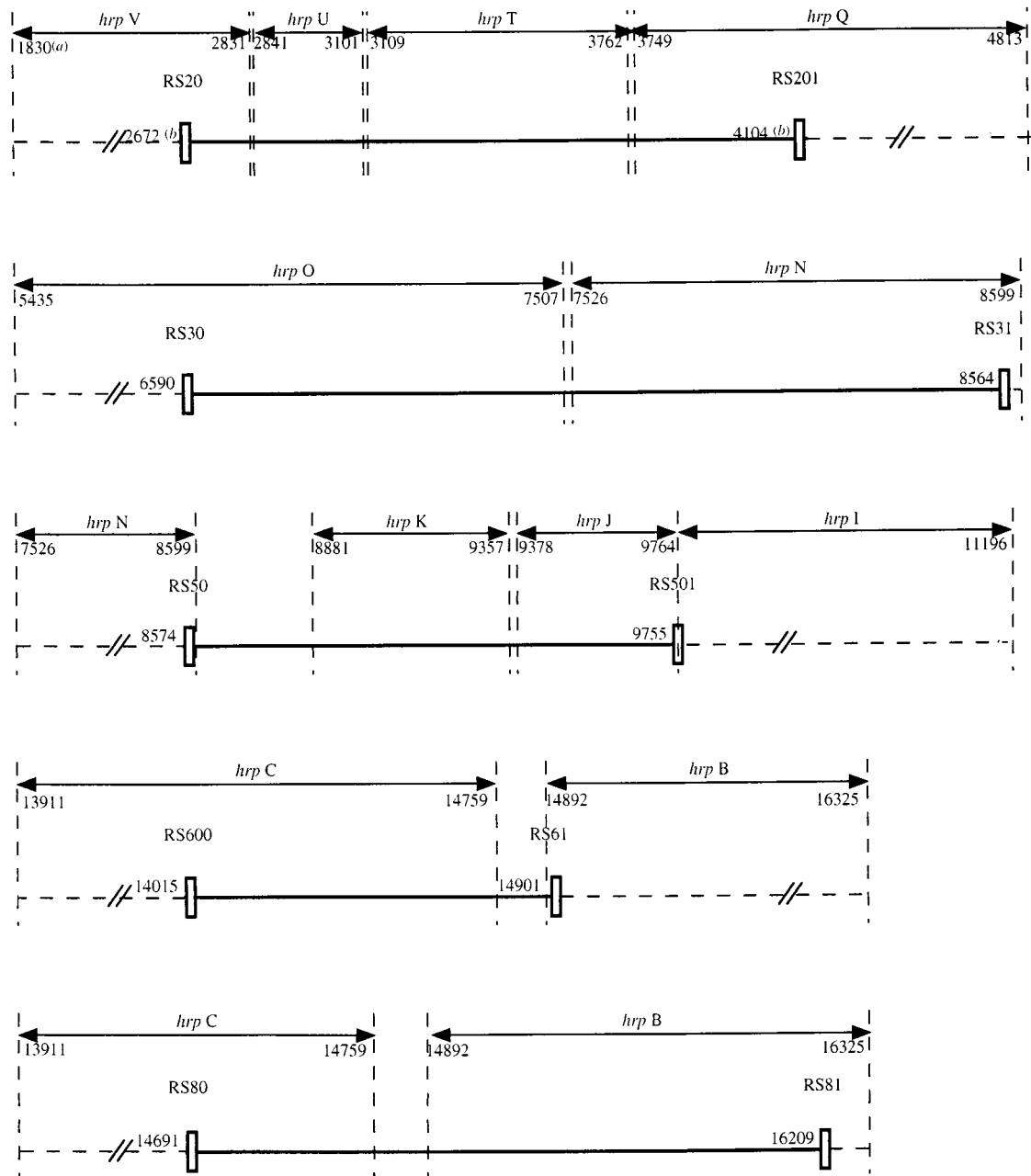
FIG. 1. Location of the five selected pairs of primers (RS20-RS201, RS30-RS31, RS50-RS501, RS600-RS61, and RS80-RS81) within the *hrp* genes of *R. solanacearum* (strain GMI1000). (*a*), number of the base on the 5′-3′ DNA sequence; (*b*), number of the base at the 5′ end of the primer.

and 4 strains and about 36% of biovar 1 strains). The diversity within biovar 1 appeared to be correlated with geographic origin since all strains belonging to cluster VII were isolated from Africa, mainly from the southern part (Angola, Zimbabwe, Madagascar, and Reunion Island), while most (85%) of those included in clusters I, II, III, and V originated from the Americas. Some strains isolated from northern Africa (Burkina Faso and Kenya) belonged, however, to clusters I and II. The 36 biovar 2 strains fell into cluster IV regardless of geographic origin: Africa, Americas, Asia, Europe, or Oceania. There were no differences in profile between biovar 3 and biovar 4 strains, and all (one exception) were gathered in one cluster (VI), but five profiles were identified, which separated five

subclusters. The subcluster VIa included many Asiatic strains (46%), VIb included most African strains (83%), and 69% of the American strains were included in subcluster VIc.

Cluster V contained four strains which were isolated from hosts of the *Musaceae* family and was characterized by a profile which included the specific restriction patterns DHa6, EBs6, ENo3, and EPs4. It must be noted that the sum of sizes of the restriction fragments included in DHa6 and EPs4 appeared to be higher (41 and 30 bp, respectively) than the total size of the corresponding amplified fragment, suggesting that an inserted sequence may be present (Fig. 4). This was characteristic only of the strains belonging to cluster V. Three other strains isolated from *Musa* sp. were distributed either into cluster III or
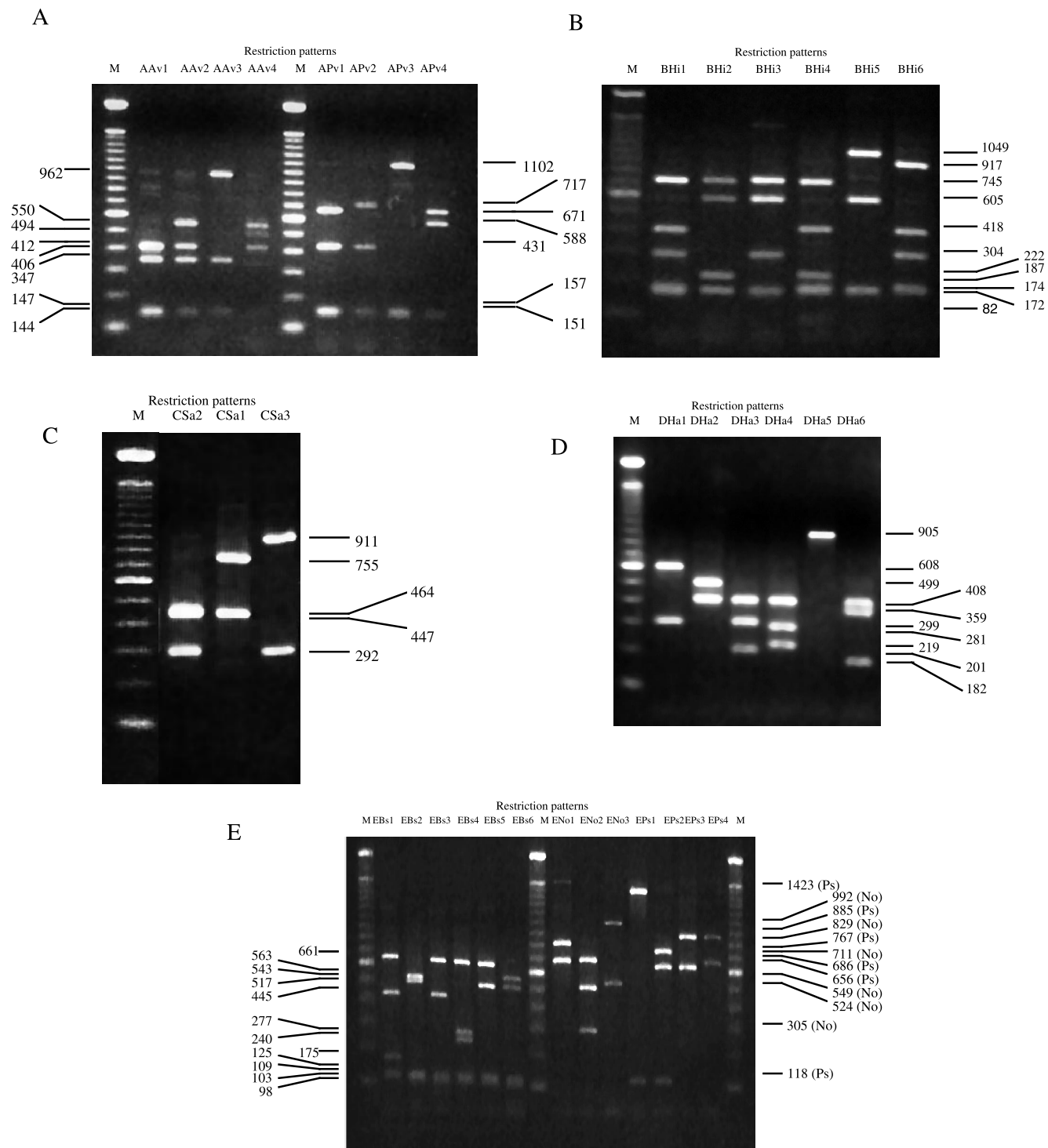
FIG. 2. Restriction patterns (see explanation in Table 2) of the five amplified fragments of the *hrp* gene region of *R. solanacearum* when digested by the designated enzymes. (A) RS20-RS201 *Ava*I (left side) and *Pvu*II (right side); (B) RS30-RS31 *Hin*dII; (C) RS50-RS501 *Sac*I; (D) RS600-RS61 *Hae*II; (E) RS80-RS81 *Bss*HII (left side), *Not*I (right side), and *Pst*I (right side). M, molecular size markers (100-bp ladder; GIBCO BRL). The size (in base pairs) of the bands was estimated from the sequence of the *hrp* gene region of the GMI1000 strain.

into cluster VI. There was no obvious correlation between the host origin of strains and their distribution into clusters.

Variability in restriction sites in the *hrp* gene region of *R. solanacearum*. Thirty-six restriction sites were identified within the five amplified fragments when the eight selected enzymes

were used (Fig. 4). Eight sites appeared to be common to all 120 strains (A5, A7, B4, B5, E12, E13, E14, and E15). The occurrence of the 28 remaining sites was variable according to the RFLP clustering of the strains. However, 14 among them appeared to be particularly remarkable since they could sepa-

TABLE 2. Characterization of the 15 PCR-RFLP profiles identified among the 120 strains of *R. solanacearum*

PCR-RFLP profile[a]	Cluster or subcluster
AAv2; APv1; BHi4; CSa2; DHa4; EBs4; ENo2; EPs3	Ia
AAv3; APv1; BHi4; CSa2; DHa4; EBs4; ENo2; EPs3	Ib
AAv2; APv3; BHi4; CSa2; DHa4; EBs4; ENo2; EPs3	Ic
AAv2; APv1; BHi2; CSa1; DHa5; EBs5; ENo2; EPs3	II
AAv4; APv2; BHi2; CSa2; DHa5; EBs2; ENo2; EPs2	III
AAv2; APv2; BHi2; CSa2; DHa2; EBs2; ENo2; EPs2	IV
AAv2; APv2; BHi2; CSa2; DHa6; EBs6; ENo3; EPs4	V
AAv2; APv1; BHi3; CSa1; DHa3; EBs3; ENo1; EPs1	VIa
AAv3; APv1; BHi3; CSa1; DHa3; EBs3; ENo1; EPs1	VIb
AAv2; APv3; BHi3; CSa1; DHa3; EBs3; ENo1; EPs1	VIc
AAv3; APv4; BHi3; CSa1; DHa3; EBs3; ENo1; EPs1	VId
AAv2; APv1; BHi6; CSa1; DHa3; EBs3; ENo1; EPs1	VIe
AAv1; APv4; BHi1; CSa1; DHa1; EBs1; ENo1; EPs1	VIIa
AAv1; APv1; BHi1; CSa1; DHa1; EBs1; ENo1; EPs1	VIIb
AAv4; APv2; BHi5; CSa3; DHa1; EBs3; ENo1; EPs1	VIII

[a] A profile was 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-R61, and 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.

rate the biovars and/or the geographic or botanical origins (Table 4).

A1 was identified within all but one biovar 3 or biovar 4 strain and within all biovar 1 strains except those isolated from musaceous plants. A2 characterized the African biovar 1 strains (cluster VII). In contrast, E11 was absent only from these African biovar 1 strains. B1 was present in all biovar 2 strains and also in all biovar 1 strains except those originating

TABLE 3. Distribution of the restriction patterns generated by *Hae*II on the RS600-RS61-amplified fragment and by *Bss*HII on the RS80-RS81-amplified fragment of the *hrp* gene region of *R. solanacearum* according to the PCR-RFLP cluster and to the biovar

Biovar	PCR-RFLP cluster	Restriction pattern with:	
		*Hae*II	*Bss*HII
1	I	DHa4	EBs4
1	II	DHa5	EBs5
1	III	DHa5	EBs2
1	V	DHa6	EBs6
1	VII	DHa1	EBs1
2	IV	DHa2	EBs2
3/4	VI	DHa3	EBs3
3	VIII	DHa1	EBs3

from Africa (cluster VII). D2 was found in biovar 3 (and biovar 4) strains and in the African biovar 1 strains (cluster VII). B2 and C1 were present in all strains except one strain (cluster VIII). D3 and E7 were characteristic of American biovar 1 strains (cluster I). E2 characterized all biovar 2 strains and also biovar 1 strains isolated from musaceous plants (clusters III and V). These musaceous clusters could be separated by four sites characterizing strains grouped in cluster V: D4, E5, E6, and E8.

An additional HCA, based on the presence or absence of discriminating restriction sites, gave a slightly different cluster distribution. The truncation then separated six groups, one joining the PCR-RFLP clusters I and II and the other joining clusters III and IV.

DISCUSSION

The exploration of the *hrp* gene region with 11 selected pairs of primers gave six amplicons which were confirmed to be specific to *R. solanacearum*. Indeed, no amplification was observed with DNA from strains belonging to other bacterial species and even from such closely related species as *R. pickettii*, *R. eutropha*, and *B. cepacia*. Consequently, the *hrp* region seems to be useful for the identification and specific detection of strains of *R. solanacearum*. However, since we did not succeed in getting strains of *Pseudomonas celebense* and *Pseudomonas syzygii* from laboratory collections, which are also species close to *R. solanacearum*, the amplification within their *hrp* region when the selected primers were used was not checked. Nevertheless, since our main objective was to develop molecular tools for the detection of populations of *R. solanacearum* on Reunion Island and since these particular pathogenic species (*P. celebense* and *P. syzygii*) were recorded only in Indonesia on bananas and cloves, respectively, a lack of specificity in that case would be of no consequence.

Although we concentrated mainly on the strains originating from Reunion Island (28 strains), the remaining 92 isolates were chosen to represent the broad host range, wide geographic distribution, and metabolic diversity (biovars) of *R. solanacearum*. Although our analysis gave a lower resolution level than that seen after genomewide RFLP analysis (12–14), we found that it gave reliable estimates of phylogenetic relationships among strains of *R. solanacearum*. Whereas the 46 described RFLP profiles were correlated with geographic origin, biochemical typing, and host origin, we identified 15 PCR-RFLP profiles distributed into eight clusters, these clusters being correlated with biochemical typing and to a lesser degree with geographic origin. Since some PCR-RFLP patterns cor-
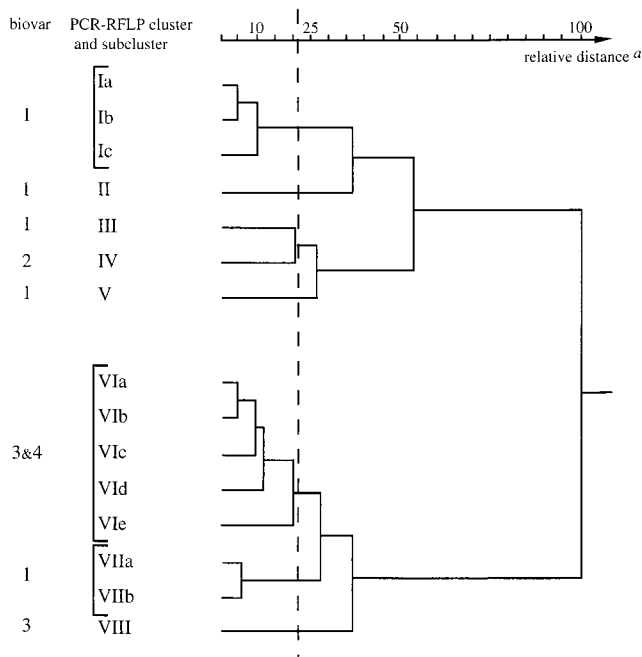

FIG. 3. Dendrogram resulting from an HCA based on the restriction patterns of the five amplified fragments within the *hrp* gene region of 120 strains of *R. solanacearum*. *a*, the relative distance between the farthest clusters was assumed to be 100.
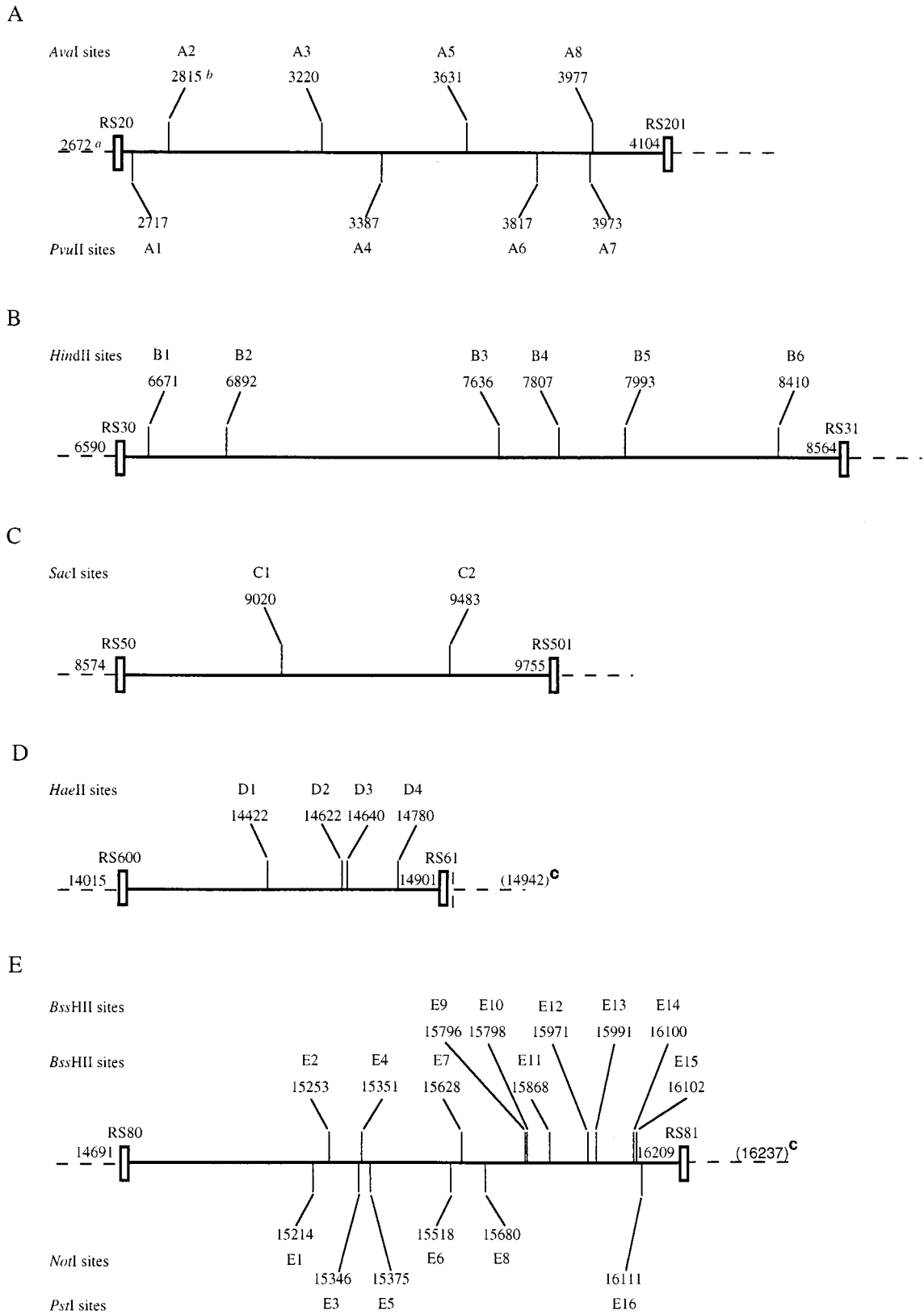
FIG. 4. Location of the 36 restriction sites identified in the five amplified fragments of the *hrp* gene region of *R. solanacearum* when digested by *Ava*I and *Pvu*II for RS20-RS201 (A); by *Hind*II for RS30-RS31 (B); by *Sac*I for RS50-RS501 (C); by *Hae*II for RS600-RS61 (D); and by *Bss*HII, *Not*I, and *Pst*I for RS80-RS81 (E), as estimated from the DNA sequence of strain GMI1000 and the size of the bands of the restriction patterns. *a*, number of the base at the 5′ end of the primer; *b*, number of the base at left of the restriction site; *c*, the size of the amplified fragment was higher for the four strains of cluster V.

TABLE 4. Occurrence of 14 discriminating restriction sites identified within the five amplified fragments of the *hrp* gene region of
R. solanacearum according to biovar typing, geographic or host origin, and PCR-RFLP clustering

Biovar	Origin	PCR-RFLP cluster	Result for restriction site:													
			A1[b,i]	A2[a,i]	B1[c,j]	B2[c,j]	C1[d,k]	D2[e,l]	D3[e,l]	D4[e,l]	E2[f,m]	E5[g,m]	E6[h,m]	E7[f,m]	E8[h,m]	E11[f,m]
1	Americas[n]	I	+	−	+	+	+	−	+	−	−	−	+	+	−	+
1	Americas[o]	II	+	−	+	+	+	−	−	−	−	−	+	−	−	+
1	Africa	VII	+	+	−	+	+	+	−	−	−	−	+	−	−	−
1	*Musaceae*[p]	III	−	−	+	+	+	−	−	−	+	−	+	−	−	+
1	*Musaceae*	V	−	−	+	+	+	−	−	+	+	+	−	−	+	+
2	Worldwide	IV	−	−	+	+	+	−	−	−	+	−	+	−	−	+
3	Worldwide	VI	+	−	−	+	+	+	−	−	−	−	+	−	−	+
3	Asia	VIII	−	−	−	−	−	+	−	−	−	−	+	−	−	+
4	Asia, Oceania	VI	+	−	−	+	+	+	−	−	−	−	+	−	−	+

[a] *Ava*I site.
[b] *Pvu*II site.
[c] *Hin*dII site.
[d] *Sac*I site.
[e] *Hae*II site.
[f] *Bss*HII site.
[g] *Not*I site.
[h] *Pst*I site.
[i] Located between the RS20 and RS201 pair of primers.
[j] Located between RS30 and RS31.
[k] Located between RS50 and RS501.
[l] Located between RS600 and RS61.
[m] Located between RS80 and RS81.
[n] All strains originated from the Americas except for one from Africa.
[o] All strains originated from the Americas except for three from Africa.
[p] All strains were isolated from musaceous plants except for one from potato.

related well with biovar typing, the PCR-RFLP procedure provides a complementary or alternative method for biovar determination.

The PCR-RFLP analysis confirmed the great variability within *R. solanacearum*. Biovar 1 strains showed the greatest diversity since they were distributed into five of the eight clusters. Six biovar 1 strains originating from the *Musaceae* family were distributed into two specific clusters, one (cluster V) including four of these strains and the other (cluster III) comprising the two remaining strains together with one strain isolated from potato. Among the 36 restriction sites identified on the five amplified fragments, only 25 were common to the musaceous strains, whereas there were 28 sites common to biovar 2 and biovar 3 strains. Moreover, among the 12 discriminating restriction sites located on the fragment delineated by the RS80-RS81 primer pair, only three were common to both clusters III and V while all were shared by clusters III and IV. These features suggest that there are important differences between the musaceous strains distributed in two separate clusters. All of these strains came from Central America or northern South America, but nothing was known of their pathogenicity, and no clear indication of the race to which they belonged was reported. The strains of cluster V could belong to race 2, whereas those of cluster III might be associated with race 1, which could explain the presence of the Colombian strain isolated from potato within the cluster. One particular strain isolated from *Musa* sp. and characterized as belonging to biovar 3 (36) fell into cluster VI, as most of the strains were related to the same biovar. Further studies incorporating more strains isolated from *Musaceae* and belonging either to race 1 or to race 2 are required.

Clusters I and II included all biovar 1 strains originating from the Americas, more specifically, either from North America for cluster I or from Central America for cluster II. The fact that four African isolates fell into these clusters suggests that they could have been introduced from the Americas. Most

biovar 1 strains isolated from African countries, however, were included in cluster VII. All of these strains originated from southern Africa, including Reunion Island, Madagascar, Zimbabwe, and Angola, whereas the African isolates from clusters I and II came from the northern part of Africa (Burkina Faso and Kenya). Thus, Africa may have two different biovar 1 populations, either endemic and commonly isolated in southern countries or introduced from the Americas through direct or indirect commercial exchanges. Although both populations belonged to the same biovar, there was no indication that they have similar host ranges (and/or similar virulence).

The 36 strains of biovar 2 displayed a similar profile which was characterized by the specific restriction pattern DHa2 and were included in cluster IV, close to those encompassing the biovar 1 strains of American origin. The consistent homogeneity of biovar 2 strains, although they were collected from 20 countries distributed worldwide, could be attributed to their narrow host range, including only potato and tomato plants. The result agrees with the commonly accepted hypothesis of a common origin for all the biovar 2 strains. South America is the presumed origin, and the wide distribution of these strains is probably due to the dissemination of latently infected plant material (particularly potato tubers) by humans (8, 10, 22). The biovar 2 strains (cluster IV) are closely related to cluster III strains, since 34 of the 36 restriction enzyme sites were common to both clusters, underlining the proximity of some musaceous isolates to race 3 strains.

Compared to biovar 1 strains, biovar 3 strains showed rather modest genetic diversity since they could be assigned to one major cluster. An additional cluster with a unique strain originating from Japan was also described. The few biovar 4 strains fell into the same cluster as most biovar 3 strains, indicating that there were only slight differences between these biovars. However, six different profiles more or less correlated with geographic origin (Asia, cluster VIa; Reunion Island, cluster VIb; America, cluster VIc) were identified.

The dendrogram resulting from an HCA revealed the separation of *R. solanacearum* into two major divisions. This result confirmed the conclusion of many previous studies on DNA homologies and physiological characterization of strains (20, 34) and more recently of RFLP analysis (12–14), of 16S rRNA sequencing (30, 40, 43), or of PCR amplification with tRNA consensus primers (39). The first division, Americanum sensu Cook et al. (12), contains biovar 1 and 2 strains, and the second division, Asiaticum sensu Cook et al. (12), includes biovar 3 and 4 strains. Thus, compared to other genomic regions (16S rRNA and tRNA), the *hrp* gene region, which is involved in host-pathogen interactions, revealed the same major trend of diversity, suggesting that *hrp* genes have evolved in parallel with 16S rRNA and tRNA.

The amplified fragment delineated by the RS80-RS81 pair of primers provided much more polymorphism than all the others: 12 discriminating restriction sites were identified and permitted separation of certain groups of strains. All of these polymorphisms were located within the *hrpB* regulatory gene (16). This observation confirms that regulatory systems of bacteria seem to be less conserved than those genes whose function they govern (5). Furthermore, this result suggests that the *hrp* regulatory gene may have other metabolic functions besides its role in pathogenic diversity of *R. solanacearum*. More precise analysis of the *hrpB* gene in different strains in the future might provide a useful way of relating pathogenicity gene function to genetic diversity.

Although the dendrogram confirms the separation of *R. solanacearum* into two groups, the distribution of biovar 1 strains, which displayed a rather wide variability, did not agree completely with the scheme proposed by Cook et al. (12). Clusters I, II, III, and V (American biovar 1 strains) were close to cluster IV, which included biovar 2 strains, and would thus belong to the Americanum division, whereas cluster VII (African biovar 1 strains) near cluster VI (biovar 3 and 4 strains) would be separated and connected rather to the Asiaticum division. The African strains included in cluster VII could have evolved separately as a result of geographic isolation and thereby have contributed to increasing the diversity of the species. Clearly, further analysis with other techniques such as DNA probes for RFLP analysis (12–14) and/or 16S rRNA sequencing (30, 40, 43) to confirm other characteristic features of these strains would be of interest. Preliminary results obtained with the *R. solanacearum*-specific primer pair PS96-H and PS96-I (38) support the hypothesis of separate evolution of these strains, since these primers never led to amplification of any biovar 1 strain originating from Reunion Island, Madagascar, Zimbabwe, or Angola (data not shown). Whatever the explanation, these African biovar 1 strains shared more sites with biovar 3 strains (23 sites) than with the American biovar 1 strains (13 to 19 sites according to the cluster), and the conclusion is that American and African biovar 1 strains are phylogenetically distinct, the latter being more closely related to Asiatic (biovar 3 and 4) strains.

Our study of the genetic diversity of the *hrp* gene region of *R. solanacearum* thus provides discriminating tools which besides being useful for fundamental research can also be used for diagnostic purposes. For example, biovars 1 and 2 and the combination of biovars 3 and 4 can easily be distinguished from each other by the restriction pattern generated after amplification with the RS600 and RS61 primers when digested by *Hae*II: DHa3 for biovars 3 and 4; DHa2 for biovar 2; and DHa1, DHa4, DHa5, or DHa6 for biovar 1. Moreover, the restriction pattern could give useful information about the geographic origin of the biovar 1 strain. As PCR amplification is known to be a very sensitive technique, such primers could

be used to detect the populations of *R. solanacearum* in plant, irrigation water, or soil extracts. They could also be employed to clarify some aspects of the epidemiology of bacterial wilt regarding, for instance, seed as a vehicle of disease spread or some weeds or resistant plants as possible carriers of low levels of infectious populations.

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