Running head: R. solanacearum *population structure*

Phylogenetic relationships and population structure of *Ralstonia solanacearum* isolated from diverse origins in Taiwan

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Bacterial wilt caused by *Ralstonia solanacearum* is a destructive disease for many crops. The aim of this study was to investigate the phylogenetic relationships and genetic structure of a *R. solanacearum* population from diverse origins in Taiwan. All 58 tested isolates belonged to phylotype I, except the two biovar 2 isolates from potato. These belonged to phylotype IIB sequevar 1 and were identical to the known potato brown rot strains, which were probably introduced. Phylotype I isolates were grouped into 10 sequevars. Sequevar 15 was predominant (34 out of 56 isolates). Its distribution covered the whole island and it was largely associated with solanaceous crops such as tomato, and with tomato field soil. A total of 14 haplotypes were identified based on a partial endoglucanase gene sequence. Parsimony network analysis revealed that haplotype A was the oldest haplotype in the local population. It encompassed all but one of the sequevar 15 isolates. Large variation in virulence on tomato was observed among the 58 isolates, and seven pathotypes were identified. Significant genetic differentiation was detected among pathotypes. Moreover, genetic differentiation was detected between biovar 3 and biovar 4 subgroups and between the strains associated with solanaceous and non-solanaceous species, but none was detected between strains from different geographic origins. The results suggest that the phylotype I population in Taiwan is homogeneous, while mutation and local adaptation to specific ecological niches keep shaping the population.

Keywords: biovar, haplotype, pathotype, phylotype, race, *Ralstonia solanacearum*

Introduction

Bacterial wilt is caused by the soilborne plant pathogenic bacterium *Ralstonia solanacearum*. It is a destructive disease and a major production constraint for many economically important crops, including tomato, potato, aubergine, tobacco, pepper, peanut, banana and ginger in tropical, subtropical and warm temperate regions of the world. The pathogen can infect more than 200 species and 50 families of plants. New hosts are still being reported (Denny, 2006). The pathogen has been recorded on 28 plant species from 19 families in Taiwan, where tomato is the most important crop in economic terms (Lin, 2008). During the summer or hot-wet seasons, disease incidence values of 15 to 55% have been reported on fresh market tomato cultivars, causing annual losses of more than $12 million (Hartman *et al*., 1991). The pathogen can survive in the soil for long periods in the absence of host plants in sheltered sites such as plant debris and latently infected plant tissues, deeper soil layers, and the weed rhizosphere (Denny, 2006). For long-distance transmission, the pathogen can be carried via contaminated water and infected planting materials (Caruso *et al*., 2005; Denny, 2006; Lin *et al*., 2009).

Traditionally, *R. solanacearum* strains are separated into five races and six biovars on the basis of host range and carbon source utilization, respectively (Denny, 2006). The *R. solanacearum* species complex can be divided into four phylotypes consistent with four genetic groups based on the sequence analysis of the inter-transcribed spacer (ITS) region between the 16S and 23S ribosomal RNA genes, the *hrpB* gene (a conserved pathogenicity factor), and the endoglucanase gene (*egl*, a conserved virulence factor; Fegan & Prior, 2005). These phylotypes correlate with the geographical origin of the strains: phylotype I includes strains originating primarily from Asia, phylotype II from America, phylotype III from Africa and surrounding islands in the Indian Ocean, and phylotype IV from Indonesia (Prior &

Fegan, 2005). In Taiwan, *R. solanacearum* strains associated with different reported hosts belong to race 1 and biovars 3 or 4 (Hsu, 1991; Lin, 2008). A large variation in the virulence of race 1 Taiwanese strains on different plant species or tomato has been demonstrated (Jaunet & Wang, 1999). Race 3 biovar 2 strains isolated from potato were only reported in 2002 (Chiou, 2002). This group of strains is thought to have been introduced after 1999. It later spread to other potato-growing regions and has remained in Taiwan (Wu *et al*., 2011).

Genetic variation in plant pathogen populations can be shaped by biotic and abiotic factors such as host diversity, soil type and cropping practice (Gilbert, 2002). Moreover, the risk of emerging disease or repeated pandemics increases with anthropic transportation, such as seedling transport, which facilitates pathogen migration via infected plants or plant parts. Recurrent gene flow between diverse origins or populations across geographical barriers is thought to result in a homogenous genetic structure within a country. Thus, understanding how continental or regional genetic diversity is partitioned into distinct genetic structures helps to determine the biotic or abiotic factors that affect the evolution of pathogen populations.

Genetic diversity of *R. solanacearum* has been investigated mainly by genotyping fingerprints produced by repetitive element palindromic polymerase chain reaction (rep-PCR) with multiple primers (e.g. ERIC, REP and BOX). On the basis of shared rep-PCR patterns among *R. solanacearum* strains, Xue *et al*. (2011) concluded that in China, site- or host plant-dependent factors do affect the distribution of genotypic diversity and determine the pathogen's clonal distribution, as nearly half of the normalized BOX clusters were found within a single province. In addition, in the Philippines, all *R. solanacearum* strains isolated from aubergine grown in different provinces displayed the same BOX patterns as those originating from other countries; this indicates a successful clonal spread across aubergine fields (Ivey *et al*., 2007). Studies on the genetic diversity of the pathogen populations in Taiwan, Japan and India failed to reveal any relationship between genotypic variation and geographic origin, or between pathogen virulence and host origin (Jaunet & Wang, 1999; Horita & Tsuchiya, 2001; Kumar *et al*., 2004). However, these studies did not examine microevolution dynamics to evidence selection as a force that shapes local pathogen populations. Ramsubhag *et al*. (2012) used rep-PCR data to examine the population structure of *R. solanacearum* strains affecting tomato in Trinidad, and found that the strains from the central zone stood significantly apart from the strains from other three zones. The application of nucleotide variation of selected genes, together with advanced phylogenetic algorithms, would be useful to determine the unresolved evolutionary relationship between *R. solanacearum* populations in order to identify ecological clusters with similar genetic characteristics. This kind of information would make it possible to reconstruct endemic pathogen history, which would in turn be helpful for the development of holistic disease management strategies.

The objective of this study was to understand the phylogenetic relationships between *R. solanacearum* strains isolated from diverse origins around Taiwan. The presence of differentiation was examined according to biovar, host origin, geographic origin and virulence variation on tomato on the basis of the *egl* gene sequence. The evolutionary process of the local population was also examined.

Materials and methods

Bacterial isolates and biovar test

A total of 58 *R. solanacearum* isolates from various sources in Taiwan between 1987 and 2003 was selected as a representative population (Table 1). These were isolated from four geographical zones in Taiwan and from 22 plant species. Four isolates were obtained from farm soils collected from tomato fields with a historical record of bacterial wilt. CLw1488 was the only isolate obtained from irrigation water in an endemic disease area in Hsinchu. All isolates were preserved in 30% glycerol at –80°C for further use. They were classified into biovars, based on their ability to oxidize three disaccharides (lactose, maltose and cellobiose) and three hexose alcohols (mannitol, sorbitol and dulcitol), as previously described (Hayward, 1964).

Virulence assay

All tested isolates were inoculated individually on three tomato varieties, L390, L180-1 and Hawaii 7996, a susceptible, a moderately resistant and a resistant genotype, respectively. Virulence was assessed following the soil drenching method described by Hanson *et al.* (1996) in the greenhouse under natural light with a mean temperature of 27–29°C. Tomato seedlings with four to six true leaves in individual pots were inoculated by pouring 20 mL of inoculum ($OD_{600} = 0{\cdot}3$; at 10^8 colony-forming units, CFU, mL^{-1}) around the stem base. The experiment was conducted using a split-plot design with two replications and six plants per replication per variety. Isolate was used as the main factor and variety as the subfactor. The percentages of wilted plants were recorded once a week for up to 28 days after inoculation. The final percentage of wilted plants for each isolate on the three tomato varieties was analysed by principal component analysis using the PRINCOMP procedure of SAS (SAS Institute). Clusters of isolates with similar virulence were determined using the average linkage method and the three clustering criteria (cubic clustering criterion, pseudo-F and pseudo-t^2) using the SAS CLUSTER procedure.

DNA assay and phylotype identification

Genomic DNA from each isolate was extracted following the method described by Chen & Kuo (1993). We used the genomic DNA of reference strains representing each phylotype, which is kept at the CIRAD, France.

The phylotype identity of each isolate was determined following a multiplex PCR protocol reported by Fegan & Prior (2005). Four forward primers, Nmult21:1F, Nmult21:2F, Nmult23:AF, Nmult22:InF and one reverse primer, Nmult22:RR and a species-specific primer pair, AU759f and AU760r, were used for multiplex PCR amplification.

egl gene sequencing and genetic analysis

An 850-bp fragment of the *egl* gene was amplified from each isolate using the primer pair Endo-F (5′-ATGCATGCCGCTGGTCGCCGC-3′) and Endo-R (5′-GCGTTGCCCGGCACGAACACC-3′) (Fegan *et al*., 1998). PCR products of the *egl* gene were purified using a purification kit (Gel Advanced Gel Extraction Miniprep System, Viogene-Biotek Corporation), then sequenced (Genomics BioSci & Tech Co., Taiwan). The sequences were aligned and manually adjusted with BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Finally, a 734-bp fragment of the *egl* consensus region of 58 *R. solanacearum* isolates was obtained for sequevar determination (Fegan & Prior, 2005) and the subsequent genetic analysis. All nucleotide sequences were deposited in GenBank under accession numbers EU407264–EU407304 and EU407315–EU407331.

Phylogenetic trees were constructed by neighbour joining (NJ) and maximum likelihood (ML) methods embedded in MEGA v. 5.0 (Tamura *et al*., 2011) and PHYML v. 3.0 (Guidon & Gascuel, 2003), respectively. Bootstrapping was performed with 100 replicates for ML and 1000 replicates for NJ. DNASP v. 5.0 (Librado & Rozas, 2009) was used to estimate genetic diversity, i.e. nucleotide diversity (π, the average number of nucleotide differences per site between sequences), number of haplotypes (h, a set of alleles inherited by an individual from a single parent), and haplotype diversity (Hd, the number and frequency of different haplotypes in the sample). Pairwise genetic distance among populations and the population differentiation index (F_{ST}, the proportion of genetic diversity due to allele frequency differences among populations) were calculated using ARLEQUIN (Excoffier *et al*., 2005). The hierarchical structural analysis of genetic diversity (AMOVA: analysis of molecular variance) among *R. solanacearum* isolates at levels corresponding to virulence and geographic origins, respectively, was implemented in ARLEQUIN and was also used to hierarchically assess the relative distribution of genetic variation. The estimated values from

ARLEQUIN were tested for 95% statistical significance by running 1000 permutation steps. To identify the evolutionary relationship among the 58 *R. solanacearum* isolates, a haplotype network was built with TCS v. 1.2 software (Clement *et al*., 2000) using statistical parsimony with a 95% confidence interval (Templeton *et al*., 1992).

Results

Biovar, phylotype and pathotype of the Taiwanese population

The biovar identity of 58 *R. solanacearum* isolates was determined according to their ability to utilize three sugars and three sugar alcohols. There were 2, 22 and 34 isolates of biovar 2, 3 and 4, respectively (Table 1). Using the multiplex PCR protocol with primers designed from the ITS region, the 144-bp fragment specific to phylotype I was observed with all tested isolates except the two *R. solanacearum* isolated from potato, Pss525 and Pss526. The phylotype II-specific 372-bp fragment was observed only for Pss525 and Pss526. These two isolates were identified as biovar 2 and race 3 (Chiou, 2002).

Considerable variation in virulence was observed among the 58 tested isolates in terms of the disease incidence that occurred on three tomato varieties. The mean final percentage of wilted plants ranged from 0 to 83·4% on Hawaii 7996, 0 to 100% on L180-1 and 8·4 to 100% on L390. Cluster analysis revealed seven pathotypes among the 58 isolates (Fig. 1). The first two principal components accounted for 88·7% of the standardized variance, with 64·6 and 24·1% for the first and second components, respectively. Pathotype 1 contained isolates Pss158, Pss190, Pss525 and Pss526 and was highly virulent on all three varieties with an average final wilting of 92·4 ± 4·2%. Pathotypes 2, 3, 4, 5 and 7 demonstrated similar interactions with the tested tomato varieties. However, they displayed decreasing overall virulence. Pathotype 6 consisted of only one isolate, Pss169, obtained from custard apple in the eastern region of Taiwan. It showed a unique pattern when interacting with tomato. While the average wilting percentage was 27·8%, this isolate had low virulence on L390 and L180-1 (25·0% final wilting), but relatively high virulence on Hawaii 7996 (33·4% final wilting).

Phylogenetic relationships and sequevar of the Taiwanese *R. solanacearum* population

Partial nucleotide sequences of the *egl* gene from 58 Taiwanese *R. solanacearum* isolates were determined and aligned. The phylogenetic relationships according to *egl* gene sequences were analysed together with 10 reference strains. The two phylogenetic methods (NJ and ML) yielded similar results; only the NJ tree is displayed for discussion. The NJ tree showed a distinct phylogenetic relationship with high bootstrap values among four phylotypes. It even distinguished phylotype IIA from IIB (Fig. 2). Pss525 and Pss526 from potato belonged to phylotype IIB. Within phylotype I, although two major phylogenetic clusters were obtained, the short branch length indicates low genetic diversity among the isolates. Clear correlation was not readily observed between phylotype I clusters and geographical origin, host species or pathotypes.

A total of 10 sequevars was determined within phylotype I. Sequevar 15 was the most frequent (34 out of 56 isolates), and was found mostly on tomato and soil samples from tomato fields. The other sequevars were detected mainly on non-solanaceous hosts, such as radish (*Raphanus sativus*), bitter gourd (*Momordica charantia*) and comfrey (*Symphytum officinale*). Interestingly, nearly all sequevars were identified from more than two host species and were randomly distributed across geographic zones (Fig. 3). The two phylotype II isolates found in Taichung belonged to sequevar 1, together with the reference strain for potato brown rot. A total of 14 haplotypes were defined. Haplotype A, corresponding to sequevar 15 was predominant, apart from Pss190 (haplotype B). Overall, the sequevar and haplotype grouping results were consistent with each other. However, haplotype grouping is more discriminating than sequevar grouping.

The short phylogenetic relationships within phylotype I provided limited evolutionary information on the Taiwanese population. To determine the evolutionary relationships among the 56 *R. solanacearum* isolates, a haplotype network characterised by statistical parsimony analysis was constructed based on *egl* gene sequences. The result of the statistical network yielded a similar topology to the consensus phylogenetic tree (Fig. 4). The 13 haplotypes were connected and split into 3 haplotype groups: (1) group 1 contained the putative ancestral haplotype A, and included 33 *R. solanacearum* isolates mainly from tomato (21 out of 33 isolates), and haplotype B; (2) group 2 consisted of haplotypes H, I, J, K, L and M that all belong to biovar 3, except Pss71 and Pss166 of haplotypes H and J, respectively; (3) group 3 consisted of haplotypes C, D, E, F and G, which contained biovars 3 and 4 isolated from diverse hosts. Moreover, when the spectrum of mutation steps on the network was examined, five transition sites were found that were shared between groups 2 and 3, and one transversion site with reversal mutation (C413G) was located within group 2.

Genetic structure of Taiwanese phylotype I strains

The presence of genetic differentiation among the Taiwanese phylotype I isolates was examined according to pathotype, biovar, host origin and geographic zone. The seven pathotypes formed three groups based on the virulence reactions on the susceptible tomato variety L390. AMOVA results (Table 2) showed that most of nucleotide variation appeared within groups (80·2%) compared to variation between groups (24·8%). Significant genetic differentiation between groups ($\Phi c_T = 0{\cdot}248$, $P < 0{\cdot}05$) was detected, and indicated a correspondence between virulence phenotype and genetic variation. Thus, sequence variation within the *egl* gene correlates with the variation in virulence.

Pairwise comparison of genetic distances and differentiation between pathotypes based on inherent nucleotide variation provided an alternative method for the quantitative characterization of the relationships between pathotypes. Pathotype 6 was excluded from the analysis due to its small sample size ($n = 1$). Genetic distances across the six pathotypes ranged from 0·003 to 0·007 (Table 3). Pathotype 7 displayed more genetic differences than the other pathotypes. Significant genetic differentiation was detected between pathotype 7 and the other pathotypes, except pathotypes 1 and 5.

Mean nucleotide diversity (π) and haplotype diversity (Hd) values of the 55 phylotype I isolates were 0·004 and 0·629, respectively (Table 4). Subgroups of biovars 3 and 4 consisted of eight haplotypes. However, biovar 3 harboured higher nucleotide diversity and haplotype diversity than biovar 4. *Ralstonia solanacearum* isolates obtained from non-solanaceous species ($n = 19$) displayed higher nucleotide diversity, haplotype numbers and haplotype diversity than those from solanaceous species ($n = 36$). Based on an estimation of the population differentiation index of biovar and host origins, significantly large F_{ST} values were obtained for both parameters (biovar = 0·219, $P < 0{\cdot}001$; host origin = 0·242, $P <$

0·001), indicating significant genetic differentiation among biovar or host populations. Four geographical zones, as well as the Central Mountain Range (the major dispersal barrier between the eastern and western regions), were assessed to determine whether genetic variation distribution correlated with geographic origins. The AMOVA showed that most genetic variation occurred within geographic zones (104·2%), not within or between regions (Table 5). In addition, no significant genetic differentiation was detected among any hierarchical level. The results confirm that *R. solanacearum* populations in Taiwan basically consist of two separate genetic clusters in relation to phenotypic characteristics (biovar and host preference), rather than geographic origin.

Discussion

Ralstonia solanacearum is a species complex displaying large phenotypic and genotypic variation. The pathogen was first reported in Taiwan on tobacco in 1942 (Tsai, 1991). Its host range currently includes 28 plant species belonging to 19 families. It is present in all geographic zones with different climatic environments. In this study, a population of *R. solanacearum* isolates obtained from different host plants or sources and geographical origins in Taiwan was characterized for its biovars, virulence and phylogenic origins. Sequence variation of the *egl* gene was used to infer the phylogenetic relationships and genetic structure of the Taiwanese population.

The 58 *R. solanacearum* isolates used in this study belonged to biovars 2, 3 and 4, as well as phylotypes I and II. The two biovar 2 isolates were obtained from potato in central Taiwan in 1999, where outbreaks of potato brown rot occurred. Studies indicated that the pathogen that caused the outbreak was biovar 2 race 3 of *R. solanacearum*, and it had probably been introduced (Chiou, 2002). These biovar 2 race 3 isolates were grouped under sequevar 1, phylotype IIB, together with the typical potato brown rot strains. They formed the unique haplotype N. This confirms the different phylogenic origins of phylotypes I and II (Fegan & Prior, 2005).

Haplotype network analysis based on *egl* partial sequence revealed the history of allelic changes of *egl* gene among phylotype I isolates in Taiwan. Isolates of haplotype A (sequevar 15) were predominant and were mostly obtained from tomato plants present around Taiwan. Furthermore, no significant genetic differentiation was detected among geographic zones. Trading of tomato seedlings might facilitate the movement of the soilborne pathogen over geographical barriers to homogenise genetic makeup across the island. Haplotype A might have spread endemically to become the reservoir for bacterial wilt epidemics on tomato and other species. Phylotype I strains of tomato in Taiwan are known to be highly variable in genetic fingerprints (Jaunet & Wang, 1999). Although frequent recombination could take place within a geographically isolated bacterial population (Smith & Smith, 2000), in this study the non-reticulated topology of the haplotype network suggested the lack of horizontal gene transfer at an intraspecies level. However, this should be confirmed with multilocus sequence typing owing to its higher discrimination on the clonal-like bacterial pathogen structure (Urwin & Maiden, 2003).

Phylotype I isolates have a wide host range. In Taiwan, hosts range from annual herbaceous plants to perennial woody plants. In this study, the haplotypes branching out from haplotype A displayed greater sequevar and host origin diversity. This suggests that host origin could be the main factor affecting the genetic dynamics of *R. solanacearum* populations in the agroecosystem. The detection of significant genetic differentiation between isolates originating from solanaceous and non-solanaceous groups further supports the hypothesis. Furthermore, phylotype I isolate Pss190 differentially requires virulence genes to colonize tomato or *Arabidopsis* (Lin *et al*., 2008). From an ecological perspective, the ability to infect and colonize diverse plant species could ensure the survival of *R. solanacearum*. Thus, the pathogen population would be under selection pressure when encountering a new plant species.

The virulence of the 58 isolates was examined on tomato. Large, yet continuous variation in virulence was observed. Significant genetic differentiation was detected between groups of isolates that exhibited different degrees of virulence (Table 2). Moreover, pathotype 7, the population's least virulent subgroup, was genetically distinct from the pathotypes with intermediate virulence (Table 3). The results imply that virulence could be associated with the pathogen's evolution. The gene-for-gene model of host–pathogen co-evolution is a well-recognized concept (Brown & Tellier, 2011). Resistance loci against *R. solanacearum* have been identified in tomato (Wang *et al*., 2013). However, the significance of tomato genotypes acting as a selection force remains to be demonstrated. Earlier studies show that Pss190, a highly virulent strain (pathotype 1) on tomato, is not better than the less virulent strains at colonizing the weed species *Solanum nigrum*, *Erechtites valerianifolius*, and *Cyperus rotundus* (Wang & Lin, 2005). Whether the ability to colonize a susceptible host such as tomato in order to rapidly increase population size or to colonize diverse weeds for better survival has a broader effect on the evolution of *R. solanacearum* remains to be determined.

The findings show that genetic diversity as mirrored by the endoglucanase gene was higher within biovar 3 isolates than biovar 4 isolates. Previous reports stated that biovar 3 isolates, rather than biovar 4, were predominant in Taiwan (Hsu, 1991; Jaunet & Wang, 1999). The higher proportion of biovar 4 isolates in the population studied may be explained due to sampling artefacts that covered a broad host range. According to Lin *et al*. (1999), most weed species can be latently infected by biovar 3 rather than biovar 4 isolates. This is consistent with the idea that biovar 3 has long been established, resulting in a larger effective population size in the field. If we consider the impact of the genetic drift of alleles, in which generation and loss of mutation randomly occur among populations, the chance of allele fixation increases with greater population size. Thus, greater genetic variation is preserved than in a smaller population (Excoffier *et al*., 2009). Therefore, the higher genetic diversity of biovar 3 might correlate with their larger effective population in the field.

Wicker *et al*. (2012) suggest that features of free recombination, broad host range, patterns of dissemination and plastic virulence endow phylotype I with a higher evolutionary potential to spread quickly over long distances. This study reveals for the first time that the formation of *R. solanacearum* phylotype I genetic structure is driven by a large effective population size and host origin. In the light of these results, it is suggested that suppressing the pathogen field population would be a key component in the sustainable management of bacterial wilt. Weed management, removal of plant debris or regular rotation with non-host crops can achieve this. At the same time, these crop management practices would maintain the durability of resistant cultivars. In the future, correlating genetic information with more ecological parameters such as cropping practices, soil type and weed diversity, will further develop understanding of *R. solanacearum* microevolution.

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Figure legends

Figure 1 Principal component and cluster analysis of the final percentages of wilted plants on three tomato varieties caused by 58 *Ralstonia solanacearum* isolates collected from different sources and locations in Taiwan. Each point represents one isolate plotted in a plane defined by the first two components (C1 × C2). Each pathotype is characterized by a histogram of the final percentage of wilted plants (W%) on three varieties. Histograms were constructed from the average final percentage of wilted plants from all isolates within pathotypes. The number of isolates of each pathotype is indicated in brackets.

Figure 2 Neighbour-joining phylogenetic tree of 58 *R. solanacearum* isolates of a Taiwanese population plus 10 reference strains obtained from four distinct phylotypes. Bootstrap values above the branch were obtained from neighbour-joining (left) and maximum likelihood (right), respectively.

Figure 3 Map of Taiwan showing the distribution of sequevars of phylotype I strains. *y*-axis of the bar chart indicates the numbers of isolates, and *x*-axis indicates specific phylotype I sequevars.

Figure 4 Parsimony network of 58 *R. solanacearum* isolates of a Taiwanese population constructed using TCS v. 1.21 software. Each haplotype is depicted with a different name inside rectangles and circles that represent the putative ancestral haplotypes and the derived haplotypes, respectively. Inferred intermediate haplotypes are shown with small empty circles connected by a line to the nucleotide variation at 95% statistical significance. Mutation steps are represented by a line with the indication of the mutation from the immediate ancestral haplotype. The code along the line indicates the nucleic acid change at the specific position of the *egl* gene. The size of each haplotype does not correspond to haplotype frequency.

Table 1 Number of *Ralstonia solanacearum* isolates used in this study under each grouping category (shown in parentheses)

Geographic zone[a]	Source	Biovar	Pathotype	Phylotype/ sequevar	Haplotype[b]
N (7)	Tomato (28)	2 (2)	1 (4)	I/13 (5)	A (33)
S (9)	Other solanaceaous species (6)	3 (22)	2 (6)	I/14 (6)	B (1)
C (33)	Non-solanaceaous species (19)	4 (34)	3 (11)	I/15 (34)	C (1)
E (9)	Field soil (4)		4 (20)	I/17 (1)	D (2)
	Irrigation water (1)		5 (6)	I/18 (2)	E (1)
			6 (1)	I/30 (1)	F (5)
			7 (10)	I/32 (1)	G (1)
				I/34 (4)	H (4)
				I/44 (1)	I (4)
				I/45 (1)	J (1)
				II/1 (2)	K (1)
					L (1)
					M (1)
					N (2)

[a] The geographical zones of the isolates were grouped into four regions: northern (N), central (C), southern (S) and eastern (E) regions.

[b] Haplotype determined by partial *egl* sequencing.

Table 2 Analysis of molecular variance (AMOVA) between *Ralstonia solanacearum* phylotype I isolates based on virulence phenotype on tomato L390[a]

Source of variation	% of variation	Φ statistic	*P* value[b]
Between groups	24·8	0·248 (Φ_{CT})	0·013
Between clusters within groups	–5·0	–0·066 (Φ_{SC})	0·894
Within cluster [a]	80·2	0·199 (Φ_{ST})	0·022

[a] The seven pathotypes defined in Table 2 were grouped into three groups according to the virulence reactions on L390: pathotypes 1–4, pathotype 5, and pathotypes 6–7.

[b] The hierarchal statistical significance among different Φ values was calculated using probabilities derived from 1000 permutations.

Table 3 Pairwise genetic distance (above diagonal) and genetic differentiation (below diagonal) among *Ralstonia solanacearum* pathotypes (P) based on the *egl* nucleotide sequences

	P1	P2	P3	P4	P5	P7
P1	–	0·003	0·003	0·003	0·005	0·007
P2	0·036	–	0·003	0·003	0·004	0·006
P3	0·013	–0·116	–	0·003	0·005	0·006
P4	0·028	–0·041	–0·036	–	0·004	0·006
P5	0·070	0·073	0·013	–0·007	–	0·006
P7	0·252	0·279**	0·226**	0·202*	0·017	–

Pathotype 6 was not included for the analysis for the presence of only one isolate, as well as phylotype II isolates Pss525 and Pss526.

*$P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$ indicate various degrees of significance of F_{ST} value based on 1000 permutation tests.

Table 4 Descriptive genetic analysis of nucleotide diversity (π), haplotype number (h) and haplotype diversity (Hd) according to the biovar type and host origin of *Ralstonia solanacearum* phylotype I isolates

	n[a]	π	h	Hd	F_{ST}
Biovar 3	21	0·006	8	0·843	0·219***
Biovar 4	34	0·003	8	0·419	
Solanaceous origin[b]	36	0·003	6	0·432	0·242***
Non-solanaceous origin[b]	19	0·006	19	0·871	
Total/mean	55	0·004	12	0·629	

*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ indicate various degrees of significance of F_{ST} value based on 1000 permutation tests.

[a] Both Pss525 and Pss526 were excluded from the analysis due to incongruent biovar type (biovar 2). CLw1448 isolated from irrigation water was also excluded.

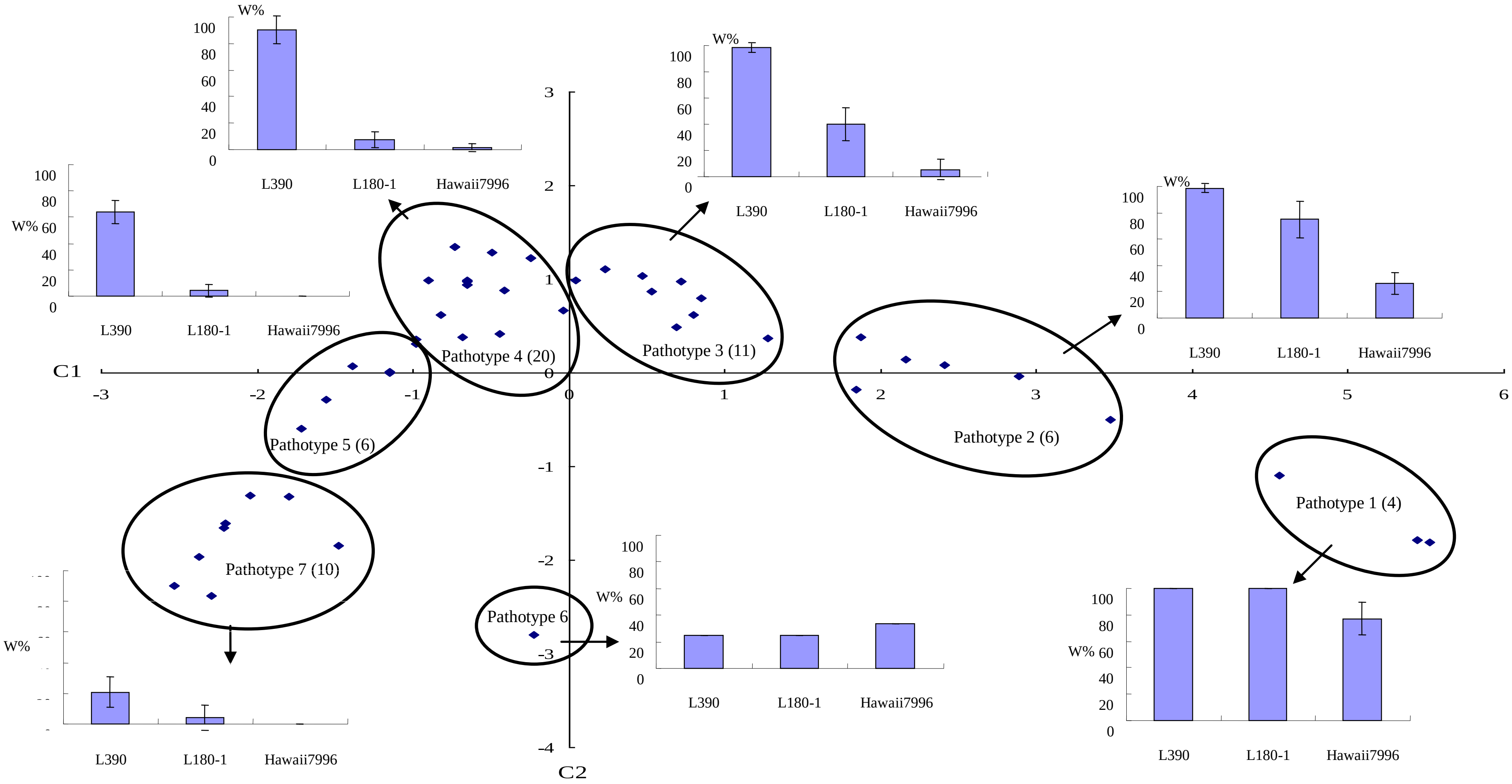
[b] Isolates from species belonging to solanaceous crops or farm soils collected from tomato fields were grouped as solanaceous origin; the other isolates were grouped into non-solanaceous origin.

Table 5 Analysis of molecular variance (AMOVA) of *Ralstonia solanacearum* phylotype I isolates from four geographic zones

Source of variation	Percentage of variation	Φ statistic	*P* value[a]
Between regions[b]	–3·1	–0·031 (Φ_{CT})	0·756
Between geographic zones within regions	–1·1	–0·011 (Φ_{SC})	0·510
Within geographic zones	104·2	–0·042 (Φ_{ST})	0·674

[a] The hierarchical statistical significance among different Φ values was calculated using probabilities derived from 1000 permutations.

[b] According to the orientation of Taiwan's Central mountain range, four geographical zones can be further grouped into the western region (North, Central and South), and the eastern region (East).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40
20
0
L390
L180-1
Hawaii7996
Pathotype 4 (20)
Pathotype 3 (11)
Pathotype 5 (6)
Pathotype 2 (6)
Pathotype 1 (4)
Pathotype 7 (10)
Pathotype 6
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C2
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-2
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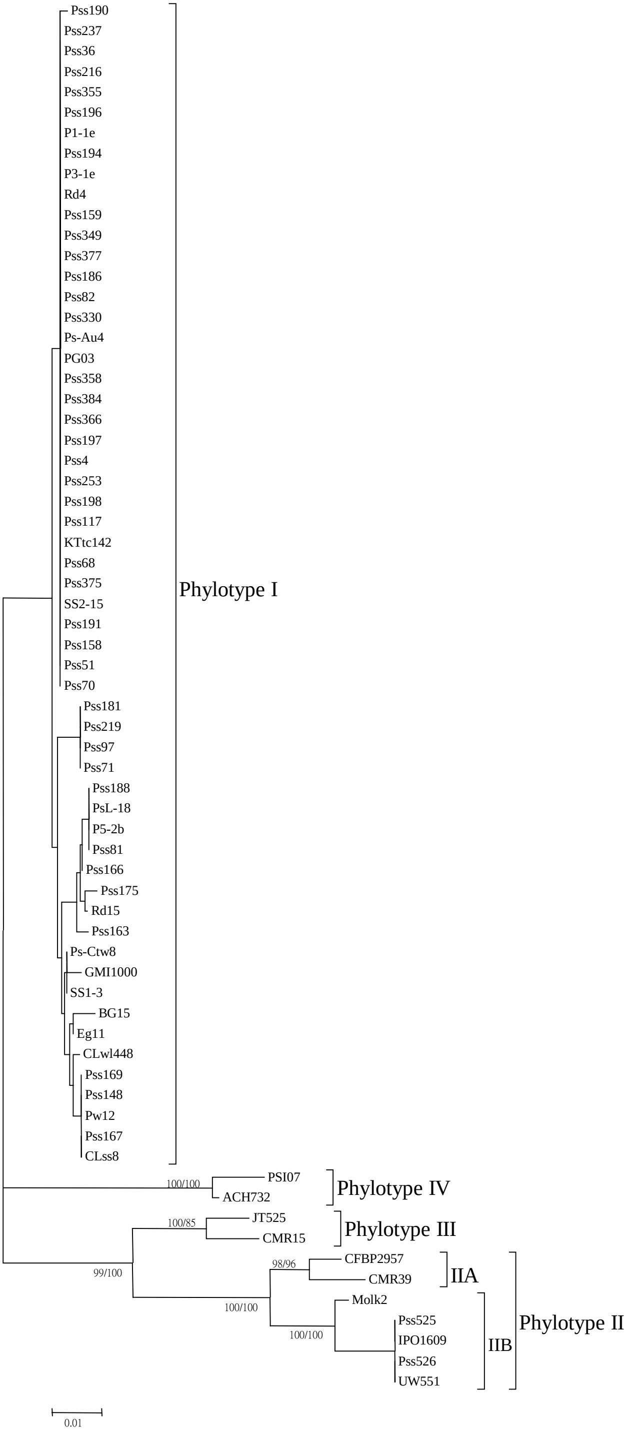
1
2
3
4
5
6
-4

1

2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Pss377
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Pss82
Pss330
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PG03
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Pss384
Pss366
Pss197
Pss4
Pss253
Pss198
Pss117
KTtc142
Pss68
Pss375
SS2-15
Pss191
Pss158
Pss51
Pss70
Pss181
Pss219
Pss97
Pss71
Pss188
PsL-18
P5-2b
Pss81
Pss166
Pss175
Rd15
Pss163
Ps-Ctw8
GMI1000
SS1-3
BG15
Eg11
CLwl448
Pss169
Pss148
Pw12
Pss167
CLss8
Phylotype I
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PSI07
ACH732
Phylotype IV
100/85
JT525
CMR15
Phylotype III
99/100
98/96
CFBP2957
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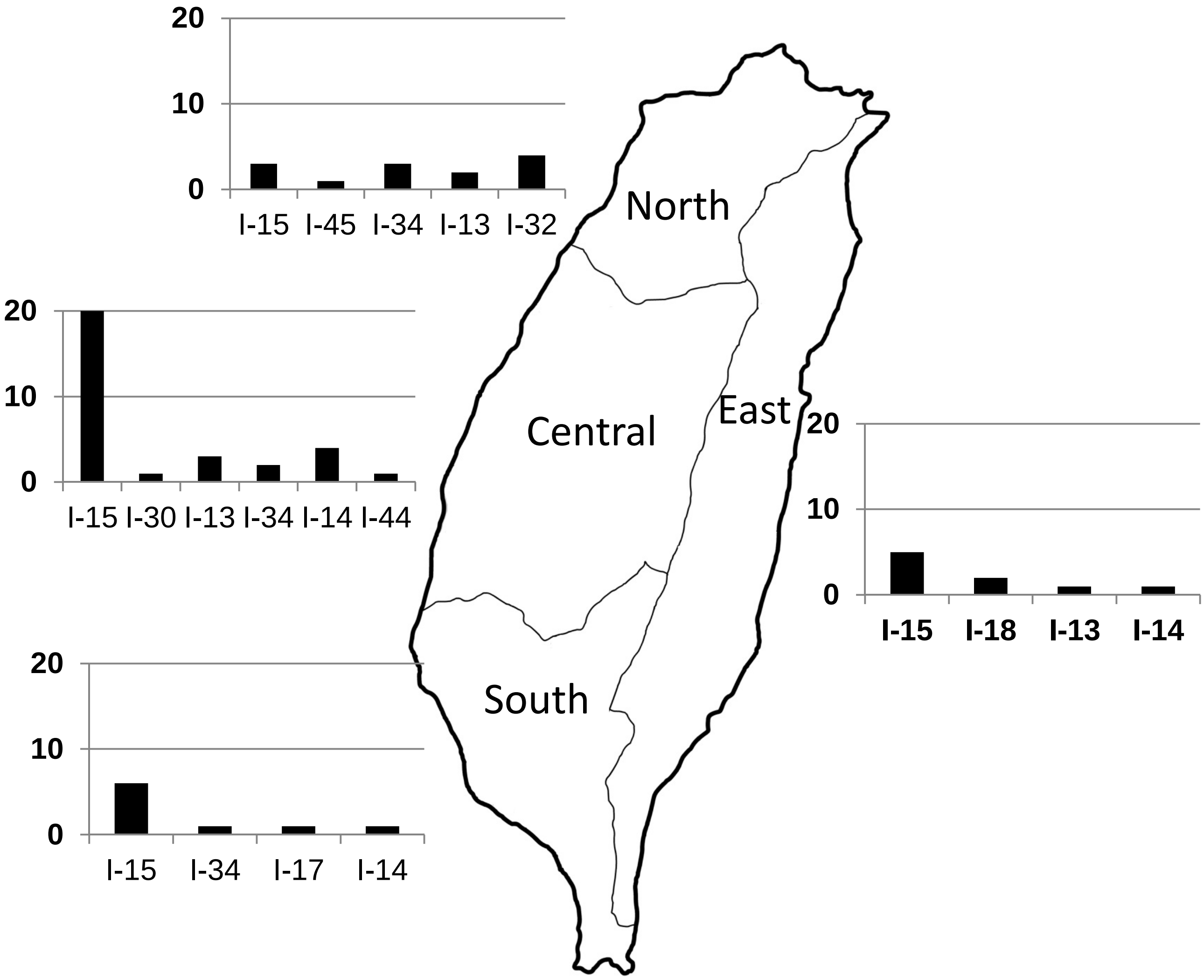
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Central
East
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20
10
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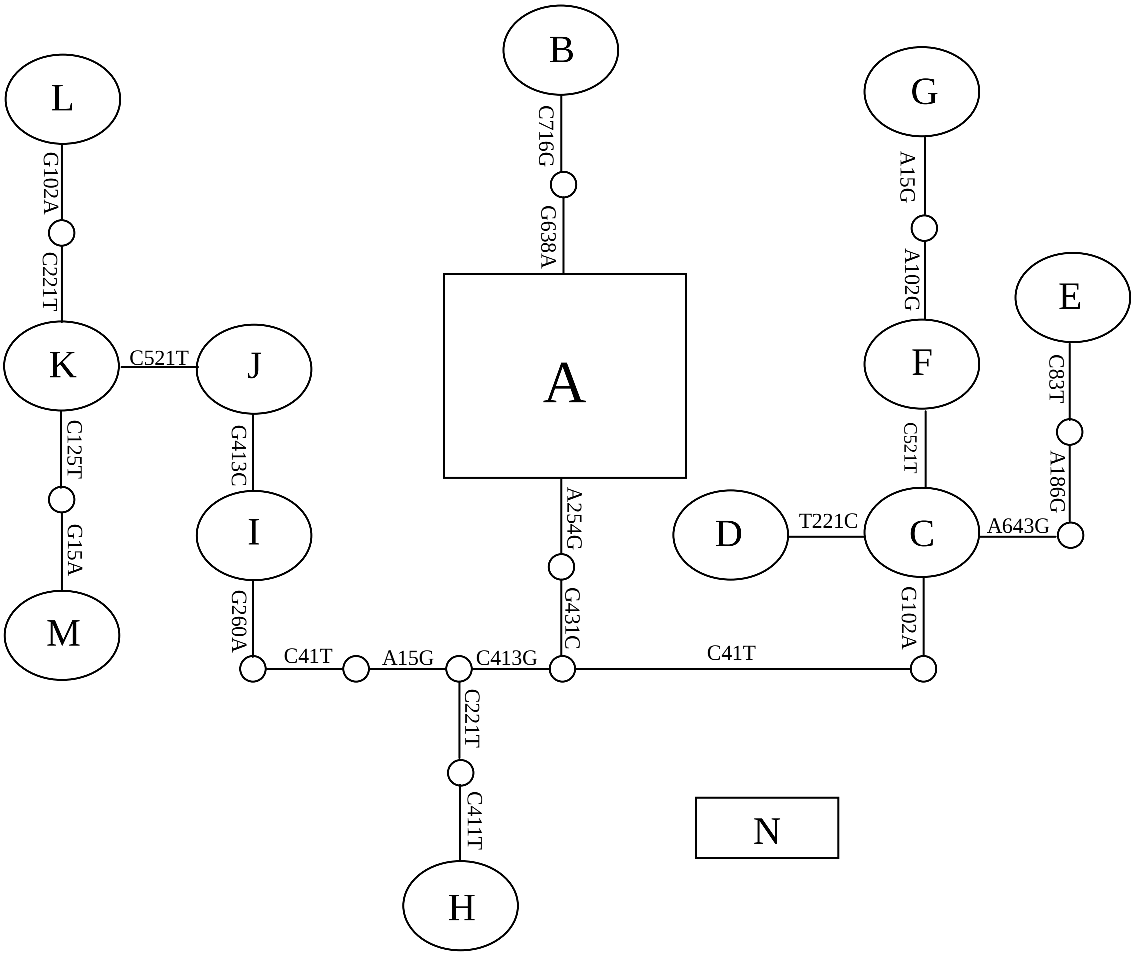
South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F
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E
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D
T221C
C
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C41T
N

1