How Complex is the "*Ralstonia solanacearum* Species Complex"?

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R. solanacearum is a heterogeneous species; as Buddenhagen (2) pointed out, "there are many bacterial wilts and there are many '*Pseudomonas solanacearums*'", but is *R. solanacearum* a species complex? A species complex is defined as a cluster of closely related isolates whose individual members may represent more than one species. The term "species complex" was first applied to *R. solanacearum* by Gillings and Fahy (7) to reflect the phenotypic and genotypic variation within the species. Taghavi *et al.* (22) then expanded the concept of the *R. solanacearum* species complex to include two closely related organisms, the blood disease bacterium (BDB) and *Pseudomonas syzygii* as both of these organisms were found to fall within the diversity of *R. solanacearum* as defined by 16S rDNA sequence analysis. Studies of DNA-DNA homology of *R. solanacearum* strains have revealed that the relatedness between isolates of this species is often less than the 70% threshold level commonly expected within a species (15,20). Therefore we define *R. solanacearum* as a species complex.

A stable and meaningful taxonomy and nomenclature which accurately defines subspecific groups of *R. solanacearum* has to be the aim of taxonomists working on the *R. solanacearum* species complex. Such a taxonomic system will aid plant breeders, plant pathologists and quarantine officials who require a system of classification where strains can be grouped into clusters of isolates that relate to epidemiology, pathogenicity, host range and/or geographic origin. The taxonomic framework and methodology proposed below allows identification of subspecific groups within the *R. solanacearum* species complex and will improve our ability to predict the properties of *R. solanacearum* strains.

Diversity of *R. solanacearum*

Traditionally *R. solanacearum* has been classified into five races on the basis of differences in host range (1,12,16) and six biovars on the basis of biochemical properties (9,10,11). The work of Cook *et al.* (4) and Cook and Sequeira (3) employing restriction fragment length polymorphism (RFLP) analysis showed that *R. solanacearum* can be divided into two divisions: division 1 comprising strains belonging to biovars 3, 4 and 5, primarily isolated in Asia and division 2 comprising strains belonging to biovars 1, 2 and N2, primarily isolated in the Americas. Several other investigations employing molecular methods have confirmed this dichotomy within *R. solanacearum* (6,21,22). Taghavi *et al.* (22), using 16S rDNA sequence analysis, also revealed the existence of a subdivision within division 2 comprising isolates of *R. solanacearum* from Indonesia including the closely related organisms the blood disease bacterium (BDB) and *P. syzygii.* Further sequencing of the 16S-23S rRNA gene intergenic spacer region (ITS), the polygalacturonase gene and the endoglucanase gene (5) has supported the existence of the two divisions and the existence of the group of strains originating in Indonesia.

A recent PCR-RFLP analysis of the *hrp* gene region (17) demonstrated that certain African biovar 1 strains did not cluster with other biovar 1 isolates as was expected. An extended PCR-RFLP analysis of the *hrp* gene region complemented by amplified fragment length polymorphism (AFLP) and sequencing of the 16S rRNA gene (19) has provided further support for the existence of this group of strains. Phylogenetic analysis of the endoglucanase and *hrpB* genes has confirmed the presence of a group of strains originating in Africa (18).

Hence the picture has emerged that the *R. solanacearum* species complex is comprised of four broad genetic groups corresponding with geographic origin.

The Phylotyping Scheme: A New Scheme for Classifying *R. solanacearum*

A hierarchical classification scheme is proposed to reflect the known diversity within the *R. solanacearum* species complex. The scheme is outlined in Table 1. Under this classification system members of the *R. solanacearum* species complex can be subdivided into four phylotypes

Table 1. Hierarchical classification scheme for *R. solanacearum.*

Taxonomic level	Taxonomic equivalent	Nomenclature	Method of identification
Species	Species	*Ralstonia solanacearum* species complex	PCR Primers (eg 759/760 (14)
Phylotype	Subspecies	Phylotypes I, II, III and IV	Phylotype specific multiplex PCR based upon the ITS region
Sequevar	Infrasubspecific groups	Sequevars 1-23	Endoglucanase gene sequencing
Clone	Clonal Lines		Genome fingerprinting methods e.g. rep-PCR, RAPD, AFLP, PFGE, etc.

corresponding to the four genetic groups identified via sequence analysis (Figure 1). A phylotype is defined as a monophyletic cluster of strains revealed by phylogenetic analysis of sequence data, in this case the ITS region, the *hrpB* gene and the endoglucanase gene. Phylotype I is equivalent to division 1 defined by Cook *et al.* (3). This phylotype includes all strains belonging to biovars 3, 4, and 5; strains are isolated primarily from Asia. Phylotype II is equivalent to division 2 (1989), and includes strains belonging to biovars 1, 2 and 2T isolated primarily from America. Phylotype II contains the *R. solanacearum* race 3 potato pathogen, which has a world wide distribution, and the race 2 banana pathogens. Phylotype III contains strains primarily isolated from Africa and surrounding islands, strains belong to biovars 1 and 2T. Phylotype IV contains strains isolated primarily from Indonesia belonging to biovars 1, 2 and 2T. *R. solanacearum* strains in Phylotype IV have also been found in Australia and Japan. This phylotype also contains the two close relatives of *R. solanacearum*, *P. syzygii* and the BDB.

The phylotype to which a strain belongs can be rapidly identified using a multiplex PCR based upon sequence information from the ITS region. This phylotype-specific multiplex-PCR employs four forward primers - one specific for each phylotype and a single reverse primer that is specific for the species (Table 2, Figure 1 and Appendix) and also includes the 759/760 primer pair described by Opina *et al.* (14). All *R. solanacearum*, BDB and *P. syzygii* strains generate the 280bp *R. solanacearum* species complex-specific fragment produced by amplification of template DNA by the 759/760 primer pair. All *R. solanacearum*, BDB and *P. syzygii* strains tested with the multiplex PCR produce a phylotype specific amplicon with the exception of strain ACH0732. Strain ACH0732 is the only *R. solan-*

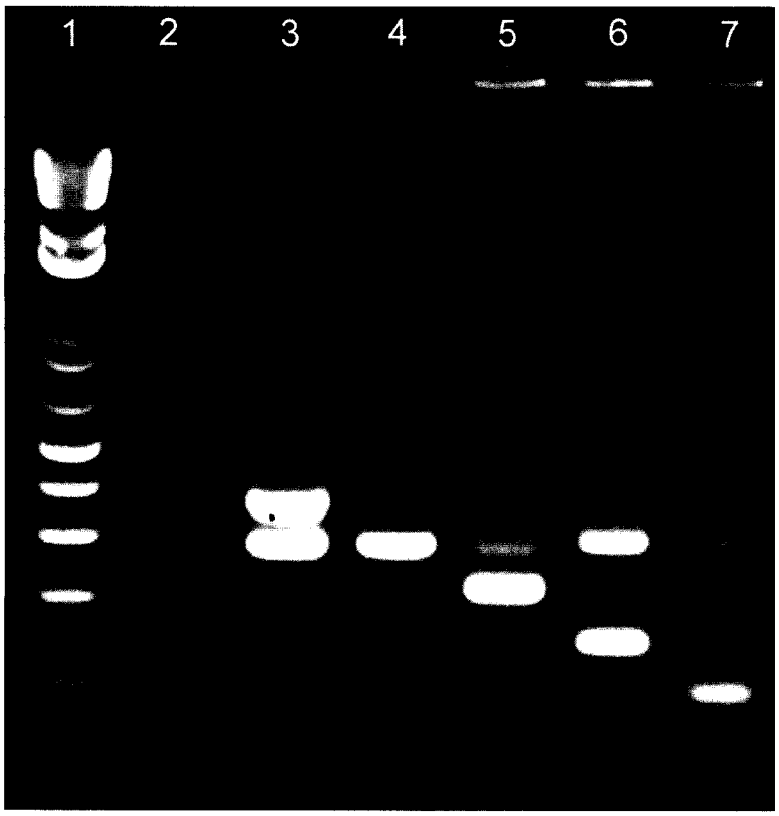

Fig. 1. Example of a multiplex PCR gel. Lane 1 molecular weight marker (1kb plus DNA ladder; Life Technologies); Lane 2 negative control; Lane 3 a representative phylotype II strain; Lane 4 ACH0732; Lane 5 a representative phylotype IV strain; Lane 6 a representative phylotype I strain; Lane 7 is a representative phylotype III strain.

acearum strain that varies in its phylogenetic position depending on the genomic region sequenced. Therefore using the phylotype specific multiplex PCR ACH0732 only produces the 280bp *R. solanacearum* species complex-specific fragment (Lane 4 in Figure 1).

Each phylotype is composed of a number of sequevars. A sequevar, or sequence variant, is defined as a group of strains with a highly conserved sequence within the area sequenced. Only if two or more strains sequenced have similar sequences has a sequevar been defined. Therefore single sequence clusters have not been given sequevar status (for example CIP10 in Figure 2). Sequevars are primarily defined upon partial endoglucanase gene sequences as a large number of strains have been sequenced in this region.

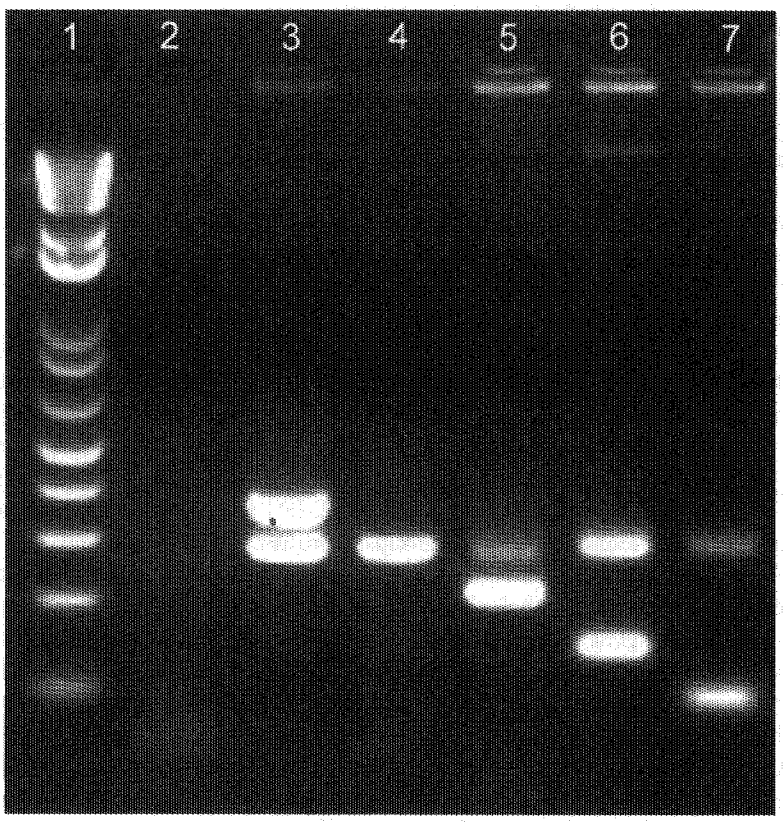

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Table 2. Primers designed from the ITS region used in the phylotype specific multiplex PCR

Primer	Sequence (5' to 3')	Specificity	Amplicon size when paired with Nmult:22:RR
Nmult21:1F	CGTTGATGAGGCGCGCAATTT	Phylotype I	144bp
Nmult21:2F	AAGTTATGGACGGTGGAAGTC	Phylotype II	372bp
Nmult23:AF	ATTACSAGAGCAATCGAAAGATT	Phylotype III	91bp
Nmult22:InF	ATTGCCAAGACGAGAGAAGTA	PhylotypeIV	213bp
Nmult22:RR	TCGCTTGACCCTATAACGAGTA	All phylotypes	NA[1]

[1] Not applicable.

In the future it is hoped that sequence information from more strains from other areas of the genome, such as the *hrpB* gene, will be generated to confirm these sequevars. The endoglucanase gene of greater than 140 *R. solanacearum* isolates has been sequenced and over 20 sequevars have been identified (Figure 2).

Each sequevar may be composed of a number of clonal lines that may be identified using genomic fingerprinting methods such as PFGE, AFLP's or rep-PCR. In our experience rep-PCR is a fast and reproducible method for identification of clonal lineages within a sequevar.

The phylotyping scheme is highly discriminatory, flexible and additive allowing identification of further sequevars or even phylotypes. This phylotyping scheme is based upon genetic variation that accumulates relatively slowly in the genome of organisms at the level of the phylotypes and sequevars thus giving a long term global epidemiological perspective. However, the scheme also incorporates the finer resolving power of the genomic fingerprinting techniques to identify clonal lines below the level of the sequevar.

Because PCR facilities are not yet routinely applicable worldwide, an attempt has also been made to identify phenotypes associated with phylotypes or sequevars. This will allow phenotypic identification of these genetic groups. This approach is fundamentally different from the biovar scheme as it is attempting to identify a phenotype associated with an already identified genetic cluster of isolates. This work is ongoing but it is hoped that we will be able to use biochemical tests to identify the major genetic groups and thus allow laboratories that do not have ready access to sequencing technologies to accurately identify and place unknown isolates into this new typing scheme.

The biotyping scheme described here is based upon the work of Harris (8) and Hayward (9). Our results have confirmed the great degree of pheno-

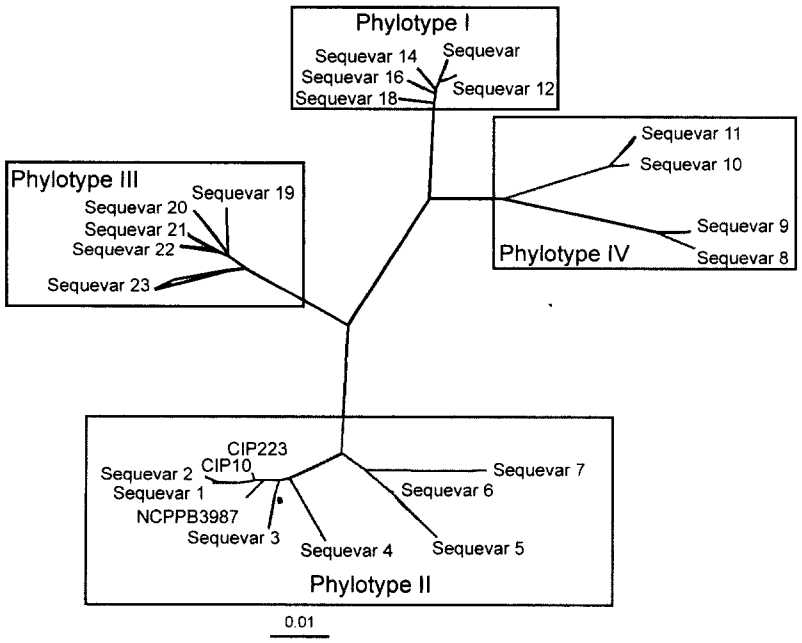

Fig. 2. Phylogenetic tree generated from partial endoglucanase gene sequence data showing the phylogenetic relationships of sequevars and phylotypes. The bar indicates 1 nucleotide change per 100 nucleotide positions.

typic diversity within biovar 1 isolates identified by Harris (8). This diversity is not surprising as this biovar is based on negative criteria for both disaccharides and hexose alcohols. The biotype scheme generates a unique metabolic profile for many sequevars based on a set of six substrates, namely maltose, manitol, malonate, trehalose inositol and hippurate (Table 2). Of the 56 strains tested in our collection, only the strain UW170 did not provide a biotyping profile consistent with the sequevar to which the strain belongs. Genetically this strain falls in phylotype II/sequevar 4 and it was expected that it would be of biotype 4. However, UW170 produced a biotype 3 profile which is characteristic of phylotype II/sequevar 3. The biotyping system needs to be fully validated on many other strains. Nevertheless, the scheme has already been useful in typing unknown strains of *R. solanacearum* isolated from pothos and from anthurium. The biotype of the isolates from these hosts was used to predict the phylotype and sequevar to which they belonged. The predicted phylogenetic positions were confirmed later by sequence analysis.

Table 2. Relationship of biotyping profiles and phylotype(s)

Biotype	Substrate[1]						Phylo-type(s)
	Maltose	Mannitol	Malonate	Trehalose	Inositol	Hippurate	
1	+	-	+	-	-*	+	II/1&2
2	+	-	+	+	+	+	II/ND
3	-	-	-	-	+	+++	II/3
4	-	-	-	-	-	+	II/4
5	-	-	+	-*	+	+	II/5
6	-	-	-	-	-	-	II/6
7	-	-	-	-	+	+	II/7
8	+/-	+	-	-	+	-	I/12-18
9	+	-	-	+	-	-	III/22
10	-	-	-	+	+	-	III/ND
11	+	-	-	+/-	+	+	III/8&9

[1] Substrates were filter sterilized and tested at a final concentration of 1% w/v in Ayers minimal medium with pH indicator. Tests were read after 14 days incubation at 28°C; NA, not applicable; *, alkalinisation; -, no acidification; +, acidification; +++, strains were considered positive for Hippurate if the media turned a deep black colour.

Comparison of the Phylotyping Classification Scheme to Previous Schemes

In comparison to race and biovar classification schemes we feel the phylotyping scheme proposed here more accurately reflects the diversity that we now know to be present in the *R. solanacearum* species complex.

Race 1, defined as strains "affecting tobacco, tomato, many solanaceous and other weeds, and certain diploid bananas" (1) is a very broad definition. Strains belonging to race 1 are found in phylotypes I and II and probably in phylotypes III and IV if host of origin can be used as a guide to which race a strain belongs. In contrast to race 1, races 2 and 3 have narrow host ranges and this is reflected in the narrow genetic diversity included within races. Race 3 strains belong to phylotype II, sequevars 1 and 2. Strains belonging to race 2 belong to phylotype II, sequevars 3, 4 and 6.

Strains representing biovars 1 and 2T are present in three of the four phylotypes and it is clear that simply identifying a strain as biovar 1 or 2T does not tell you much about the strain. The large degree of variation within strains of biovar 1 has previously been recognised phenotypically (8) and this is mirrored in the genetic variation found in biovar 1 strains. Most strains belonging to biovar 2 are equivalent to race 3 and therefore belong

to phylotype II sequevars 1 and 2. However, some biovar 2 strains do not belong to race 3 and are found in phylotype IV sequevars 8 and 9.

This new scheme largely confirms the RFLP typing scheme (4). Phylotypes I and II are equivalent to the divisions 1 and 2 defined by Cook *et al.* (3). Phylotypes III and IV were not recognised by Cook *et al.* (4) as they did not study strains belonging to these two phylotypes. It would be expected that if strains belonging to these two phylotypes had been analysed using RFLP's then these two groups would have been identified earlier. Below the level of the phylotype at the sequevar level the RFLP and phylotyping schemes are also congruent with strains that belong to different MLG's also belonging to distinct sequevars. For example strains belonging to MLG's 24, 25, and 28, which contain moko disease causing strains of *R. solanacearum* are equivalent to sequevars 3, 4 and 6 respectively.

How Useful is the Phylotyping Scheme?

The DNA-DNA hybridisation values of certain strains of *R. solanacearum* are lower than would be expected of organisms belonging to the same species (15). Palleroni and Doudoroff (15) used strains representing biovars 1-4; the puzzling result from their analysis was the low DNA-DNA hybridisation values between strains representing biovar 1. The biovar 1 strains used by these authors originated from the USA, South America, Zimbabwe, and Reunion Island. When the same strains were analysed using the phylotyping scheme the strains from the USA and South America were found to belong to Phylotype II, whereas the strains from Zimbabwe and Reunion Island belonged to Phylotype III. This probably explains the low hybridization values. However, it does raise the question as to the taxonomic relationship of strains belonging to each phylotype. Are strains representing each phylotype related at the species or subspecies level? To adequately answer this question we require further information on the genotypic and phenotypic differences/similarities of representative strains of these two genetic groups.

The phylotyping scheme has also been used to help elucidate the unexplained results of Marín and El-Nashaar (13). These authors isolated biovar 1 strains from potato in Peru that apart from the biochemical tests used to differentiate the biovars were phenotypically and in pathogenicity the same as biovar 2/race 3 strains. When these strains were placed in the phylotyping scheme they were found to belong to Phylotpe II, sequevar 1 which contains only *R. solanacearum* biovar 2/race 3 pathogens of potato. When the strains were compared to other strains belonging to sequevar 1 using rep-PCR they produced a fingerprint the same as other strains within sequevar 1.

Table 3. List of strains with their PCR and biotype characteristics

Strain No	Origin	Host	Other No	Biovar[1]	Phylotype[2]	MLG	Biotype
UW20	Honduras	Banana		1	II/6	28	6
UW21	Honduras	Banana	R371, CIP21	1	II/6	28	6
UW181	Venezuela	Plantain	JT649, K261	1	II/6	28	6
DAR64836	Australia	*Musa* sp.		1	II/6	28	6
A3907	Hawaii	Heliconia		1	II/6	ND	6
A3909	Hawaii	Heliconia		1	II/6	ND	6
A3911	Hawaii	Heliconia		1	II/6	ND	6
UW 127	Peru	Plantain	CIP4	1	II/4	25	4
UW129	Peru	Plantain		1	II/4	25	4
UW131	Peru	Plantain		1	II/4	ND	4
UW160	Peru	Plantain	R282	1	II/4	25	4
UW162	Peru	Plantain	JT 648	1	II/4	25	4
UW163	Peru	Plantain		1	II/4	ND	4
CFBP1419	Costa Rica	*Musa* sp.	K163, JS847	1	II/4	ND	4
CIP20	Peru	Plantain	UW156, S249	1	II/4	25	4
UW70	Colombia	Plantain	CIP70	1	II/4	25	4
UW179	Colombia	Plantain	R368, CIP30	1	II/4	ND	4
UW175	Colombia	Plantain		1	II/4	25	4
CFBP2144	Colombia	Plantain	IW1509	1	II/4	ND	4
CFBP1412	Colombia	Plantain	NCPPB2314	1	II/4	ND	4
R368	Colombia	Plantain		1	II/4	ND	4
P515	USA	Pothos		1	II/4	ND	4
P548	USA	Pothos		1	II/4	ND	4
ANT307	FWI	Anthurium		1	II/4	ND	4
ANT11212	FWI	Anthurium		1	II/4	ND	4
UW170	Colombia	Heliconia	PD1453	1	II/4	ND	3
UW09	Costa Rica	Heliconia	JT644	1	II/3	24	3
UW11	Costa Rica	Heliconia		1	II/3	ND	3
UW135	Honduras	Banana	CIP7, S228	1	II/3	24	3
UW138	Costa Rica	Plantain		1	II/3	24	3
UW166	Costa Rica	Plantain	CIP27	1	II/3	ND	3
UW167	Costa Rica	Banana	R283, CIP125	1	II/3	24	3
CFBP1183	Costa Rica	Heliconia	JS793	1	II/3	ND	3
CFBP1409	Honduras	*Musa* sp	K135, JS77	1	II/3	ND	3
CFBP1482	Panama	*Musa* sp	K168, JS730	1	II/3	ND	3
CIP418	Indonesia	Peanut	MOH6	1	II/3	ND	3
CFBP2957	FWI	Tomato	MT5	1	II/5	ND	5
CFBP2958	FWI	Tomato	GT4	1	II/5	ND	5
CFBP3057	Burkina Faso	Tomato	JS912	1	II/5	ND	5
CIP301[D1]	Peru	Potato	R311	1	II/5	3	5

Strain No	Origin	Host	Other No	Biovar[1]	Phylotype[2]	MLG	Biotype
CIP239	Brazil	Potato	R306	1	II/5	38	5
CFBP3858	Netherlands	Potato	JS907	2	II/1	ND	1
CIP 117	Nigeria	Potato	UW453	2	II/1	34	1
CIP309	Colombia	Potato	UW80, S206	2	II/2	27	1
CIP10	Peru	Potato	UW477, R569	N2	II/ND	29	2
ICMP7969	Kenya	Potato		1	II/7	ND	7
K60	USA	Tomato	UW25	1	II/7	1	7
JT528	Reunion Is.	Potato		1	III/19	ND	10
CFBP3059	Burkina Faso	Eggplant	JCG.AU28	1	III/23	ND	10
J25	Kenya	Potato		N2	III/22	ND	9
GMI1000	F-Guyana	Tomato		3	I/12	ND	8
CFBP765	Japan	Tomato	JS771	4	I/ND	ND	8
R288	China	*Morus alba*	JT659	5	I/18	ND	8
PSI07	Indonesia	Tomato		2	IV/8	ND	11
ACH0732	Australia	Tomato	UW433	2	IV/7	ND	11
R24	Indonesia	Clove	*P. syzygii*	NA	IV/10	ND	NA

[1] Biovar and ecotypes of *Musa* sp strains of *R. solanacearum*; [2] Phylotype/Sequevar; known or not determinate (ND) RFLP-multi locus groups (3); 3. PCR primers (see Table 1) and products generated (+) or not (-); 4. Biotype NA, not applicable, see Table 2 for details of biotypes; UW; University of Wisconsin USA; R, Rothamsted Experimental Station UK; CFBP, Collection Française des Bactéries Phytopathogènes, France; CIP, International Potato Center, Peru; ACH, C. Hayward, University of Queensland, Australia

Conclusions, Predictions, and Speculations

The *R. solanacearum* species complex is composed of at least four genetic groups or phylotypes. Within these phylotypes there are subgroupings, sequevars, which correspond to clusters of isolates with similar pathogenicity or isolates of common geographic origin. By employing the phylotyping scheme outlined in this paper a view of the evolutionary relationships of the *R. solanacearum* species complex is gained. The additive nature of the scheme gives it great flexibility and allows addition of more genotypes as they are discovered. It is hoped that this scheme will be of use to plant breeders, plant pathologists and quarantine officials as it is able to distinguish epidemiological and ecological groupings of *R. solanacearum* strains and will thus help predict the biological properties of unknown strains. The vast majority of the *R. solanacearum* strains used in this study were collected from other strain collections and all were isolated from diseased crop plants. As more strains are isolated from environmental sources and other natural hosts of *R. solanacearum* it is expected that greater

genetic diversity will be uncovered. Although it is possible that this new genetic diversity may lead to the description of new phylotypes it is, in our opinion, unlikely. However, it is almost certain that as more strains are sequenced more sequevars will be described.

Collecting and cataloguing strains of *R. solanacearum*, although very important, are relatively simple endeavours. However, it is more difficult to gather information on the biological, ecological and epidemiological properties of strains. Without both pieces of the puzzle it is impossible to use any taxonomic scheme to predict pathogenicity of strains or to aid in control of the disease. We suggest that research into the biological and ecological properties of strains should have high priority. It is our hope that the taxonomic framework based upon the evolutionary history of *R. solanacearum* outlined in this paper will be used to better predict the properties of strains and aid in the successful control of the many bacterial wilts caused by *R. solanacearum*.

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Appendix

MULTIPLEX PCR METHOD

Reactions were carried out in a total volume of 25 µl containing 1 x PCR buffer (supplied by the manufacturer of the polymerase) 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2U of *Taq* Polymerase (Biotech International, Perth WA, Australia), 6 pmoles of the primers Nmult:21:1F, Nmult:21:2F, Nmult:22:InF, 18 pmoles of the primer Nmult:23:AF and 4 pmoles of the primers 759 and 760 (14). Reactions were heated to 96°C for 5 min and then cycled through 30 cycles of 94°C for 15s, 59°C for 30s and 72°C for 30s, followed by a final extension period of 10 min at 72°C. Samples (5 µl) of reaction mixtures were examined by electrophoresis through 2% agarose gels and bands were revealed by staining in 0.5 µg mL-1 ethidium bromide.