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Bacteriology

e-Xtra*

MLST Reveals a Separate and Novel Clonal Group for *Acidovorax avenae* Strains Causing Red Stripe in Sugarcane from Argentina

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ABSTRACT

Acidovorax spp. cause a wide range of economically important diseases in monocotyledonous and dicotyledonous plants, including sugarcane, corn, rice, oat, millet, foxtail watermelon, and orchid. In Argentina, the red stripe disease of sugarcane caused by *Acidovorax avenae* affects 30% of the milling stems with important economic losses. To explore the genetic diversity of this bacterium associated with red stripe in Argentina, multilocus sequence typing (MLST) was applied. This study included 15 local strains isolated from four different sugarcane planting regions and selected after random amplified polymorphic DNA analysis and reference strains of *A. citrulli*, *A. avenae*, and *A. oryzae* to investigate their phylogenetic relationships. MLST analysis resulted in five sequence types among the sugarcane *A. avenae* strains which constitute a clonal complex, meaning a common and close origin. Sugarcane strains were related to *A. avenae* from other hosts and distant to *A. citrulli.* Signals of frequent recombination in several lineages of *A. avenae* was detected and we observed that *A. oryzae* is closely related to *A. avenae* strains. This study provides valuable data in the field of epidemiological and evolutionary investigations of novel clone of *A. avenae* strains causing sugarcane red stripe. The knowledge of the genetic diversity and strain-host specificity are important to select the genotypes with the best response to the red stripe disease.

Sugarcane is an important commercial crop worldwide, and one of the main sources of sugar and ethanol (FAO 2017). Due to the increasing demand for its use as biofuel, sugarcane has great potential for expansion to new cropping areas (de Vries et al. 2010). In Argentina, sugarcane production is geographically distributed in three regions—Tucumán, Northern (Salta and Jujuy), and Littoral (Santa Fe and Misiones)—extending over a 365,000-ha approximate area (Wallberg and Minetti 2015). Tucumán is the main sugarcane production province of Argentina, with 68% of total national production (Pérez et al. 2007). Sugarcane diseases have caused significant direct and indirect losses to the sugar industry (Rott et al. 2013). Pathogenic bacteria such as *Leifsonia xyli* subsp. *xyli*, *Xanthomonas albilineans*, and *Acidovorax avenae* are the etiologic agents of the three most important bacterial sugarcane diseases: ratoon stunting, leaf scald, and red stripe, respectively (Rott et al. 2000). Sugarcane red stripe, also known as "polvillo", affects sugarcane crops practically worldwide. Symptoms appear on the leaves as water-soaked stripes that gradually turn reddish and may extend to the plant apical meristem, which becomes wet, resulting in top rot in severe infections (Rott and Davis 2000). New agricultural techniques implemented in Argentina such as green-cane harvesting and crop rotation with soybean resulted in a significant increase of red stripe disease incidence. Severe symptom occurrence in commercial varieties of the Northwest production areas was observed in the last 15 years. The causal agent of this infective outbreak in sugarcane was identified for the first time by Fontana et al. (2013) as *A. avenae*. In addition, the whole-genome sequence of a virulent strain, *A. avenae* T10_60 for sugarcane, has been recently announced (Fontana et al. 2016). Currently, ongoing studies are focused on providing information on the molecular mechanisms involved in the pathogenesis of this sugarcane pathogen.

Acidovorax spp. cause a wide range of economically important diseases in monocotyledonous and dicotyledonous plants (Giordano et al. 2012). According to Willems and Gillis (2015), three subspecies for *A. avenae* were described*: A. avenae* subsp. *cattleyae, A. avenae* subsp. *citrulli*, and *A. avenae* subsp. *avenae*. The three subspecies have different host ranges: *A. avenae* subsp. *citrulli* infects Cucurbitaceae family members; *A. avenae* subsp. *cattleyae* infects only *Cattleya* and *Phalaenopsis* spp.; and *A. avenae* subsp. *avenae* infects Poaceae family members, including maize, rice, sorghum, corn, oat, barley, rye, various millet strains, vasey grass, and sugarcane (Fontana et al. 2013; Martin and Wismer 1989; Song et al. 2003; Willems and Gillis 2015). However, even now, several authors adopted the reclassification up to species level proposed formerly by Schaad et al. (2008) as *A. avenae*, *A. cattleyae*, *A. citrulli*, and *A. oryzae* sp. nov. (for the rice isolates). According to phylogenetic analysis based on 16S ribosomal RNA gene sequences, the plant-pathogenic *Acidovorax* spp. cluster together and the nonplant-pathogenic strains cluster together as a separate clade (Giordano et al. 2012). The ability to accurately identify and differentiate *Acidovorax* pathogenic strains causing disease is of critical importance for epidemiological surveillance and for designing efficient crop management procedures. The development of molecular typing methods based on nucleic acid fingerprint has contributed to accurately distinguish *Acidovorax* strains; among these, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism, restriction fragment length polymorphism, and pulsed-field gel electrophoresis (PFGE) have been largely applied (Fontana et al. 2013; Dhkal et al. in press; Li et al. 2017; Pulawska et al. 2013; Silva et al. 2016; Stead 1995; Walcott et al. 2000; Yan et al. 2013). Moreover, the combination of these methods with techniques based on sequence analysis such as multilocus sequence

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typing (MLST) introduced valuable information to the field of epidemiological investigation of these bacterial pathogens (Feng et al. 2009; Silva et al. 2016; Yan et al. 2013).

In this study, MLST was applied to explore genetic diversity among *A. avenae* strains from sugarcane associated with red stripe disease and to understand phylogenetic relationships with other *Acidovorax* strains from different hosts and geographical origins.

MATERIALS AND METHODS

Plant material. Leaf samples from sugarcane exhibiting red stripe symptoms were collected from 2008 to 2014 in Tucumán, Salta, Santa Fe, and Misiones Provinces, representing the main sugarcane production areas from Argentina (Supplementary Fig. S1). Young plants ($n = 50$), less than 4 months after harvesting, were sampled starting when the initial symptoms were more easily identified. In this study, samples collected from Salta, Santa Fe, and Misiones were placed on filter paper into Ziplock plastic bags; one portion of these was placed at 4 to 7°C for 24 to 48 h, then used for the isolation of *A. avenae*. Remaining samples were kept at –20°C for long preservation time. Five sugarcane *A. avenae* strains previously isolated from Tucuman (T10_61, T8_45, T6_50, and T4_53) and Salta (S11_3) were also included in this work. Sample codes, sugarcane genotype, cultivation regions, and strains used in this study are indicated in Table 1.

Isolation, identification, and typing of *A. avenae* strains. Leaves stored at 4 to 7°C were cut into small pieces (approximately 1 cm), disinfected twice with 70% ethanol (1 min), and rinsed with sterile water (1 min). Leaf material (approximately 0.5 g) was manually macerated with a pestle-polypropylene pellet (Sigma) in sterile 2-ml tubes using 1 ml of saline solution (NaCl at 0.9 g/liter). With the supernatant, 0.1 ml of each dilution was plated on the surface of nutritive agar (NA), prepared using peptone at 5.0 g/liter, meat extract at 3.0 g/liter, NaCl at 3.0 g/liter, and agar at 17.0 g/liter. After incubation for 48 h at 37°C, colonies with distinct morphologic characteristics (circular, translucent, white- to cream-colored colonies with entire margins) were selected, streaked onto yeast-glucose agar (yeast extract at 10.0 g/liter, glucose at 20.0 g/liter, calcium carbonate at 20.0 g/liter, and agar at 15.0 g/liter) and incubated for 48 h at 37°C. Typical *Acidovorax* colonies (circular, translucent, and beige colored with entire margins) were retained. Taxonomic identification was achieved by species-specific polymerase chain reaction (PCR) according to Fontana et al. (2013) from a pure culture grown on lysogeny broth (Bertani 2004) overnight at 30°C in a shaking incubator. For this PCR and other molecular testing, total genomic DNA was extracted and purified according to the cetyltrimethylammonium bromide method described by Ausubel et al. (1992). The bacterial DNA was quantified with Qubit (Invitrogen), visualized by electrophoresis through 0.7% (wt/vol) agarose gel, and stained with Gel Red (Genbiotech). RAPD reactions were carried out using primer M13 GAGGGTGGCGGTTCT (Huey and Hall 1989) according to Fontana et al. (2005) in 50 µl of reaction volume containing 3 mM $MgCl_2$, reaction buffer (1×), deoxynucleoside triphosphate (200 µM each), 1 µM each primer, 20 ng of DNA, and 0.5 U of Taq polymerase (Promega). PCR products were electrophoresed at 100 V on 2.5% agarose gel and stained with Gel Red (Genbiotech). RAPD profiles were normalized and submitted to Cluster Analysis with BioNumerics software, (version 5.0; Applied Maths) Dice similarity coefficient was used for similarity matrix calculation and dendrograms were obtained by the unweighted pair-group method with arithmetic averages.

MLST analysis. *PCR amplification and sequencing.* Fragments of seven housekeeping genes (Table 2), representing a total of 3,247 bp, were used for the MLST analysis, as previously described (Feng et al. 2009). PCR amplifications were carried out in a final volume of 25 µl containing 1× Master Mix PCR (Promega), 0.8 to 1.0 µM each primer, and 10 to 20 ng of sample DNA. Reaction conditions included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s and 60°C for 30 s for primer annealing, and an extension step at 72°C for 30 s. The final step was an extension period at 72°C for 5 min. Purification of the PCR products was performed with the ExoSap-IT Clean-up system (USB Co.). Sequencing with forward and reverse primers was performed in a 3130xl Genetic Analyzer (INTA Castelar).

MLST data analysis. MLST analysis included sequences downloaded from GenBank from strains of *A. avenae* ($n = 9$), *A. citrulli* ($n = 93$), and *A. oryzae* ($n = 1$) and from 15 strains isolated from sugarcane (Table 2). The analyzed housekeeping gene sequences are available under GenBank accession numbers MF623064 to MF623168 and EU928004 to EU928726 for *Acidovorax* strains isolated from sugarcane in Argentina and other hosts, respectively. Sequences were aligned with MEGA7.0.26 (https://www.megasoftware.net/); allelic profiles for each strain were calculated using MLSTest software (Tomasini et al. 2013). Based on the allelic profile, a sequence type (ST) was assigned to each strain (McCombie et al. 2006). A BURST analysis (Feil et al. 2004) was performed using MLSTest to identify clonal complexes with a group definition of at least six shared alleles (Tomasini et al. 2013). In addition, to build a neighbor-joining (NJ) tree with different node support measures, MLSTest was used. Consensus trees summarizing the information of individual tree fragments (based on branch frequency into the NJ tree for each locus) were also built. Multidimensional scaling plots from pairwise distance matrices were created. Topological incongruence between locus trees and consensus networks were calculated by MLSTest to address recombination into the *Acidovorax* spp., and the statistical significance was addressed using the Templeton test (Tomasini et al. 2013).

Seedling virulence assays. The virulence of sugarcane *A. avenae* strains representing the five ST determined by MLST analysis on a susceptible sugarcane variety TucCP 77-42 was evaluated (Rago 2005). *A. avenae* strains T10_61, S11_3. S22_3, SF17_4, and SF18_1 (ST5, ST1, ST4, ST2, and ST3 respectively) were used to inoculate young plants (less than 2 months old). *A. avenae* T10_61 (Fontana et al. 2016) was also used as virulent positive control. Inoculum was prepared from a pure bacterial culture grown on lysogeny broth on a shaking incubator for 48 h at 30°C. Bacterial

TABLE 1. Sample descriptions and strains used in this study

Sample ID	Sugarcane genotypes	Strains	Cultivation region	Province	Year
4	INTA NA 89-686	T4_53	La Trinidad, south	Tucumán	2008
6	INTA NA 91-209	T6_50	Cruz Alta, central	Tucumán	2008
8	TucCP 77-42	T8_45	Las Piedritas, central	Tucumán	2008
10	INTA NA 89-686	T10_61	Famaillá, central	Tucumán	2008
11	NA 85-1602	S11_3	Colonia Santa Rosa	Salta	2008
17	NA 85-1602	SF17_1, SF17_2, SF17_3, SF17_4, SF17_5, SF17_6, SF17_7	Tacuarendí	Santa Fe	2013
18	NA 85-1602	SF18_1	Tacuarendí	Santa Fe	2014
19	INTA 04-1604	SF19_1_SF19_2, SF19_3, SF19_4	Tacuarendí	Santa Fe	2014
20	INTA CP 98-828	SF20_1, SF20_2, SF20_3, SF20_4	Villa Ocampo	Santa Fe	2014
21	Unknown	SF21_1, SF21_2, SF21_3, SF21_4, SF21_5	Las Toscas	Santa Fe	2014
22	NA 02-2320	S22_1, S22_2, S22_3:S22_4	Tabacal	Salta	2014
23	Unknown.	M23_1, M23_2, M23_3, M23_4	San Javier	Misiones	2014

suspensions, adjusted to approximately 10^8 CFU/ml, were applied on adaxial and abaxial surfaces by rubbing the leaves manually. Plants used as the control were inoculated in an identical way with sterile water. In total, 20 biological replicates (potted plants) were assessed for each treatment and the experiment was carried out once. Plants were placed in 300-ml pots with a mixture of nonpasteurized soil and substrate (INTA) in a ratio of 70/30 and were maintained under high relative humidity (>90%) in plastic tunnels at constant temperature (30°C). A completely randomized experimental design was used. Red stripe occurrence on leaves from seedlings was evaluated every day up to 10 days postinoculation (dpi). The severity was evaluated once at 10 dpi as follows: 0 = no symptoms, 1 = localized infection and less than three red stripes per leaf, 2 = advanced infection and more than three red stripes per leaf, 3 = severe infection with red stripe that reaches the apical bud, and 4 = apical top rot or death of the apical top. This scale was developed by Fontana (2010) based on a similar scale described by Rott et al. (1994) with minor modifications and adapted to the red stripe disease characteristics. Data were used to calculate the mean of severity for each plant. One-way analysis of variance was performed for severity data analysis using the InfoStat software (Di Rienzo et al. 2018). Leaves showing red stripe were subjected to

TABLE 2. Allelic profiles and sequence types (ST) obtained by multilocus sequence typing analysis in this study

ST	Strains[z]	*glt*A	*gmc*	*lep*A	*pha*C	*pil*T	*trp*B	*ugp*B	Host	Geographic origin	Reference
1	S11-3	1	1	1	1	1	1	1	Sugarcane	Argentina	Fontana et al. 2013
1	SF21-2	1	1	1	1	1	1	1	Sugarcane	Argentina	In this study
1	M23-1	1	1	1	1	1	1	1	Sugarcane	Argentina	In this study
1	M23-4	1	1	1	1	1	1	1	Sugarcane	Argentina	In this study
1	T4-53	1	1	1	1	1	1	1	Sugarcane	Argentina	Fontana et al. 2013
1	T6-50	1	1	1	1	1	1	1	Sugarcane	Argentina	Fontana et al. 2013
2	SF17-4	1	1	1	1	2	1	1	Sugarcane	Argentina	In this study
2	SF17-5	1	1	1	1	2	1	1	Sugarcane	Argentina	In this study
2	SF19-4	1	1	1	1	2	1	1	Sugarcane	Argentina	In this study
2	SF20-1	1	1	1	1	2	1	1	Sugarcane	Argentina	In this study
2	S22-1	1	1	1	1	2	1	1	Sugarcane	Argentina	In this study
2	T8-45	1	1	1	1	2	1	1	Sugarcane	Argentina	Fontana et al. 2013
3	SF18-1	1	1	2	1	2	1	1	Sugarcane	Argentina	In this study
4	S22-3	1	1	3	1	2	1	1	Sugarcane	Argentina	In this study
5	T10-61	1	1	4	1	2	1	1	Sugarcane	Argentina	Fontana et al. 2013, 2016
6	AAA19860	2	2	5	1	3	2	2	Maize	United States	Lucas et al. 2011
7	AC30002	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30042	3	3	6	2	4	3	3	Watermelon	Japan	Feng et al. 2009
7	AC30046	3	3	6	2	4	3	3	Watermelon	Nigeria	Feng et al. 2009
7	AC30073	3	3	6	2	4	3	3	Melon	Korea	Feng et al. 2009
7	AC30084	3	3	6	2	4	3	3	Watermelon	Nigeria	Feng et al. 2009
7	AC30087	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30091	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30092	3	3	6	2	4	3	3	Watermelon	Brazil	Feng et al. 2009
7	AC30107	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30119	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30120	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30121	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30137	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30139	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30142	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30144	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30146	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30147	3	3	6	2	4	3	3	Watermelon	China	Feng et al. 2009
7	AC30248	3	3	6	2	4	3	3	unknown	China	Feng et al. 2009
7	AC30249	3	3	6	2	4	3	3	unknown	China	Feng et al. 2009
7	AC30287	3	3	6	2	4	3	3	Melon	China	Feng et al. 2009
7	AC30288	3	3	6	2	4	3	3	Watermelon	Japan	Feng et al. 2009
7	AC30290	3	3	6	2	4	3	3	Melon	China	Feng et al. 2009
7	AC30293	3	3	6	2	4	3	3	Watermelon	Malaysia	Feng et al. 2009
7	AC30294	3	3	6	2	4	3	3	Watermelon	Malaysia	Feng et al. 2009
7	AC30353	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30354	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30355	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30356	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30358	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30372	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30375	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30376	3	3	6	2	4	3	3	Melon	United States	Feng et al. 2009
7	AC30377	3	3	6	2	4	3	3	Watermelon	United States	Feng et al. 2009
7	AC30381	3	3	6	2	4	3	3	Watermelon	Australia	Feng et al. 2009
7	AC_W1	3	3	6	2	4	3	3	Watermelon	Australia	Feng et al. 2009
7	AC_W2	3	3	6	2	4	3	3	Watermelon	Brazil	Feng et al. 2009
7	AC_W4	3	3	6	2	4	3	3	Watermelon	China	Feng et al. 2009
7	AC_W6	3	3	6	2	4	3	3	Watermelon	China	Feng et al. 2009
8	AAA30003	4	4	7	3	5	4	4	Rice	China	Feng et al. 2009

(*Continued on next page*)

[z] Letters on the strain names represent the sugarcane-producing province as follows: Salta (S), Santa Fe (SF), Misiones (M), and Tucumán (T). For example, *Acidovorax avenae* T10_61 represents strain number 61 isolated from sample number 10 (INTA NA 89-686 sugarcane genotype) from Tucuman. AAA = *A. avenae* from other hosts (9 strains), AC = *A. citrulli* (93 strains), and AO = *A. oryzae* (1 strain).

microbiological and molecular analysis as described above to confirm that red stripe symptoms were caused by the inoculated *A. avenae* strains (data not shown).

RESULTS

Identification and differentiation of *A. avenae* isolates. In all, 100 colonies exhibiting the typical morphology of *Acidovorax* on NA (circular, translucent, white- to cream-colored with the entire margin) were isolated. After a first characterization by microscopy examination and Gram staining, only Gram-negative, typical colonies with single- or two- or three-rod chain morphology were selected for molecular assays. The species-specific PCR (s-sPCR) from all white-creamy colonies showed that approximately 50% of the isolates exhibited a positive signal for a specific product of 550 bp in size. This result indicated the presence on the plates of other bacterial groups with morphology and color similar to *A. avenae* colonies. The isolates identified as *A. avenae* by means of s-sPCR were analyzed by RAPD to investigate their genetic relatedness. The dendrogram drawn by the cluster analysis

TABLE 2. (*Continued from previous page*)

ST	Strains[z]	*glt*A	*gmc*	*lep*A	*pha*C	*pil*T	*trp*B	*ugp*B	Host	Geographic origin	Reference
9	AAA30015	5	5	8	4	3	5	5	Rice	China	Feng et al. 2009
10	AAA30044	6	6	9	5	6	6	2	Millet	China	Feng et al. 2009
11	AC30064	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30081	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30082	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30118	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30123	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30145	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30148	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30150	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30151	7	7	6	2	7	3	6	Rockmelon	China	Feng et al. 2009
11	AC30152	7	7	6	2	7	3	6	Rockmelon	China	Feng et al. 2009
11	AC30224	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30226	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30229	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30231	7	7	6	2	7	3	6	Melon	United States	Feng et al. 2009
11	AC30235	7	7	6	2	7	3	6	Melon	United States	Feng et al. 2009
11	AC30237	7	7	6	2	7	3	6	Melon	United States	Feng et al. 2009
11	AC30238	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30240	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30243	7	7	6	2	7	3	6	Melon	Japan	Feng et al. 2009
11	AC30250	7	7	6	2	7	3	6	Melon	Japan	Feng et al. 2009
11	AC30251	7	7	6	2	7	3	6	Melon	Japan	Feng et al. 2009
11	AC30254	7	7	6	2	7	3	6	Melon	United States	Feng et al. 2009
11	AC30289	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30291	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30292	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30357	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30359	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30360	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30361	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30362	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30363	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30364	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30365	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30366	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30370	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30371	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30378	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30379	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30380	7	7	6	2	7	3	6	unknown	China	Feng et al. 2009
11	AC30382	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC30383	7	7	6	2	7	3	6	Watermelon	China	Feng et al. 2009
11	AC30384	7	7	6	2	7	3	6	Watermelon	United States	Feng et al. 2009
11	AC30385	7	7	6	2	7	3	6	Watermelon	United States	Feng et al. 2009
11	AC_M1	7	7	6	2	7	3	6	Melon	China	Feng et al. 2009
11	AC_M6	7	7	6	2	7	3	6	Melon	Turkey	Feng et al. 2009
12	AC30080	2	7	6	2	8	3	6	Watermelon	China	Feng et al. 2009
13	AC30090	8	3	6	2	4	3	3	Watermelon	China	Feng et al. 2009
14	AC30093	9	3	6	2	4	3	3	Watermelon	China	Feng et al. 2009
15	AC30140	3	3	6	6	4	3	3	Watermelon	China	Feng et al. 2009
15	AC30143	3	3	6	6	4	3	3	Watermelon	United States	Feng et al. 2009
16	AAA30179	10	8	10	7	9	7	7	Sorghum	United States	Feng et al. 2009
17	AC30228	3	3	6	8	4	3	3	Melon	United States	Feng et al. 2009
18	AAA30296	11	9	11	9	10	8	2	Rice	United States	Feng et al. 2009
18	AAA30297	11	9	11	9	10	8	2	Rice	Israel	Feng et al. 2009
18	AAA30298	11	9	11	9	10	8	2	Rice	Israel	Feng et al. 2009
19	AAA30305	12	10	12	10	11	3	8	Vasey grass	Israel	Feng et al. 2009
20	AC30367	13	7	6	2	7	3	6	Melon	Israel	Feng et al. 2009
21	AC30373	14	7	6	11	7	3	6	Melon	Israel	Feng et al. 2009
22	AC30374	15	3	6	2	4	3	3	Watermelon	Israel	Feng et al. 2009
Q:12 23	AO19882	16	11	13	3	5	4	4	Rice	United States	Kyrpides et al. 2014

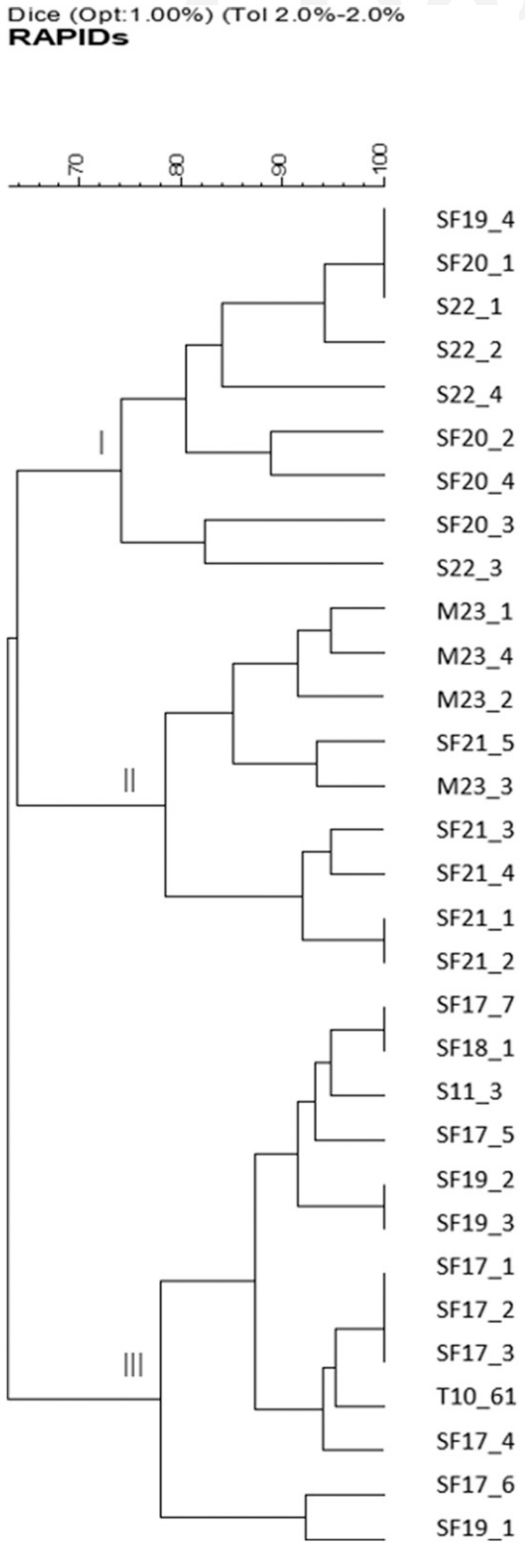

Fig. 1. Dendrogram obtained from random amplified polymorphic DNA polymerase chain reaction patterns of sugarcane *Acidovorax avenae* strains generated with M13 primer and analyzed by BioNumerics software. Similarity matrix was calculated using the Dice coefficient and the dendrogram was constructed by unweighted pair-group method with arithmetic averages analysis. Letters on the strain code represent the sugarcane-producing province, as follows: Salta (S), Santa Fe (SF), Misiones (M), and Tucumán (T). For example, T10_61 represents strain number 61 isolated from sample number 10 (INTA NA 89-686 sugarcane genotype) from Tucuman, while SF20_1 represents strain number 1 isolated from sample number 20 (INTA CP 98-828 sugarcane genotype) from Santa Fe.

performed based on RAPD profiles of 31 strains is shown in Figure 1. At a similarity level of approximately 75%, three main clusters were observed. Cluster I included five strains isolated from Santa Fe (sugarcane genotype INTA 04-1604 and INTA CP 98-828) and four from Salta (sugarcane genotype NA 02-2320) provinces; the *A. avenae* strains isolated from an "unknown" sugarcane variety cultivated in Misiones were only allocated in cluster II, together with five strains from Santa Fe also isolated from an "unknown" genotype of sugarcane; and cluster III contained only one strain from Tucumán (sugarcane genotype INTA NA 89-686), one strain from Salta, and one from Santa Fe isolated from the sugarcane genotype NA 85-1602. The remaining 10 Santa Fe strains were obtained from NA 85-1602 and INTA 04-1604. Because the number of genotypes of sugarcane sampled in the province of Santa Fe was higher compared with the other provinces (Table 1), the number of strains isolated was also higher, with these strains placed in the three clusters according to the sugarcane genotype from which they were isolated. Regarding the year of sampling, cluster I and II contained only isolates obtained in 2014 while cluster III grouped strains in 2008, 2013, and 2014 sampling years. *A. avenae* strains (three, four, and five strains from clusters I, II, and III, respectively) isolated from different sugarcane genotypes from different production regions were subjected to MLST analysis. The *A. avenae* strains T4_53, T6_50, T8_45, T10_61, and S11_3 isolated in previous work were also included in the MLST (Table 2).

Sugarcane strains have a recent clonal origin. MLST allelic profiles are reported in Table 2. Five STs, not previously described, were defined among the 15 *A. avenae* strains from sugarcane analyzed in this study; most of them were typed as ST1 or ST2 (each ST composed of six strains), whereas ST3, ST4, and ST5 were singletons. As indicated by the allelic profile analysis, the greatest variability for *A. avenae* sugarcane strains corresponded to the *lepA* gene (Table 2). The BURST algorithm clustered such sequences in a single clonal complex, indicating a common and close origin for all of them (Supplementary Fig. S2). In addition, the NJ tree was made to analyze the relationships with other *A. avenae* strains. Sugarcane strains were clustered together and separated from other strains with a high bootstrap value and four loci supporting the split, suggesting possible host specificity (Fig. 2). Topological incongruence between trees for each locus was not detected in these strains,

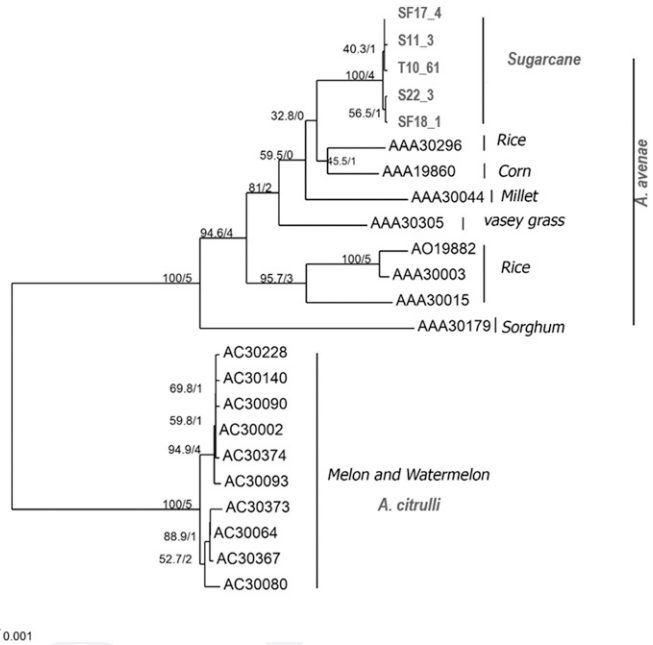

Fig. 2. Neighbor-joining tree for analyzed sugarcane strains and other *Acidovorax* strains. The tree was built based on nucleotide p-distance of seven concatenated loci. Support values (based on 1,000 bootstrap replications) are shown at each branch.

supporting the clonal behavior (Supplementary Fig. S3). A Fisher exact test showed that no significant association was found between the strains analyzed and their geographic origin.

Genetic exchange in *A. avenae*. Sugarcane strains and *A. citrulli* conformed to different clonal complexes, while other strains were not clustered together by a BURST analysis (i.e., singletons). In addition, the NJ analysis showed that such singletons were clustered in branches with low support (Fig. 2) and with high and statistically significant topological incongruence. These results indicate frequent recombination among strains (Tomasini et al. 2014). Additional information about the recombination for *A. avenae* strains was obtained by building a consensus network (Fig. 3). The network shows several square patterns indicating recombination. From the NJ tree (Fig. 2), incongruence tests, and the multidimensional scaling plot (Fig. 4), it was possible to observe that *A. oryzae* grouped with *A. avenae* from rice.

Seedling virulence assays. Sugarcane strains T10_61, S11_3. S22_3, SF17_4, and SF18_1 successfully reproduced the red stripe symptoms on sugarcane leaves. Significant differences in the severity of symptoms were observed among strains from different STs (F = 520.82; $P < 0.0001$). Strains S22_3 and S11_3 were more virulent (mean severity ratings of 3.65 and 3.11, respectively) than strains SF17_4 and SF18_1 (mean severity ratings of 2.20 and 2.30, respectively) (Table 3). Strains S22_3 and S11_3 developed lesions on leaves considered to be severe and generalized striations, affecting apical bud in some cases. Strains SF17_4 and SF18_1 exhibited an intermediate virulence, developing typical red stripe lesions on leaves. The positive control, *A. avenae* T10_61, showed a lower level of symptom severity compared with the rest of the strains (mean severity ratings of 1.60). In all cases, first symptoms were observed after 48 h of inoculation but the severity was more evident for *A. avenae* strains S22_3 and S11_3. Seedling death by apical bud rot (top rot) due to infection was not observed up to 10 dpi. *A. avenae* was successfully reisolated from inoculated sugarcane leaves.

DISCUSSION

Red stripe of sugarcane is a bacterial disease distributed among most sugarcane-producing areas in the world. In Argentina, for the

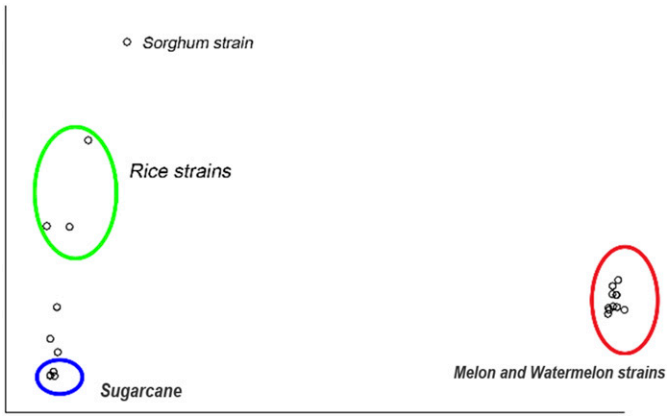

Fig. 4. Multidimensional scaling of *Acidovorax* strains based on the concatenated sequences. The two axes represent more than 90% of the variability into the data.

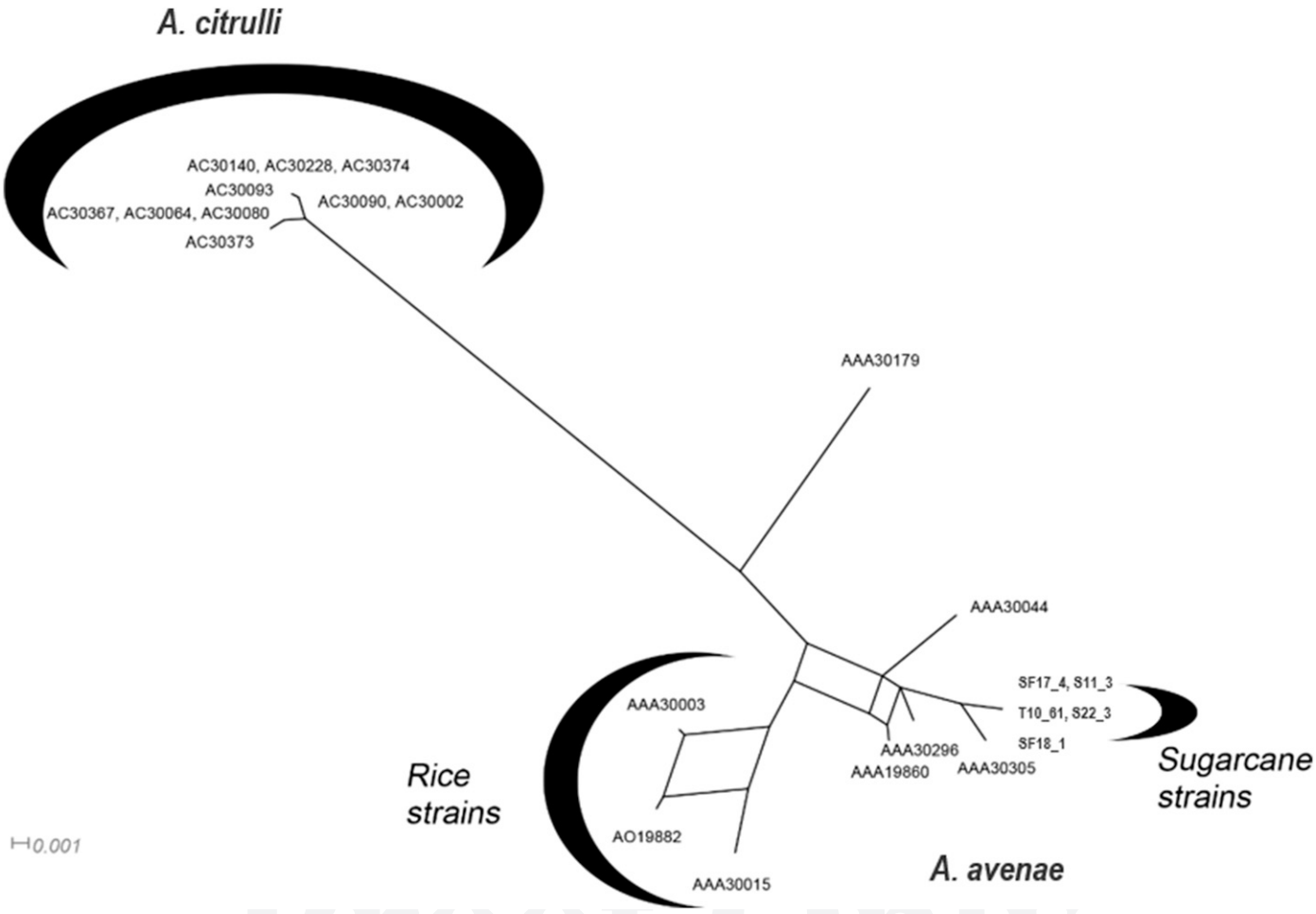

Fig. 3. Consensus network of seven loci showing possible genetic exchange. Each split in the network is shown if at least two trees had such a split. Network regions with square patterns indicates probable recombination. Sugarcane *Acidovorax avenae* strains are circled on the right side.

last 15 years, red stripe has become the most serious plant disease, causing industrial losses of 30% due to the occurrence of severe infections on susceptible sugarcane genotypes. Fontana et al. (2013) reported for the first time the isolation and identification of *A. avenae* as the causal agent of red stripe affecting sugarcane in Argentina. The main strategy adopted currently to manage this disease, after repeated infection cycles, is the replacement of the susceptible sugarcane variety by a resistant one. Due to this, knowledge of the genetic diversity among *A. avenae* is an important factor to be considered for improving an accurate diagnosis or for the selection of tolerant sugarcane varieties. To investigate their genetic similarity, *A. avenae* isolated from different sugarcane varieties infected with red stripe in 2008, 2013, and 2014 in four provinces of northern Argentina were analyzed by RAPD. The cluster analysis grouped 31 strains—29 isolated in this study and 2 previously isolated by Fontana et al. (2013)—in three main clusters. No association was observed with years of sampling and geographical origin of the strains. Based on RAPD profiles, intraspecies diversity among *A. avenae* strains isolated from sugarcane commercial varieties was observed. In accordance with Fontana et al. (2013), the presence of *A. avenae* strains adapted to sugarcane genotypes was detected. Fontana et al. (2013) analyzed *A. avenae* strains from Tucumán and Salta (northwest region) by RAPD, and these strains grouped in two main clusters by their geographical origin. The northwest region is the bigger sugarcane producer, containing 98% of total hectares of cultivation from Argentina (Benedetti 2018). Due to the increasing demand to use sugarcane as biofuel, the northeast region (Santa Fe and Misiones) is an expanding production area with great potential (Wallberg and Minetti 2015), having small growers who often cultivate different sugarcane varieties as a way to select the best adapted, representing a source of different and more diverse strains. In the present work, no clear geographical association was observed, perhaps due to the greater and different area of sampling.

A. avenae strains representative of different sugarcane genotypes covering all the sampling production areas were selected to explore their genetic diversity by applying an MLST scheme already described by Feng et al. (2009). MLST databases for other *Acidovorax* strains from different hosts and geographical origins were also included to understand the phylogenetic relationships. The MLST analysis showed that strains from sugarcane clustered together, and they have a relatively recent origin and clonal behavior, suggesting host specificity. Such host specificity in different clades of *A. avenae* was also observed for other groups (Yan et al. 2017). It was already demonstrated that there is a strong association of *A. avenae* more with the host than with the geographical origin (Feng et al. 2009; Yan et al. 2013), In this study, *A. avenae* strains from sugarcane were clustered separately from *A. citrulli* from watermelon and melon strains, and closer to *A. avenae* from species of Poaceae origin (millet, rice, corn, vasey grass, and sorghum).

Because we applied an MLST scheme design by Feng et al. (2009), in accordance with their findings, the presence of two clonal complexes grouping the *citrulli* was observed, with a clear separation from the other *A. avenae* strains and *Acidovorax* spp. Similarly, MLST analysis of 118 strains of *A. citrulli* from Chinese watermelon resulted in 73 STs that were typed into three clonal groups (Yan et al. 2013). Even though a new taxon for the *A. avenae* from rice (*A. oryzae*) was proposed by Schaad et al. (2008), we observed that *A. oryzae* is closely related to other *A. avenae* strains from rice. We also detected phylogenetic incongruence in *A. avenae*, suggesting frequent recombination in some clades. Recombination between different lineages has been described for virulence genes in some *A. avenae* that share the same host (Zeng et al. 2017). This is relevant because new, highly virulent strains may originate in such clades, where recombination is frequent (Feil et al. 1999). Recombination in other plant pathogens was also reported. Timilsina et al. (2015) found evidence of multiple recombination events between *X. euvesicatoria* and *X. perforans,* which indicate that there have been shifts in the species composition of bacterial spot pathogen populations due to the global spread of dominant genotypes and that recombination between species has generated genetic diversity in these populations.

It is important to highlight the fact that, despite their close relationships by MLST, the sugarcane strains showed virulence differences when virulence assays were performed. However, this is not contradictory because virulence factors are codified by genes that mutate faster than housekeeping genes (Moxon et al. 1994). Consequently, there is much more relevant genetic diversity that is hidden from the resolution power of MLST.

In this study, based on allelic profile analysis of seven housekeeping genes, five STs were defined among the 15 sugarcane *A. avenae* strains analyzed; most of them were typed as ST1 (containing strains from Misiones, Tucumán, Salta, and Santa Fe), whereas ST2 and its derivatives (ST3, ST4, and ST5) were in Santa Fe, Tucumán, and Salta. It could be inferred that the dominant STs are ST1 and ST2; however, for more conclusive information about more predominant STs in Argentina, it will be necessary to analyze more isolates.

The most virulent *A. avenae* strains on sugarcane genotype TucCP 77-42 were the strains S22_3 (ST4) and S11_3 (ST1) from Salta, strains SF17_4 (ST2) and SF18_1 (ST3) from Santa Fe exhibited an intermediate virulence, and the T10_61 strain (ST5) of Tucumán was less virulent. Similar results were reported by Fontana et al. (2013) when investigating *A. avenae* cross pathogenicity, observing that red stripe symptoms developed earlier in Tucumán sugarcane variety (TucCP 77-42) inoculated with a pathogenic strain from another province. Recently, Silva et al. (2016) reported high variability in disease severity when selected *A. citrulli* strains representing the most abundant PFGE-determined haplotypes observed in Brazil were used to infect watermelon seedlings.

Molecular typing methods are powerful tools to differentiate between genetically closely related organisms with acceptable reproducibility, good performance, and easy interpretation. The MLST data reported in this study provide an invaluable platform for epidemiological and evolutionary investigations of novel clones of *A. avenae* strains. The knowledge of genetic diversity and strain-host specificity has great value at the time of selecting genotypes with the best response to the red stripe disease.

TABLE 3. Mean severity and standard error (SE) values for each strains are reported[z]

Strains	Means severity ± SE
Control	0.00 ± 0.05 a
T10_61	1.61 ± 0.05 b
SF17_4	2.20 ± 0.05 c
SF18_1	2.30 ± 0.05 c
S11_3	3.11 ± 0.05 d
S22_3	3.56 ± 0.05 e

[z] Values followed by different letters are significantly different according to Fisher's least significant difference test ($P < 0.05$).

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