A MLSA-MLST scheme to investigate the evolutionary dynamics within the *Ralstonia solanacearum* species complex

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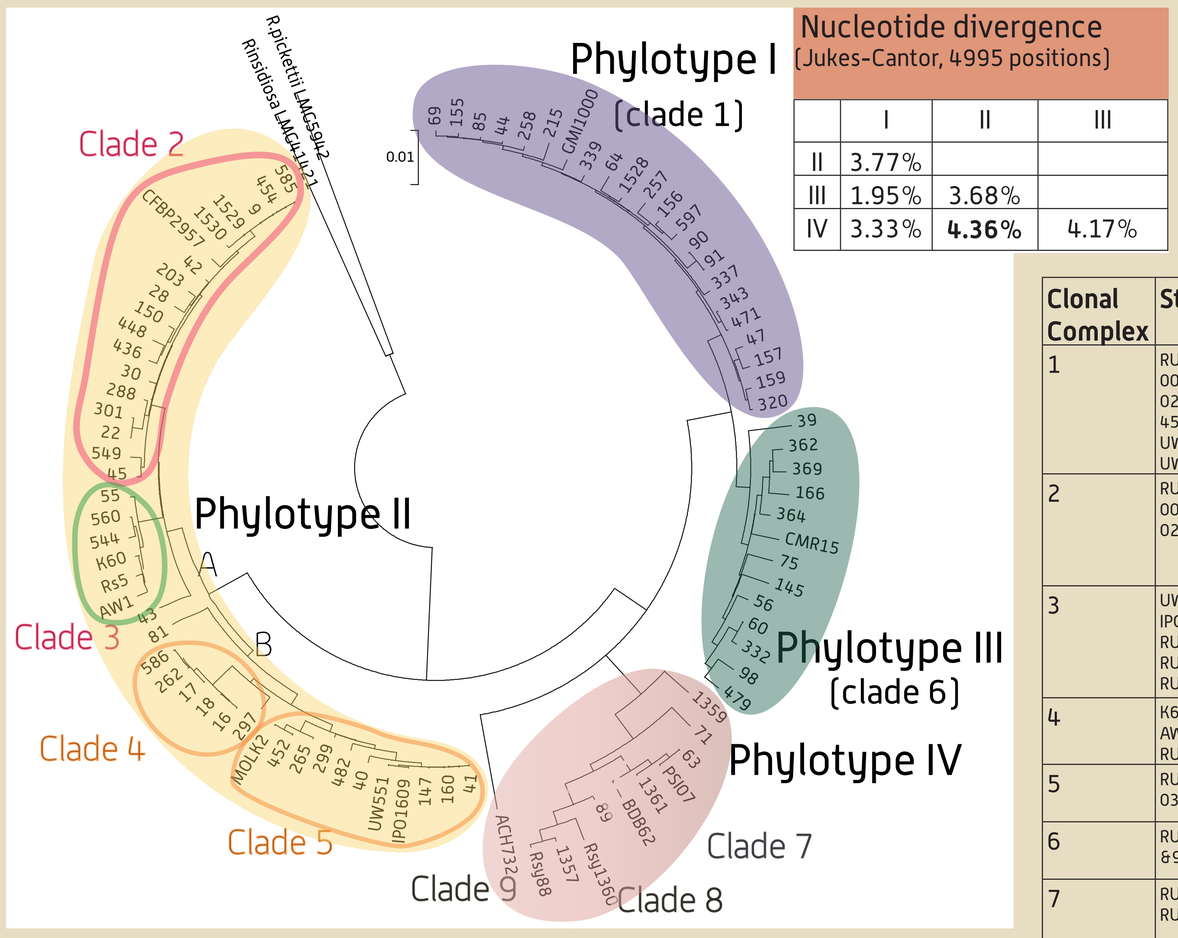
- *Ralstonia solanacearum :* responsible for heavy losses on cash and subsistence crops throughout tropical and temperate areas (bacterial wilt, potato brown rot, Moko disease)
- High genotypic and phenotypic plasticity :
  - emergence of new devastating pathotypes, plant resistance breakdown
  - occurence of horizontal gene transfer *in planta* BUT no information available on the real population structures
- Phylogeny based on endoglucanase (Egl) partial sequence analysis, confirmed by CGH data => 4 phylotypes

Objectives

- Precise the intraspecific phylogeny: are phlyotypes furtther subdivided in distinct clades ?
- Assess the contributions of recombination in the evolutionary dynamics of *R.solanacearum*

Multi-Locus Sequence Analysis

- 92 *Ralstonia* strains : (i) 90 *R.solanacearum* of the 4 phylotypes and various ecotypes, (ii) type strains of *R.insidiosa* and *R.pickettii*
- 8 single-copy and evenly distributed genes : housekeeping, chromosome (*leuS*, *rplB*, *gdhA*, *gyrB*, *adk*, *mutS*) ; virulence-related, megaplasmide (*Egl*, *fliC*)
- Test for recombination (RDP), phylogeny reconstruction by NJ, ML, MP



Nucleotide divergence
(Jukes-Cantor, 4995 positions)

	I	II	III
II	3.77%		
III	1.95%	3.68%	
IV	3.33%	**4.36%**	4.17%

Maximum Likelihood tree estimated under the TIM+I+G model using the concatenated sequences of six housekeeping genes (chromosome) from 92 *Ralstonia strains*. Robustness of the nodes was evaluated by bootstraping (1000 replications).
Clades were defined within phylotypes according to their phylogenetic relevance (bootstrap values over 80%)

Phylotype		Clade	EGL (Fegan&Prior ,2005, unpublished 2008)	Features
I (Asian)		1	Sequevars 12-34	bacterial wilt
II (American)	*A*	2	Sequevar 7	US biovar 1, bacterial wilt
	A	3	Sequevars 6, 24, 35,36,39..	Moko disease, bacterial wilt
	B	4	Sequevar 4	Moko disease, bacterial wilt (emerging strains)
	B	5	Sequevar 1-2, 3	Brown rot Moko disease
III (African)		6	Sequevars 19-23,29, 42-44,49	Brown rot and bacterial wilt
IV		7	Sequevar 9	*R.syzygii*
		8	Sequevar 10	BDB & BW
		9 ?	Sequevar 11	ACH432

Gene	Length (bp)	No. haplotypes	% polymorphic sites	θ[a]	π[b] Total	π[b] Synonymous	π[b] Non synonymous	Tajima's D[c]	Ka/Ks[d]
adk	468	54	6.838	0.013	0.020	0.063	0.007	0.800	0.120
gyrB	423	65	22.695	0.045	0.028	0.091	0.011	-1.451	0.117
gdhA	966	59	22.981	0.045	0.027	0.091	0.009	-1.633	0.096
leuS	735	49	18.503	0.036	0.025	0.086	0.009	-1.244	0.099
rplB	738	42	13.686	0.027	0.033	0.124	0.005	0.340	0.033
mutS	651	47	28.111	0.055	0.036	0.133	0.009	-1.692	0.076
fliC	348	39	12.356	0.024	0.023	0.063	0.011	-0.431	0.230
Egl	666	60	26.426	0.052	0.055	0.125	0.036	-0.053	0.273

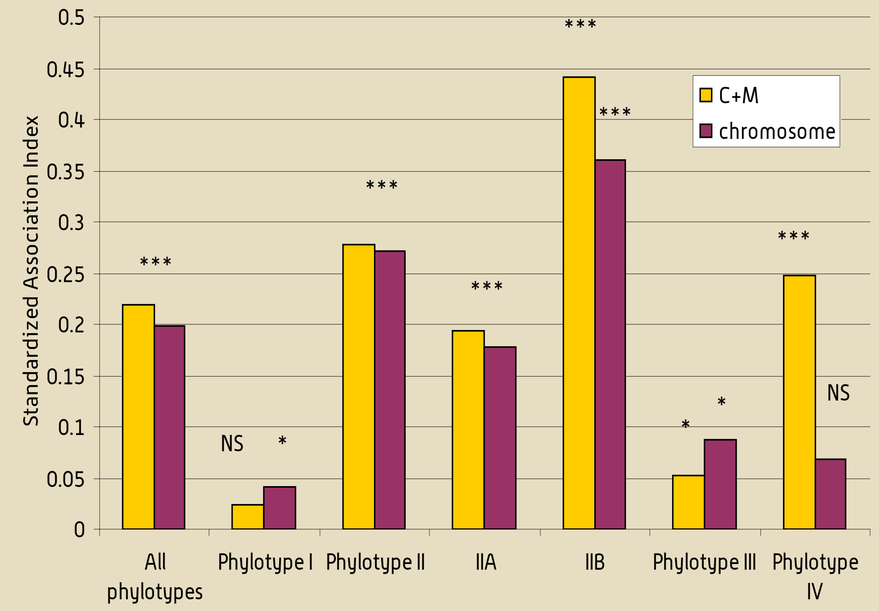
[a] Theta per site (Watterson estimate)
[b] Nucleotide diversity, after Jukes&Cantor correction
[c] Not significant (P>0.05)
[d] Synonymous (s) et non-synonymous (a) substitution rate calculated by the Nei&Gojobori (1986) method

Multi-Locus Sequence Typing

- Determination of allele profiles (sequence types) for each gene (DnaSP)
- Detection of homologous recombination by assessment of linkage disequilibrium (IS_A)
- BURST approach (START2, E-BURST)

Clonal Complex	Strains	Phylotype/ecotype
1	RUN0009, 0030, 0203,0 454, 0585, UW588, UW595	IIA, clade 2 (seqvar6, 35, 52) BW and Moko strains from Guatemala, Venezuela, Carribean, Cameroon
2	RUN0016, 0017, 0018, 0262, 0568	IIB, clade 4 (sequevar 4&4NPB) Moko strains from Peru, emerging strains from Martinique
3	UW551, IPO1609, RUN0041, RUN0160, RUN147	IIB, clade 5 (sequevar 1) Brown rot strains from Kenya, Cameroon, Nigeria, Réunion, Netherlands)
4	K60, AW1, Rs5, RUN0055	IIA, clade 3 (sequevar 7) BW strains from Kenya, USA
5	RUN0022, 0301	IIA, clade 2 (seqvar24) Moko strains from Brazil
6	RUN0090 &91	I Mulberry strains, China
7	RUN0265, RUN0452	IIB, clade 5 (sequevar 3) Moko strain/Honduras, peanut/ Indonesia
8	RUN0320, 0343	I BW,China & Madagascar

Clonal complexes identified by BURST approach on 87 multilocus sequence types (6 housekeeping genes). 60 singletons were also identified.



Levels of linkage disequilibrium within the different phylotypes of *R.solanacearum*, as assessed by «standardized association index ($\bar{r}_A^S$) (Maynard-Smith *et al* 1993), calculated on allelic profiles (grouped STs) of chromosome (6 loci) and chromosome+megaplasmide (C+M, 8 loci). $\bar{r}_A^S$ values significanty different from 0 indicated that population was clonal (linkage disequilibrium), whereas not significant values indicated a recombining population structure. All phylotypes= 87 ST; phylotype I = 21 ST; Phylotype II = 41 ST (24 within IIA, 17 within IIB); phylotype III = 14 ST; phylotype IV= 11 ST. NS = not-significant ; *= 0.05>*P*>0.01, ***=P<0.001 (Monte Carlo and parametric methods, 1000 random resamplings) All calculations were done with LIAN software (Haubold &Hudson, 2000).

Conclusions

- The 4 phylotypes are subdivided in different clades, but DNA divergences observed between phylotypes are below species delineation values
- Phylotypes display different population structures : recombining (phylotype I), slightly clonal (phylotype III), highly clonal (phylotype II)
  - confirm LD analyses done on field populations in Trinidad (REP-PCR data, Ramsubhag *et al* in prep)
  - clonality in phylotype II = associated to long distance dissemination by ornementals and tubers ?
- Consequences in resistance durability, emergence risk ?