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Development of variable number of tandem repeats typing schemes for *Ralstonia solanacearum*, the agent of bacterial wilt, banana Moko disease and potato brown rot

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ABSTRACT

Ralstonia solanacearum is an important soil borne bacterial plant pathogen causing bacterial wilt on many important crops. To better monitor epidemics, efficient tools that can identify and discriminate populations are needed. In this study, we assessed variable number of tandem repeats (VNTR) genotyping as a new tool for epidemiological surveillance of *R. solanacearum* phylotypes, and more specifically for the monitoring of the monomorphic ecotypes "Moko" (banana-pathogenic) and "brown rot" (potato-pathogenic under cool conditions). Screening of six *R. solanacearum* genome sequences lead to select 36 VNTR loci that were preliminarily amplified on 24 strains. From this step, 26 single-locus primer pairs were multiplexed, and applied to a worldwide collection of 337 strains encompassing the whole phylogenetic diversity, with revelation on a capillary-electrophoresis genotype. Four loci were monomorphic within all phylotypes and were not retained; the other loci were highly polymorphic but displayed a clear phylotype-specificity. Phylotype-specific MLVA schemes were thus defined, based on 13 loci for phylotype I, 12 loci for phylotype II, 11 loci for phylotype III and 6 for phylotype IV. MLVA typing was significantly more discriminative than *egl*-based sequevar typing, particularly on monomorphic "brown rot" ecotype (phylotype IIB/sequevar 1) and "Moko disease" clade 4 (Phylotype IIB/sequevar 4). Our results raise promising prospects for studies of population genetic structures and epidemiological monitoring.

1. Introduction

Ralstonia solanacearum is a soilborne betaproteobacterium, capable of inducing disease on more than 250 plant species belonging to 55 botanical families (Hayward and Hartman, 1994). Its prevalence in most of the tropical and subtropical areas, affecting both cash (potato, banana, ginger) and subsistence crops (eggplant, tomato, pepper), and its huge phenotypic and genotypic diversity and plasticity, make it a major plant pathogen. Due to its host range and its ability to survive and induce disease at cool temperatures, it has been listed as a quarantine organism in many European countries (OEPP/EPPO, 2004), and considered as a Bioterrorism select Agent in the USA (Lambert, 2002).

R. solanacearum is regarded as a species complex (Gillings and Fahy, 1994), composed of four phylotypes of different geographical origins (I: Asian, II: American, III: African, IV: Indonesian), as previously defined based on *hrpB*, *egl* and *mutS* sequence analysis (Fegan and Prior, 2005; Poussier et al., 2000a,b; Prior and Fegan, 2005). Previous classifications were based on host range variations [races (Buddenhagen et al., 1962)] and metabolic properties [biovars (Hayward, 1964)], but these systems were neither predictive nor meaningful. The four phylotypes were further subdivided into sequevars based on *egl* sequence similarities (Fegan and Prior, 2005). Based on this classification, strains are assigned to a specific phylotype and sequevar. A recent multilocus sequence analysis (MLSA) demonstrated that the *R. solanacearum* species complex (RSSC) was actually structured in five evolutionary lineages corresponding to phylotypes (I, IIA, IIB, III, IV), and eight clades: clades 1 and 6 corresponding respectively to phylotype I and III, clades 2 and 3 within the phylotype IIA, clades 4 and 5 within phylotype IIB, clade 7 and 8 within the phylotype IV. Each clade encompasses one to several *egl*-based sequevars (Wicker et al., 2012). MLSA was particularly

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valuable to reconstruct evolutionary dynamics and history of the different phylotypes within the RSSC, but was poorly resolutive on some epidemiologically major groups. For instance, clade 5 (encompassing the potato brown rot strains, race 3/biovar 2) was highly monomorphic; and within IIB/clade 4 two phenotypically divergent groups, i.e. banana wilt-inducing strains ("Moko" ecotype) and banana-nonpathogenic emergent strains (formerly named "sequevar 4NPB" strains, "emerging" ecotype), were not distinguished (Wicker et al., 2012). Several genomes of *R. solanacearum* from the four phylotypes have been sequenced (Gabriel et al., 2006; Guidot et al., 2009; Remenant et al., 2010, 2011; Salanoubat et al., 2002), giving insights into the gene composition specificities and allowing the development of new tools based on genome-wide screens. However, the scientific community still seeks for powerful and reliable genetic tools to address key questions related to disease introduction and emergence, and for monitoring population dynamics at fine scales related to adaptation to plant resistance or specific suppressive crops. The comprehension of adaptive dynamics of this fascinating plant pathogen implies to identify patterns of genetic structuring of populations at spatial scales ranging from plant to field and cropping area, as well as time scales ranging from months to years. In other words, focus must be done on *R. solanacearum* molecular ecology.

Molecular ecology and population genetics of *R. solanacearum* remain poorly investigated and understood. Several questions remain unanswered such as how plant species shape the genetic structure of *R. solanacearum*? or how related are soil-inhabiting populations and xylem-infecting populations? Very few studies addressed *R. solanacearum* microevolution questions at the local scale (region and field), due to lack of adequate typing tools. A field population of *R. solanacearum* was studied by RAPD-PCR (Grover et al., 2006), but the low repeatability and portability of this technique renders these results difficult to interpret (Roumagnac et al., 2007). Regional population structures and reproductive modes were compared at the scale of the Trinidad island using REP-PCR (Ramsubhag et al., 2012). However, despite its high repeatability and reproducibility within a laboratory, REP-PCR is most frequently not portable between different laboratories (Roumagnac et al., 2007).

Multi-Locus VNTR [acronym for "Variable Number of Tandem Repeats"] Analysis (MLVA), a technique based on the detection of tandem repeat polymorphism within genomes, has been revealed as a source of very informative markers (Van Belkum, 2007; Vergnaud and Pourcel, 2006). Indeed, MLVA have been extensively used in medicine for tracking pathogens transmission (Le Flèche et al., 2002, 2006) (Pourcel et al., 2007) and typing monomorphic bacterial pathogens including *Bacillus anthracis* (Keim et al., 2000), *Yersinia pestis* (Le Fleche et al., 2001; Li et al., 2009), *Mycobacterium tuberculosis* (Jiang et al., 2012; Le Flèche et al., 2002), and methicillin-resistant *Staphylococcus aureus* (Pourcel et al., 2009). To date however, few MLVA schemes have been published on plant bacterial pathogens (Bui Thi Ngoc et al., 2009a,b; Colleta-Filho and Machado, 2002; Gironde and Manceau, 2012; Zhao et al., 2012).

In this study, we explored the potential of multilocus VNTR analysis (MLVA) as a genotyping method for *R. solanacearum* and to assess its usefulness for future epidemiological studies. More specifically we aimed to determine whether (i) the different phylotypes of *R. solanacearum* could be typed by one single or by several specific MLVA schemes, (ii) haplotype diversity could be found within monomorphic groups.

2. Material and methods

2.1. Bacterial strains

A total of 337 worldwide strains of *R. solanacearum* belonging to phylotypes I, IIA, IIB, III and IV were used (Table S1). Most of these strains are maintained in the RUN collection (Saint Pierre, Reunion Island), stored on microbeads (Microbank®, PRO-LAB DIAGNOSTICS, Neston, Wirral, U.K.) at −80 °C. After growth on LB broth and Kelman medium, DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Courtaboeuf, France) using the routine protocol with addition of a RNase A step. DNA solutions were then quantified and checked for quality using a NanoDrop® ND-8000 device (NanoDrop Technologies Inc., Wilmington, DE 19810 USA). DNA solutions were diluted to 2 ng.μL^{-1} in milliQ water, and stored at −30 °C until use.

2.2. Detection of tandem repeats

Six genomes of *R. solanacearum*, publicly available via the MaGe interface (RalstoniaScope, http://www.genoscope.cns.fr/agc/mage/ralstoniascope), were screened for the presence of Tandem repeats (TR), using the Microorganisms Tandem Repeats Database (http://minisatellites.u-psud.fr/GPMS/) (Denoeud and Vergnaud, 2004; Grissa et al., 2008): GMI1000 (phylotype I-sequevar 14, Guyane) (Salanoubat et al., 2002), MOLK2 (Phylotype IIB- sequevar 3 ["Moko" ecotype], Philippines) and IPO1609 (Phylotype IIB-sequevar 1 ["Brown Rot" ecotype], Netherlands) (Guidot et al., 2009; Poueymiro and Genin, 2009), PSI07 (phylotype IV-sequevar 10), CMR 15 (phylotype III-sequevar 29, Cameroun) and CFBP2957 (phylotype IIA-sequevar 36) (Remenant et al., 2010). Detection criteria were derived from (Bui Thi Ngoc et al., 2009b): the total length was set in a range of 50–600 bp, the length of tandem repeats in a range of 5–100 bp, the copy number in a range of 5–30, matching percentage above 80%, and other parameters set as default. Additional TR were found within IPO1609 and MOLK2 genomes by using the Boston University's Tandem Repeat DataBase (TRDB, https://tandem.bu.edu/egi-bin/trdb/trdb.exe) (Gelfand et al., 2006), using default parameters (alignment weights at 2, 7, 7 respectively for match, mismatch and indels; the two latter parameters are considered least permissive [see detailed explanation at http://tandem.bu.edu/trf/trf.definitions.html]). From this screening, 30 loci were identified within GMI1000, seven loci within IPO1609 and MOLK2, 11 loci within CMR15, nine loci within CFBP2957, and four loci within PSI07.

PCR primers (18–25 bp long) were designed within the 300 bp TR loci-flanking regions, using Primer3 (Rozen and Skaletsky, 2000); and submitted as queries against the six *R. solanacearum* genomes sequences deposited in MaGe, using the BLAST program (BLASTN) available at the MaGe website. From this step, only primer pairs matching (i) at a single locus per genome, and (ii) on at least one genome of each phylotype, were retained for further analyses. Thirty-six loci and primer pairs were thus selected.

First preliminary experiments were then done to check for amplificability, repeatability and polymorphism of these different loci. Simplex PCRs of each locus were performed on a collection of 24 strains (eight phylotype I, five phylotype IIA, five phylotype IIB, four phylotype III and two phylotype IV), using QIAGEN Multiplex Master Mix (see below) or regular PCR mix (in 15 μL: 1XPCR buffer, 1.5 mM MgCl2, 0.2 μM of each dNTPs [Ozyme, Saint Quentin en Yvelines, France], 3 pmol of each primer, 1 U RedGoldStar DNA polymerase [Eurogentec, Seraing, Belgium], 2 μL of bacterial DNA template [2 ng.μL^{-1}]). PCR reactions were performed on 9700 GenAmp thermocycler (Applied BioSystems, Life Technologies, Carlsbad, California, USA), with the following program: (i) initial denaturation step at 96 °C for 5 min, (ii) 30 cycles of 94 °C for 30 s, the annealing temperature for 90 s, 72 °C for 90 s, and (iii) 72 °C for 10 min, followed by a stand step at 12 °C. Electrophoreses (5 V.cm^{-1}) were performed on high resolution agarose gels (3% MetaPhor + 1% Seakem agarose in TAE 1X), along with 50 bp size ladder (BioLabs Inc, Ipswich, MA, USA). Gels were visually assessed for band presence and intensity, and loci were evaluated for their specificity (positive amplifications per total number of strains of each phylotype) and their polymorphism (number of band sizes). From these preliminary experiments, 26 loci were selected over 36