Colletotrichum species associated to ripe rot disease of grapes in the "Serra Gaucha" region of Southern Brazil

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Abstract. Ripe rot disease caused by *Colletotrichum* (*Glomerella*) has become a serious problem for viticulture in Southern Brazil. Global warming contributes to the increase of this devastating and difficult to control disease. Several species of *Colletotrichum*, with different phytopathological characteristics, have been associated with ripe rot disease in different viticultural regions. In this article, a total of 63 fungi were isolated from grapes showing symptoms of ripe rot disease, and classified by sequencing of ITS region, and compared with the sequences deposited in GenBank. The isolates were included in three clades of *Colletotrichum*: 84.1% belonged to the "gloeosporioides" clade, 3.2% to the "boninense" clade, and 12.7% to the "acutatum" clade. Of the 53 isolates included in the "gloeosporioides" clade, 44.4% were classified as *C. viniferum*/*C. ampelinum*, 37.1% as *C. fruticola*, 13.0% as *C. kahawae* and 5.5% as a species related to *C. fruticola*. In turn, the two isolates of the "boninense" clade were classified as *C. kartii*/*C. phyllanthi*, and the six "acutatum" isolates were similar to *C. acutatum* and *C. nymphaeae* reference materials. The identified species were previously linked to ripe rot disease in other viticulture regions of the world, but the frequency of some species in southern Brazil is particularly different.

1. Introduction

Viticulture is economically important in at least six Brazilian states (Rio Grande do Sul, Santa Catarina, São Paulo, Minas Gerais, Bahia and Pernambuco). Statistical data (2017) showed that the highlands of Rio Grande do Sul, known as "Serra Gaucha" region, is responsible for the production of about 750 thousand tons of grapes, and 480 million liters of wines, representing almost 90% of Brazilian wine production. Ninety percent of the wines are produced with *V. labrusca* and hybrid varieties, and 10% are considered as "fine" wines elaborated with *V. vinifera* varieties, like Chardonnay, Cabernet Sauvignon, Merlot, among others [1].

The "Serra Gaucha" region (29°10′S/51° 32′W) has an mean altitude of 640 m and a humid temperate/subtropical climate with a mean annual temperature of 17.2 °C, and a precipitation of 1736 mm per year. During vintage (February/April) the maximum temperatures reach 27 to 28 °C with thermal amplitude of approximately 10 °C, and precipitation of 120 to 150 mm per month [2]. The high temperatures and humidity during the summer season is responsible for a high incidence of bunch diseases, which control requires the application of fungicides near the harvest. The most common grape bunch diseases in the "Serra Gaucha" region are the Botrytis rot, black rot, sour rot, bitter rot, and ripe rot.

Surveys of grape diseases [3] showed that ripe rot was relatively rare until 2000/2001, but has increased significantly in recent decades, especially in years with high humidity and high temperatures near the harvesting time. Moreover, predictions based on climate change indicate in a near future, ripe rot will be one of the most important diseases of grapes in the highlands of Southern Brazil [4]. Aside from yield losses, ripe rot disease negatively impacts on grape and wine quality parameters including color, flavor and chemical composition leading to an increase residual sugar, volatile acidity, glycerol, gluconic acid, and malic acid [5].

The grape ripe rot disease is caused by the fungus *Glomerella cingulata* (Stonemam) Spauld & Schrenk, the perfect or sexual phase of *Colletotrichum gloeosporioides* (Penz.) [6], but many other species of this genus, eg. *C. acutatum* [6–8], *C. fruticola*, *C. viniferum* [9] and *C. capsici* [10] have been associated with the disease in several winegrowing regions of the world.

In grapes *Colletotrichum* shows a hemibiotrophic behavior characterized a quiescent or biotrophic phase that evolved to a necrotrophic phase under appropriate conditions. During disease development, *Colletotrichum* alkalinizes host-tissue by the active secretion of ammonia [11]. The alkalinization of host tissue stimulates appressorium formation [12], and the secretion of pathogenicity factors, such as pectate lyase [13], cutinase, and peptidases [14]. In the necrotrophic phase berries rot and develop the classical disease symptoms that include circular, reddish brown spots on skin, which enlarge to include entire berry. With disease progression, berries are covered by salmon/orange-colored conidia from fruiting bodies (or acervuli) that further spread the disease.

Although most *Colletotrichum* species share an overall similar behavior, each species have particular microbial-host interactions that result in certain host specificity. Moreover, *Colletotrichum* species can be more or less

Table 1. Isolates, geographical origin and grape varieties.

Code	Grape variety	Geographic origin (county)	Code	Grape variety	Geographic origin (county)
CI001	Isabella	Bento Gonçalves	CA035	Concord	Garibaldi
CA002	Niagara Rosa	Bento Gonçalves	CI036	Isabella	Monte Belo do Sul
CI003	Isabella	Bento Gonçalves	CI037	Isabella	Bento Gonçalves
CA004	Moscato hybrid	Bento Gonçalves	CI039	Isabella	Caxias do Sul
CI005	Isabella	Bento Gonçalves	CI040	Isabella	Pinto Bandeira
CI006	Isabella	Veranópolis	CA041	Cabernet Franc	Pinto Bandeira
CA007	Trebbiano	Veranópolis	CI042	Isabella	Pinto Bandeira
CI008	Isabella	Monte Belo do Sul	CA043	Moscato hybrid	Pinto Bandeira
CI009	Isabella	Monte Belo do Sul	CA044	Malvasia	Pinto Bandeira
CA010	Trebbiano	Monte Belo do Sul	A001-18	Isabella	Caxias do Sul
CI011	Isabella	Bento Gonçalves	A002-18	Moscato Branco	Farroupilha
CI012	Isabella	Bento Gonçalves	A004-18	Cabernet Sauvignon	Farroupilha
CI013	Isabella	Cotiporã	A21-17	Merlot	Bento Gonçalves
CI014	Isabella	Bento Gonçalves	44-17	Ives	Antonio Prado
CI015	Isabella	Bento Gonçalves	A056-17	Isabella	Veranópolis
CA016	Niagara Rosa	Bento Gonçalves	A031-18MF	Merlot	Nova Pádua
CA017	Niagara Branca	Bento Gonçalves	A031-18M9	Ives	Nova Pádua
CA018	Malvasia	Bento Gonçalves	LMF18-1	Lorena	Flores da Cunha
CI019	Isabella	Bento Gonçalves	LMF18-2	Lorena	Farroupilha
CI020	Isabella	Bento Gonçalves	LMF18-3	Lorena	Ipê
CI021	Isabella	Bento Gonçalves	LMF18-4	Moscato EMBRAPA	Pinto Bandeira
CI022	Isabella	Bento Gonçalves	LMF18-6	Lorena	Farroupilha
CI023	Isabella	Pinto Bandeira	LMF18-7	Moscato EMBRAPA	Pinto Bandeira
CA024	Cabernet Sauvignon	Pinto Bandeira	LMF18-8	Moscato	Pinto Bandeira
CI025	Isabella	Veranópolis	LMF18-10	Merlot	Flores da Cunha
CI026	Isabella	Veranópolis	LMF18-12	Merlot	Caxias do Sul
CI027	Isabella	Veranópolis	LMF18-14	Merlot	Caxias do Sul
CI028	Isabella	Cotiporã	LMF18-16	Cabernet Sauvignon	Caxias do Sul
CI029	Isabella	São Valentim do Sul	LMF18-17	Cabernet. Sauvignon	Caxias do Sul
CI030	Isabella	São Valentim do Sul	CA045	APPLE	Vacaria
CI031	Isabella	São Valentim do Sul	A004-17 (19)	APPLE	Vacaria
CI032	Isabella	Cotiporã	A006-14	PERSIMMON	Farroupilha
CI033	Isabella	Bento Gonçalves	A60-18	PERSIMMON	Caxias do Sul
CI034	Isabella	Garibaldi	A61-18	PERSIMMON	Vacaria

pathogenic, aggressive, or resistant to fungicides, which implies differences on agricultural practices and control systems.

Traditionally, identification of *Colletotrichum* species has been based on the shape and size of conidia, appressoria characteristics, colony morphology, presence of setae, physiological traits, among others [15]. These criteria are variable and led to misidentification of species in numerous cases. To resolve this problem, several molecular techniques has been used with more or less success. Among these, the analysis of internal transcribed spacer (ITS) region of rDNA has been found to be efficient and reliable for *Colletotrichum* classification [15].

In this context, the present work aimed to determine the *Colletotrichum* species associated to ripe rot disease of grapes in the “Serra Gaucha” region, in order to contribute to epidemiologic studies and disease management.

2. Material and methods

2.1. Samples

Grape fruits showing typical symptoms of ripe root were collected from vineyards of the most important grape-growing counties of the “Serra Gaucha” region (Table 1). Two apple and three persimmon fruits with anthracnose symptoms were included as representatives of other fruits produced in the region. Skin tissues of approximately 5 mm in diameter were collected, surface-sterilized with 1% NaClO for 1 min, washed twice with sterile distillated water, and partially dried with filter paper. The tissues were plated on PDA (potato-dextrose-agar) amended with gentamicin (50 mg/L). The plates were incubated at 27 °C for 4 days. Single-spore cultures were maintained in PDA slants, and stored at −80 °C with 25% glycerol.

2.2. DNA extraction

DNA was extracted from fungal mycelia using the method proposed Tapia-Tussell et al., [16]. DNA was quantified by absorbance at 260 nm, and the quality estimated by 260/280 ratio.

2.3. Amplification of the ITS region

The ITS1-5.8S-ITS2 region of rDNA of fungal isolates were amplified by PCR using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [17]. PCR amplifications (25 µl) were performed in a Veriti (Applied Biosystems) thermocycler programmed for 35 cycles of denaturation at 94 °C for 60 min, annealing at 55 °C for

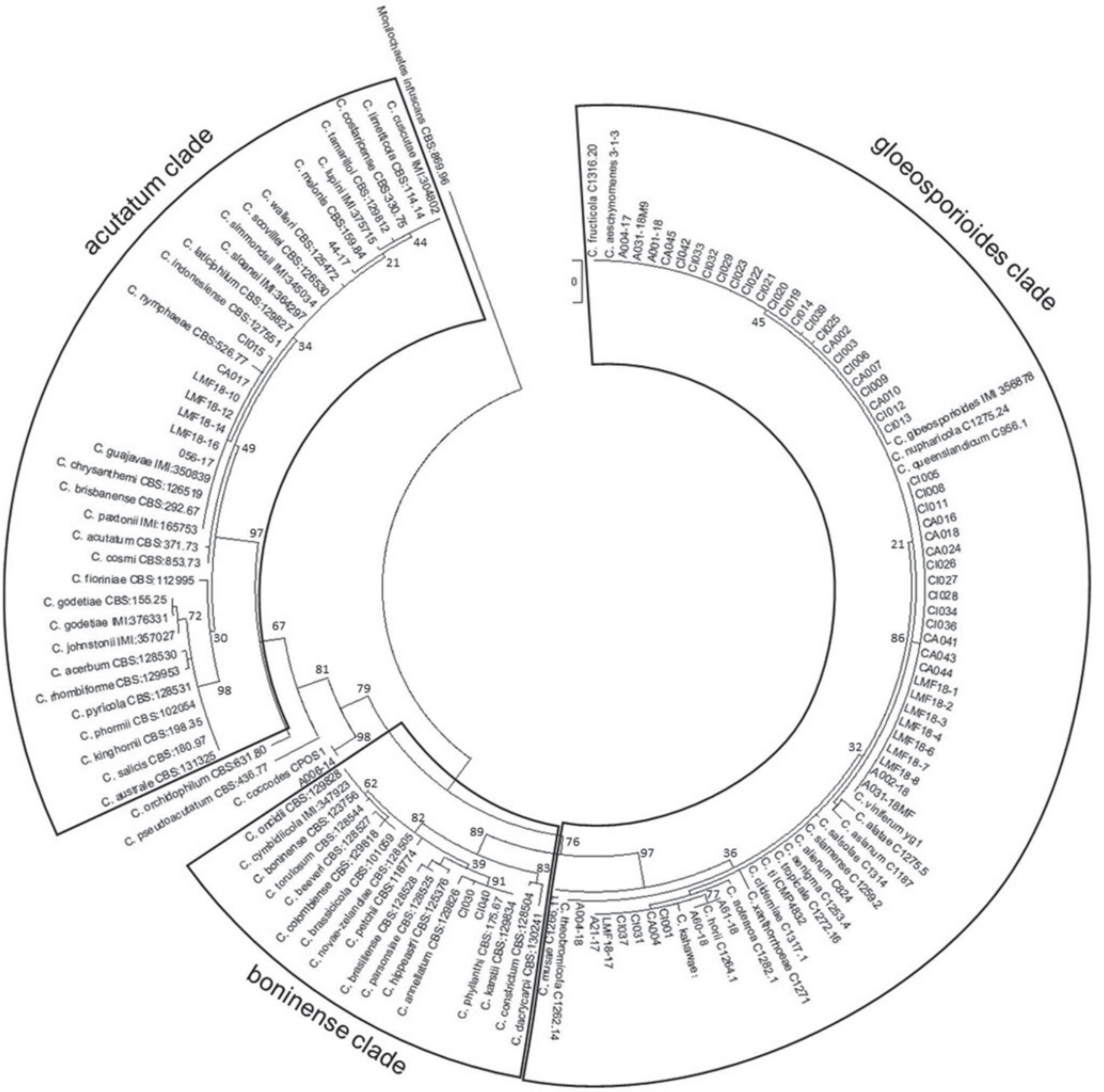

Figure 1. Phylogram generated by Maximum Likelyhood algorithm based on the alignment of ITS1-5.8S-ITS2 rDNA partial sequences, showing the phylogenetic relationship of *Colletotrichum* species isolated in Southern Brazil and reference strains from GenBank.

1 min, and externsion at 72 °C for 1.5 min, with an initial denaturation of 5 min at 94 °C and a final externsion of 5 min at 72 °C. Amplicons (530–550 pb) were stained with GelRed (Unisicence), separated in 2% agarose gels with TBE (Tris-Borate-EDTA) and visualized under UV light.

2.4. DNA sequencing of the ITS region

ITS amplification products were enzymatically purified with EXOI/SAP (USB) following manufacture instructions. Purified amplicons (50–60 ng) were sequenced using Big Dye Terminator V.3.1 sequencing kit (Thermo) and analyzed with a 3500 Genetic Analyzer (Thermo). Data were collected by Data Collection software (Thermo).

2.5. Molecular classification and phylogenetic analysis

The DNA sequences were compared with those deposited in GenBank using the BLAST similarity test. Alignment of the ITS sequences of the isolates, references of *Colletotrichum* species, and *Monilochaetes infuscans* (out group) was performed with CLUSTAL X. MEGA X was used to build a distance tree with the Maximum Likelyhood algorithm.

3. Results and discussion

A total of 63 cultures of *Colletotrichum* were obtained from 39 *Vitis labrusca* (Isabella, Ives, Concord, and Niagara), 19 *Vitis vinifera* (Cabernet Sauvignon, Cabernet Franc, Trebbiano, Malvasia, Moscato, and Merlot), and 8 hybrids (Moscato hybrids, Moscato EMBRAPA, and Lorena) mature grapes with ripe rot lesions (Table 1). Samples were obtained during 2017 and 2018 vintages in vineyards of 13 counties that represent the “Serra Gaucha” region located in the highlands of Rio Grande do Sul State, Southern Brazil.

Isolates exhibited good growth on PDA medium varying between 4.7 to 8.05 mm/day, with yellow, salmon, and grey color. All the isolates showed typical *Colletotrichum* cylindrical conidia with 10.7 to 16.6 µm length per 4.9 to 7.6 µm width. produced conidial appressoria, and most of them exhibited acervuli.

The BLAST comparison of the ITS sequences with those deposited in GenBank allowed classifying the isolates obtained from grapes in several ambiguous *Colletotrichum* species: *C. fruticola*, *C. viniferum*, *C. amplenium*, *C. simensis*, *C. gloeosporioides, C. kartii, C. boninense, C. nymphaeae, C. acutatum, C. melonis, C. acutatum,* and *C. simmonosii*, all of them with e-values of 0.0 and 98–100% identity. Isolates obtained from apples and persimmon with anthracnose symptoms classified as *C. fruticola* and *C. horii* and *C. coccodes*, respectively, species previously reported on these host plants [15].

ITS data set comprised 478 characters after alignment, with 139 variations including the out-group species *Monilochaetes infuscans*. Within *Colletotrichum* species variaitions (139/478) involved 29% of the analyzed sequences.

Phylogram generated based on the ITS1-5.8S-ITS2 rDNA partial region (Fig. 1) revealed that, according to [15], the samples clustered within three clades: 84.1% (53/63) within the "gloeosprioides clade", 12.7% within the "acutatum clade", and 3.2% within the "boninense clade". The prevalence of *Colletotrichum* clades and species were not significantly different among *V. labrusca*, hybrids, and *V*. vinifera, or among the counties of the "Serra Gaucha" region, indicating that the clades and species are uniformly distributed throughout the region, and affect both *Vitis* species.

As can be observed in Fig. 1, the most prevalent species isolated from grape ripe rot at the "Serra Gaucha" region were *C. viniferum* (34.9%), and *C. fruticola* (34.9%). These species that belongs to the "gloeosporioides clade" has been previously detected in Korea [18,19], and China [20–22]. However, in Chinese vineyards *C, vineferum* was more prevalent (76.9 to 96%) than in Southern Brazil, and *C. fruticola* was referred in only one report and represents just 15.4% of the isolates [20].

Colletotrichum species of the "acutatum clade" associated to grape ripe rot disease has been reported in Korea [18], Japan [23], Australia [6], England [24], and India [25]. Except Australia [6], *C. acutatum* sensu latu represent less than 10% of *Colletotrichum* isolated from grape with ripe rot disease. In our survey, species of this clade represented 12.7%, with 11.1% of *C. nympheaea*, and 1.6% of *C. melonis/C. lupine*.

Two out of 63 isolates were classified as *C. kartii* or *C. phyllanthi*, two species of the "boninense clade". As far as we know this is the first report of species of this clade causing ripe rot on grapes. *C. boninense* sensu latu had been reported in almond, avocado, tropical trees, and *Euonymus japonicas*, among other host plants [15].

Other *Colletotrichum* species reported in China (*C. citri, C. cliviae, C. hebeiense, C. aenigma, C. capsici*) [21,22], Australia (*C. godetiae*) [24], were not found in Southern Brazil. However, *Colletotrichum* species diversity associated to grape ripe rot disease in the "Serra Gaucha" region was higher than that reported in other winegrowing regions of the world, which can be associated to the particular climatic conditions and the amplitude of the ripening and harvesting season, which extends from early summer to mid-autumn, considering both *V. labrusca* and *V. vinifera* varieties.

4. Conclusion

Grape ripe rot caused by *Colletotrichum* is one of the most devastating bunch diseases of Brazilian viticulture, and responsible for the application of fungicides near the harvest period. A survey of *Colletotrichum* species associated to grapes ripe rot disease in the "Serra Gaucha" regions showed high diversity with the presence species of the "gloeosporioides", "acutatum", and "boninense" clades. The most prevalent species were of *C. fruticola* and *C. vineferum*. The characterization of fungal diversity and species responsible for grapes ripe rot can assist in the choice of more efficient conventional or alternative control systems.

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