Genetic Structure of *Ralstonia solanacearum* and *Ralstonia pseudosolanacearum* in Brazil

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Abstract

Bacterial wilt-causing *Ralstonia* threaten numerous crops throughout the world. We studied the population structure of 196 isolates of *Ralstonia solanacearum* and 39 isolates of *Ralstonia pseudosolanacearum*, which were collected from potato- and tomato-growing areas in 19 states of Brazil. Regardless of the species, three groups of isolates were identified. One group encompassed *R. pseudosolanacearum* isolates. The other two groups comprise isolates of *R. solanacearum* (phylotype II) split according to geographic regions, one made of isolates from the North and Northeast and the other made of isolates from the Central, Southeast, and South regions (CSS). Among the isolates collected in CSS, those from tomato were genetically distinct from the potato isolates. The genetic variability in the population of *R. pseudosolanacearum* was lower than that of *R. solanacearum*, suggesting that the former was introduced in Brazil. Conversely, the high genetic variability of *R. solanacearum* in all regions, hosts, and times supports the hypothesis that this species is autochthonous in South America, more

precisely in Brazil and Peru. For *R. solanacearum*, higher variability and lower migration rates were observed when tomato isolates were analyzed, indicating that the variability is caused mainly by the differences of the local, native soil population. The North subpopulation was distinct from all others, possibly because of differences in environmental features of this region. The proximity of some geographic regions and the movement of potato tubers could have facilitated migration and therefore low genetic differentiation between geographic regions. Finally, geography, which also influences host distribution, affects the structure of the population of *R. solanacearum* in Brazil. Despite quarantine procedures in Brazil, increasing levels of trade are a threat to biosecurity, and these results emphasize the need for improving our regional efforts to prevent the dispersal of pathogens.

Keywords: bacterial wilt, genetic variability, phylotype, population biology

Ralstonia solanacearum is one of the most threatening pathogens affecting crops in tropical and subtropical regions (Hayward 1994). The pathogen causes bacterial wilt in staple foods such as banana; vegetable crops such as potato, which is also staple food; tomato, eggplants, and sweet pepper; and woody plants such as eucalypt, among many other plant species of economic importance. The disease is particularly devastating in the tropics, to which the pathogen is well adapted (Hayward 1994). *R. solanacearum* is allegedly responsible for annual losses of almost \$1 billion and for partial or complete yield losses in more than 4 million ha of solanaceous crops, ornamentals, fruit trees, and *Eucalyptus* that are cultivated in about 40 countries (Elphinstone 2005). The economic losses are difficult to quantify but are high. Although evaluation of direct yield losses is possible, estimating indirect losses commonly associated with bacterial wilt is not straightforward. For example, the occurrence of bacterial wilt in certain areas may hamper the cultivation of susceptible hosts. This limitation imposes severe restrictions on the economic

viability of land use because *R. solanacearum* can survive for years in soils and associates with the root systems of naturally occurring plants (Denny 2006).

R. solanacearum is considered a species complex with four distinct phylogenetic lineages defined based on the partial sequences of internal transcribed spacers, *hrpB*, and the endoglucanase (*egl*) genes. These lineages are commonly called phylotypes. Interestingly, the phylotypes are somewhat associated with the geographic origin of the isolates (Fegan and Prior 2005). Phylotype I isolates are found predominantly in Asia, phylotypes IIA and IIB in the Americas, phylotype III in Africa, and phylotype IV in Indonesia. However, because of long-distance dispersal by water and infected propagative material or substrates, different phylotypes can be found in distinct geographic areas (Ramsubhag et al. 2012; Sanchez Perez et al. 2008). Recently, the *R. solanacearum* complex was split into three species. Individuals of phylotypes I and III are now considered as belonging to *Ralstonia pseudosolanacearum* sp. nov. Phylotype IV isolates were divided into *Ralstonia syzygii* subspecies *indonesiensis*, *R. syzygii* subsp. nov. *celebesensis* and *R. syzygii* subsp. *syzygii*. Finally, phylotype II individuals maintained the name *R. solanacearum*. The separation in three genospecies was based on 16S–23S rRNA intergenic space, *egl* gene, DNA–DNA hybridization test, and genomic analyses (Prior et al. 2016; Safni et al. 2014).

Brazil and Peru are considered ancient centers of origin of the pathogen, more specifically of phylotype II (Clarke et al. 2015; Wicker et al. 2012). In Brazil, *R. solanacearum* was first reported affecting tobacco in the state of Rio Grande do Sul in 1922 by Von Parseval (Takatsu and Lopes 1997). Since this first report, severe yield losses caused by bacterial wilt have been recorded in different crops and several regions. In the past few years, different studies aimed at determining the distribution and frequency of genetic variants of *R. solanacearum* have been conducted in Brazil (Albuquerque et al. 2014; Fonseca et al. 2014; Garcia et al. 2013; Santiago et al. 2017). However, isolates of two species, *R. solanacearum* (phylotypes IIA and IIB)

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and *R. pseudosolanacearum* (phylotype I), have been reported in the country. Thus, there are at least two populations of *Ralstonia* spp. causing bacterial wilt in different hosts in Brazil.

Breeding for host resistance is the best management strategy to control bacterial wilt. However, the high genetic variability of the pathogen hampers the effective development of resistant cultivars. For example, tomatoes resistant to *R. solanacearum* in one region can be susceptible when planted in another area (Lin et al. 2008). The strategic use of resistant cultivars depends on basic information about the environmental conditions, the characteristics of the host, and the genetic variability of the pathogen population, which is often subject to change. Therefore, successful breeding programs require knowledge of the mechanisms that affect the evolution of *R. solanacearum*. Molecular markers have been used effectively to investigate the variability and genetic structure of populations of *R. solanacearum* at local and regional scales. For the species complex *R. solanacearum* (*sensu lato*), many investigations were conducted using multilocus sequence typing (MLST) based on repetitive sequences (repetitive element PCR [rep-PCR]) as BOX-AIR-based repetitive extragenic palindromic-PCR (BOX), enterobacterial repetitive intergenic consensus (ERIC), and REP; amplified fragment length polymorphism (AFLP); simple sequence repeats (SSR), and restriction fragment length polymorphism (Cardozo et al. 2010; Sakthivel et al. 2016; Yu et al. 2003). For *R. pseudosolanacearum*, in addition to the techniques listed above, a study was conducted to develop a new genotyping alternative to *R. pseudosolanacearum* isolates: multilocus variable number of tandem repeat analysis (Ravelomanantsoa et al. 2016). It includes tandem repeats, microsatellites, and minisatellites regions, and it could differentiate isolates at global, regional, and local scales.

Population genetics studies were conducted in different countries, including Brazil, and the general emerging picture is of high genetic variability. In Trinidad and Tobago, the genetic diversity of 71 isolates causing bacterial wilt in tomato was studied via rep-PCR DNA fingerprinting; no structure was found. Intense gene flow between vegetable crop production areas was suggested as an important factor for panmixis of the pathogen population in the island (Ramsubhag et al. 2012). On the other hand, 30 strains sampled from banana production areas in Malaysia revealed a geographically structured population of *R. solanacearum*: Cluster 1 contained isolates of Kedah, Selangor, Negeri Sembilan, and Johor districts, and cluster 2 contained strains of Pahang (Zulperi et al. 2016). In Colombia, the genetic variability of *R. solanacearum* was associated with the host plants (Cardozo et al. 2010). Isolates obtained from banana plants, soil, and weeds revealed high genetic diversity, and the population was structured according to the host (Cardozo et al. 2010). In another study conducted in Hawaii with AFLP, the isolates from ginger crops grown on a farm with fields established from imported rhizomes were genetically different from local isolates obtained from tomato and heliconia, revealing the effects of different introductions (Yu et al. 2003). In contrast, multilocus sequence typing analysis with isolates of tomato, eggplant, and chili from the Andaman Islands revealed a small number of clones, possibly from a recent introduction from mainland India (Sakthivel et al. 2016).

Despite the importance of Brazil as an ancient center of origin of the pathogen, the studies conducted so far used a small number of isolates from few host crops. Six studies of population genetics of

R. solanacearum have been conducted in Brazil in the past decade, and in most cases high genetic variability of the pathogen was reported. Silveira et al. (2005) assessed genetic variability based on the patterns of random amplification of polymorphic DNA (RAPD) and biovar markers for 51 isolates of *R. solanacearum* sampled from 25 fields in 10 municipalities of the state of Rio Grande do Sul. Isolates were grouped according to region. High polymorphism and no correlation with host, biovar, ecotype, or geographic origin were detected for 70 isolates collected from different hosts in the Amazon region (Costa et al. 2007). Another study of 33 isolates from different hosts (banana, heliconia, tomato, eggplant, bell pepper, and potato) that were genotyped with RAPD markers found no correlation between genetic distance and geographic origin (Pinheiro et al. 2011). Likewise, no structure was observed in a study of 41 isolates of *R. solanacearum* from ornamental plants, eucalypt, Solanaceae, banana, and heliconia (Rodrigues et al. 2012) plus 120 isolates from soil and wilted tobacco plants (Viana et al. 2012). Using ERIC-PCR as genetic marker detected population structure according to geographic region for eucalypt isolates (Fonseca et al. 2014). In summary, a body of evidence points to the fact that whenever isolates from different hosts are analyzed, high genetic variability and lack of population structure appear the norm. However, no studies were conducted with a collection of isolates that represent country-wide geographic coverage and from a more diverse host range.

The Brazilian population of *R. solanacearum* coevolved for millions of years with a high diversity of plant species, but the opposite seems to have occurred with the population of *R. pseudosolanacearum* (Santiago et al. 2017). Thus, in Brazil, two closely related *Ralstonia* spp. with distinct evolutionary histories coexist, causing wilt in different hosts. In the current study, we assessed the genetic diversity of the populations of *R. solanacearum* and *R. pseudosolanacearum*, the population structure according to geographic region or host plant (potato or tomato), and the change in diversity over time.

Materials and Methods

Isolates and DNA extraction. A total of 235 isolates from the culture collection of EMBRAPA Hortaliças (Brasília) and Universidade Federal de Viçosa were analyzed in the current study (Supplementary Table S1). The isolates were previously characterized as *R. solanacearum* ($n = 196$; phylotype IIA and IIB) and *R. pseudosolanacearum* ($n = 39$; phylotype I), both obtained from tomato and potato plants (Table 1) (Santiago et al. 2017). Isolates from potato (99) and tomato (136) were collected in different years and from different regions of Brazil. *R. pseudosolanacearum* isolates were obtained from infected plants collected in two regions: 36 isolates from the North and 3 from the Northeast. Isolates of *R. solanacearum* were obtained from infected plants collected in five regions: 25 from the North, 27 from the Northeast, 33 from the Central region, 35 from the Southeast, and 76 from the South (Table 1; Fig. 1A). χ^2 tests were performed to investigate the association between *R. solanacearum* phylotypes IIA and IIB and geographic regions or hosts by using the package Mass in R software (R Development Core Team 2014).

Isolates were grown in Kelman medium (Kelman 1954) in a rotary shaker at 180 rpm for 48 h at 28°C. One milliliter of cell suspension was centrifuged at 12,500 rpm for 1 min, and genomic DNA was extracted according to the instructions of the DNA purification kit. Each DNA preparation was kept in Tris-EDTA buffer at -20°C after treatment with RNase. DNA concentration was checked and adjusted with a spectrophotometer. Bacterial cultures were stored in tubes containing sterilized water or cryogenically in 40% glycerol at 80°C.

Genomic fingerprinting. Aliquots of genomic DNA were used as templates to generate rep-PCR genomic fingerprints with the BOX AIR primer (Louws et al. 1994). PCR products were separated by electrophoresis on 2% agarose gels at 80 V for 2 h, stained with an intercalating nucleic acid stain and photographed under ultraviolet light. Digital images of the stained agarose gels were stored, and the banding pattern was scored as presence or absence in the BioNumerics 4.5 software package. The first eight isolates of the collection (RS01, RS02, RS03, RS04, RS05, RS06, RS08, and RS09) were selected to evaluate the reproducibility of BOX-PCR. These eight isolates were run on

Table 1. Occurrence and distribution of phylotypes IIA and IIB of *Ralstonia solanacearum* collected in wilted tomato and potato plants in five regions of Brazil (North, Northeast, Central, Southeast, and South)

Region	Tomato		Potato		Total
	IIA	IIB	IIA	IIB	
North	23	2	0	0	25
Northeast	15	0	1	11	27
Central	12	3	5	13	33
Southeast	13	2	6	14	35
South	8	19	7	42	76
Total	71	26	19	80	196

three separate occasions in different electrophoresis agarose gels together with other isolates to check the consistency of the results. The expected banding pattern for the eight isolates should be consistent between runs.

Population structure and genetic analysis. The dataset of each *Ralstonia* species was analyzed to investigate potential subdivision of the population according to host or geographic region. Additionally, clonality, genotypic diversity, and genotypic differentiation were quantified. The clonal fraction of the subpopulations was estimated as:

$$\{1 - [(N - G)/N]\} \times 100$$

where G is the number of genotypes within a population and N is the total number of isolates. Genotypic diversity was assessed via the Shannon–Wiener and Simpson indices of BOX-PCR multilocus genotypes for each population of *Ralstonia* species. The effective

numbers of genotypes, $N1$ and $N2$, from each index, were scaled with the Hill number to allow comparisons between populations with different sample sizes (Jost 2007). The Nei gene diversity (Nei 1978) and evenness index (E_5) for each population were also estimated in the poppr package (Kamvar et al. 2014). E_5 was calculated to estimate the genotype distribution in the population. This index consists of the ratio between the numbers of abundant genotypes and rare genotypes (Grünwald et al. 2003). Allelic richness was estimated after rarefaction considering the smallest subpopulation (25 individuals when considering different regions and 97 when considering hosts). All previous analyses were conducted in the adegenet and vegan packages and by jackknife rarefaction methods in poppr package version 2.2.0 for the R program. Rarefaction was conducted to allow comparisons between sample sizes. Bootstrap standard error of the estimates was obtained with 1,000 replicates. Migration rates and directional movement between regions and hosts were estimated in BayesAss version 1.0, based on data for isolates collected between

Fig. 1. Discriminant analysis of principal components of Brazil. **A**, Sampling locations of tomato and potato isolates of *Ralstonia solanacearum* and *Ralstonia pseudosolanacearum* in Brazil. Circles in red and orange represent isolates of tomato and potato, respectively. **B**, Bayesian information criterion (BIC) values in K-means clustering. **C**, Scatter plot of the distribution of *R. solanacearum* and *R. pseudosolanacearum* phylotype I isolates, showing clustering into four genetic subgroups: 1, tomato isolates of *R. solanacearum* collected in Central, Southeast, and South regions (blue circles); 2, isolates of potato and tomato of *R. solanacearum* collected in Northeast, Central, Southeast, and South regions (brown circles); 3, *R. pseudosolanacearum* (orange circles); and 4, isolates of *R. solanacearum* collected in the North region (red circles). Eigenvalues: axis 1 = 72.12, axis 2 = 16.28.

2008 and 2012 (Wilson and Rannala 2003). We used 1 million Markov chain Monte Carlo iterations with a sampling frequency of 10,000 generations, and 20% of the samples were discarded as burn-in.

Evidence of population structure was initially assessed by inspecting the pattern of clustering of isolates. BOX-PCR data were grouped via discriminant analysis of principal components (DAPC) (Jombart et al. 2010) in the adegenet package (Jombart 2008) for R, version 2.11 (R Development Core Team 2014) and the unweighted pair group method with arithmetic mean (UPGMA) of Nei's unbiased genetic distances. The number of *K*-means was calculated in DAPC with the *find.clusters* function and Bayesian information criterion (BIC). We analyzed *K* varying from 1 to 20, with 10 runs at each value. The *dapc* function was executed with the lowest BIC. The first two axes of principal component analysis explained 88% of the total variance. A phylogenetic tree was constructed with UPGMA in PopGen version 1.32 (Yeh and Boyle 1997). The SEQBOOT, GENDIST, CONSENSE, and NEIGHBOR programs of PHYLIP version 3.6 were used to calculate the bootstrap values for branches of the trees from 100 replicates (Felsenstein 1993). Genotype relationships were studied via Nei's genetic distance matrices, UPGMA trees, and 1,000 bootstrap replications generated with PAUP* (Swofford 2002).

Genetic differentiation of subpopulations potentially structured according to geographic region or host was estimated based on *G''ST* values calculated with GENODIVE (Meirmans and Hedrick 2011). A complementary analysis of molecular variance (AMOVA) based on Euclidean distance was used to partition the variability for *R. solanacearum* population by host. All tomato isolates of the South, Southeast, and Central regions were pooled and compared with the potato isolates distributed in the same regions. It was not possible to conduct this analysis for each region because of

unbalanced sample sizes: Potatoes are not grown in the North region, and this crop is economically important in a single state of the Northeast region (the state of Bahia). The AMOVA was conducted in *poppr* (Kamvar et al. 2014). Finally, the linkage disequilibrium was assessed to infer random mating and, consequently, recombination. The index of multilocus association, *rD*, was used for this purpose.

Results

Overall, the BOX-PCR fingerprints of the 235 isolates produced 35 bands that ranged from 200 to 3,500 bp (data not shown). The banding pattern allowed the identification of 185 haplotypes. In reproducibility assay, the banding pattern of the tested isolates remained the same.

Diversity of *R. pseudosolanacearum*. For *R. pseudosolanacearum*, 15 haplotypes were identified among the 39 isolates obtained from the North and Northeast regions. The clonal fraction in the *R. pseudosolanacearum* population was 61.5%, and the most common haplotype occurred four times in the North region. High values of *H_E* and evenness were estimated for *R. pseudosolanacearum* isolates (*H_E* = 0.99 and *E₅* = 0.71). Population structure was not studied because of the low number of isolates from the Northeast region. Furthermore, all isolates were collected from wilted tomato plants, which prevented any analysis by host.

Diversity in *R. solanacearum*. A total of 196 isolates of *R. solanacearum* were obtained, and 170 haplotypes were derived from the multilocus pattern. The overall clonal fraction of the population was 13.3%. The clonal fraction varied from 3.7% (*E₅* = 0.96) in the Northeast region to 17.1% (*E₅* = 0.96) in the Southeast region (Table 2). The clonal fractions for tomato and potato were 7.2 (*E₅* = 0.98) and 12.1% (*E₅* = 0.99), respectively. Two isolates from tomato shared a haplotype that was found in the Central and North regions. Considering the isolates from potato, two isolates shared a haplotype that was found in the Central and Northeast regions, and two other isolates shared another haplotype in the Central and Southeast regions. The most common genotype was observed three times in each state (data not shown).

High levels of genotypic diversity were detected when analyses were partitioned by host (Table 2). The effective number of genotypes was higher in tomato (*N₂* = 83.3) than in potato (*N₂* = 77.2). The highest genotypic diversity was detected in the South, followed by the Central, Northeast, Southeast, and North regions. The genotypic richness in the North and Northeast regions was higher than in other regions. The highest estimated average heterozygosity per locus (gene diversity) values were for the North and Northeast (*H_E* = 0.98), followed by the Central region (*H_E* = 0.97) and in the tomato host (*H_E* = 0.95). The potato subpopulation from the Southeast region had the lowest gene diversity (*H_E* = 0.89). The rarefied genotypic richness, *E(g_n)*, was similar between regions and host populations. We found *E(g_n)* of 90 for tomato and 85.4 for potato. By region, the values varied from 21.7 in the Southeast population to 24.1 in the Northeast population.

Population structure of *R. solanacearum*. Genotypes were clustered into well-supported groups (Fig. 1C; Supplementary Fig. S1; Supplementary Table S2). In DAPC, a structure by region was observed. There was also an accentuated decline in BIC values with *K* values varying from 4 to 9 (Fig. 1B). When *K* = 4, the first axis differentiated isolates of potato and some strains of tomato collected in the South, Southeast, and Central regions. The second axis separated the Northeast, Central, Southeast, and South regions from the North.

In UPGMA, the first group included isolates from the Northeast, Central, Southeast, and South regions, mainly those of phylogroup IIA (Supplementary Fig. S1; Supplementary Table S2). The second group consisted mainly of isolates from the North and a few isolates from tomato plants sampled in the Northeast regions and of phylogroup IIA. The third group consisted of *R. pseudosolanacearum* isolates. The difference in cluster number corresponded to a separation of haplotypes of phylogroup IIB, mainly from tomato in the South,

Table 2. Descriptors of population variability, genetic diversity indices, and linkage disequilibrium estimates for subpopulations of *Ralstonia solanacearum* sampled from distinct regions and hosts^a

Region ^b	Subpopulation					Total
	North	Northeast	Central	Southeast	South	
<i>N</i>	25	27	33	35	76	196
Haplotypes	24	26	31	29	65	170
Clonal fraction	4.0	3.7	6.1	17.1	14.5	13.2
<i>H</i>	3.16	3.24	3.41	3.29	4.12	5.08
<i>G</i>	23.1	25.1	29.4	24.0	56.6	150.1
Lambda	0.96	0.96	0.97	0.98	0.99	0.99
<i>E(g₂₅)</i>	24	24.1	23.9	21.7	23.7	24.5
<i>H_E</i>	0.98	0.98	0.97	0.89	0.92	0.93
<i>rD</i>	0.111	0.137	0.254	0.213	0.088	0.130
<i>E₅</i>	0.96	0.96	0.97	0.96	0.98	0.99

Host ^c	Subpopulation		Total
	Tomato	Potato	
<i>N</i>	97	99	196
Haplotypes	90	87	170
Clonal fraction	7.2	12.1	13.2
<i>H</i>	4.47	4.42	5.08
<i>G</i>	83.3	77.2	150.1
Lambda	0.988	0.987	0.99
<i>E(g₉₇)</i>	90	85.4	90.2
<i>H_E</i>	0.953	0.931	0.930
<i>rD</i>	0.133	0.089	0.130
<i>E₅</i>	0.98	0.99	0.99

^a *N*, sample size; haplotypes, number of haplotypes; *H*, Shannon index of genotypic diversity; *G*, Stoddart and Taylor index of genotypic diversity; lambda, Simpson index of genotypic diversity; *E(g_n)*, genotypic richness estimated considering the smallest *N*; *H_E*, Nei's 1978 average heterozygosity per locus; *rD*, association index and multilocus estimate of linkage disequilibrium; *E₅*, evenness index. Significant at *P* < 0.05.

^b Isolates collected in North, Northeast, Central, Southeast, and South.

^c Isolates of potato and tomato.

Southeast, and Central regions, that were assigned to a separate cluster in DAPC. Isolates from tomato of the South region also clustered together, though with low bootstrap support in UPGMA analysis. Other than that, similar region, phylotype, and host groupings were detected. The UPGMA tree and DAPC analysis revealed distinct groups such as those formed by isolates of phylotypes I and II and by phylotype II isolates from the South, Southeast, and Central regions from populations of the Northeast and North regions (Supplementary Fig. S1; Supplementary Table S2). Movement of isolates between regions was unidirectional. Gene flow seems to take place between the Central and Southeast regions, mainly between Minas Gerais and São Paulo (Southeast) and Goiás and Distrito Federal (Central) (0.14) (Table 3 and Fig. 2). There is evidence for the occurrence of unidirectional migration from the Central to South and Southeast to South region (0.06 and 0.12, respectively). Migration was detected from the North to the Northeast and Central region (0.08 and 0.20, respectively). Migration from the Northeast to the South region (0.12) was also detected. Analysis by host indicated that migration rates were influenced mainly by movement of isolates from potato (0.04) and occurred at lower intensity with tomato isolates (0.01).

When populations were analyzed according to geographic region, the North population differed from the other regions, mainly from the Southeast and South, with $G''ST$ values of 0.37 and 0.39, respectively (Table 4). The populations from the Northeast, Central, Southeast and South regions were more similar to one another (Table 4). Overall, the population was moderately structured according to host ($G''ST = 0.24$, $P < 0.05$). The $G''ST$ values between the tomato and potato populations in the Central and Southeast regions varied from 0.81 to 0.50. In contrast, no population structure according to host was detected in the South region. The AMOVA results support the lack of genetic structuring between the Central, Southeast, and South regions. Approximately 73.0% of the genetic variation occurs with fields sampled in each region. However, there was evidence of population structuring according to host, especially between tomato and potato subpopulations (36% of the total variability) (Table 5). In contrast to the host population, the Brazilian population of *R. solanacearum* was not structured over the years or decades (data not shown).

Discussion

There is limited information about the genetic history of the Brazilian populations of *R. solanacearum* and *R. pseudosolanacearum*. Here we find higher gene and genotypic diversity in *R. solanacearum* compared with *R. pseudosolanacearum*. These results were expected because *R. solanacearum* is postulated to have originated in South America, whereas *R. pseudosolanacearum*, which putatively originated in Asia, was probably introduced in the continent by unknown routes. The population of *R. solanacearum* was weakly structured, and human-mediated transportation of propagative material as well as natural dispersal by rivers could have contributed to pathogen spread across regions, influencing the pattern of genetic variation in space. However, different analyses support the influence of the host in the genetic structure of the Brazilian population of *R. solanacearum*. When populations of both species of *Ralstonia* were analyzed, three groups of isolates were clearly identified. One group was formed by *R. pseudosolanacearum* isolates, a second group was formed by isolates predominantly from the South, Southeast,

and Central regions, and a third group was formed by isolates of *R. solanacearum* from the North and Northeast regions. An extra group with isolates of tomato from the South, Southeast, and Central regions could also have been considered. We hypothesize that the spatial distribution of the population of *R. solanacearum* is affected largely by ecological differences between regions, which in turn affect the distribution of both host crops under investigation. The subpopulation of the North region can be clearly differentiated from those of all other geographic regions. The difference between the North subpopulation can be explained by environmental properties such as temperature, humidity, altitude, and geographic distance affecting immigration rates.

The absence of regional structure in the Central, Southeast, South, and Northeast regions suggested no isolation by distance and gene flow between these large spatial groups. Transportation of

Fig. 2. Migration rates between geographic regions of Brazil estimated in BayesAss. Rates $>5\%$ were represented in the map (dates of movement between all geographic regions of Brazil are given in Table 3). Encircled numbers in the map indicate geographic regions: 1, North; 2, Northeast; 3, Central; 4, Southeast; and 5, South between 2008 and 2012.

Table 4. Genetic differentiation ($G''ST$) between regional subpopulations of *Ralstonia solanacearum* from Brazil

Subpopulation	North	Northeast	Central	South
North				
Northeast	0.11			
Central	0.21	0.06		
South	0.37	0.14	0.13	
Southeast	0.39	0.14	0.19	0.03

Table 3. Movement rate between geographic regions (with 95% confidence intervals) in Brazil between 2008 and 2012, estimated in BayesAss

	Movement from				
	North	Northeast	Central	Southeast	South
Movement into					
North	0.95 (0.92–1)	0.02 (0.02–0.03)	0.01 (0.00–0.02)	0.01 (0.00–0.02)	0.01 (0.0–0.02)
Northeast	0.08 (0.03–0.132)	0.79 (0.73–0.84)	0.04 (0.01–0.06)	0.04 (0.01–0.08)	0.04 (0.0–0.08)
Central	0.20 (0.15–0.25)	0.03 (0.01–0.06)	0.70 (0.67–0.73)	0.03 (0.02–0.05)	0.03 (0.0–0.06)
Southeast	0.04 (0.01–0.09)	0.05 (0.01–0.08)	0.14 (0.08–0.20)	0.71 (0.67–0.75)	0.05 (0.01–0.09)
South	0.04 (0.03–0.06)	0.02 (0.00–0.03)	0.06 (0.04–0.08)	0.12 (0.009–0.06)	0.74 (0.71–0.75)

contaminated propagative materials and the presence of *R. solanacearum* in river waters may have distributed variants of the pathogen across regions and influenced the genetic structure of the population. This fact can be observed in some haplotypes found associated with potato sampled in different geographic regions. Potato haplotypes were probably more efficiently dispersed than those found in other crops. It was previously suggested that the occurrence of biovar 2 in potato fields in the Northeast and Central regions of Brazil may be associated with the movement of latently infected tubers from colder southern regions to warmer northern regions (Santana et al. 2012). We detected biovar 2 potato isolates in the Central and Northeast regions of Brazil, and haplotype 72 was found in the South (the state of Paraná) and was also detected in the Northeast region (the state of Bahia). Similarly, haplotype 111 was found in the South and in the Central regions, in the states of Paraná and Goiás, respectively. In Europe and in the United States, *R. solanacearum* was detected in river water, and the origins of introduction of *R. solanacearum* in cultivated fields have been traced to contaminated water used in irrigation (Hong et al. 2008; López and Biosca 2005). The spatial distribution of sequevars 28, 41, and 50 over distinct regions may have been facilitated by flowing water of river affluents in distinct regions (Santiago et al. 2017). We believe that the movement between Central–Northeast and South–Northeast regions took place before 2008, because we did not detect migration between in the last 4 years of sampling. However, the recent movement of isolates found in the North region will increase the genetic variability observed in Brazil and reduce regional structure.

A previous study conducted in the Agreste and Zona da Mata meso-regions of the state of Pernambuco (Northeast region) reported no separation of isolates according to biovar, phylotype, or area (Garcia et al. 2013). At a much smaller geographic scale, contrary to what we found, no clustering by area was detected in the island of Trinidad. This finding can be explained by the relative close proximity of the most distant sampled areas, which were separated by approximately 60 km. In contrast, the two farthest areas of our study were separated by 3,393 km. Short distances would allow more intense gene flow and prevent genetic differentiation (Ramsubhag et al. 2012). The decrease in community similarity with geographic distance is a universal biogeographic pattern observed for all domains of life, mainly for organisms with limited dispersal (Martiny et al. 2011). When analyzing the genetic structure at a regional scale such as the South, Southeast, Central, and Northeast regions, we detected weak population structure. The lower genetic diversity among potato isolates compared with that found among tomato isolates supports the role played by transportation of infected or infested seed tubers. Despite the fact that *R. solanacearum* can be spread by infected tomato fruits, this does not seem to occur very often (Sanchez Perez et al. 2008).

Genetic divergence of *R. solanacearum* isolates collected in potato and tomato occurs between populations that are geographically in close proximity. This “sympatric-like” process can lead to pathogen adaptation to host plants (Stukenbrock and McDonald 2008). The

subpopulations of *R. solanacearum* associated with potato and tomato in different regions of Brazil are genetically different, but the highest fraction of molecular variation was attributed to variation in fields followed by differences within hosts.

The high genetic diversity of the Brazilian population of *R. solanacearum* raises important issues for disease management. The high genetic variability of the population is one of the reasons for the limited success of several management strategies aimed at controlling bacterial wilt in several hosts. It can be anticipated that this constitutes a major challenge to the control of bacterial wilt, particularly for the deployment of both biological control strategies and resistant varieties. Additionally, the high variability associated with selection exerted by the host can contribute to host adaptation and emergence of new ecotypes in the pathogen population (Jacques et al. 2016). The findings of the present study prompt for the need of greater caution during the commercialization and use of propagation materials and the quality of irrigation water in clean areas. The high genetic variability of *R. solanacearum* strains in Brazil supports the claim that South America is a center of origin and diversity of *R. solanacearum*. In addition to the high genotypic diversity, high gene polymorphism, the occurrence of bacterial wilt in native host plants, and the occurrence of the pathogen in uncultivated areas of different biomes such as the Cerrado and in the Amazon rainforest strengthen the claim of the origin of phylotype II in Brazil (Takatsu and Lopes 1997).

To conclude, our study is the first analysis of the genetic structure of the highly diverse populations of *R. solanacearum*. These populations can be separated according to host and geographic region. Breeding programs for durable resistance to bacterial wilt in Brazil should aim to develop genotypes for the different agroecosystems. However, there is much to learn about microbial diversification and pathogenesis. In future studies, we will use multilocus sequence typing to study the influence of recombination, explore isolate variability in crop fields, determine the influence of the environment, and reconstruct patterns of descent in genealogical analyses of Brazilian isolates of *R. pseudosolanacearum* and *R. solanacearum*.

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Table 5. Analysis of molecular variance for the BOX-PCR fingerprint of 100 isolates of *Ralstonia solanacearum* sampled from tomato and potato plants collected in the Central, Southeast, and South regions of Brazil

Source of variation	Degrees of freedom	Sum of squared deviations	Variance components	Proportion of variance components (%)
Variations between region	4	126.60	–0.64	–9.67
Variations between hosts	4	200.00	2.42	36.64
Variation within fields in each region	170	821.70	4.84	73.03
Total	178	1,148.30	6.62	100.00

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