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Identification of Genomic Species in *Agrobacterium* Biovar 1 by AFLP Genomic Markers▽

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Biovar 1 of the genus *Agrobacterium* consists of at least nine genomic species that have not yet received accepted species names. However, rapid identification of these organisms in various biotopes is needed to elucidate crown gall epidemiology, as well as *Agrobacterium* ecology. For this purpose, the AFLP methodology provides rapid and unambiguous determination of the genomic species status of agrobacteria, as confirmed by additional DNA-DNA hybridizations. The AFLP method has been proven to be reliable and to eliminate the need for DNA-DNA hybridization. In addition, AFLP fragments common to all members of the three major genomic species of agrobacteria, genomic species G1 (reference strain, strain TT111), G4 (reference strain, strain B6, the type strain of *Agrobacterium tumefaciens*), and G8 (reference strain, strain C58), have been identified, and these fragments facilitate analysis and show the applicability of the method. The maximal infraspecies current genome mispairing (CGM) value found for the biovar 1 taxon is 10.8%, while the smallest CGM value found for pairs of genomic species is 15.2%. This emphasizes the gap in the distribution of genome divergence values upon which the genomic species definition is based. The three main genomic species of agrobacteria in biovar 1 displayed high infraspecies current genome mispairing values (9 to 9.7%). The common fragments of a genomic species are thus likely "species-specific" markers tagging the core genomes of the species.

Agrobacteria are common soil and root inhabitants. They are generally inoffensive plant commensals. However, agrobacteria may cause crown gall or hairy root diseases in many plants, including economically important crops, when they harbor a large Ti or Ri plasmid (8, 36). Ti and Ri plasmids can be experimentally transferred by conjugation (13) to numerous bacterial species, most of which are in the family *Rhizobiaceae* (17, 34, 35). In most cases, new transconjugant species or genera become pathogenic (5, 17, 34, 35). Probably due to in situ plasmid transfer, pathogenic populations of agrobacteria involved in crown gall outbreaks are characterized by a high degree of chromosomal background diversity in terms of genotype, serotype, ribotype, and biovar, while the same Ti plasmids can be associated with various chromosome backgrounds (25, 26, 28, 30). Therefore, elucidating crown gall epidemiology and Ti plasmid ecology requires identification of genuine or potential bacterial Ti plasmid reservoirs in soil and plants at various taxonomic levels.

The current consensus for bacterial species identification (40) is based on DNA-DNA hybridization of whole genomes and indicates that "the phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔTm." This definition has recently been supported by an international committee (32), but the committee also indicated that delineation of genomic species can now be obtained with alternative molecular methods that sample parts of the genome instead of DNA-DNA hybridization of whole genomes alone. Among the alternative genome sampling methods, AFLP (38) easily identifies genomic species (24). Multilocus sequence typing (22) or, more generally, multilocus sequence analysis (MLSA) has also been proposed for identification of bacterial species (14). AFLP has the advantage of identifying bacterial species on a genomic basis. The AFLP method is based on analysis of markers which are massively and randomly sampled along the entire genome. Thus, AFLP targets not only genes in the conserved core genome but also accessory genes that have likely ecological relevance for the species. This is important for the concept of ecological species (6, 37), which have been described as clusters of strains adapted to the same ecological niches (e.g., ecotypes). In addition, the AFLP methodology provides high resolution and reveals differences between strains not distinguished by MLSA (1).

Agrobacterium taxonomy is still strongly debated. First, it was proposed that so-called "biovars 1 and 2," which are defined by biochemical characteristics (18, 19, 20), should be redefined at the species level as *A. radiobacter* and *A. rhizogenes*, respectively (31). However, based on the *International Code of Nomenclature of Bacteria* (20a), Bouzar (2) indicated that *A. tumefaciens* was the type species of the genus and thus should be retained. Controversially, in a request for an opinion, Young et al. (44) decided that the epithet "*radiobacter*" was proposed before the epithet "*tumefaciens*" and that *A. ra-*

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diobacter has priority over *A. tumefaciens*. Moreover, although *A. rhizogenes* (i.e., "biovar 2") is a true genomic species, as revealed by DNA-DNA hybridization, and therefore a bona fide species, "biovar 1" was found to be a complex of several genomic species (9, 10, 29). Researchers identified nine genomic species or genomospecies in biovar 1 by DNA-DNA hybridization, which were subsequently designated genomic species G1 to G9 (24). The splitting of "biovar 1" into several genomic species does not solve the terminology problem. Actually, the type strain of *A. tumefaciens* (strain B6 [= ATCC 23308]) belongs to genomic species G4; thus, only genomic species G4 corresponds to the bona fide species *A. tumefaciens* sensu stricto. Unfortunately, the type strain of *A. radiobacter* (ATCC 19358) also belongs to the same genomic species (genomic species G4). A choice between *A. tumefaciens* and *A. radiobacte*r must be made for the name of this taxon. Meanwhile, strain C58, which was completely sequenced twice (15, 41), belongs to genomic species G8, which has not received an accepted species name yet. Genomic species G1 is another unnamed genomic species frequently found in crown gall outbreaks (unpublished results). Second, there is also a long-standing quarrel about the existence of the genus *Agrobacterium* itself (11, 42, 43). Briefly, Young et al. (42) proposed that the genus *Agrobacterium* should be eliminated by including all agrobacteria in *Rhizobium*, essentially to solve the *rrs* (i.e., 16S rRNA gene) polyphyly problem encountered with the former classification. Farrand et al. (11) recognized *Agrobacterium* as a definable genus, leaving the *rrs* polyphyly question pending. The subcommittee on the taxonomy of *Agrobacterium* and *Rhizobium* suggested that it is up to individual authors to choose which name they want to use (21). A compromise position is used in this paper. We refer to *Agrobacterium* as the *rrs* monophyletic genus, which includes "biovar 1" members and three closely related species, *A. larrymoorei* (3), *A. vitis* (formerly known as biovar 3 [27]), and *A. rubi* (33). Based on *rrs* phylogeny, strain NCPPB1650 belonging to an undefined related species should also be included in the genus *Agrobacterium*. The remote "biovar 2" is considered *Rhizobium rhizogenes*.

The present study was done to facilitate high-throughput identification of agrobacteria using the AFLP methodology, which is useful for genomic species identification and infraspecific typing of a large number of strains. Catalogues of specific AFLP genomic markers, which are conserved among all genomic species members, were established using a set of 52 agrobacterial strains from international collections, and there was a particular emphasis on the three most frequent genomic species of *Agrobacterium* biovar 1, genomic species G1, G4, and G8. This approach was validated by subsequent DNA-DNA hybridization of some environmental isolates identified as members of genomic species G1, G4, and G8 by AFLP.

MATERIALS AND METHODS

Bacterial strains. The strains analyzed (Table 1) represent almost all the genomic species richness of the genus *Agrobacterium* known so far. We placed special emphasis on genomic species G1, G4, and G8 because the numbers of strains of these genomic species identified by DNA-DNA hybridization were greatest (9, 11, and 5 strains, respectively) and because these three species are so closely related that they cannot be distinguished by conventional biochemical tests, leading to their inclusion in a single biovar called "biovar 1." All strains are available from the Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; most are also available from the Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium. The clinical isolates of agroabacteria are available from the Collection de l'Institut Pasteur.

Eight environmental strains isolated from galls, bulk soil, or root tissue identified as members of biovar 1 after conventional biochemical tests (production of 3-keto-lactose) were included in the study. The AFLP classification in genomic species for six of them was later confirmed by DNA-DNA hybridization.

DNA extraction and purification. DNA used in AFLP experiments was extracted with a DNeasy tissue kit (QIAGEN, Hilden, Germany) by following the manufacturer's instructions. Genomic DNAs used in DNA-DNA hybridization were extracted as described by Brenner et al. (4).

AFLP analyses. The AFLP methodology used in this study was adapted from that of Vos et al. (38). The EcoRI and MseI endonucleases were used for genomic DNA restriction as previously described (24), using adaptors and PCR primers shown in Table 2. The digestion-ligation step was performed for 2 h at 37°C with an 11-μl (final volume) mixture by incubating 55 ng of genomic DNA with EcoRI (5 U), MseI (5 U), T4 DNA ligase (1 U), the appropriate quantity of T4 DNA ligase buffer (Boeringer-Manheim, Germany), 0.5 μg of bovine serum albumin, 50 μM NaCl, the EcoRI-specific adaptor (0.18 μM) prepared with F1363-adEco+ hybridized with F1931-adEco−, and the MseI-specific adaptor (1.8 μM) prepared with F1365-adMse+ hybridized with F1931-adMse−. Each adapted DNA (4 μl) was then subjected to a nonselective PCR performed in a 20-μl (final volume) mixture containing 15 μl of the AFLP amplification CoreMix (Perkin-Elmer Applied Biosystems, Foster City, Calif.), 0.25 μM primer F1247-coreEco, and 0.25 μM primer F1248-coreMse. A PE-9600 thermocycler (Perkin-Elmer) was used with the following PCR program: denaturation at 94°C for 20 s, annealing at 56°C for 30 s, and elongation at 72°C for 2 min for 20 cycles. The quality of nonselective PCRs was controlled by agarose gel electrophoresis before storage of well-amplified products at −20°C. A well-amplified product was then typically diluted 1/30 before it was used as a template (1.5 μl) in a selective PCR mixture (final volume, 10 μl) with 7.5 μl of AFLP amplification CoreMix, primer F1248-coreMse (0.25 μM), and fluorescently labeled primers (0.05 μM). The following fluorescently labeled primers were designed with the F1247-coreEco sequence plus two discriminant nucleotides at the 3′ end: F1598-EcoCA-FAM, F1599-EcoCC-HEX, F1601-EcoCG-HEX, and F1915-EcoCT-FAM for selective nucleotides CA, CC, CG and CT, respectively. Selective PCRs were performed using a touchdown procedure consisting of denaturation at 94°C for 20 s, annealing at temperatures ranging from 66 to 57°C (the temperature was decreased 1°C per cycle) for 30 s, and elongation at 72°C for 2 min for 10 cycles, followed by a conventional PCR consisting of denaturation at 94°C for 20 s, annealing at 56°C for 30 s, and elongation at 72°C for 2 min for 20 cycles.

The total selective PCR products (10 μl) were purified on a Sephadex G-50 column (Amersham Biosciences, Orsay, France) before separation of the AFLP fragments by electrophoresis with a capillary sequencer (MegaBACE 1000; Amersham Pharmacia Biotech Europe, Orsay, France). The device automatically calculated the length of the fluorescent fragments by comparison to the MegaBACE ET-900-R size standard (Amersham). A genetic profiler (model 1.5; Molecular Dynamics Inc., Sunnyvale, Calif.) was used to display the results and to export data in text format. Data were transferred to a spreadsheet with the program Thresholdfilter 1.3 (Yann Legros, Amersham). A threshold fluorescence value of 200 arbitrary units was generally used to eliminate the background before subsequent bioinformatic treatment.

For each strain, experiments under the four AFLP conditions (EcoRI+CA/MseI+0, EcoRI+CC/MseI+0, EcoRI+CT/MseI+0, and EcoRI+CG/MseI+0) were performed in duplicate. AFLP fragments were coded as in the following example: the CA109 fragment corresponded to a 109-bp fragment experimentally obtained under the Eco+CA/Mse+0 AFLP conditions.

Predictive AFLP. A predictive AFLP analysis was performed with the complete genome sequence of C58 essentially as previously described by Mougel et al. (24) by simulating digestion with EcoRI and with MseI and then selecting restriction fragments based on the selective nucleotides added to selective primers. The lengths of the predicted AFLP fragments corresponded to the lengths of the restriction fragments plus 27 bp for the adaptors.

Phylogenomic analyses. The LecPCR and Align2 programs were used to transform raw data into tabular binary data, and the DistAFLP program was used to calculate the evolutionary genome divergence (rate of nucleotide substitution) and current genome mispairing (CGM) essentially as described by Mougel et al. (24), except that fragments were placed in length classes for genomic species instead of classes for all the strains together, which was done in the previous study. The LecPCR, Align2, and DistAFLP programs are accessible at http://pbil.univ-lyon1.fr/ADE-4/microb/. Dendrograms were calculated with the Neighbor/UPGMA module of the PHYLIP package (12) using evolutionary genome divergence data as distance data and were displayed with NJ-Plot

TABLE 1. Genomic species and strains of *Agrobacterium* used in this study

Strain	Other designation(s)[a]	Biological source, geographical origin, and other information	DNA-DNA hybridization results (reference)[b]
Genomic species G1[c]			
TT111[d]	CFBP 5767, LMG 196	Crown gall, United States	10
ATCC 4720	CFBP 5493, LMG 182	Black raspberry, Iowa	29
NCPPB 396	CFBP 5765, LMG 176	*Dahlia* sp., Germany	10
S377	CFBP 5768, LMG 326	Plant	29
S56	CFBP 5491, LMG 321	Plant	29
S4	CFBP 5492, LMG 318	Plant	29
CFBP 5622		*Solanum nigrum*, root tissue commensal, LCSA,[e] France	This study
CFBP 2517		Hybrid poplar, *Leuce* section, gall, France	This study
CFBP 5771		Bulk soil, LCSA, France	This study
CFBP 2712		*Prunes persica* × *Prunus amygdalus* cv. GF677 gall, France	ND
Genomic species G2			
CIP 497-74	CFBP 5494, CFBP 2884	Human blood, France	29
Bernaerts M2/1	CFBP 5876, LMG 147	Ditch water, Belgium	29
CIP 28-75	CFBP 5495	Human urine, France	29
CIP 43-76	CFBP 5496	Human urine, France	29
Genomic species G3			
CIP 107443	CFBP 6623, CIP 493-74	Antiseptic flask, France	29
CIP 107442	CFBP 6624, CIP 111-78	Human, France	29
Genomic species G4 (*A. tumefaciens* sensu stricto)			
B6[T]	CFBP 2413[T], LMG 187[T]	Apple seedling, Iowa; *A. tumefaciens* type strain	29
CIP 67-1	CFBP 2413[T], LMG 187[T]	Other designation for B6	29
ATCC 4452	CFBP 5766, LMG 181	*Rubus idaeus*, Iowa	10
ATCC 4718	CFBP 5764, LMG 139	Soil, United States	10
Kerr 14	CFBP 5761, LMG 15	Soil around *Prunus dulcis*, South Australia	10
Hayward 0322	CFBP 5770, LMG 1687	*Prunus persica* stock gall, South Australia	10
ATCC 19358	CFBP 5522, LMG 140	Soil; *A. radiobacter* type strain	29
LMG 340	CFBP 5769, ICPB TT11	*Librocedrus* sp. gall, United States	10
LMG 62		No information available	10
CFBP 5621		*Lotus corniculata*, root tissue commensal, LCSA, France	This study
CFBP 5627		Bulk soil, LCSA, France	This study
CFBP 2514		*Vitis vinifera* gall, Spain	ND
Genomic species G5			
CIP 107444	CFBP 6626, CIP 120-78	Human cephalorachidian liquid, France	29
CIP 107445	CFBP 6625, CIP 291-77	Patient food, France	29
Genomic species G6			
NCPPB 925	CFBP 5499, LMG 225	*Dahlia* sp., South Africa	29
Zutra F/1	CFBP 5877, LMG 296	*Dahlia* sp., Israel	29
Genomic species G7			
Zutra 3/1	CFBP 6999, LMG 298	*Malus* sp., Israel	29
RV3	CFBP 5500, LMG 317	No information available	29
NCPPB 1641	CFBP 5502, LMG 228	*Flacourtia indica*, United Kingdom	29
Genomic species G8			
C58	CFBP 1903, LMG 287	*Prunus* sp. cv. Montmorency (cherry), New York	9
TT9	CFBP 5504, LMG 64	Likely hop, United States	29
T37	CFBP 5503, LMG 332	*Juglans* sp. gall, California	29
Mushin 6	CFBP 6550, LMG 201	*Humulus lupulus* gall, Victoria, Australia	29
LMG 75	CFBP 6549	*Euonymus alata* cv. Compacta gall, United States	ND
LMG 46	CFBP 6554	*Rubus macropetalus*, Oregon	ND
AW137	LMG R-10181	Transmitted by A. Willems, LMG	ND
J-07	CFBP 5773	Bulk soil, LCSA, France	This study
Genomic species G9			
Hayward 0363	CFBP 5506, LMG 27	John Innes potting soil, Australia	29
Hayward 0362	CFBP 5507, LMG 26	John Innes potting soil, Australia	29

Continued on following page

TABLE 1—*Continued*

Strain	Other designation(s)[a]	Biological source, geographical origin, and other information	DNA-DNA hybridization results (reference)[b]
Agrobacterium sp. strain NCPPB 1650	CFBP 4470, LMG 230	*Rosa* sp., South Africa	ND
A. larrymoorei AF 3.10^T	CFBP 5473, LMG 21410	*Ficus benjamina*; type strain	3
A. rubi LMG 17935^T	CFBP 5509^T	*Rubus ursinus* var. Loganobaccus, United States; type strain	20
A. vitis K309^T	CFBP 5523^T	*Vitis vinifera*; type strain	27
Rhizobium rhizogenes			
LMG 150^T	CFBP 2408^T, CFBP 5520^T	Apple tree; type strain	29
K84	CFBP 1937, LMG 138	*Prunus persica*, soil around gall, Australia	ND
CFBP 2519		Hybrid poplar gall, *Leuce* section, France	ND

[a] CFBP, Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; LMG, Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium; CIP, Collection de l'Institut Pasteur.
[b] Reference for strain assignment to a genomic species as determined by DNA-DNA hybridization. ND, DNA-DNA hybridization not done.
[c] Genomic species G1 to G9 based on the nomenclature of Mougel et al. (24).
[d] The first strain of each genomic species is the reference strain or the accepted type strain of the described species.
[e] LCSA, maize field at La-Côte-Saint-André, Insère, France.

(http://pbil.univ-lyon1.fr/software/njplot.html). Bootstrap values were calculated by using the bootstrap option of DistAFLP and the Neighbor/UPGMA and Consense modules of the PHYLIP package.

Determination of common AFLP fragments. A newly developed program, AlignK (http://pbil.univ-lyon1.fr/ADE-4/microb/), was used to align fragment patterns in length classes in a spreadsheet by gathering fragments with the same length ± 0.5 bp in the same line (one line for each fragment length class). After visual correction, the program calculated how frequent a fragment of a given length was, its average length, and the length standard deviation. The program was used to determine the AFLP fragments common to all members of the same species.

DNA-DNA hybridization. Native DNAs were labeled in vitro by random priming with tritium-labeled nucleotides using the Megaprime DNA labeling systems (Amersham Biosciences). DNA-DNA hybridization was performed by using the S1 nuclease-trichloroacetic acid method (7, 16). Reassociation was performed at 70°C in 0.42 M NaCl. DNA-DNA hybridizations were carried out using labeled DNAs from strains TT111, C58, and B6^T.

TABLE 2. AFLP oligonucleotides used to construct adaptors and to prime PCRs

Oligonucleotide	Sequence
EcoRI-specific adaptors	
F1363-adEco+	CTCGTAGACTGCGTACC
F1931-adEco−	AATTGGTACGCAGTCTAC
MseI-specific adaptors	
F1365-adMse+	GACGATGAGTCCTGAG
F1931-adMse−	TACTCAGGACTCAT
Core primers	
F1247-coreEco	GACTGCGTACCAATTC
F1248-coreMse	GATGAGTCCTGAGTAA
Selective primers	
F1598-EcoCA-FAM	GACTGCGTACCAATTCCA
F1599-EcoCC-HEX	GACTGCGTACCAATTCCC
F1601-EcoCG-HEX	GACTGCGTACCAATTCCG
F1915-EcoCT-FAM	GACTGCGTACCAATTCCT

RESULTS

Experimental AFLP analyses. The average numbers of fragments per AFLP pattern obtained using the EcoRI+CA/MseI+0, EcoRI+CC/MseI+0, EcoRI+CT/MseI+0, and EcoRI+CG/MseI+0 conditions were 69, 45, 76, and 66 fragments, respectively. The phylogenomic analysis separated groups of strains on long branches supported by significant bootstrap values (>80%) (Fig. 1). These clusters are in accordance with genomic species assignments described previously (29). The maximum CGM value obtained for two strains in a genomic species was 10.8% (for Zutra 3/1 and RV3 in genomic species G7) (Fig. 1). The three major genomic species, genomic species G1, G4, and G8, exhibited comparable high levels of diversity; the greatest CGM values within these genomic species were 9.7%, 9.7%, and 9.0%, respectively, and the average CGM values were 7.5%, 6.5%, and 7.2%, respectively. A relevant finding is that the smallest CGM value found for comparisons of genomic species was 15.2% (for strain Zutra 3/1 in genomic species G7 and strain Hayward 0362 in genomic species G9) (Fig. 1).

Confidence interval for AFLP fragment length. The AFLP fragment lengths provided by the sequencer were subject to variation due to experimental conditions and sequence differences. The *rrs* gene was used to estimate the variation in fragment length, because fragment lengths are identical within all members of a given genomic species but there are small sequence differences between genomic species G1, G4, and G8 (24). The lengths of two fragments (CA109 and CG221) flanking the EcoRI site occurring in the *rrs* genes of genomic species G1, G4, and G8 strains (accession numbers of reference strains, AJ389895, AJ389904, and AJ012209, respectively) were studied within and between genomic species. The lengths and sequences of the CG221 fragment, predicted from *rrs*

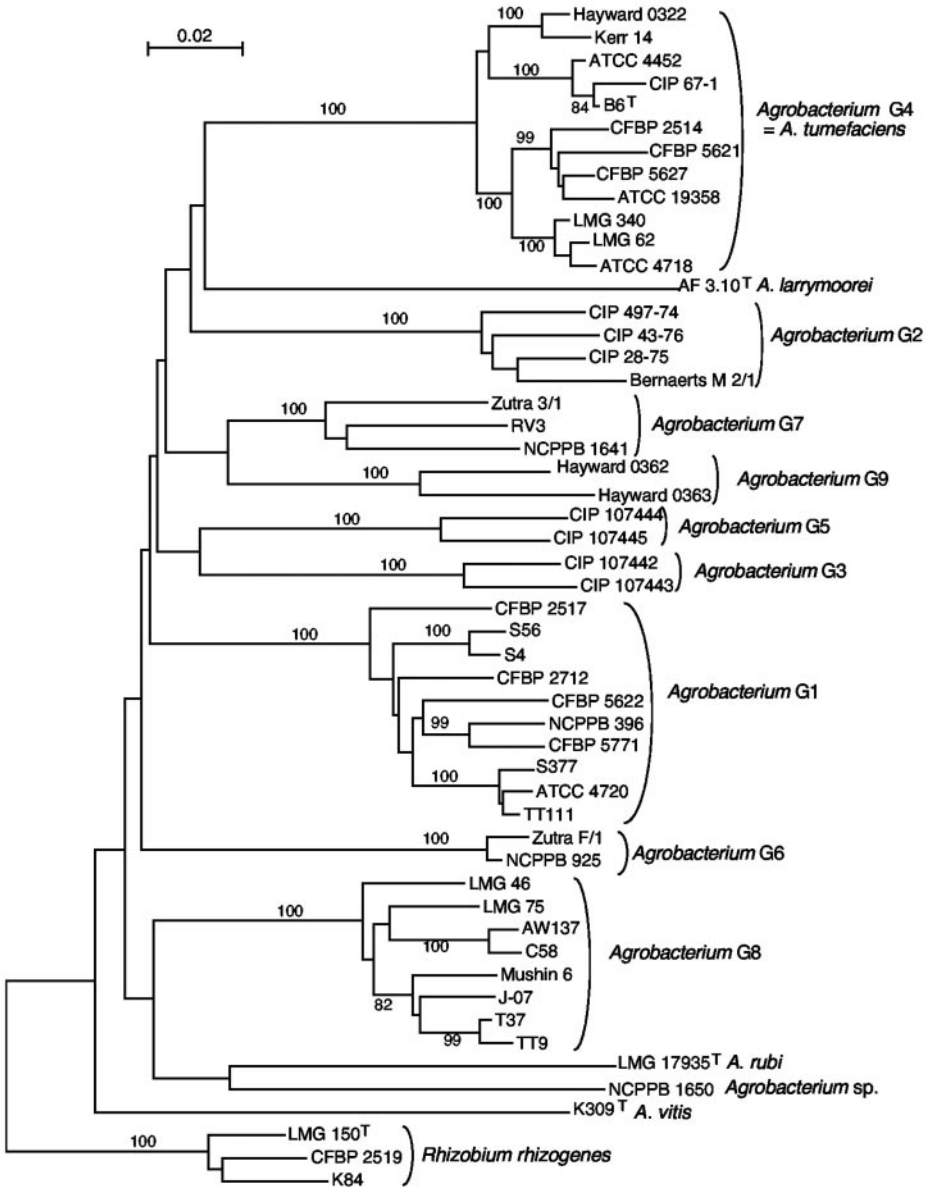

FIG. 1. Phylogenomic relatedness of *Agrobacterium* strains, species, and genomic species, calculated by the neighbor-joining method using pooled data for four AFLP conditions. The values are significant bootstrap values (i.e., >80%) obtained from 100 data resamplings. The unit of evolutionary distance is the rate of nucleotide substitution per nucleotide site calculated with DistAFLP.

sequences, were identical in all members of the three genomic species. The lengths of the experimental *rrs* fragments were 221.40 ± 0.04, 221.38 ± 0.03, and 221.38 ± 0.04 bp (averages ± standard deviations) in genomic species G1, G4, and G8, respectively (Table 3), indicating that the lengths of fragments with identical sequences could differ by a maximum of 0.10 bp ($P < 0.05$). The sequence of the *rrs* CA109 fragment differed by four nucleotides at positions 522, 523, 533, and 534 in genomic species G8 and G1 or G4 (5% mispairing), but the fragments were identical within genomic species. The lengths of the experimental *rrs* fragments were 108.37 ± 0.05, 108.40 ± 0.02, and 107.95 ± 0.05 bp (averages ± standard deviations) for genomic species G1, G4, and G8, respectively (Table 3), indicating that there was a difference of about 0.60 bp ($P < 0.05$) between the estimated lengths of experimental fragments having about 5% different nucleotides. Taking this uncertainty into account and considering that the maximum level of whole-genome mispairing within a genomic species is greater (9 to 10%) (see above), we assumed that the estimated lengths of fragments originating from the same genome region in different strains could differ by 1 bp (i.e., average length ± 0.5 bp).

AFLP markers of genomic species G1, G4, and G8. The fragments common to all members of a genomic species were determined by aligning the AFLP patterns of all members of the genomic species with AlignK (Table 3). The total numbers of common fragments for the four AFLP conditions were 57, 55, and 57 for genomic species G1, G4, and G8, respectively. The genomic markers of a genomic species were generally

TABLE 3. AFLP fragments common to all members of genomic species G1, G4, and G8

Genomic species	Fragment size (bp)							
	EcoRI+CA/MseI+0		EcoRI+CC/MseI+0		EcoRI+CG/MseI+0		EcoRI+CT/MseI+0	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1	52.10	0.07	48.94 (G4)[b]	0.12	48.16	0.18	65.70 (G8)	0.08
	65.91	0.23	64.35	0.09	55.90 (G4, G8)	0.09	128.12	0.08
	67.04	0.04	89.22 (G4, G8)	0.08	63.11	0.07	150.23	0.07
	89.31	0.08	161.35	0.12	91.53	0.29	156.55	0.12
	108.37 (*rrs*)[a]	0.05	184.75	0.21	94.68	0.25	171.60 (G8)	0.22
	116.93	0.30	573.97 (G8)	0.13	108.51 (G8)	0.04	186.06	0.16
	131.91	0.05			176.49	0.12	191.17 (G4)	0.12
	136.59	0.08			195.83	0.17	204.87	0.17
	146.81	0.33			213.68	0.08	246.84	0.63
	163.08 (G8)	0.26			221.40 (*rrs*)	0.04	386.72	0.28
	177.57 (G4)	0.19			232.70	0.12	525.49 (G4)	0.12
	191.68	0.28			236.35	0.29	528.16	0.19
	271.39	0.49			244.49	0.13	589.69 (G4, G8)	0.23
	295.09	0.12			250.73	0.11		
	339.75	0.43			253.91	0.18		
	418.95	0.21			260.71 (G8)	0.14		
	423.55	0.21			299.40	0.16		
	504.42	0.30			308.22	0.11		
	525.96	0.18			542.74	0.15		
G4	81.06	0.30	49.13 (G1)	0.07	50.81 (G8)	0.06	62.35	0.06
	108.40 (*rrs*)	0.02	89.48 (G1, G8)	0.06	55.74 (G1, G8)	0.05	74.74	0.06
	144.36	0.05	167.22	0.17	71.53	0.08	93.73	0.07
	171.92	0.03	219.43	0.18	104.90	0.28	148.39	0.25
	174.63	0.03	271.83	0.08	124.80	0.13	191.11 (G1)	0.06
	178.03 (G1)	0.05	286.36	0.10	131.20	0.06	197.20	0.05
	183.05	0.09	333.43 (G8)	0.30	157.89	0.04	201.10	0.03
	190.48	0.29	372.56	0.37	199.50	0.05	212.16	0.26
	210.70	0.15	433.68	0.23	204.49	0.11	251.25	0.21
	238.33	0.07	454.53	0.17	206.99	0.04	435.75	0.15
	243.79 (G8)	0.30	518.13	0.08	221.38 (*rrs*)	0.03	524.95 (G1)	0.07
	263.43	0.26	588.61	0.15	245.76 (G8)	0.38	589.77 (G1, G8)	0.37
	270.03	0.42			255.65	0.06		
	379.76	0.33			321.66	0.07		
	422.31	0.25			430.58	0.14		
	426.37	0.12						
G8	36.62	0.45	82.60	0.07	40.70	0.16	40.78	0.13
	68.20	0.30	84.90	0.13	50.80 (G4)	0.06	66.80 (G1)	0.07
	95.63	0.26	89.30 (G1, G4)	0.07	55.70 (G1, G4)	0.14	88.48	0.08
	106.20	0.31	213.00	0.14	103.92	0.08	136.80	0.07
	108.00 (*rrs*)	0.05	334.30 (G4)	0.05	108.78 (G1)	0.05	172.70 (G1)	0.27
	121.54	0.06	520.10	0.16	120.00	0.04	266.91	0.54
	164.40 (G1)	0.32	574.10	0.16	180.50	0.30	292.30	0.21
	210.24	0.10			190.70	0.13	300.60	0.24
	231.34	0.24			221.38 (*rrs*)	0.04	302.09	0.45
	233.23	0.05			223.53	0.26	336.00	0.14
	244.37 (G4)	0.05			246.23 (G4)	0.32	374.34	0.09
	355.6	0.07			252.80	0.13	441.70	0.59
	463.25	0.11			260.56	0.33	590.46 (G1, G4)	0.22
	507.26	0.15			275.40	0.06		
	627.13	0.11			318.10	0.08		
					357.18	0.12		
					363.03	0.12		
					370.14	0.09		
					374.54	0.30		
					391.20	0.19		
					400.30	0.26		
					510.40	0.21		

[a] *rrs*, fragments whose lengths corresponds to the lengths of fragments predicted to be liberated from *rrs* genes in all members of genomic species G1, G4, and G8.
[b] G1, G4, and G8, fragments also found in genomic species G1, G4, and G8 members, respectively.

TABLE 4. Genomic species assignment of environmental agrobacteria by DNA-DNA hybridization

Source of unlabeled DNA	Genomic species	Relative binding ratio (%) with ^{3}H-labeled DNA from strain:		
		TT111	B6^T	C58
TT111	G1	100	45	45
CFBP 5622	G1	77	ND[a]	45
CFBP 2517	G1	78	ND	45
CFBP 5771	G1	75	ND	41
B6^T	G4	42	100	52
CFBP 5621	G4	ND	76	ND
CFBP 5627	G4	37	75	ND
C58	G8	39	58	100
CFBP 5773	G8	ND	ND	87

[a] ND, hybridization not done.

found to be common only to members of that genomic species, even if they could be found in some strains belonging to another genomic species (data not shown). Thus, combinations of these genomic markers could be used as discriminative characteristics for genomic species. Nevertheless, as expected from the *rrs* sequence analysis, predicted fragments CA109 and CG221 flanking the EcoRI site found in the *rrs* genes of genomic species G1, G4, and G8 strains were detected in all members of the three genomic species, and the sizes of the fragments detected ranged from 108.00 to 108.47 bp for CA109 and from 221.38 to 221.40 bp for CG221 (Table 3). Three other fragments, CC89 (range, 89.22 to 89.48 bp), CG56 (range, 55.70 to 55.90 bp), and CT590 (range, 589.69 to 590.46 bp), were found to be common to the three genomic species, and some other fragments were common to pairs of genomic species. Moreover, the two putative *rrs* fragments (CA109 and CG221) and the CT590 fragment were found in all biovar 1 members, while fragments CC89 and CG56 were common to most but not all biovar 1 members (CC89 was not found in genomic species G3 and G9; CG56 was not found in genomic species G2 and G9). Thus, the presence of all five of these fragments is unique for genomic species G1, G4, G5, G6, G7, and G8, and these fragments did not occur outside biovar 1, while a combination of CT590, CA109, and CG221 was found both in all biovar 1 members and also in *A. vitis* (data not shown).

An attempt was made to identify fragments conserved in biovar 1 by predictive AFLP. This investigation revealed that CA109 and CG221 probably originated from the four *rrs* copies, ATU0053, ATU2547, ATU3937, and ATU4180, since there are four ribosomal operons in C58 with identical *rrs* copies (15, 41). The other conserved fragments, CG56, CC89, and CT590, presumptively originated from ATU0223, ATU2571, and a region spanning the ATU3980 and ATU3981 genes, respectively. Remarkably, ATU0223, ATU2571, and ATU3980 are not distributed at random over the genomes but are located in the vicinity of the ATU0053, ATU2547, and ATU3937 *rrs* gene copies at 170 kbp, 25 kbp, and 53 kbp, respectively.

Confirmation of AFLP species identification by DNA-DNA hybridization. Eight strains isolated from plants or soil samples whose classification in genomic species was not previously determined were used in this study. The AFLP patterns of these strains unambiguously placed them in genomic species G1, G4, or G8. The AFLP placement of six of them (CFBP 5622, CFBP 2517, CFBP 5771, CFBP 5621, CFBP 5627, and J-07) in genomic species G1, G4, and G8 was confirmed a posteriori by DNA-DNA hybridizations performed with TT111, B6^T, and C58, respectively (Table 4).

DISCUSSION

The AFLP methodology clearly delineates all genomic species presently known in the genus *Agrobacterium* when enough AFLP conditions are used to obtain clusters supported by significant bootstrap values (24). Some genomic species, such as genomic species G7 and G9, display great infraspecies diversity. The DNA-DNA hybridization values for these genomic species are close to or even less than 70%, and to ascertain the genomic species status, it is necessary to verify that differences in melting temperatures for members of a species are less than 5°C (29). These highly diverse genomic species were significantly separated from their close neighbors if up to four AFLP conditions were used (bootstrap values, >80%), while only one AFLP condition was enough to correctly place a strain in a less diverse genomic species (data not shown). In all cases, the AFLP approach rapidly and unambiguously determined the genomic species status of agrobacteria isolated from various biotopes, as well as strains deposited in international bacterial collections. Moreover, the results were unambiguously confirmed by further DNA-DNA hybridizations for six environmental strains. As a consequence, AFLP has been proven to be reliable and has rendered DNA-DNA hybridization unnecessary for placing environmental agrobacteria into genomic species.

In spite of the power of AFLP for species identification, AFLP data are not easily compared by phylogenetic methods when they are obtained with different sequencers (gel versus capillary; different molecular weight markers; different data extraction software). It would be easier to identify a species by a set of well-defined molecular markers. In order to facilitate the analysis and to examine the universal applicability of the method, AFLP fragments common to all members of a genomic species were determined for the three major genomic species of agrobacteria, genomic species G1, G4, and G8 (Table 2). We verified that most of these "species-specific" markers were present in AFLP profiles obtained previously with another sequencer and with smaller sets of strains (http://pbil.univ-lyon1.fr/ADE-4/microb/IJSEM2001), provided that a larger confidence interval for the fragment size determination was used since different molecular weight ladders were used. Thus, the combination of the fragments listed Table 3 is sufficient to place an isolate into genomic species G1, G4, or G8. Thus, we describe here a reliable and portable method for identifying the three major *Agrobacterium* genomic species even in the absence of databases and phylogenomic analyses. With the present list of "species-specific" markers, this approach is accessible to all laboratories without a need for phylogenetic analyses.

At higher taxonomic levels, only five AFLP fragments, including two fragments of *rrs*, were found to be common to all or almost all biovar 1 members. This small number of markers is not enough to allow significant and robust identification of a

new isolate as a member of biovar 1 by AFLP. Nevertheless, these conserved fragments likely originated from the core genome of biovar 1 strains and are good candidates for definition of markers for sequence-based approaches, such as PCR-restriction fragment length polymorphism analysis or MLSA. Identification of the fragments conserved in biovar 1 was attempted. We used predictive AFLP because fragments detected by fluorescent AFLP (which provides only virtual images of fragments) are difficult to clone and sequence. Remarkably, all the biovar 1 markers were presumptively found to originate from genes located close to the ribosomal operons. Housekeeping genes, such as *recA*, *gyrB*, *groEL*, or *mutS*, which are frequently used in MLSA, were found in the vicinity of the ribosomal operons. This confirmed that AFLP markers readily tag the core genome of biovar 1 strains and that standard housekeeping genes are the most relevant genes for MLSA.

One of the advantages of AFLP is that it measures diversity by estimating maximal and average current genome mispairing of populations (23). This provided a minimal set of strains which displayed the highest diversity in a given species and which were the strains that were best suited for determining the most conserved markers in a given species. Using strains from various collections, the three main genomic species of agrobacteria in biovar 1 displayed infraspecies CGM values of 9 to 9.7%, which were close to the maximum value found in another genomic species in biovar 1 (10.8%). As a result, the list of markers in Table 3 is likely to be "species specific," and the markers tag the core genomes of the genomic species.

Current genome mispairing values are key parameters for species delineation as they are highly correlated to the data obtained by the conventional DNA-DNA hybridization procedure (24). In the present study, the maximum CGM value found for two strains in a genomic species was 10.8%, while the smallest CGM value found for two genomic species was 15.2%. This emphasizes the gap in the distribution of genome divergence values on which the current genomic species definition is based. These results establish the CGM threshold values that could be used to delineate genomic species. The values are actually slightly different but likely much more accurate than the values reported previously (24), because they were calculated by using a more relevant procedure for assignment of AFLP fragments to fragment length classes.

In order to determine whether the CGM threshold values determined for *Agrobacterium* spp. are relevant for delineating genomic species in other taxa, it is necessary to perform extensive studies before the method can be generalized. However, as an indication, the minimal interspecies CGM value for agrobacteria as determined by AFLP (15.2%) appears to be close to the value for whole-genome mispairing estimated by Vulic et al. (39) using DNA-DNA hybridization data for *Escherichia coli* and *Salmonella enterica* (16%). These two species are very closely related but are sexually isolated, as measured by the dramatic drop in the rate of homologous recombination within and between species (39). This strengthens the idea that the genome divergence threshold, which delineates genomic species, including agrobacterial genomic species (70% for DNA-DNA, corresponding to 15% genome mispairing), is a critical parameter of the sexual isolation of bacterial species.

In this study, we developed an original method to place rapidly an agrobacterial strain into one of the three major *Agrobacterium* genomic species (genomic species G1, with reference strain TT111; genomic species G4 or bona fide *A. tumefaciens*, including type strain B6; and genomic species G8, including sequenced strain C58) by determining lists of common AFLP markers detected in all strains of the three species. This procedure is thought to allow high-throughput identification and genomic species classification of agrobacteria isolated from field samples.

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