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Application of Amplified Fragment Length Polymorphism Fingerprinting for Taxonomy and Identification of the Soft Rot Bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*

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The soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi* are important pathogens of potato and other crops. However, the taxonomy of these pathogens, particularly at subspecies level, is unclear. An investigation using amplified fragment length polymorphism (AFLP) fingerprinting was undertaken to determine the taxonomic relationships within this group based on their genetic relatedness. Following cluster analysis on the similarity matrices derived from the AFLP gels, four clusters (clusters 1 to 4) resulted. Cluster 1 contained *Erwinia carotovora* subsp. *carotovora* (subclusters 1a and 1b) and *Erwinia carotovora* subsp. *odorifera* (subcluster 1c) strains, while cluster 2 contained *Erwinia carotovora* subsp. *atroseptica* (subcluster 2a) and *Erwinia carotovora* subsp. *betavasculorum* (subcluster 2b) strains. Clusters 3 and 4 contained *Erwinia carotovora* subsp. *wasabiae* and *E. chrysanthemi* strains, respectively. While *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* showed a high level of molecular diversity (23 to 38% mean similarity), *E. carotovora* subsp. *odorifera*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *atroseptica*, and *E. carotovora* subsp. *wasabiae* showed considerably less (56 to 76% mean similarity), which may reflect their limited geographical distributions and/or host ranges. The species- and subspecies-specific banding profiles generated from the AFLPs allowed rapid identification of unknown isolates and the potential for future development of diagnostics. AFLP fingerprinting was also found to be more differentiating than other techniques for typing the soft rot erwinias and was applicable to all strain types, including different serogroups.

The genus *Erwinia* is a member of the family *Enterobacteriaceae* and consists of 18 species that fall into two main groups, the necrogenic, or Amylovora, group and the soft rot, or Carotovora, group (15, 26, 34). Within the soft rot group, *Erwinia carotovora* and *Erwinia chrysanthemi* are the most commercially important soft rotting pathogens. Until 1981, *E. carotovora* contained only two subspecies, *Erwinia carotovora* subsp. *carotovora* (causing soft rot diseases, mainly in storage, on a wide variety of plant species, including potato) and *Erwinia carotovora* subsp. *atroseptica* (causing a vascular disease [blackleg] of potato plants and storage rot of potato tubers). More recently, a number of new subspecies have been included, namely, *Erwinia carotovora* subsp. *betavasculorum* (causing soft rot of sugar beet [37]), *Erwinia carotovora* subsp. *wasabiae* (originally isolated from Japanese horseradish but causing soft rot disease of various vegetables, including potato [16]), and *Erwinia carotovora* subsp. *odorifera* (isolated from and causing disease in chicory, leeks, and celery [15]). Hauben et al. (18) reclassified *E. carotovora* and *E. chrysanthemi* into the genus *Pectobacterium* based on 16S ribosomal DNA (rDNA) sequence analysis. Although both *Erwinia* and *Pectobacterium* are validly published names for this group of pathogens, throughout the remainder of this manuscript they will be referred to as the soft rot erwinias, *E. carotovora* (with subspecies *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *odorifera*, and *E. carotovora* subsp. *wasabiae*) and *E. chrysanthemi*.

Understanding the diversity within and relationships among pathogenic taxa is an important prerequisite to meaningful taxonomic classification, accurate identification, pathogen detection, and epidemiology studies. This is particularly important when a number of closely related subspecies cause disease on the same host, e.g., *E. chrysanthemi*, *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *E. carotovora* subsp. *wasabiae* on potato. Several taxonomic and identification studies of the soft rot erwinias have been undertaken using molecular techniques, such as DNA-DNA hybridization (3–5, 15), PCR-restriction fragment length polymorphism (RFLP) (2, 9, 20, 31), ribotyping (30), and 16S rDNA analyses (18, 27), and phenotypic techniques, including biochemistry (12–14) and API identification strips (11, 29, 41). Although a small number of studies have included the more recent subspecies *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *odorifera*, and *E. carotovora* subsp. *wasabiae* (15, 18, 27), they have been limited by the number of strains used. Studies using the highly conserved 16S rDNA gene (18, 27) have confirmed the heterogeneity of the genus *Erwinia*, which forms four clusters intermixed with other genera. They have also shown that the soft rot erwinias fall into a single cluster, in one case leading to the renaming of members of this group as *Pectobacterium* (18). However, the technique approaches its limit of taxonomic resolution at subspecies level (40), and the accuracy of the groupings for the *E. carotovora* subspecies is thus questionable. Furthermore, one study (27) did not investigate all subspecies, and

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in both studies only one strain of each subspecies was used, offering no information about the genetic variation within each subspecies.

Amplified fragment length polymorphism (AFLP) is a genomic fingerprinting method first described by Vos et al. (42), with an effective taxonomic resolution from species to strain level (40), which has been used to study the taxonomy and genetic diversity of a number of organisms, including a growing list of bacteria (6, 7, 21, 23–25, 36, 38). The aims of this study were to investigate the utility of the AFLP technique for the taxonomic classification of the soft rot erwinias at species and subspecies levels and as a method of identification, to generate markers for the development of diagnostics, and to type strains for epidemiological investigations.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Tables 1 and 2. They consist of over 130 well-characterized soft rot erwinias and other strains from both established and recent culture collections from around the world. In addition, over 200 recent isolates from potato, collected both locally and from Australia, were included in the study. The reference strains are species type strains obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), York, United Kingdom. In cases where type strains were not available from the NCPPB, strains identified by the NCPPB as belonging to certain species or subspecies were used. The bacterial strains were stored in freezing medium at −80°C (1), and all cultures were maintained on nutrient agar (code CM3; Oxoid) at 18°C. When required, *Erwinia* species were grown at 27°C, while other enterobacteria were grown at 37°C in Luria broth medium for 18 h with shaking.

AFLP. Bacterial genomic DNA was extracted and purified using a DNeasy tissue kit (Qiagen) as described by the manufacturer. The DNA was stored at −20°C until it was required. The AFLP reactions were performed as described previously (42) with minor modification. Bacterial genomic DNA (0.5 μg) was digested with 5 U each of the restriction enzymes *Eco*RI and *Mse*I in OnePhorAll buffer (Amersham Pharmacia Biotech) at 10 μg ml^{-1} for 3 h at 37°C. *Mse*I (50 pmol; 5′-GACGATGAGTCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′) and *Eco*RI (5 pmol; 5′-CTCGTAGACTGCGTACC-3′ and 5′-AATTGGTACGCA GTC-3′) double-stranded adapters were ligated to the digested DNA in a total volume of 35 μl using 1 U of T4 DNA ligase in OnePhorAll buffer (10 μg ml^{-1}) plus 1 mM ATP for 3 h at 37°C. Following ligation, the DNA was first amplified by PCR using nonselective *Mse*I (M00) (5′-GATGAGTCCTGAGTAA-3′) and *Eco*RI (E00) (5′-GACTGCGTACCAATTC-3′) primers in a 25-μl total volume. Each reaction mixture contained 2 μl of the ligation mixture, 2.5 μl of AmpliTaq LD buffer, 1 U of AmpliTaq LD (Perkin-Elmer), all four deoxynucleoside triphosphates at 200 μM, and 50 ng each of the *Mse*I and *Eco*RI primers. The PCR was performed under the following conditions: 35 cycles of 30 s of denaturing at 94°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C. All amplifications were performed in a PE-9600 thermocycler (Perkin-Elmer). The amplification products were then diluted threefold, and 0.5 μl was used as a template for selective PCR with primers M00 and E19 (an *Eco*RI primer with the extension GA). In each selective PCR, the *Eco*RI primers were radiolabeled for 1 h at 37°C. The labeling reaction contained 3.5 ng of primer, 0.125 U of T4 polynucleotide kinase (Invitrogen), 0.1 μl of 5× forward reaction buffer provided with the enzyme, and 0.5 μCi (18.5 kBq) of [γ-^{33}P]ATP (Amersham), and the total volume was adjusted to 0.5 μl with sterile distilled water. The selective PCR mixture in a total volume of 11 μl contained 1 μl of Perkin-Elmer AmpliTaq LD buffer, all four deoxynucleoside triphosphates at 200 μM, 15 ng of M00 primer, 3.5 ng of labeled *Eco*RI primer, and 1 U of *Taq* polymerase (Invitrogen). The selective PCR was performed under the following conditions: (i) 1 cycle of 30 s of denaturing at 94°C, 30 s of annealing at 65°C, and 1 min of extension at 72°C; (ii) 11 cycles over which the annealing temperature was reduced from 65°C by 0.7°C each cycle; and (iii) 23 cycles of 30 s of denaturing at 94°C, 30 s of annealing at 56°C, and 1 min of extension at 72°C. To the completed reactions, 10 μl of gel loading buffer (94% formamide, 10 mM EDTA, 0.5 mg of xylene cyanol FF ml^{-1}, 0.5 mg of bromophenol blue ml^{-1}) was added. Samples were heated to 90°C for 5 min and cooled on ice. The AFLP products were electrophoresed through a 6% polyacrylamide denaturing gel (sequencing gel; Severn Biotech Ltd.) at 100 W. After being dried, the gels were exposed to autoradiographic film (Kodak) for 24 to 72 h to visualize the results. The autoradiographs were converted to TIF format, and the data were analyzed using GelCompar version 4.1 software (Applied Maths) as described by the manufacturer. *E. carotovora* subsp. *atroseptica* strain SCRI 1039 was used as a reference strain to normalize tracks from different gels.

Biochemical and phenotypic tests. Biochemical tests, including production of phosphatase and indole; utilization of citrate; acid production from α-methyl glucoside, palatinose, sorbitol, melibiose, and lactose; reducing substances from sucrose; and growth in 5% NaCl and on nutrient agar at 37°C were performed as described previously (13). Cavity formation on crystal violet pectate medium at 27, 33.5, and 37°C was assessed as described previously (33).

Data analysis. Following electrophoresis of polyacrylamide gels, the autoradiographs were digitized and band profiles were analyzed using GelCompar software. The Pearson product-moment correlation coefficient was used to estimate levels of similarity between densitometric profiles for each isolate. Unweighted pair-group method of averages (UPGMA) and neighbor-joining algorithms within GelCompar were then used to construct dendrograms from the similarity matrices (22).

RESULTS AND DISCUSSION

Reproducibility of AFLP and choice of restriction enzymes. To determine the reproducibility of AFLP profiles, a standard strain (*E. carotovora* subsp. *atroseptica* SCRI 1039) was used for each AFLP amplification and on each electrophoresis gel. The AFLP profile of this strain was also used to normalize gels in GelCompar. Following normalization, the similarity between profiles from this strain was at least 90% within each gel and 85% between gels, using the Pearson product-moment correlation. This variation appeared to be in the intensities of the lanes and may be due to differences in exposure of the autoradiograph, as previously noted (6, 21, 24).

The restriction enzymes *Eco*RI and *Mse*I, together with different combinations of 0-, 1-, and 2-bp primer extensions, were tested on representative strains of *E. carotovora* and other *Erwinia* and enterobacterial species. Following these tests, primers M00 (no extensions) and E19 (2-bp extensions), which generated between 30 and 50 clearly distinguishable bands for each strain tested (with the total number of bands for cluster analysis being considerably higher), were chosen for the remainder of the study.

AFLP fingerprinting of soft rot erwinias and other *Erwinia* and enterobacterial species. When different species and subspecies of *Erwinia* and other genera (selected as outgroups) were compared using AFLP fingerprinting under the conditions chosen for the study, a high level of heterogeneity was observed (Fig. 1). The infrequency of shared AFLP bands provided little information on the molecular relationships among species. However, this has not been the case for all genera, e.g., *Acinetobacter* (24), *Bacillus* (25), *Burkolderia* (6), *Ralstonia* (7), *Stenotrophomonas* (19), and *Xanthomonas* (36). Where bands of similar size were observed, this may simply have arisen due to comigration of unrelated bands on the gel (homoplasy), a possibility that is increasingly likely with more distantly related organisms (8). In the majority of cases where more than one strain of a species was analyzed, related but nonidentical profiles were obtained (Fig. 1), indicating the utility of AFLPs to differentiate within species. As expected, the soft rot erwinias did group, and thus, these conditions were used for a more detailed analysis of the group.

AFLP fingerprinting of the soft rot erwinias. A selection of type strains and other well-characterized strains from the soft rot erwinias were investigated (Tables 1 and 2) and, in all cases, known species and subspecies were clearly distinguished by

TABLE 1. Soft rot erwinia strains used in this study

Bacterial strain	Host	Location	Source
E. carotovora subsp. *carotovora*			
SCRI 108	Potato	Finland	P. Harju
SCRI 112 (NCPPB 1746)[a]	Potato	Japan	D. C. Graham
SCRI 134	Cyclamen	Israel	V. M. Lumb
SCRI 167	Water	Israel	M. C. M. Pérombelon
SCRI 174	Potato	Peru	E. French; J. G. Elphinstone
SCRI 193	Potato	United States	M. P. Starr
SCRI 196	Caladium	United States	D. C. Graham
SCRI 212	Potato	Scotland	P. Harju
SCRI 244, SCRI 295	Potato	Scotland	R. Lowe; L. J. Hyman
SCRI 258	Potato	Israel	V. M. Lumb
AU3[b], AU6[b], AU8[b], AU9[b], AU12[b], AU14[b], AU43[b], AU44[b], AU55[b], AU56[b]	Potato	Australia	T. Wicks
E. carotovora subsp. *atroseptica*			
SCRI 13, SCRI 47, SCRI 53, SCRI 54, SCRI 59, SCRI 83, SCRI 93, SCRI 1035, SCRI 1039, SCRI 1040, G999P6[b], G994A7[b], G998F1[b], G98292[b], G998V8[b], G991N8[b], G98218[b]	Potato	Scotland	L. J. Hyman
SCRI 33	Water	Scotland	L. J. Hyman
SCRI 37	Soil	Scotland	L. J. Hyman
SCRI 49	Insect	Scotland	L. J. Hyman
SCRI 135	Potato	Arizona	M. Stanghellini
BSB 9202, BSB 9205, BSB 9207, BSB 9302, H1	Potato	Germany	F. Neipold
IPO 161, N88.30, N88.37, N88.40, N88.41, N88.45, N88.46, N88.49, N88.52, J12, IPO 723, IPO 848, IPO 850, IPO 852, IPO 856, IPO 861, IPO 862, IPO 1005, IPO 1008	Potato	Netherlands	J. M. Van der Wolf
Z141, Z144, Z147, Z148, Z149, Z150, Z153, Z406, Z413, Z414	Potato	Sweden	P. Persson
494, 495, 310 (88.33), 311 (88.45), 312 (87.7), 315 (86.14.11)	Potato	France	Y. Bertheau
581-1.1, 1342-47, 1346-8, 1346-11, 1347-39, 1348-37, 1366-19	Potato	Spain	M. Lopez
E. carotovora subsp. *betavasculorum*			
SCRI 479 (NCPPB 2795)[a,c], SCRI 908 (NCPPB 2792)[a], SCRI 909 (NCPPB 2793)[a], SCRI 910 (NCPPB 2794)[a], SCRI 911 (NCPPB 3075)[a]	Sugar beet	United States	NCPPB
E. carotovora subsp. *odorifera*			
SCRI 482 (NCPPB 3840), SCRI 912 (NCPPB 3839), SCRI 913 (NCPPB 3841), SCRI 914 (NCPPB 3842), SCRI 915 (NCPPB 3843), SCRI 916 (NCPPB 3844) SCRI 487	Chicory	France	NCPPB
E. carotovora subsp. *wasabiae*			
SCRI 481 (NCPPB 3701), SCRI 917 (NCPPB 3702), SCRI 918 (NCPPB 3703), SCRI 919 (NCPPB 3704)	Horseradish	Japan	NCPPB
SCRI 488	Horseradish	Japan	Y. Bertheau
E. chrysanthemi			
pv, unspecified			
SCRI 417, SCRI 4039	Potato	Peru	E. French; J. G. Elphinstone
SCRI 4000 (IPO 645)	Potato	Netherlands	E. Cother
SCRI 4044 (IPO 502) (biovar 7)	Potato	Netherlands	J. M. Van der Wolf
SCRI 4076 (NCPPB 1125)[a,d]	Pineapple	Malaysia	Y. Bertheau
E. chrysanthemi			
pv. dianthicola			
SCRI 401 (NCPPB 426), SCRI 409 (NCPPB 518)[a,d]	Carnation	Denmark	D. C. Graham
E. chrysanthemi			
pv. zeae			
SCRI 4071 (NCPPB 2541) (biovar 7)[a,d]	Maize	United States	NCPPB
SCRI 4072 (NCPPB 2547) (biovar 7)[a,d]	Maize	India	NCPPB
SCRI 4078 (NCPPB 1065)[a,d]	Maize	Egypt	D. C. Graham
E. chrysanthemi			
pv. dieffenbachiae			
SCRI 4074 (NCPPB 1514)[a,d]	*Dieffenbachia*	Germany	Y. Bertheau

[a] Authenticity of culture verified by NCPPB.
[b] Previously uncharacterized isolate.
[c] Type strain.
[d] Pathogenicity of culture confirmed by NCPPB.

AFLP (Fig. 2). Following numerical analysis of the AFLP banding profiles, whether by UPGMA or neighbor joining (the results shown are for UPGMA only), the taxa clustered into four broad groups (clusters 1 to 4) (Fig. 2). Cluster 1 contained all *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera* strains, which linked at a relatively low similarity (38.4% ± 12.6%). However, the cluster was further divided into three subclusters, 1a (n = 9), 1b (n = 12), and 1c (n = 7),

TABLE 2. Other strains used in this study

Bacterial strain	Host	Location	Source
Pantoea agglomerans			
SCRI 435	Red deer; human	United States	D. C. Graham
SCRI 462	Gypsophila	Israel	H. Vigodsky-Haas
Pantoea stewartii			
SCRI 475 (NCPPB 449)[a,b]	Maize	United States	NCPPB
Pantoea ananatis			
SCRI 485 (NCPPB 1846)[a,b,c]	Pineapple	Brazil	NCPPB
SCRI 922 (NCPPB 544)	Pineapple	Hawaii	NCPPB
Enterobacter cancerogenus			
SCRI 489 (NCPPB 2176)[c], SCRI 920 (NCPPB 2177)[a]	Carolina poplar	Not known	NCPPB
Enterobacter dissolvens			
SCRI 921 (NCPPB 1850)[a,c]	Maize	United States	NCPPB
Enterobacter nimipressuralis			
SCRI 491 (NCPPB 2045)[a,c], SCRI 901 (NCPPB 440)	Elm	United States	NCPPB
Erwinia rhapontici			
SCRI 421 (NCPPB 139)[a]	Rhubarb	United Kingdom	D. C. Graham
SCRI 423 (NCPPB 1578)[a,c]	Rhubarb	United Kingdom	NCPPB
Erwinia uredovora			
SCRI 432 (NCPPB 800)[a,c]	Wheat	United States	D. C. Graham
Erwinia cypripedii			
SCRI 478 (NCPPB 2636)[b]	Orchid	Germany	NCPPB
Erwinia quercina			
SCRI 442, SCRI 477	Oak	United States	D. C. Graham
Erwinia rubrifaciens			
SCRI 445 (NCPPB 2020)[a,c], SCRI 446 (NCPPB 2021)[a]	Persian walnut	United States	D. C. Graham
Erwinia nigrifluens			
SCRI 476 (NCPPB 564)[a,c]	Persian walnut	United States	NCBBP
Erwinia amylovora			
SCRI 444	Pear	United States	D. C. Graham
SCRI 906 (NCPPB 595)[a,d]	Pear	United Kingdom	NCPPB
SCRI 907 (NCPPB 686)[a]	*Crataegus* spp.	United Kingdom	NCPPB
Erwinia salicis			
SCRI 474 (NCPPB 447)[a,c], SCRI 905 (NCPPB 2535)[a]	Cricket-bat willow	United Kingdom	NCPPB
Erwinia persicinus			
SCRI 480 (NCPPB 3375)	French bean	United States	NCPPB
Erwinia cacticida			
SCRI 484 (NCPPB 3849)	Giant cactus	United States	NCPPB
Erwinia mallotivora			
SCRI 490 (NCPPB 2851)[a,b,c], SCRI 903 (NCPPB 2852)[a,b]	*Mallotus japonicus*	Japan	NCPPB
Erwinia psidii			
SCRI 492 (NCPPB 3555)[a], SCRI 902 (NCPPB 3556)[a]	Guava	Brazil	NCPPB
Erwinia tracheiphila			
SCRI 900 (NCPPB 2133)	Cucumber	United States	NCPPB

[a] Authenticity of culture verified by NCPPB.
[b] Pathogenicity of culture confirmed by NCPPB.
[c] Type strain.
[d] Authenticity and virulence have been well confirmed.

with all *E. carotovora* subsp. *carotovora* strains in subclusters 1a and 1b (38.4% ± 12.6% similarity) and all *E. carotovora* subsp. *odorifera* strains in subcluster 1c (75.8% ± 2.8% similarity). Although *E. carotovora* subsp. *carotovora* appeared in two subclusters, this did not reflect any obvious subdivision in terms of host or geographic origin. Such intragroup diversity in *E. carotovora* subsp. *carotovora* had been noted previously with PCR-RFLP (9, 20) and may simply reflect the high molecular diversity within the subspecies. *E. carotovora* subsp. *odorifera*-specific banding profiles clearly distinguished *E. carotovora* subsp. *odorifera* from *E. carotovora* subsp. *carotovora*, supporting previous results using DNA-DNA hybridization (15) and 16S rDNA sequencing (18). However, the latter study, which included representatives from all subspecies, used only one strain of each, offering no information on diversity within these subspecies. API tests (11, 41) also led to the formation of a distinct cluster for all chicory strains of "atypical" *E. carotovora* subsp. *atroseptica* ("atypical" now *E. carotovora* subsp. *odorifera*), related to but separate from *E. carotovora* subsp. *carotovora*.

Cluster 2 contained all *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum* strains, with the two subspecies linking at 49.0% ± 7.5% similarity. This cluster was further divided into two subclusters, 2a (n = 21) and 2b (n = 5), which were composed of all *E. carotovora* subsp. *betavasculorum* strains, grouped at 70.2% ± 3.8% similarity, and all *E. carotovora* subsp. *atroseptica* strains, grouped at 56.6% ± 10.4% similarity, respectively. The results clearly showed that *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum* were related but distinct taxa (Fig. 2). 16S rDNA

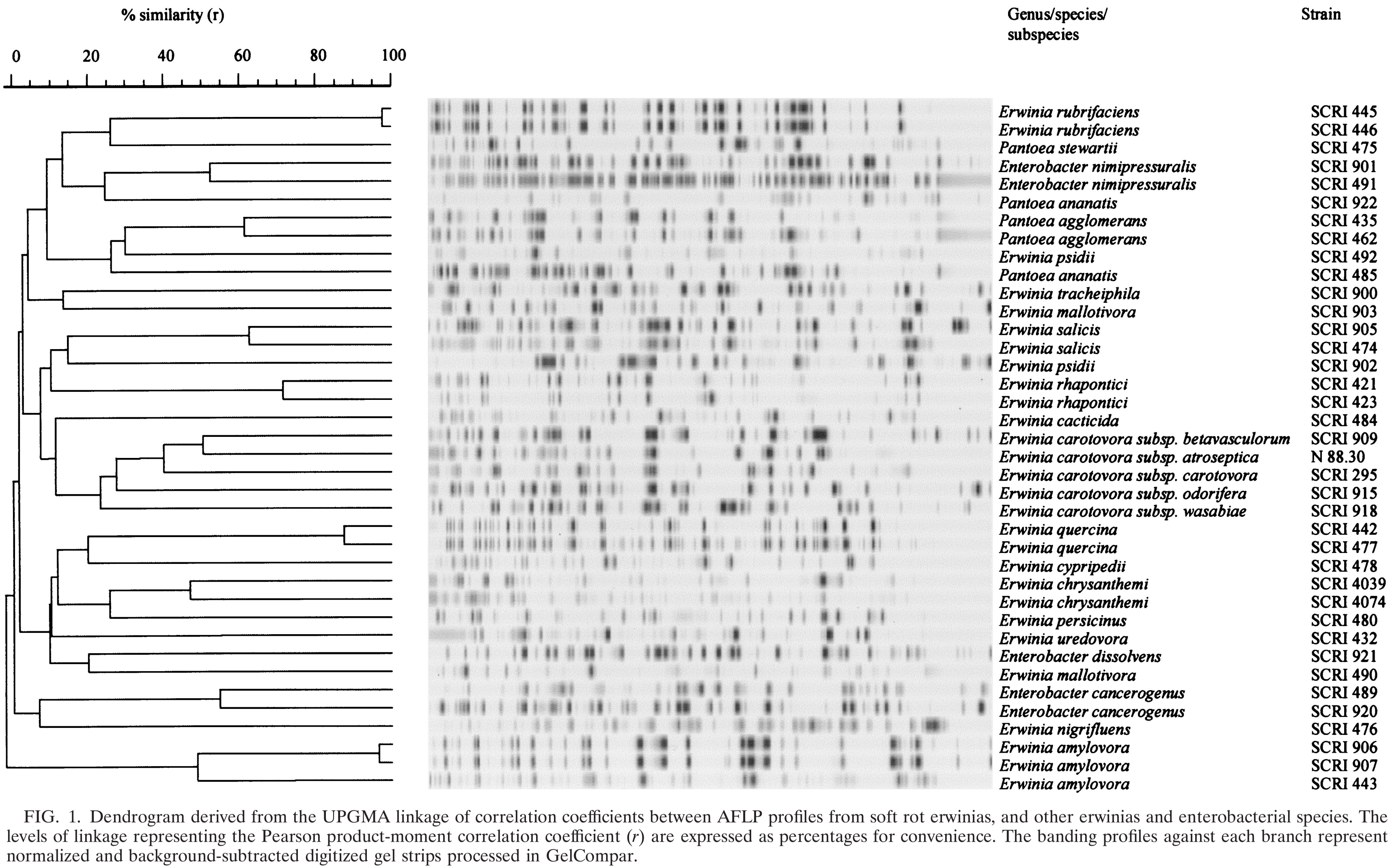

FIG. 1. Dendrogram derived from the UPGMA linkage of correlation coefficients between AFLP profiles from soft rot erwinias, and other erwinias and enterobacterial species. The levels of linkage representing the Pearson product-moment correlation coefficient (r) are expressed as percentages for convenience. The banding profiles against each branch represent normalized and background-subtracted digitized gel strips processed in GelCompar.

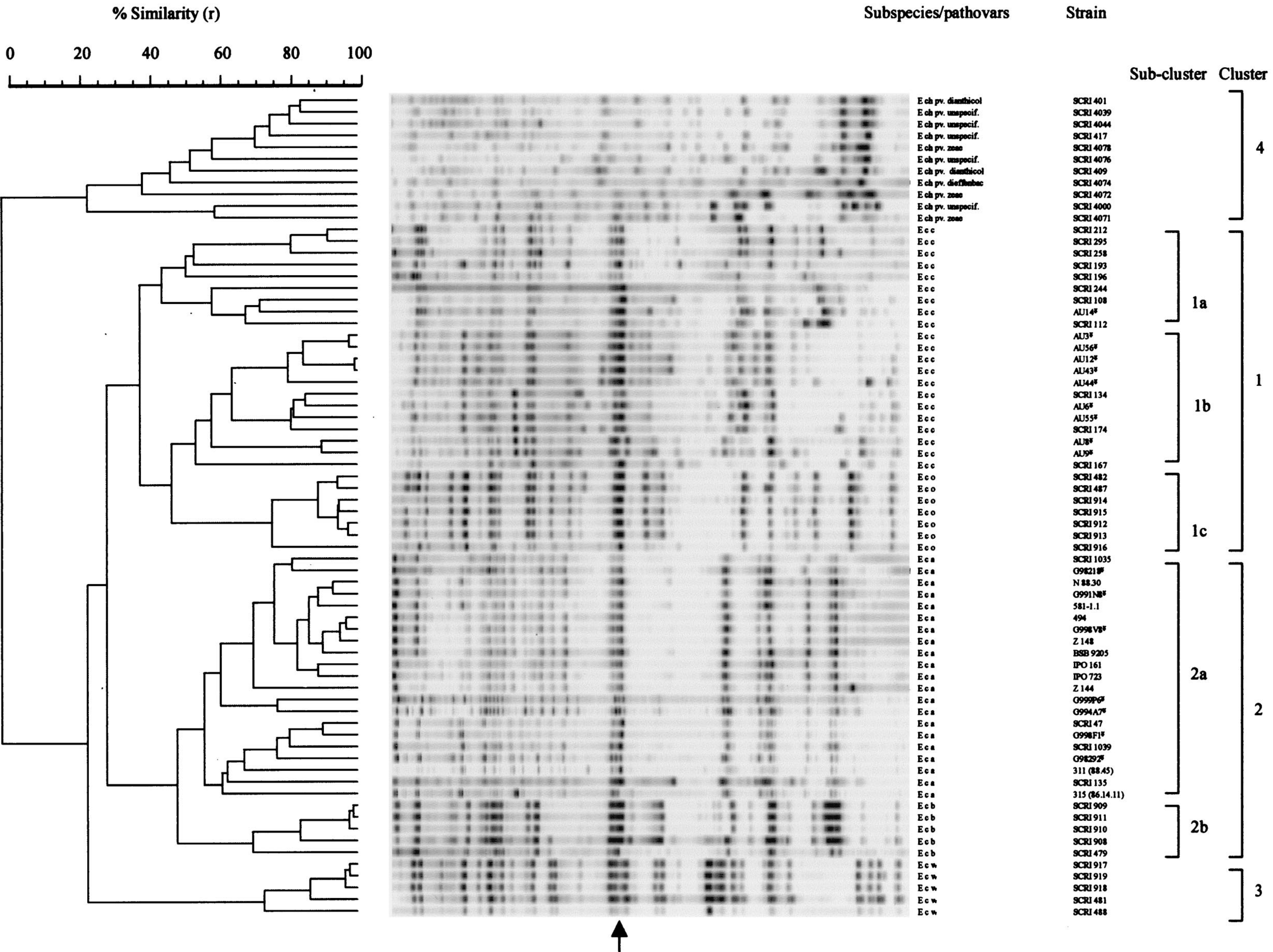

FIG. 2. Cluster analysis of *E. chrysanthemi* and *E. carotovora* subspecies together with suggested species and subspecies clusters (clusters 1 to 4). ¥ indicates a previously uncharacterized strain. The dendrogram is derived from the UPGMA linkage of correlation coefficients between AFLP profiles. The levels of linkage representing the Pearson product-moment correlation coefficient (*r*) are expressed as percentages for convenience. The banding profiles against each branch represent normalized and background-subtracted digitized gel strips processed in GelCompar. The arrow indicates *E. carotovora*-specific AFLP bands. Ech, *E. chrysanthemi*; Ecc, *E. carotovora* subsp. *carotovora*; Eco, *E. carotovora* subsp. *odorifera*; Eca, *E. carotovora* subsp. *atroseptica*; Ecb, *E. carotovora* subsp. *betavasculorum*; Ecw, *E. carotovora* subsp. *wasabiae*.

sequencing (18) showed *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, and *E. carotovora* subsp. *wasabiae* to be more closely related to each other than to *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *odorifera*, and *E. chrysanthemi*, while *E. carotovora* subsp. *atroseptica* was more closely related to *E. carotovora* subsp. *wasabiae* than to *E. carotovora* subsp. *betavasculorum*. Similarly, phenotypic tests (11, 41) showed that *E. carotovora* subsp. *betavasculorum* strains formed a group distinct from other subspecies. However, unlike AFLP and 16S rDNA analyses, these tests linked *E. carotovora* subsp. *atroseptica* more closely with *E. carotovora* subsp. *carotovora* than with *E. carotovora* subsp. *betavasculorum* (Fig. 2), showing a discrepancy between the molecular and phenotypic studies. No clustering data were obtained for *E. carotovora* subsp. *betavasculorum* by PCR-RFLP due to problems with amplification of *E. carotovora* subsp. *betavasculorum* DNA (9, 20).

Cluster 3 ($n = 5$) was composed of all *E. carotovora* subsp. *wasabiae* strains and grouped at 73.5% ± 3.5% similarity. It was distinct from all other subclusters, although marginally more closely related to clusters 1 (*E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera*) and 2 (*E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum*) than to cluster 4 (*E. chrysanthemi*). In both 16S rDNA sequence (18, 27) and RFLP (20) analyses, *E. carotovora* subsp. *wasabiae* was more closely related to *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum* (subcluster 2) than to members of clusters 1 (*E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera*) and 4 (*E. chrysanthemi*) and, in the case of 16S rDNA sequencing, clustered between *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum* (18). Given the close grouping between *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum* using AFLP, the discrepancies between AFLP and 16S rDNA sequencing may be related to the number of strains used.

E. chrysanthemi, not unexpectedly, formed the most distantly related and genetically diverse cluster (cluster 4; $n = 11$) within the soft rot erwinias and grouped at 23.6% ± 10.3% (Fig. 2). API tests (11, 29, 41) found that *E. chrysanthemi* formed a cluster distinct from the *E. carotovora* subspecies. In addition, however, these tests (41) showed that *E. chrysanthemi* pv. dieffenbachia strains, which are known to constitute a well-defined group corresponding to DNA hybridization group II and biovar II (3, 12), clustered separately from other *E. chrysanthemi* strains. *E. chrysanthemi* strains have also been characterized using ribotyping (30, 31) and PCR-RFLP (2), which suggest that *E. chrysanthemi* clusters show some correlation with other intraspecific levels, such as pathovar, biovar, and, to a lesser extent, geographical origin, although these correlations are by no means universal. There were no such correlations in our study, although only 11 *E. chrysanthemi* strains were used.

Polymorphisms were demonstrated both among and within species and subspecies, with some amplified bands being shared among subspecies. In all isolates of *E. carotovora* subspecies, a characteristic pattern of two to three bands was clearly visible (Fig. 2), providing potential molecular markers for identification and targets for the development of diagnostics. For individual subspecies, other characteristic bands were present. In most cases, the species- and subspecies-specific bands appeared more intense than others (Fig. 2), perhaps reflecting their higher genomic copy numbers. It has been suggested that rDNA genes could be the sources of these bands (25).

To investigate the utility of AFLP fingerprinting as a method of identification, the dendrogram produced from well-characterized strains was used to compare profiles from over 200 additional unidentified soft rot erwinia strains, freshly isolated from potato material both locally and from Australia. To verify these identifications, all strains, including a number of different serogroups, were tested in parallel using biochemical and phenotypic methods (13, 33). In all cases, the unidentified strains grouped with either *E. carotovora* subsp. *atroseptica* or *E. carotovora* subsp. *carotovora* on the dendrogram, and these identifications were confirmed using the biochemical and phenotypic methods (only 17 of these strains are included in Fig. 2). In addition to showing the utility of AFLPs for identification, the results also show that the reproducibility of the method, in terms of comparing different gels produced at different times, had little effect on strain identification. It also showed that using entire banding profiles in GelCompar, a process much faster than marking individual bands, was sufficient for an accurate identification of the soft rot erwinias and could be used routinely. Finally, strain SCRI 135, originally identified by biochemical and phenotypic tests as *E. carotovora* subsp. *carotovora*, was identified as *E. carotovora* subsp. *atroseptica* by AFLP. On retesting by the former methods, the new identification was confirmed, again highlighting the utility of AFLP for identification purposes.

Investigating diversity within subspecies. *E. carotovora* and *E. chrysanthemi* are phenotypically and genetically diverse, and this was reflected in the AFLP study, with mean similarities of ca. 23% for both species (Fig. 2). *E. carotovora* subsp. *carotovora* was the most diverse subspecies at 34% similarity, while *E. carotovora* subsp. *odorifera*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *atroseptica*, and *E. carotovora* subsp. *wasabiae* were considerably more homogeneous, with mean similarities of between 59 and 75%. This homogeneity is well known for *E. carotovora* subsp. *atroseptica* (9–11, 17, 32, 35) and has been reported for *E. carotovora* subsp. *odorifera* (11), but it is less clear for *E. carotovora* subsp. *betavasculorum* (11) and is unknown for *E. carotovora* subsp. *wasabiae*. Such relatively low levels of genetic diversity may be due to a subspecies having more recent origins, limited population divergence, and/or limited host range, e.g., due to recent spread of a New World crop such as the potato. From our current knowledge of these subspecies, and including data from this study, all three possibilities may apply. This is not the case for *E. chrysanthemi* and *E. carotovora* subsp. *carotovora*, however, where an earlier divergence, wider geographical distribution, and wider host range (see the introduction) may explain their genetic diversity.

As detailed studies of the diversity within *E. carotovora* subsp. *atroseptica* have been carried out using a number of physiological and molecular methods (39), we chose *E. carotovora* subsp. *atroseptica* to investigate the applicability of AFLP fingerprinting for studying diversity within a subspecies. Fifty-nine strains of *E. carotovora* subsp. *atroseptica* were compared by AFLP fingerprinting, and a high level of diversity among these strains was seen (Fig. 3). Thus, AFLP fingerprinting is a powerful technique for determining genetic variation in a large number of *E. carotovora* subsp. *atroseptica* isolates and shows improvements over present methods, such as phage typ-

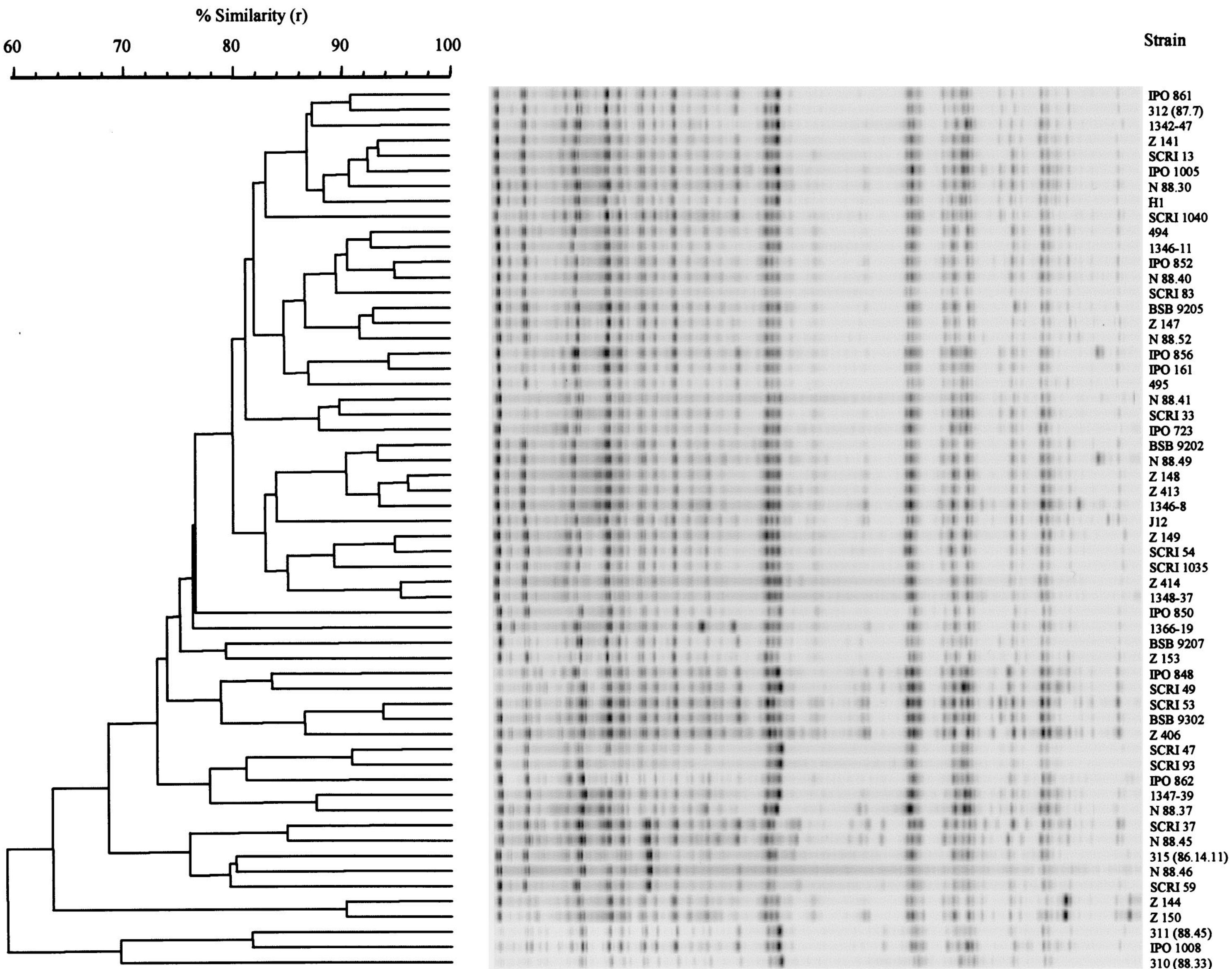

FIG. 3. Dendrogram derived from the UPGMA linkage of correlation coefficients between AFLP profiles from strains of *E. carotovora* subsp. *atroseptica*. The levels of linkage representing the Pearson product-moment correlation coefficient (r) are expressed as percentages for convenience. The banding profiles against each branch represent normalized and background-subtracted digitized gel strips processed in GelCompar.

ing and randomly amplified polymorphic DNA (28, 39), i.e., the ability to work on all serogroups and an improved resolving power.

Conclusions. This is the first study to investigate the taxonomic and phylogenetic relationships among the soft rot erwinias using multiple strains from *E. chrysanthemi* and all *E. carotovora* subspecies. Although interspecific relationships among more distantly related taxa could not be determined using AFLP fingerprinting, at least under the conditions chosen, the method did generate subspecies-specific banding profiles that allowed four clusters to be delineated. In addition, the method was used to identify a number of unknown isolates, to discriminate between closely related strains for epidemiological investigations, and to provide species- and subspecies-specific banding profiles that are now being used in our laboratory to develop molecular diagnostics.

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