Genetic Structure of *Colletotrichum fruticicola* Associated to Apple Bitter Rot and Glomerella Leaf Spot in Southern Brazil and Uruguay

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ABSTRACT

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Colletotrichum fruticicola is the main species causing apple bitter rot (ABR) and Glomerella leaf spot (GLS) in southern Brazil, and ABR in Uruguay where GLS remains unnoticed. Thus, this work aimed to determine the genetic structure of *C. fruticicola* isolates of both the countries. A total of 28 out of 31 Brazilian isolates (90.3%) caused typical symptoms of GLS, while only 6 of 25 Uruguayan isolates (24.0%) originating from fruits were able to infect leaves, but causing atypical symptoms. Both populations showed similar levels of Nei's gene diversity ($h = 0.088$ and 0.079 , for

Brazilian and Uruguayan populations, respectively), and Bayesian cluster analysis inferred two genetic clusters correlated with the geographical origin of isolates. A principal coordinates analysis scatter plot and an unweighted pair group method with arithmetic mean-based dendrogram also grouped Brazilian and Uruguayan isolates into two groups. By pairwise comparison of nitrate-nonutilizing (*nit*) mutants with a proposed set of testers, all Uruguayan isolates were grouped into a unique vegetative compatibility group (namely VCG 1), while Brazilian isolates were grouped into four VCGs (VCG 1 to 4). Brazilian and Uruguayan populations of *C. fruticicola* were found to be genetically distinct. Our results suggest that isolates of *C. fruticicola* from Brazil capable of causing GLS and ABR arose independently of those from Uruguay. Possible causes leading to the evolutionary differences between populations are discussed.

Colletotrichum fruticicola Prihast., L. Cai & K.D. Hyde (2009) (Prihastuti et al. 2009) is a widely distributed fungal species that often causes plant diseases in the tropics and subtropics. Although originally described on coffee berries (*Coffea arabica* L.) (Prihastuti et al. 2009), it causes fruit rot and leaf spot in a broad range of host species around the world (Weir et al. 2012). Recently, *C. fruticicola* was found to be the main species associated with apple bitter rot (ABR) and Glomerella leaf spot (GLS) in southern Brazil, and ABR in Uruguay (Velho et al. 2015), the two important diseases of apple (*Malus domestica* Borkh.).

ABR is a fruit rot disease that causes significant losses in most countries where apple is cultivated commercially (Sutton et al. 1992). Symptoms are described as initial light brown lesions in the fruit that enlarge over time becoming brown and sunken. Each lesion usually progresses into the core of the fruit in a V-shaped pattern (Sutton et al. 1992). All apple cultivars have been considered susceptible to ABR (Denardi et al. 2003).

On the other hand, the occurrence of GLS is still limited to a few regions of the world, including southeastern United States (González et al. 2006), southern Brazil (Velho et al. 2015), and China (Wang et al. 2015). In Brazil, GLS was first described in 1983 and since then it has strongly affected the local economy (Sutton and Sanhueza 1998). Despite the relative proximity to Brazilian producing areas, this disease remains unnoticed in Uruguayan orchards (Velho et al. 2015). Symptoms of GLS consist of reddish-purple leaf spots evolving to irregular necrotic lesions. Frequently, leaves turn yellow

and fall off (Araújo and Stadnik 2013). On fruits, it can cause small sunken lesions (1 to 3 mm) that rarely develop into a rot (Velho et al. 2015). Apple cultivars descending from the 'Golden Delicious' group, in particular the cultivar Gala, are highly susceptible to GLS, whereas those from the 'Red Delicious' group, such as the cultivar Fuji, are completely resistant (Araújo and Stadnik 2013).

It is unknown how virulent GLS isolates arose in regions so far apart from each other such as southeastern United States, southern Brazil, and China, but not in others like Uruguay. In this context, genetic structure studies of *C. fruticicola* isolates causing ABR and GLS can be helpful to understand the impact of environmental conditions, and geographical barriers influencing the distribution of populations pathogenic to fruits and leaves of apple across the world (McDermott and McDonald 1993; McDonald 1997). It can also provide information on how evolutionary forces such as selection, recombination, and gene flow can affect the persistence and viability of highly aggressive isolates, and the generation and distribution of new fungal genotypes having a potential to cause disease (McDermott and McDonald 1993; McDonald and Linde 2002).

Restriction fragment length polymorphism (RFLP)- and multi-locus gene sequencing (MGS)-based analyses were already tentatively used to differentiate populations of *Colletotrichum* spp. causing apple diseases (González et al. 2006; Velho et al. 2015). Although MGS-based analysis could successfully differentiate *Colletotrichum* spp. causing ABR and GLS in Brazil and Uruguay (Velho et al. 2015), inter-simple sequences repeat (ISSR) molecular markers can be applied to resolve the genetic structure of *Colletotrichum* populations at a finer taxonomic level (Kumar et al. 2011; McKay et al. 2009; Rampersad 2013; Ratanacherdchai et al. 2010). The evolutionary rate within the microsatellite loci is generally higher than other genome regions, thus enabling the detection of polymorphism at individual locus (Wolfe and Liston 1998). ISSR markers also allow to differentiate populations according

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to their geographical origin or host preferences, and to estimate gene diversity, genetic differentiation, and gene flow among populations (Kumar et al. 2011; McKay et al. 2009; Rampersad 2013; Ratanacherchai et al. 2010).

Determination of the vegetative compatibility group (VCG) is frequently used to complement the molecular-based analysis while studying the population biology of pathogens (Brooker et al. 1991; Leslie 1993). This technique is based on complementation tests using nitrate nonutilizing (*nit*) mutants, which are impaired to utilize nitrate as nitrogen source. When two different and compatible *nit* mutants anastomose, a biochemical complementation takes place in the viable heterokaryon that is detectable as the restitution of a prototrophic growth at the contact zone (Leslie 1993). Knowledge of *nit* mutations of testers can facilitate the determination of VCGs because complementation tests can then be directed at choosing complementary *nit* mutant pairs. VCGs have been previously employed to study the relationship between *Colletotrichum* populations from North American and Brazilian apple orchards (González et al. 2006).

The objective of this work was to determine the genetic structure of the *C. fruticola* species causing ABR and GLS in southern Brazil, and ABR in Uruguay, by means of pathogenicity tests, ISSR-based analysis, and VCGs.

MATERIALS AND METHODS

Isolates collection. Fifty-six *C. fruticola* isolates were selected for this study. Among these, 31 Brazilian isolates causing ABR (14) or GLS (17) were obtained from the mycological collection (MANE) of the Federal University of Santa Catarina, and 25 ABR isolates from Uruguay were provided by the culture collection (C) of the University of the Republic. Forty-six of them were previously identified by Velho et al. (2015), and 10 further GLS isolates (derived from Fraiburgo-SC, Brazil) were likewise identified.

Pathogenicity tests. The pathogenicity of isolates was tested on susceptible apple seedlings according to Araújo and Stadnik (2013). Briefly, for each isolate, three 2-month-old seedlings were inoculated by spraying the leaves with a conidial suspension 1×10^6 conidia/ml), and maintained for 48 h at 25°C in dark and 100% relative humidity. Subsequently, seedlings were transferred to a greenhouse (25°C $\pm$ 5°C) for further 15 days, and then assessed for incidence of symptoms of GLS. The assays were arranged in a completely randomized design.

ISSR analysis. Fungal DNA was extracted from monospore cultures according to Gupta et al. (2013). Six isolates (MANE147, MANE34, MANE76, MANE38, C5, and C29) were used in preliminary screening to determine primers enabled to generate polymorphic and reproducible ISSR fingerprints (Table 1). PCR was performed with a 20- μ l mixture containing 1 $\times$ PCR buffer, 0.4 mM dNTPs, 0.4 μ M primers, 1 U of *Taq* DNA polymerase (SBS, Genetech Co., Ltd., China), and 5 to 15 ng of fungal genomic DNA. Standard PCR conditions were as follows: an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 48°C (primers (AG)₈ and (CT)GA₈) or 51.5°C (primer (CAG)₅), and 90 s at 72°C; with a final extension step of 5 min at 72°C. The reactions were carried out in a thermal cycler (Labnet International, Inc.), amplicons were separated on 1.5% (wt/vol) agarose gel, stained with GelRed (Biotium, Hayward, CA), and visualized under UV light. A gene ruler 100-bp DNA ladder plus (Fermentas Life Science, Leon-Rot, Germany) was used as molecular weight marker. ISSR profiles were analyzed digitally using GelAnalyzer 2010 Software (<http://www.gelanalyzer.com>). Only bands of sizes between 100 and 1,500 bp that could be digitally detected were used, with the assumption that each band represented a distinct locus. Amplicons that shared the same molecular weight were considered the same locus, and data were transformed into a binary matrix (1 = presence, 0 = absence).

Genetic Analysis in Excel (GenAIE) v.6.501 Software (Peakall and Smouse 2006) was used to calculate allele frequencies, bands

polymorphism, and Nei's gene diversity (Nei 1973), and to generate a principal coordinates analysis (PCoA) scatter diagram. Bayesian clustering was implemented with the STRUCTURE 2.3 Software (Pritchard et al. 2000) to determine the probability of each isolate belongs to a distinct genetic cluster, assuming admixture and correlated allele frequency. Model was first run for 5×10^4 Markov chain Monte Carlo iterations after an initial burn-in period of 1×10^4 generations, from $K = 1$ to $K = 10$.

An unweighted pair group method with arithmetic mean (UPGMA)-based dendrogram was performed by Paleontological Statistics package (PAST) v.2.17b software (Hammer et al. 2001) to determine phylogenetic relationships between isolates. Dendrogram was generated using Dice's genetic similarity coefficient, and nodes weights were calculated with 1×10^4 bootstrap resamplings performed with WinBoot software (Yap and Nelson 1996).

Complementation tests and identification of *nit* mutants.

Generation of all *nit* mutants was carried out according to Brooker et al. (1991). Isolates were first grown on Petri plates containing potato dextrose agar (PDA) medium supplemented with 1.5% of potassium chlorate (KClO₃) for 15 to 20 days at 25°C with 12 h photoperiod white fluorescent light. Three-millimeter-diameter agar discs with mycelia were picked up from the sectors showing dense growth, and arranged on Petri plates containing basal medium (BM) supplemented with sodium nitrate at 3.0 g/liter (minimum medium [MM]). Plates were incubated at 25°C in dark for 15 to 20 days, and *nit* mutants were identified by characteristic thin growth.

Complementation tests were carried out on 12-well Corning Costar cell culture plates (Sigma-Aldrich, St. Louis) containing MM. Two 3-mm-diameter agar discs containing mycelia of the generated *nit* mutants and testers were placed into a well about 1 cm away from each other. Plates were incubated at 25°C in dark for 15 to 20 days, and isolates that generated a white and dense heterokaryotic mycelium at the contact zone were considered compatible.

Phenotypic identification of *nit* testers was based on visual observation of their growth on BM supplemented with each one of the following nitrogen sources: (i) nitrate (3.0 g/liter); (ii) nitrite (0.5 g/liter); (iii) hypoxanthine (0.2 g/liter); and (iv) uric acid (0.2 g/liter). Petri plates were kept at 25°C in dark for 15 to 20 days, and phenotypes were identified as described by Brooker et al. (1991).

RESULTS

Pathogenicity tests. Twenty-eight Brazilian isolates (90.3%) caused typical GLS symptom on seedling leaves (Table 2). Symptoms started with appearance of reddish-purple irregular shaped spots at 48 h after inoculation that became necrotic within 7 days (Fig. 1A). Only three isolates (MANE34, MANE40, and MANE75) obtained from ABR did not cause any symptom. In contrast, six of the Uruguayan isolates (C27, C28, C29, C31, C33, and C39) infected leaves causing GLS-like symptoms. However,

TABLE 1. Characteristics of inter-simple sequence repeat primers screened

Primer ^a	Anchor	Ta (°C) ^b	Polymorphic	Reproducible
*(AG) ₈	Anchorless	48	Yes	Yes
(AC) ₈	Anchorless	48	Yes	No
(GACA) ₄	Anchorless	51.5	No	No
(GTG) ₅	Anchorless	51.5	Yes	No
(GACAC) ₃	Anchorless	48	Yes	No
(TGTC) ₄	Anchorless	51.5	No	No
*(CAG) ₅	Anchorless	51.5	Yes	Yes
BBD(AAC) ₅	5' anchor present	53	No	No
BDB(ACA) ₅	5' anchor present	53	No	No
(AG) ₈ TG	5' anchor present	48	Yes	No
*CT(GA) ₈	5' anchor present	48	Yes	Yes

^a * indicates primers selected to analyze all isolates.

^b Annealing temperature.

these isolates were less aggressive, provoking fewer and smaller reddish diffuse lesions on leaves, which appeared for more than 10 days after inoculation, but seldom progressed to necrotic symptoms (Fig. 1B and C). Leaf infection was confirmed with these isolates by re-isolating the fungus from atypical spots.

ISSR markers and bands characteristics. The three primers CT(GA)₈, (AG)₈, and (CAG)₅ could generate polymorphic and reproducible band patterns in the screening, and were used to analyze all isolates (Table 1). A total of 109 polymorphic bands (PB) were generated with these primers (Table 3). The highest number of PB

were produced by (AG)₈ primer (47 PB), followed by CT(GA)₈ (36 PB) and (CAG)₅ (26 PB) (Supplementary Fig. S1). A high level of polymorphism was observed by considering Brazilian and Uruguayan isolates as a whole (95.41%). However, when considered as two independent populations, the level of polymorphism was 32.11 and 22.02% for Brazilian and Uruguayan groups, respectively (Table 3).

Gene diversity. Gene diversity (*h*), unbiased gene diversity (*uh*), and number of different (*Na*) and effective (*Ne*) alleles are given in Table 3. For a whole population, *h* = 0.084 and *uh* = 0.087,

TABLE 2. Leaf pathogenicity of *Colletotrichum fructicola* isolates fruits and leaves exhibiting apple bitter rot (ABR) and Glomerella leaf spot (GLS) symptoms in Brazil and Uruguay

Species	Geographical origin ^a	Source			Isolate code	Pathogenicity to leaves ^b
		Cultivar	Disease	Host tissue		
<i>C. fructicola</i>	RS/Brazil	Gala	ABR	Fruit	MANE34	–
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE35	+
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE36	+
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE37	+
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE38	+
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE39	+
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE40	–
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE41	+
<i>C. fructicola</i>	RS/Brazil	Gala	ABR	Fruit	MANE51	+
<i>C. fructicola</i>	RS/Brazil	Gala	ABR	Fruit	MANE54	+
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE71	+
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE73	+
<i>C. fructicola</i>	RS/Brazil	Gala	ABR	Fruit	MANE75	–
<i>C. fructicola</i>	SC/Brazil	Gala	ABR	Fruit	MANE90	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE5	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE7	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE8	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE9	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE12	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE16	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE17	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE26	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE28	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE31	+
<i>C. fructicola</i>	RS/Brazil	Gala	GLS	Leaf	MANE55	+
<i>C. fructicola</i>	RS/Brazil	Gala	GLS	Leaf	MANE57	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE60	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE70	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE76	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE91	+
<i>C. fructicola</i>	SC/Brazil	Gala	GLS	Leaf	MANE147	+
<i>C. fructicola</i>	MO/Uruguay	Cripps Pink	ABR	Fruit	C1	–
<i>C. fructicola</i>	MO/Uruguay	Cripps Pink	ABR	Fruit	C2	–
<i>C. fructicola</i>	MO/Uruguay	Cripps Pink	ABR	Fruit	C3	–
<i>C. fructicola</i>	MO/Uruguay	Oregon spur	ABR	Fruit	C5	–
<i>C. fructicola</i>	MO/Uruguay	Oregon spur	ABR	Fruit	C7	–
<i>C. fructicola</i>	MO/Uruguay	Oregon spur	ABR	Fruit	C8	–
<i>C. fructicola</i>	CA/Uruguay	Granny Smith	ABR	Fruit	C9	–
<i>C. fructicola</i>	CA/Uruguay	Granny Smith	ABR	Fruit	C10	–
<i>C. fructicola</i>	CA/Uruguay	Fuji	ABR	Fruit	C13	–
<i>C. fructicola</i>	CA/Uruguay	Fuji	ABR	Fruit	C14	–
<i>C. fructicola</i>	CA/Uruguay	Granny Smith	ABR	Fruit	C17	–
<i>C. fructicola</i>	CA/Uruguay	Cripps Pink	ABR	Fruit	C22	–
<i>C. fructicola</i>	MO/Uruguay	Granny Smith	ABR	Fruit	C27	+
<i>C. fructicola</i>	CA/Uruguay	Red delicious	ABR	Fruit	C28	+
<i>C. fructicola</i>	CA/Uruguay	Cripps Pink	ABR	Fruit	C29	+
<i>C. fructicola</i>	CA/Uruguay	Cripps Pink	ABR	Fruit	C30	–
<i>C. fructicola</i>	CA/Uruguay	Cripps Pink	ABR	Fruit	C31	+
<i>C. fructicola</i>	CA/Uruguay	Granny Smith	ABR	Fruit	C33	+
<i>C. fructicola</i>	CA/Uruguay	Granny Smith	ABR	Fruit	C35	–
<i>C. fructicola</i>	CA/Uruguay	Granny Smith	ABR	Fruit	C36	–
<i>C. fructicola</i>	CA/Uruguay	Fuji	ABR	Fruit	C38	–
<i>C. fructicola</i>	CA/Uruguay	Fuji	ABR	Fruit	C39	+
<i>C. fructicola</i>	CA/Uruguay	Fuji	ABR	Fruit	C40	–
<i>C. fructicola</i>	CA/Uruguay	Fuji	ABR	Fruit	C41	–
<i>C. fructicola</i>	CA/Uruguay	Fuji	ABR	Fruit	C42	–

^a RS, Rio Grande do Sul; SC, Santa Catarina; MO, Montevideo; CA, Canelones.

^b Leaf pathogenicity tests were performed on seedlings of Gala. +, typical GLS symptoms (Fig. 1); –, no symptom; and +*, atypical symptoms characterized by reddish diffuse lesions with longer incubation period (10 days) and seldom necrotizing of leaves (Fig. 1).

and when considering two populations, $h = 0.088$ and $uh = 0.091$ were estimated for Brazilian population, and $h = 0.079$ and $uh = 0.082$ for Uruguayan population. Number of different and effective alleles were $Na = 0.734$ and $Ne = 1.139$ for a whole population, and considering two populations, $Na = 0.835$ and $Ne = 1.141$ were estimated for Brazilian population, and $Na = 0.633$ and $Ne = 1.136$ for Uruguayan population.

Population stratification. Plot of ancestry assignment resulted in grouping Brazilian and Uruguayan isolates of *C. fructicola* into two distinct genetic clusters ($P > 0.8$) (Fig. 2). The individual's estimated membership proportion of Brazilian and Uruguayan isolates to each group were 55.4 and 44.6%, respectively. PCoA scatter plot also distributed Brazilian and Uruguayan isolates forming two independent groups, which showed a clear correlation with the geographical origin instead of host tissue preference (Fig. 3).

Phylogenetics relationships. UPGMA-based dendrogram grouped Brazilian and Uruguayan populations separately with 100% of BS value, and shared up to 78% of genetic similarity (Fig. 4). Moreover, Uruguayan and Brazilian isolates shared up to 80 and 85% of genetic similarity, respectively, and no internal node within each group was found to be significant when bootstrap analysis was performed.

Vegetative compatibility grouping. Vegetative complementation grouping of isolates with the proposed set of testers allowed the classification of 55 (98.2%) of the 56 isolates into four VCGs named VCG 1, 2, 3, and 4 (Fig. 4). All Uruguayan and two Brazilian isolates (MANE39 and MANE76) were included in VCG 1. On the other hand, 18 Brazilian isolates were included in VCG 2, seven in VCG 3, and three in VCG 4. One Brazilian isolate (MANE37) did not grow in PDA/KClO₃ mutagenic medium, and therefore, it was not possible to produce its *nit* mutants (Fig. 4).

Nit mutant phenotypes identification. Three types of *nit* mutants (*nit1*, *nit2*, and *nit3*) were identified for the set of testers used in the complementation assays (Table 4). Thus, pairs of testers representing VCG 1, 2, and 3 were identified as *nit1* and *nit3*; and VCG 4 as *nit2* and *nit3* (Supplementary Fig. S2).

DISCUSSION

ISSR analysis has been successfully employed in a number of studies on genetic diversity involving *Colletotrichum* spp. from different hosts (Kumar et al. 2011; McKay et al. 2009; Rampersad 2013). In our study, it allowed a separation of isolates of *C. fructicola* from southern Brazil and Uruguay, but not according to the disease that they preferentially cause. Brazilian isolates were grouped into a unique genetic cluster, and the majority caused typical GLS symptoms. On the other hand, all Uruguayan isolates were grouped into another cluster

and only a few were able to infect and cause symptoms on leaves, but not typical ones.

Differences in the ability to cause GLS observed between Uruguayan and Brazilian populations may have been a result of growing different cultivars in both countries for many years. Whereas the Gala (highly susceptible to GLS) is the main cultivar produced in Brazil, in Uruguay there is a predominance of those descending from 'Delicious' group such as 'Red Delicious' (53% of total production) (MGAP 2015), which are considered to be resistant to disease (Araújo and Stadnik 2013). Thus, it is possible to assume that susceptible apple Gala, but also others descending from 'Golden Delicious' such as 'Cripps Pink', could act as a selection force on fruit-rotting populations of *C. fructicola* favoring the emergence of leaf disease, namely GLS (González et al. 2006; Velho et al. 2015; Wang et al. 2015). Interestingly, although most Uruguayan isolates did not cause typical GLS symptoms on apple leaves, a few of them could infect plants and provoke spots resembling the initial stages of disease and with a longer incubation period, but seldom resulting in leaf tissue necrotization (Velho et al. 2015). This observation suggests that such Uruguayan leaf-infecting isolates of *C. fructicola* are in a transitional evolutionary phase, because they are not well adapted to leaf tissue colonization yet (McDonald and Linde 2002).

TABLE 3. Band characteristics and gene diversity inferred from inter-simple sequence repeat profiles of Brazilian and Uruguayan isolates of *Colletotrichum fructicola* causing apple bitter rot and Glomerella leaf spot on apple

Index	Overall	Brazilian population	Uruguayan population
Number of isolates	56	31	25
Polymorphic bands	109	56	53
Bands (frequency >5%)	72	55	44
Private bands	109	12	1
Local bands (<50%)	0	0	0
Mean Na^a	0.734	0.835	0.633
SE Na	0.058	0.085	0.079
Mean Ne^b	1.139	1.141	1.136
SE Ne	0.019	0.026	0.028
Mean h^c	0.084	0.088	0.079
SE of Mean h	0.011	0.015	0.015
Mean uh^d	0.087	0.091	0.082
SE of Mean uh	0.011	0.015	0.016
Polymorphism (%)	95.41	32.11	22.02

^a Na : number of different alleles.

^b Ne : number of effective alleles ($Ne = 1/(p^2 + q^2)$, where p = band frequency and $q = 1 - p$, for haploid binary data).

^c h : gene diversity, where $h = 1 - (p^2 + q^2)$.

^d uh : unbiased gene diversity, where $uh = [N/(N - 1)] \times h$.

Fig. 1. A, Typical and B and C, atypical Glomerella leaf spot symptoms caused by Brazilian leaf-infecting isolate MANE147 (A), and Uruguayan fruit-infecting isolate C29 (B and C) of *Colletotrichum fructicola*, respectively. C, Bar = 1 mm.

Reproductive strategies can have great impact on population biology of fungal pathogens, which can show sexual and asexual

Brazilian and Uruguayan populations of *C. fruticicola* showed marked differences in terms of vegetative compatibility. Interestingly, all Uruguayan isolates were grouped into only one VCG (VCG 1) similar to ISSR-based grouping, suggesting that they derived from a common population (Leslie 1993). On the other hand, four VCGs were determined for the Brazilian population, each one including both ABR and GLS isolates. Thus, it is possible

and Brazil belonged to distinct VCGs and concluded that they emerged independently. However, we showed that Brazilian isolates MANE76 and MANE39 belonged to VCG 1, but according to ISSR-based grouping they were not different from other Brazilian isolates. Accordingly, Velho et al. (2015) through

TABLE 4. Phenotypes identification of the nitrate nonutilizing (*nit*) set of testers belonging to the four vegetative compatibility groups (VCG) of *Colletotrichum fructicola*, according to growth into four nitrogen sources (nitrate, nitrite, hypoxanthine, and uric acid)^a

Species	Isolate designation	VCG	Geographical origin	Host tissue	Nitrogen sources				<i>Nit</i> mutants
					Nitrate	Nitrite	Hypoxanthine	Uric acid	
<i>C. fructicola</i>	C29	1	CA/Uruguay	Fruit	—/—	—/+	+/+	+/+	<i>nit3/nit1</i>
<i>C. fructicola</i>	BR1	2	SC/Brazil	Leaf	—/—	—/+	+/+	+/+	<i>nit3/nit1</i>
<i>C. fructicola</i>	MANE55	3	RS/Brazil	Leaf	—/—	+/-	+/+	+/+	<i>nit1/nit3</i>
<i>C. fructicola</i>	MANE54	4	RS/Brazil	Fruit	—/—	—/—	—/+	+/+	<i>nit2/nit3</i>

^a CA, Canelones; SC, Santa Catarina; RS, Rio Grande do Sul; —, thin growth without aerial mycelium; and +, wild-type growth phenotype.

MGS-based analysis found that two Brazilian isolates, i.e., MANE76 and MANE91, were more related to Uruguayan ones, which further strengthens the fact that there is a link between both populations.

The reasons for almost simultaneous emergence of GLS disease in distant regions of the world are still far from being uncovered. One hypothesis for the origin of Brazilian leaf-infecting isolates of *C. fructicola* is that they would have originated from those pathogenic to fruits. This is supported by the fact that Brazilian isolates causing ABR and GLS were grouped into the same genetic cluster, and because the four Brazilian VCGs shared isolates causing both diseases. It is highly unlikely that isolates causing ABR have been originated from isolates causing GLS, because the former disease has been known for about 150 years, while the latter for about 3 decades. In our work, we found some evidences that potentially leaf-infecting isolates already exist in Uruguayan population at a low frequency, but possibly environmental conditions and use of resistant cultivars could have hindered their spreading.

Another hypothesis for the GLS disease emergence in different countries is that the genotypes causing GLS could have disseminated from one region to the other. This seems, however, less probable since the conidia of *Colletotrichum* spp. are dispersed by water splashing that restricts their spread to short distances (Sutton and Shane 1983). On the other hand, it has been demonstrated that *C. fructicola* can survive overwinter in apple dormant buds and twigs (Crusius et al. 2002) that would allow, theoretically, its long-distance dispersal. Nevertheless, Brazilian imports of both apple fruits and propagative plant material have been, until now, made from GLS-free countries (MAPA 2015). Hence, comparing isolates from distant world regions where GLS occur will be an exciting challenge for future research.

In this study, the *C. fructicola* isolates causing ABR and GLS in southern Brazil and Uruguay were revealed to be genetically distinct. Brazilian GLS isolates were found to be more closely related to isolates causing ABR in Brazil than those in Uruguay, suggesting that they arose independently from the Uruguayan population.

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