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Aetiology of anthracnose on grapevine shoots in Brazil

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Anthracnose is an important disease in vineyards in south and southeast Brazil, the main grape-producing regions in the country. This study aimed to identify the causal agents of grapevine anthracnose in Brazil through multilocus phylogenetic analyses, morphological characterization and pathogenicity tests. Thirty-nine *Elsinoë ampelina* and 13 *Colletotrichum* spp. isolates were obtained from leaves, stems and berries with anthracnose symptoms collected in 38 vineyards in southern and southeastern Brazil. For *E. ampelina* isolates, the internal transcribed spacer (ITS), histone H3 (*HIS3*) and elongation factor 1-α (*TEF*) sequences were analysed. *HIS3* was the most informative region with 55 polymorphic sites including deletions and substitutions of bases, enabling the grouping of isolates into five haplotypes. Colonies of *E. ampelina* showed slow growth, variable colouration and a wrinkled texture on potato dextrose agar. Conidia were cylindrical to oblong with rounded ends, hyaline, aseptate, (3.57–) 5.64 (−6.95) µm long and (2.03–) 2.65 (−3.40) µm wide. Seven species of *Colletotrichum* were identified: *C. siamense*, *C. gloeosporioides*, *C. fructicola*, *C. viniferum*, *C. nymphaeae*, *C. truncatum* and *C. cliviae*, with a wide variation in colony and conidium morphology. Only *E. ampelina* caused anthracnose symptoms on leaves, tendrils and stems of *Vitis vinifera* and *V. labrusca*. High disease severity and a negative correlation between disease severity and shoot dry weight were observed only when relative humidity was above 95%. In this study, only *E. ampelina* caused anthracnose symptoms on grapevine shoots in Brazil.

Keywords: black spot, *Colletotrichum* spp., *Elsinoë ampelina*, genetic diversity, pathogenicity, *Vitis* species

Introduction

Grapevine is cultivated in Brazil between parallels 9° and 30°S, with higher density in the southeastern and southern regions between latitudes 20° and 30°S. These regions contain 78%, representing 68 779 ha, of Brazilian grapevine production (Instituto Brasileiro de Geografia e Estatística, 2016). The American species *Vitis labrusca*, including the Niagara Rosada, Niagara Branca, Concord and Isabel cultivars, is used for juice, wine and fresh market production. *Vitis labrusca* is responsible for 49% of grape production in São Paulo State, the largest producer of the southeast region, and 80% of the production in Rio Grande do Sul State, the largest grape producer of the southern region (Oliveira *et al.*, 2008; Protas & Camargo, 2011). The annual growth cycle of Brazilian grapevines in both regions begins with bud break in September and lasts until harvest in December and January. This period is characterized by temperatures between 25 and 30 °C and by frequent rain. The warm and humid environment in these grape-growing areas is conducive to the occurrence of diseases on shoots, especially downy mildew and anthracnose (Amorim *et al.*, 2016). Overall, wine grapes are highly resistant to anthracnose, whereas table grapes belonging to the species *V. labrusca* and *V. vinifera* are susceptible (Hart *et al.*, 1993; Schilder *et al.*, 2005; Kono *et al.*, 2013). Anthracnose represents a constraint to production of susceptible cultivars in humid regions in Asia, where the disease is well established (Thind, 2015). Major outbreaks, which may cause yield losses as high as 50%, occur when there is synchronism between rain and the presence of susceptible host tissues (Anderson, 1956; Prakongkha *et al.*, 2013).

Infection occurs mostly in young tissues, including stems, leaves, petioles, tendrils, rachises and berries. The first symptoms are numerous slightly depressed circular or angular spots, dark brown in colour, on all aerial parts of the plants (Thind, 2015). As the disease develops, lesions may coalesce and form cankers or blight on stems, causing early defoliation and delaying the ripening of berries. The symptoms on the berries compromise fruit quality for the fresh market (Amorim *et al.*, 2016).

The causal agent of anthracnose is *Elsinoë ampelina* (Brook, 1973; Kumar *et al.*, 1994; Pedro Júnior *et al.*, 1999; Schilder *et al.*, 2005; Poolsawat *et al.*, 2010; Carisse & Lefebvre, 2011). However, *Colletotrichum gloeosporioides* species complex (Chowdappa *et al.*, 2009; Sawant *et al.*, 2012a), *Colletotrichum acutatum* species complex (Chowdappa *et al.*, 2009; Baroncelli *et al.*, 2014; Liu *et al.*, 2016) and *Colletotrichum capsici* (Sawant *et al.*, 2012a,b) have also been reported as causal agents of the disease. In most cases, identification of the causal agent of anthracnose was based on cultural

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and morphological characteristics and sometimes, for *Colletotrichum*, species-specific primers were also used. Consequently, few DNA sequences of *E. ampelina* have been deposited in the GenBank database and those that have, obtained from isolates from Michigan (USA), only represent the internal transcribed spacer (ITS) region (Schilder *et al.*, 2005); this hinders reliable pathogen identification and knowledge of its genetic variability. For the genus *Colletotrichum*, several genes of isolates in various pathosystems have been sequenced and deposited in GenBank. The main regions sequenced are ITS, β-*tubulin* and *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH), which allow differentiation of the species within the *C. acutatum* and *C. gloeosporioides* complexes (Baroncelli *et al.*, 2015; Velho *et al.*, 2015).

Most cultivars of *V. vinifera* and *V. labrusca* from Brazilian vineyards are susceptible to anthracnose (Giovaninni, 2001; Barros *et al.*, 2015). However, there have been no studies on the aetiology of the disease in the country. This study aimed to identify the causal agents of grapevine anthracnose in Brazil through multilocus phylogeny, morphological characterization and pathogenicity tests.

Materials and methods

Fungal isolates

Leaves, stems, and berries of grapevines with anthracnose symptoms were collected from 38 vineyards in southern and southeastern Brazil (Fig. 1). Small fragments of typical anthracnose lesions were disinfected in 70% alcohol (1 min) and 2.5% sodium hypochlorite (3 min), followed by three rinses with sterile distilled water. Subsequently, the fragments were dried on sterilized filter paper, deposited on water agar (WA; Difco) and incubated for 7 days at 25 °C with a photoperiod of 12 h. Putative colonies of *E. ampelina* (Fig. S1) and *Colletotrichum* spp. were then transferred to potato dextrose agar (PDA; Difco) amended with streptomycin sulphate (0.5 g L^{-1}). To obtain pure cultures of *E. ampelina*, mycelia were dislodged from the colony, sterile distilled water was added and they were mixed using a Drigalski spatula. Then, 150 μL of this mycelial suspension were spread over a Petri dish containing WA. After 14 h, the dishes were observed under an optical microscope, and the isolates were purified by removing a hyphal tip to a plate containing PDA. For the purification of *Colletotrichum* isolates, 150 μL of a conidial suspension were plated on WA. After 8 h, a single germinating conidium was transferred to PDA. The purified isolates were grown at 25 °C for 10 days on plates of PDA medium with fragments of 1 cm^2 sterilized filter paper on the surface. After fungal growth, the pieces of filter paper were removed from the culture medium, stored in 2 mL microtubes containing silica gel and kept at 4 °C in the Department of Plant Pathology and Nematology, ESALQ, University of São Paulo.

Molecular characterization

For each *E. ampelina* and *Colletotrichum* spp. isolate, a small amount of mycelium was collected from pure cultures grown on PDA at 25 °C with a 12 h photoperiod for 2 weeks. DNA was extracted and purified using the Wizard Genomic DNA Purification kit (Promega), following the manufacturer's instructions. For *E. ampelina* isolates, PCR-based techniques were used for

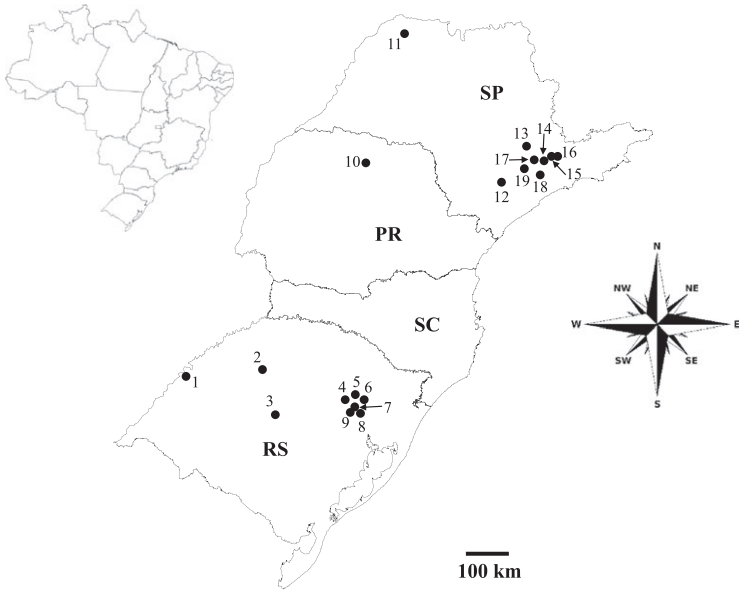

Figure 1 Collection sites of isolates of *Elsinoë ampelina* and *Colletotrichum* spp. associated with grapevine anthracnose symptoms in southern and southeastern Brazil. Municipalities where samples were collected are indicated on the map by numbers: 1-São Borja ($v = 1$; $n = 1$); 2-Augusto Pestana ($v = 4$; $n = 4$); 3-Santa Maria ($v = 4$; $n = 4$); 4-Cotiporã ($v = 2$; $n = 2$); 5-Nova Roma do Sul ($v = 1$; $n = 2$); 6-Flores da Cunha ($v = 2$; $n = 2$); 7-Bento Gonçalves ($v = 3$; $n = 4$); 8-Farroupilha ($v = 2$; $n = 2$); 9-Garibaldi ($v = 1$; $n = 1$); 10-Londrina ($v = 1$; $n = 1$); 11-Jales ($v = 1$; $n = 1$); 12-São Miguel Arcanjo ($v = 1$; $n = 1$); 13-Piracicaba ($v = 1$; $n = 3$); 14-Jundiaí ($v = 3$; $n = 6$); 15-Jarinu ($v = 1$; $n = 2$); 16-Atibaia ($v = 2$; $n = 4$); 17-Indaiatuba ($v = 2$; $n = 3$); 18-São Roque ($v = 4$; $n = 5$); and 19-Porto Feliz ($v = 2$; $n = 4$). v is the number of vineyards sampled and n is the number of isolates analysed in each municipality. PR, Paraná State; RS, Rio Grande do Sul State; SC, Santa Catarina State; SP, São Paulo State.

the amplification of the internal transcribed spacer region (ITS; spanning ITS1, ITS2 and the 5.8S ribosomal RNA gene), a partial sequence of the histone H3 gene (*HIS3*), and a partial sequence of the translation elongation factor 1-α gene (*TEF*) using, respectively, the primers pairs ITS1 and ITS4 (White *et al.*, 1990), CYLH3F and CYLH3R (Crous *et al.*, 2004) and elongation-1-F and elongation-1-R (Hyun *et al.*, 2009). For *Colletotrichum* isolates, the ITS region, a partial sequence of the β-tubulin gene (*TUB2*), and a partial sequence of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) were amplified with the primers pairs ITS1 and ITS4 (White *et al.*, 1990), Bt2a and Bt2b (Glass & Donaldson, 1995) and GDF1 and GDR1 (Guerber *et al.*, 2003), respectively.

Each PCR contained 2 μL DNA (25 ng μL^{-1}), 8.5 μL nuclease-free water, 1 μL of each primer (10 μM) and 12.5 μL GoTaq Colorless Master Mix 2 × (Promega) in a total volume of 25 μL. The amplification programme for the ITS and *TUB2* loci consisted of initial denaturation at 94 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. For *GAPDH*, the same conditions were used except the annealing step was 60 °C for 45 s. For *TEF*, initial denaturation at 94 °C for 2 min was followed by 40 cycles of amplification (94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min) and a final extension at 72 °C for 5 min. For *HIS3*, initial denaturation at 95 °C for 3 min was followed by 34 cycles of amplification (95 °C for 1 min, 56.9 °C for 1 min and 72 °C for 1 min) and a final extension at 72 °C for 4 min. PCR was carried out in a TC-512 thermal cycler (Techne).

The PCR-amplified products were analysed by electrophoresis in a 2% agarose gel stained with SYBR Safe (Invitrogen), in 0.5 × Tris borate EDTA (TBE) buffer and viewed under UV light. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega), following the manufacturer's instructions. Sequencing of PCR products was performed using a 3130xl Genetic Analyzer (Applied Biosystems).

The forward and reverse sequences were edited using SEQUENCHER v. 5.4.1 (Gene Codes Corporation, 2016) to obtain consensus sequences. Consensus sequences were compared with sequences deposited in GenBank using the basic local alignment sequence tool (BLAST; Boratyn *et al.*, 2013). The GenBank sequences with the highest similarity scores were selected and aligned with the sequences obtained from this study using the CLUSTALW algorithm in BIOEDIT (Hall, 1999). MESQUITE software was used to concatenate the multiple alignments (Maddison & Maddison, 2011).

Three phylogenetic analyses were carried out with the amplicons generated. The first phylogenetic tree was built using ITS sequences of *Elsinoë*/*Sphaceloma* species, the second tree was built using multilocus alignment (ITS and *HIS3*) of *E. ampelina*, and, finally, a third tree was built using multilocus alignment (ITS, *TUB2* and *GAPDH*) of *Colletotrichum* spp. Nucleotide substitution models for each gene were determined and included for each locus based on the Akaike information criterion (AIC) using MRMODELTEST v. 2.3 (Nylander, 2004). Bayesian inference was used to rebuild the phylogenies using a Markov chain Monte Carlo (MCMC) algorithm to generate phylogenetic trees with Bayesian posterior probabilities. Four MCMC chains were executed simultaneously for random trees with 10^7 generations. Trees were randomly sampled every 1000 generations and 25% of generations were discarded as burn-in. For each phylogenetic analysis, the Bayesian inference was conducted twice in MRBAYES v. 3.1.1 (Ronquist & Huelsenbeck, 2003). Trees were edited in TREEVIEW (Page, 1996). All sequences generated in this study were deposited in GenBank.

Morphological characterization

To assess characteristics of *E. ampelina* and *Colletotrichum* spp. colonies, mycelial plugs, 6.5 mm in diameter, were excised from the edges of actively growing colonies on PDA, deposited at the centres of Petri dishes containing PDA medium and incubated at 25 °C with a 12 h photoperiod. Colony texture, density, colour and diameter, and absence/presence of staining spore mass were used for characterization of the isolates. For *Colletotrichum* spp. isolates, the colony diameter was assessed at 5 days and the other cultural features at 7 days, and for *E. ampelina* all assessments were performed at 30 days. Colour was described using the mycological colour chart of Rayner (1970). Colony diameter (mm) was measured using a digital calliper in two perpendicular directions, and a mean colony diameter was obtained. The experimental design was completely randomized with three replicates per isolate. The experiment was performed twice.

As *E. ampelina* did not sporulate *in vitro*, mycelia from colonies grown on PDA for 30 days were fragmented and 10 mL of liquid Fries' medium were added in Petri dishes (Whiteside, 1975). The dishes were incubated at room temperature in the dark for 48 h. The suspension of microcolonies and rare conidia formed in Fries' medium was sprayed on young leaves of cv. Niagara Rosada. The plants were covered with plastic bags and kept at 25 °C in the dark for 48 h. After removal from the moist chamber, the plants were kept at 25 °C with a 12 h photoperiod for 7 days. Leaves with sporulating lesions were detached and transferred to Petri dishes. A 50 μL drop of sterile distilled water was placed on top of each lesion. After 4 h, the drop was removed and the conidia were measured with an optical microscope. Isolates of *Colletotrichum* spp. were grown on synthetic nutrient-poor agar (SNA; Nirenberg, 1976) with two 1 cm^2 pieces of filter paper at 20 °C with a 12 h photoperiod for 10 days.

For each *E. ampelina* and *Colletotrichum* spp. isolate, the lengths and widths of 30 conidia were measured. The conidial shapes were also observed. Measurements were taken at × 1000 magnification using an Axio Lab.A1 microscope (Carl Zeiss). Images were captured using an Axiocam 503 colour digital video camera (Carl Zeiss).

Pathogenicity

One-year-old plants of *V. vinifera* 'Moscato Giallo' (wine grape) grafted on SO4 rootstock and *V. labrusca* 'Niagara Rosada' (table grape) grafted on IAC 766 Campinas rootstock were inoculated with 17 isolates representative of the subclades identified in the phylogenetic analyses. *Elsinoë ampelina* isolates were grown for 30 days on PDA at 25 °C with a 12 h photoperiod, and the colonies were fragmented in sterile distilled water using scalpel and pestle. The mycelial suspension was calibrated with a Neubauer chamber at 2×10^5 CFU mL^{-1} for each isolate. Conidial suspensions of *Colletotrichum* spp. isolates were obtained from colonies grown on PDA for 7 days at 25 °C with a 12 h photoperiod and adjusted to 2×10^5 conidia mL^{-1}. The suspensions were sprayed to the point of runoff on plants at growth stage 14 (four unfolded leaves; Lorenz *et al.*, 1995). The plants were covered with plastic bags and incubated in a growth room at 25 °C in the dark for 48 h. Plants sprayed with water only were used as a control. After the initial incubation period,

Table 1 Species, isolate code, host and tissue of isolation, location of origin and GenBank accession numbers for isolates of *Elsinoë* spp. (anamorph: *Sphaceloma* spp.) used in this study in Brazil, 2014

Species	Isolate code	Host	Tissue	Location[a]	GenBank accession number[b]		
					ITS	*HIS3*	*TEF*
Elsinoë ampelina	AV20	*Vitis riparia* × (*V. rupestris* × *V. cordifolia*) 'Riparia do Traviú'[c]	Leaf	Piracicaba, SP, Brazil	KX786349	KX786466	KX786388
	AV25	*V. labrusca* 'Niagara Rosada'	Leaf	Jundiaí, SP, Brazil	KX786350	KX786467	KX786389
	AV34	*V. labrusca* 'Niagara Rosada'	Leaf	Jundiaí, SP, Brazil	KX786351	KX786468	KX786390
	AV37	*V. labrusca* 'Niagara Rosada'	Leaf	Jundiaí, SP, Brazil	KX786352	KX786469	KX786391
	AV39	*V. labrusca* 'Bordô'	Berry	Santa Maria, RS, Brazil	KX786353	KX786470	KX786392
	AV40	*V. vinifera* 'Moscato Bailey'	Leaf	Santa Maria, RS Brazil	KX786354	KX786471	KX786393
	AV44	*V. labrusca* 'Tardia de Caxias'	Leaf	Santa Maria, RS, Brazil	KX786355	KX786472	KX786394
	AV47	*V. vinifera* 'Moscato Branco'	Leaf	Augusto Pestana, RS, Brazil	KX786356	KX786473	KX786395
	AV48	*V. labrusca* 'Niagara Rosada'	Berry	Jundiaí, SP, Brazil	KX786357	KX786474	KX786396
	AV49	*V. riparia* 'Gloria de Montpellier'[c]	Leaf	Porto Feliz, SP, Brazil	KX786358	KX786475	KX786397
	AV51	*V. labrusca* 'Niagara Rosada'	Berry	Porto Feliz, SP, Brazil	KX786359	KX786476	KX786398
	AV52	*V. riparia* 'Gloria de Montpellier'[c]	Leaf	Porto Feliz, SP, Brazil	KX786360	KX786477	KX786399
	AV53	*V. labrusca* 'Niagara Rosada'	Berry	Porto Feliz, SP, Brazil	KX786361	KX786478	KX786400
	AV55	*V. labrusca* 'Niagara Rosada'	Berry	Jarinu, SP, Brazil	KX786362	KX786479	KX786401
	AV56	*V. labrusca* 'Niagara Rosada'	Berry	Jarinu, SP, Brazil	KX786363	KX786480	KX786402
	AV60	*V. labrusca* 'Niagara Branca'	Leaf	Atibaia, SP, Brazil	KX786364	KX786481	KX786403
	AV62	*V. labrusca* 'Niagara Rosada'	Leaf	Atibaia, SP, Brazil	KX786365	KX786482	KX786404
	AV63	*V. labrusca* 'Niagara Rosada'	Berry	Atibaia, SP, Brazil	KX786366	KX786483	KX786405
	AV64	*V. labrusca* 'Niagara Rosada'	Stem	Atibaia, SP, Brazil	KX786367	KX786484	KX786406
	AV67	*V. labrusca* 'Niagara Rosada'	Berry	Indaiatuba, SP, Brazil	KX786368	KX786485	KX786407
	AV71	*V. vinifera* 'Moscato Branco'	Leaf	Indaiatuba, SP, Brazil	KX786369	KX786486	KX786408
	AV72	*V. labrusca* 'Niagara Rosada'	Leaf	Indaiatuba, SP, Brazil	KX786370	KX786487	KX786409
	AV82	*Vitis* sp. hybrid 'BRS Violeta'	Stem	São Roque, SP, Brazil	KX786371	KX786488	KX786410
	AV84	Riparia do Traviú × *V. caribaea* 'IAC-766'[c]	Leaf	São Roque, SP, Brazil	KX786372	KX786489	KX786411
	AV85	*V. vinifera* × (*V. vinifera* × *V. rupestris* ×*V. lincecumii*) 'BRS Lorena'	Leaf	São Roque, SP, Brazil	KX786373	KX786490	KX786412
	AV86	*V. labrusca* 'Niagara Rosada'	Leaf	São Roque, SP, Brazil	KX786374	KX786491	KX786413
	AV93	*V. vinifera* × (*V. rupestris* × *V. lincecumii*) 'Seibel 2'	Berry	Garibaldi, RS, Brazil	KX786375	KX786492	KX786414
	AV95	*V. labrusca* 'Niagara Branca'	Berry	Nova Roma do Sul, RS, Brazil	KX786376	KX786493	KX786415
	AV97	*V. vinifera* 'Zante Currant'	Berry	Augusto Pestana, RS, Brazil	KX786377	KX786494	KX786416
	AV99	*V. labrusca* 'Bordô'	Leaf	Flores da Cunha, RS, Brazil	KX786378	KX786495	KX786417
	AV102	*V. labrusca* 'Niagara Rosada'	Berry	Augusto Pestana, RS, Brazil	KX786379	KX786496	KX786418
	AV103	*V. labrusca* 'Isabel Precoce'	Berry	Farroupilha, RS, Brazil	KX786380	KX786497	KX786419
	AV104	*Vitis* sp. hybrid 'BRS Carmen'	Berry	Farroupilha, RS, Brazil	KX786381	KX786498	KX786420
	AV107	*V. vinifera* hybrid 'BRS Morena'	Leaf	Cotiporã, RS, Brazil	KX786382	KX786499	KX786421
	AV111	*V. labrusca* 'Concord'	Berry	Bento Gonçalves, RS, Brazil	KX786383	KX786500	KX786422
	AV113	*V. vinifera* 'Moscato Giallo'	Berry	Bento Gonçalves, RS, Brazil	KX786384	KX786501	KX786423
	AV114	*V. labrusca* 'Isabel'	Berry	Bento Gonçalves, RS, Brazil	KX786385	KX786502	KX786424
	AV115	*V. vinifera* hybrid 'BRS Clara'	Berry	Cotiporã, RS, Brazil	KX786386	KX786503	KX786425
	AV117	*V. labrusca* 'Niagara Rosada'	Berry	São Borja, RS, Brazil	KX786387	KX786504	KX786426
	EAMI-1	*Vitis* sp. 'Marquis'	–	USA	AY826762	–	–
	EAMI-2	*Vitis* sp. 'Marquis'	–	USA	AY826763	–	–
	EAMI-3	*Vitis* sp. 'Marquis'	–	USA	AY826764	–	–
E. australis	70212	*Citrus unshiu* 'Satsuma mandarin'	–	Argentina	FJ010291	–	–
E. banksiae	STE-U 2678	*Banksia serrata*	–	Australia	AF227197	–	–
E. fawcettii	Jin-6	*Citrus* sp.	–	South Korea	FJ010323	–	–
E. eucalypticola	CBS 124765	*Eucalyptus* sp.	–	Australia	GQ303275	–	–
E. eucalyptorum	CBS 120084	*Eucalyptus propinqua*	–	Australia	DQ923530	–	–
Sphaceloma asclepiadis	CPC 18583	–	–	Brazil	JN943494	–	–
S. erythrinae	CPC 18542	–	–	Brazil	JN943487	–	–
S. freyliniae	CBS 128204	*Freylinia lanceolata*	–	South Africa	HQ599577	–	–

(continued)

Table 1 (continued)

Species	Isolate code	Host	Tissue	Location[a]	GenBank accession number[b] ITS	*HIS3*	*TEF*
S. krugii	CPC 18585	–	–	Brazil	JN943490	–	–
S. manihoticola		*Manihot esculenta*	–	Brazil	AY739018	–	–
S. perseae	CBS 28864	*Perseae americana*	–	Brazil	HM191255	–	–
S. tectificae	CBS 124777	*Eucalyptus tectifica*	–	Australia	GQ303294	–	–

[a]RS, Rio Grande do Sul State; SP, São Paulo State.
[b]Sequence numbers in bold were obtained in the present study.
[c]Rootstock.

for each cultivar, plants were placed either in the greenhouse with average relative humidity between 60% and 70% (environment 1) or in a growth room containing a humidifier (Britânia) to maintain the relative humidity higher than 95% (environment 2). In both environments, plants were kept at 25 °C for 10 days, with leaves and stems assessed daily for symptoms.

Five days after inoculation, the number of lesions was quantified visually in 4 cm^2 of the top two leaves using a scale from 0 to 5 (adapted from Poolsawat *et al.*, 2012): 0 = no lesions, 1 = 1–6 lesions, 2 = 7–25, 3 = 26–50, 4 = 51–100 and 5 = >100 lesions. Images of the top two leaves of each plant were captured 12 days after inoculation, and disease severity was quantified based on the percentage of leaf area covered by lesions using ImageJ (Rasband, 1997). The value of 100% disease severity was assigned for leaves that wilted or dropped. The shoot dry weight was determined by drying shoots in a forced-air oven at 60 °C until a constant weight was reached. For reisolation of the pathogen, fragments from leaves, tendrils and stems with symptoms were disinfected, as described previously, and transferred to Petri dishes containing PDA. The colonies were incubated for 21 days and the morphological characteristics of the isolates were analysed. The experimental design was completely randomized. For each experiment, four replicates per isolate were used; each replicate consisted of one plant. The variables of number of lesions, disease severity and shoot dry weight were transformed using $\sqrt{(x + 1)}$. Transformed data were subjected to analysis of variance (ANOVA) and the means were compared using the Scott–Knott test ($P < 0.05$). The relationship between the variables evaluated was verified using the Pearson correlation coefficient ($P < 0.05$). Comparisons between the experiments (environments and cultivars) were performed by F test ($P < 0.05$). All statistical analyses were performed using R v. 3.3.0 (R Core Team, 2016).

Results

Molecular characterization

The amplicons of *E. ampelina* generated by PCR were 412, 351–400 and 602 bp in length for the *TEF*, *HIS3* and ITS regions, respectively (Table 1). The phylogenetic analysis using ITS sequences was carried out to enable comparison with the only three sequences of *E. ampelina* deposited in GenBank. Even for other species of the genus *Elsinoë*/*Sphaceloma*, only ITS sequences were available. Bayesian phylogenetic analysis used the evolutionary model GTR+I+G based on the AIC criterion. *Elsinoë ampelina* isolates were grouped into three clades, two with two isolates each and a third clade with 35 isolates (Fig. 2). The alignment and analysis of 39 *TEF* sequences showed a lack of nucleotide variation at this locus, thus they were not used in the multilocus phylogenetic analysis. For the multilocus analysis, including *C. nymphaeae* (CBS 173.51) as an outgroup, the substitution models selected were HKY+I for ITS and GTR for *HIS3*. Isolates AV34, AV85, AV95 and AV111 from different locations and plant organs remained in a separate clade with high posterior probability value (Fig. 3). For *E. ampelina*, *HIS3* was the most informative locus, with 55 polymorphic sites, including deletions and substitutions of bases, enabling the grouping of isolates into five haplotypes.

The multilocus analysis for 50 *Colletotrichum* isolates, including *C. orbiculare* (CBS 57097) as an outgroup, was carried out using a sequence alignment of 1229 characters. The sequences obtained were 555–565, 252–291 and 489–497 bp in length for ITS, *GAPDH* and *TUB2*, respectively (Table 2). The substitution models used were GTR+G for ITS, HKY+I+G for *GAPDH* and GTR+I+G for *TUB2*. The isolates were separated into four major clades: *C. gloeosporioides* and *C. acutatum* species complexes, as well as *C. truncatum* and *C. cliviae*, with Bayesian posterior probability values between 0.99 and 1. Therefore, the 13 isolates analysed in this study were identified as *C. siamense*, *C. gloeosporioides*, *C. fructicola*, *C. viniferum*, *C. nymphaeae*, *C. cliviae* and *C. truncatum* (Fig. 4).

Morphological characterization

Elsinoë ampelina was the most abundant species (75% of all isolates analysed), with great diversity of colony colour ranging from white to dark vinaceous, wrinkled texture, absence of spore masses, sparse to absent aerial mycelia and slow growth on PDA, measuring 22.5 to 28.1 mm in diameter after 30 days (Table 3). None of the *E. ampelina* isolates produced diffusible pigment on PDA. *Elsinoë ampelina* isolates did not produce any conidia on PDA, oatmeal agar, corn agar, Sabourad's agar, yeast extract agar, Richard's agar or Czapek's agar media incubated at 25 °C with 0, 12 and 24 h photoperiods for 30 days (data not shown). On Fries' medium, the presence of conidia

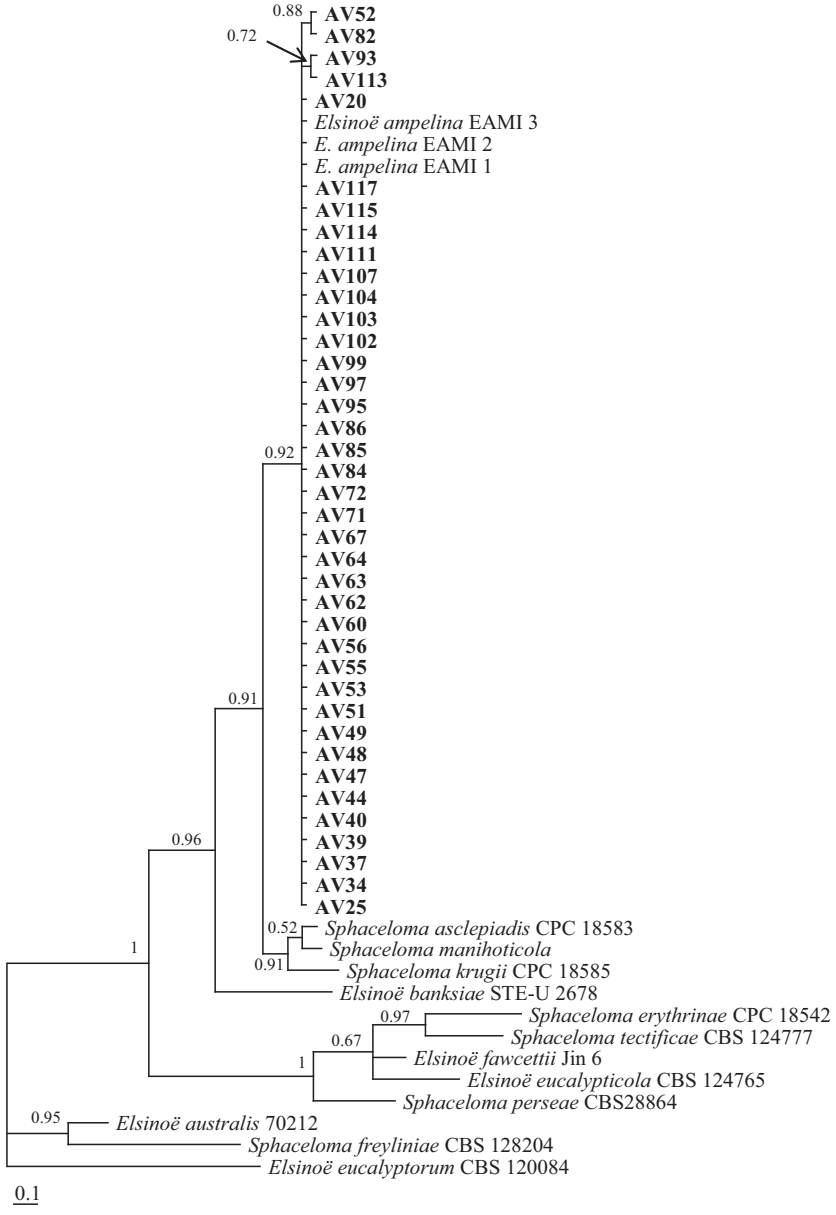

Figure 2 Phylogenetic tree derived from a Bayesian analysis of an alignment based on ITS sequences of *Elsinoë*/*Sphaceloma* isolates, run for 10^7 generations using the GTR+I+G model of nucleotide substitution. GenBank accession numbers of all isolates used in the phylogeny are listed in detail in Table 1. The numbers on the nodes are Bayesian posterior probability values. Isolates in bold were obtained in the present study in Brazil, 2014.

was rare (Fig. 5). The lesions in the inoculated leaves also presented low sporulation. *Elsinoë ampelina* isolates produced conidia that were cylindrical to oblong, hyaline, aseptate, with both ends rounded, (3.57–) 5.64 (−6.95) μm long and (2.03–) 2.65 (−3.40) μm wide.

Isolates of *Colletotrichum* showed high variation in colony colour, ranging from white to mouse grey, cottony to felty aerial mycelia and fast growth ranging from 48.8 to 76.4 mm in diameter on PDA after 5 days of incubation. Only *C. nymphaeae*, belonging to the *C. acutatum* species complex, showed abundant sporulation, forming orange concentric zones (Table 3). Conidia of *Colletotrichum* spp. isolates showed a wide variation in size and shape. *C. truncatum* had falcate conidia, which had acute ends and measured (23.32–) 26.75 (−31.21) × (3.32–) 3.92 (−4.87) μm. Meanwhile, the remaining species of *Colletotrichum* analysed showed cylindrical conidia. *Colletotrichum fructicola*, *C. siamense* and *C. viniferum* belonging to the *C. gloeosporioides* species complex and also *C. cliviae* showed conidia with rounded ends that measured (8.45–) 12.63 (−15.33) × (3.33–) 4.10 (−4.83), (9.79–) 14.36 (−17.66) × (3.73–) 4.54 (−5.58), (10.91–) 13.51 (−15.74) × (4.42–) 5.23 (−5.85) and (8.94–) 15.64 (−20.07) × (4.35–) 5.50 (−6.89) μm, respectively. *Colletotrichum gloeosporioides* isolates showed conidia with rounded and sometimes acute ends, measuring (12.35–) 15.62 (−18.56) × (4.22–) 5.04 (−5.96) μm. For *C. nymphaeae*, conidia showed acute and sometimes rounded ends, measuring (9.57–) 14.69 (−17.31) × (3.83–) 4.46 (−5.62) μm.

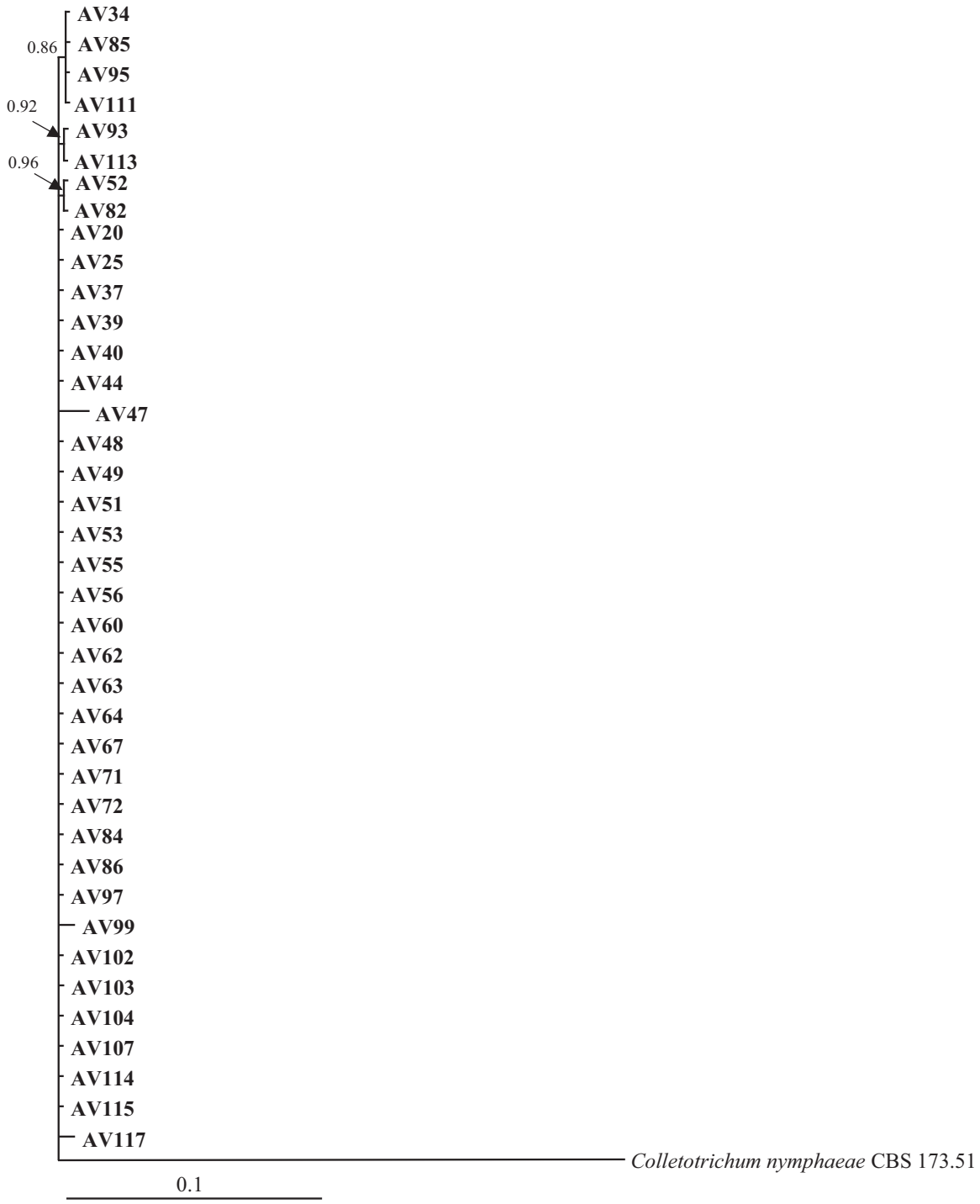

Figure 3 Phylogenetic tree derived from a Bayesian analysis of a combined ITS and *HIS3* sequence alignment of *Elsinoë ampelina* isolates, run for 10^7 generations using the HKY+I and GTR models of nucleotide substitution for ITS and *HIS3*, respectively. GenBank accession numbers of all isolates used in the phylogeny are listed in detail in Table 1. The numbers on the nodes are Bayesian posterior probability values. Isolates in bold were obtained in the present study in Brazil, 2014. *Colletotrichum nymphaeae* was used as the outgroup.

Pathogenicity

All *E. ampelina* isolates inoculated on *V. vinifera* 'Moscato Giallo' and *V. labrusca* 'Niagara Rosada' caused anthracnose symptoms on leaves, stems and tendrils (Fig. 6). The disease severity was greater in the two youngest leaves than in the other leaves. *Colletotrichum* spp. isolates did not cause any symptoms, with the

Table 2 Species, isolate code, host and tissue of isolation, location of origin and GenBank accession numbers for isolates of *Colletotrichum* spp. used in this study in Brazil, 2014

Species	Isolate code	Host	Tissue	Location[a]	GenBank accession number[b]		
					ITS	*GAPDH*	*TUB2*
Colletotrichum acutatum	CBS 127598	*Olea europaea*	–	South Africa	JQ948363	JQ948694	JQ950014
	CBS 112996	*Carica papaya*	–	Australia	JQ005776	JQ948677	JQ005860
C. aenigma	ICMP 18608	*Persea americana*	–	Israel	JX010244	JX010044	JX010389
C. alienum	ICMP 12071	*Malus domestica*	–	New Zealand	JX010251	JX010028	JX010411
C. aotearoa	ICMP 18537	*Coprosma* sp.	–	New Zealand	JX010205	JX010005	JX010420
C. asianum	ICMP 18580	*Coffea arabica*	–	Thailand	FJ972612	JX010053	JX010406
C. brisbanense	CBS 292.67	*Capsicum annuum*	–	Australia	JQ948291	JQ948621	JQ949942
C. chrysanthemi	CBS 126519	*Chrysanthemum coronarium*	Vascular tissue	Netherlands	JQ948272	JQ948602	JQ949923
C. cliviae	LC3546	*Camellia sinensis*	–	China	KJ955215	KJ954916	KJ955361
	CBS 125375	*Clivia miniata*	–	China	JX519223	JX546611	JX519249
	AV1	*Vitis riparia* × *V. labrusca* 'Baco Blanc'	Leaf	Augusto Pestana, RS, Brazil	**KX786427**	**KX786440**	**KX786453**
C. cosmi	CBS 853.73	*Cosmos* sp.	Seed	Netherlands	JQ948274	JQ948604	JQ949925
C. costaricense	CBS 330.75	*C. arabica* 'Typica'	Fruit	Costa Rica	JQ948180	JQ948510	JQ949831
C. cuscutae	IMI 304802	*Cuscuta* sp.	–	Dominica	JQ948195	JQ948525	JQ949846
C. fioriniae	CBS 125396	*M. domestica*	Fruit	EUA	JQ948299	JQ948629	JQ949950
C. fructicola	CBS 238.49	*Ficus edulis*	–	Germany	JX010181	JX009923	JX010400
	ICMP 18581	*C. arabica*	–	Thailand	JX010165	JX010033	JX010405
	AV24	*V. labrusca* 'Niagara Rosada'	Leaf	Jundiaí, SP, Brazil	**KX786433**	**KX786446**	**KX786459**
C. gloeosporioides	ICMP 18695	*Citrus* sp.	–	USA	JX010153	JX009979	–
	IMI 356878	*Citrus sinensis*	–	Italy	JX010152	JX010056	JX010445
	AV4	*V. labrusca* 'Niagara Rosada'	Leaf	Londrina, PR, Brazil	**KX786428**	**KX786441**	**KX786454**
	AV31	*V. labrusca* 'Niagara Rosada'	Leaf	Jundiaí, SP, Brazil	**KX786434**	**KX786447**	**KX786460**
C. horii	NBRC 7478	*Diospyros kaki*	–	Japan	GQ329690	GQ329681	JX010450
C. kahawae subsp. *ciggaro*	CBS 237.49	*Hypericum perforatum*	–	Germany	JX010146	JX010050	HQ596280
C. lupini	CBS 109222	*Lupinus albus*	–	Germany	JQ948170	JQ948500	JQ949821
C. melonis	CBS 159.84	*Cucumis melo*	Fruit	Brazil	JQ948194	JQ948524	JQ949845
C. musae	CBS 116870	*Musa* sp.	–	USA	JX010146	JX010050	HQ596280
C. nymphaeae	CSL 1441	*Fragaria* × *ananassa*	–	United Kingdom	KM246574	KM252179	KM251929
	CBS 173.51	*Mahonia aquifolium*	Leaf	Italy	JQ948200	JQ948530	JQ949851
	AV59	*V. labrusca* 'Isabel Precoce'	Berry	Santa Maria, RS, Brazil	**KX786435**	**KX786448**	**KX786461**
	AV78	*V. labrusca* 'Niagara Rosada'	Leaf	São Roque, SP, Brazil	**KX786437**	**KX786450**	**KX786463**
	AV87	*V. labrusca* 'Isabel'	Berry	Flores da Cunha, RS, Brazil	**KX786438**	**KX786451**	**KX786464**
	AV90	*V. labrusca* 'Niagara Branca'	Berry	Nova Roma do Sul, RS, Brazil	**KX786439**	**KX786452**	**KX786465**
C. orbiculare	CBS 570.97	*Cucumis sativus*	–	Europe	KF178466	KF178490	KF178587
C. siamense	ICMP 17795	*M. domestica*	–	USA	JX010162	JX010051	JX010393
	ICMP 12567	*P. americana*	–	Australia	JX010250	JX009940	JX010387
	AV11	Riparia do Traviú × *V. caribaea* 'IAC-766'[c]	Leaf	Piracicaba, SP, Brazil	**KX786431**	**KX786444**	**KX786457**
	AV19	*V. riparia* × (*V. rupestris* × *V. cordifolia*) 'Riparia do Traviú'[c]	Leaf	Piracicaba, SP, Brazil	**KX786432**	**KX786445**	**KX786458**
C. simmondsii	CBS 122122	*C. papaya*	Fruit	Australia	JQ948276	JQ948606	JQ949927
C. tamarilloi	CBS 129814	*Solanum betaceum*	Fruit	Colombia	JQ948184	JQ948514	JQ949835
C. theobromicola	CBS 124945	*Theobroma cacao*	–	Panama	JX010294	JX010006	JX010447
C. tropicale	CBS 124949	*T. cacao*	–	Panama	JX010264	JX010007	JX010407
C. truncatum	CTM12	*Phaseolus lunatus*	–	Malaysia	JX971135	KC109590	KC109470
	CBS 710.70	*Phaseolus vulgaris*	–	Brazil	GU227864	GU228256	GU228158
	AV5	*V. labrusca* 'Niagara Rosada'	Leaf	Jales, SP, Brazil	**KX786429**	**KX786442**	**KX786455**
	AV6	*V. labrusca* 'Niagara Rosada'	Leaf	São Miguel Arcanjo, SP, Brazil	**KX786430**	**KX786443**	**KX786456**

(continued)

Table 2 (continued)

Species	Isolate code	Host	Tissue	Location[a]	GenBank accession number[b]		
					ITS	*GAPDH*	*TUB2*
C. viniferum	GZAAS5.08601	*V. vinifera* 'Shuijing'	Berry	China	JN412804	JN412798	JN412813
	JZB330015	*V. vinifera* 'Syrah'	Fruit	China	KF156852	KF377483	KF288971
	GZAAS5.08622	*V. vinifera* 'Shuijing'	Fruit	China	JN412806	JN412796	JN412812
	AV76	*V. labrusca* 'Concord'	Leaf	Bento Gonçalves, RS, Brazil	**KX786436**	**KX786449**	**KX786462**

[a]PR, Paraná State; RS, Rio Grande do Sul State; SP, São Paulo State.
[b]Sequence numbers in bold were obtained in the present study.
[c]Rootstock.

exception of *C. viniferum* (isolate AV76), which caused small necrotic lesions on leaves of Moscato Giallo 2 days after inoculation. These lesions were similar to a hypersensitive reaction, did not show sporulation and became less apparent 5–6 days after inoculation. The control plants did not show any anthracnose symptoms. In both cultivars, kept in the greenhouse or the growth room, the first anthracnose symptoms on leaves occurred 2–3 days and on stems between 3 to 7 days after inoculation.

Elsinoë ampelina isolates showed different levels of disease severity in both cultivars assessed (Tables 4 & 5). Regardless of disease severity, plants inoculated with *E. ampelina* showed a significant decrease in shoot dry weight, which was due to shoot necrosis; this was not observed in plants inoculated with *Colletotrichum* spp. In both cultivars, *E. ampelina* isolates caused higher disease severity and higher shoot dry weight reduction ($P < 0.05$) when plants were kept at a relative humidity >95% than at 60–70%. At >95% relative humidity, there was a negative correlation between disease severity and shoot dry weight in Niagara Rosada ($r = -0.33$) and Moscato Giallo ($r = -0.59$). For Niagara Rosada, the humid environment increased the number of lesions. However, for Moscato Giallo, the numbers of lesions were not significantly different in the two environments ($P < 0.05$). Comparing the two cultivars, the greatest number of lesions and the highest disease severity were observed in Niagara Rosada in both environments ($P < 0.05$). All *E. ampelina* isolates were reisolated from the plants with symptoms on leaves, stems and tendrils.

Discussion

Elsinoë ampelina was the only causal agent of grapevine anthracnose identified in populations of isolates collected in *Vitis* spp. in Brazil. Although isolates of *Colletotrichum* spp. were isolated from anthracnose lesions, none were pathogenic to the cultivars used in this study. Multilocus DNA analysis was used for the first time for the identification of *E. ampelina* in grapevine. Sequence analysis of the ITS region has been widely used for the molecular identification of *Elsinoë/Sphaceloma* in several pathosystems (Hyun *et al.*, 2009; Everett *et al.*, 2011; Miles *et al.*, 2015; Minutolo *et al.*, 2016) and the ITS region has also been used together with the *TEF* gene (Hyun *et al.*, 2009; Miles *et al.*, 2015). Hyun *et al.* (2009) reported that isolates of *E. australis* and *E. fawcettii* associated with citrus scab in Argentina, Brazil, New Zealand and the United States showed 88 polymorphic sites within the ITS region and 34 within the *TEF* gene; thus, these isolates can be easily differentiated by analysis of ITS and *TEF* sequences.

The 39 ITS sequences of *E. ampelina* obtained in this study, together with three other *E. ampelina* sequences from the United States (Schilder *et al.*, 2005), were grouped in a main clade with a Bayesian posterior probability of 0.92, markedly distinct from other *Elsinoë/Sphaceloma* spp. sequences obtained from GenBank. Isolates of *E. ampelina* from the United States did not show any polymorphic sites within the ITS locus (Schilder *et al.*, 2005), being identical to 35 Brazilian isolates obtained in this study. Nucleotide substitutions were observed in only two isolates at position 192 and in two other isolates at position 234 within the 602 bp sequenced, which suggests low genetic variability within the ITS region. Additionally, the 39 *TEF* sequences obtained did not contain any polymorphic sites in the 412 bp analysed and was, therefore, a monomorphic locus. The low genetic variability of the ITS region and the absence of variability within the *TEF* gene indicates high conservation of these genomic regions in this fungal species. The low variability may also be related to the absence of sexual reproduction in this species in Brazil (Amorim *et al.*, 2016).

In this study, the third locus used for molecular characterization was the *HIS3* gene, which has never been used for the genus *Elsinoë/Sphaceloma*. In contrast to the low variability of the ITS and *TEF* sequences, *HIS3* sequences showed the highest degree of polymorphism (55 polymorphic sites within 351–400 bp), including deletions and substitutions of nucleotides. This result suggests that the *HIS3* region may be used for studies of genetic diversity in *E. ampelina*. In multilocus phylogenetic analysis (*TUB2*, *HIS3*, *TEF* and ITS) of the genus *Ilyonectria*, the causal agent of black foot disease of grapevine, the *HIS3* gene was also the most informative and the only gene able to separate all the species with high probabilities (Cabral *et al.*, 2012). Similarly, the *HIS3* locus was also more informative than *TUB2*, ITS

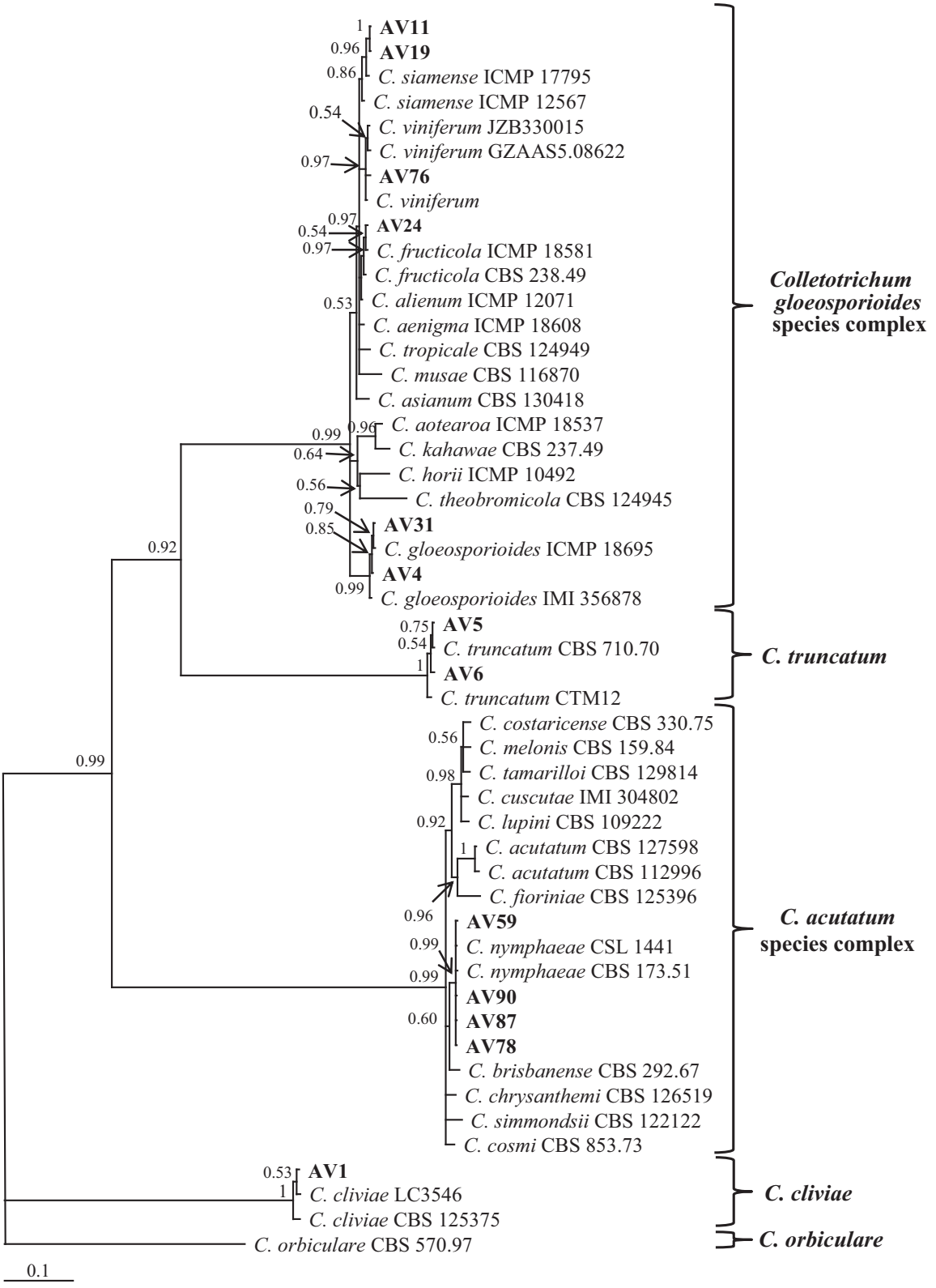

Figure 4 Phylogenetic tree derived from a Bayesian analysis of a combined ITS, *GAPDH* and *TUB2* sequence alignment of *Colletotrichum* spp. isolates, run for 10^7 generations using the GTR+G, HKY+I+G and GTR+I+G models of nucleotide substitution for ITS, *GAPDH* and *TUB2*, respectively. GenBank accession numbers of all isolates used in the phylogeny are listed in detail in Table 2. The numbers on the nodes are Bayesian posterior probability values. Isolates in bold were obtained in the present study in Brazil, 2014.

Table 3 Frequency of isolates in the population analysed and cultural characteristics including colony diameter, texture and colour of *Elsinoë ampelina* and *Colletotrichum* spp. in Brazil, 2014

		Cultural characteristics		
Species	Frequency (%)	Diameter (mm)[a]	Texture	Colour[b]
Elsinoë ampelina	1.92	23.2	Wrinkled	Violet slate with white edge
	9.62	24.5	Wrinkled	Sepia with dark brick edge
	11.54	26.6	Wrinkled	Dark vinaceous with isabelline edge
	28.88	23.9	Wrinkled	Peach with scarlet edge
	21.15	28.1	Wrinkled	Peach to white with peach edge
	1.92	22.5	Wrinkled	White with ochreous sectors
Colletotrichum cliviae	1.92	76.4	Cottony	Pale mouse grey with white to pale mouse grey edge
C. fructicola	1.92	60.6	Cottony	White to pale mouse grey
C. gloeosporioides	3.84	63.0	Cottony	White with mouse grey centre and orange masses of spores
C. nymphaeae	7.69	48.8	Felty-cottony	White to saffron with concentric rings of orange sporulation
C. siamense	3.84	70.5	Cottony	White to pale mouse grey
C. truncatum	3.84	51.3	Felty	Mouse grey to olivaceous grey
C. viniferum	1.92	58.8	Cottony	White to pale mouse grey

[a]Diameter of colony on potato dextrose agar after 30 days for *E. ampelina* and after 5 days for *Colletotrichum* spp.
[b]Colony colour was described using the mycological colour chart of Rayner (1970).

and *TEF* in a phylogenetic study of *Cercospora* spp. (Tessmann *et al.*, 2001).

Conidia of all *E. ampelina* isolates were similarly sized to those of Thai isolates (Sompong *et al.*, 2012). In the present study, *E. ampelina* colonies showed slow growth and high variability in colour, which was also reported for *E. ampelina* (Sompong *et al.*, 2012) and for *E. australis* (Miles *et al.*, 2015), *S. coryli* (Minutolo *et al.*, 2016) and *S. perseae* (Everett *et al.*, 2011). In the present study, *E. ampelina* grew slowly and did not sporulate on culture media. Lesions produced by artificial inoculation sporulated poorly 9 days after inoculation. However, it is reported that, under field conditions, infected canes are the main inoculum source of grapevine anthracnose (Thind, 2015). In spring, sclerotia or mycelium surviving in cane lesions become active and produce abundant conidia resulting in severe epidemics during rainy years (Thind *et al.*, 2004; Amorim *et al.*, 2016).

The incubation period of the disease on leaves was short, 2 to 3 days, as reported by Brook (1973) and Poolsawat *et al.* (2010). Leaves and young stems are highly susceptible to the disease, requiring 3–4 h of wetness at 21 °C for infection, while expanded leaves are highly resistant (Brook, 1973). In the greenhouse, with an average relative humidity of 60–70%, many lesions quantified on the fifth day after inoculation did not

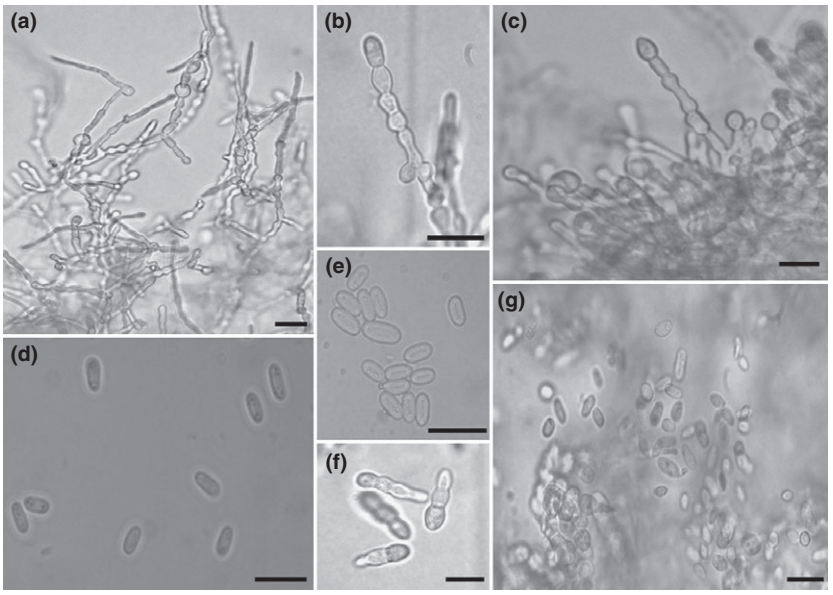

Figure 5 *Elsinoë ampelina* (isolate AV25): (a) monilioid hyphae developed on potato dextrose agar; (b, c) monilioid hyphae developed on microcolony in Fries' medium; (d, e) conidia obtained from leaf lesions; (f) germination of hyaline conidia in Fries' medium showing development of septa at 24 h; and (g) conidia produced in Fries' medium. Scale bars: 10 μm.

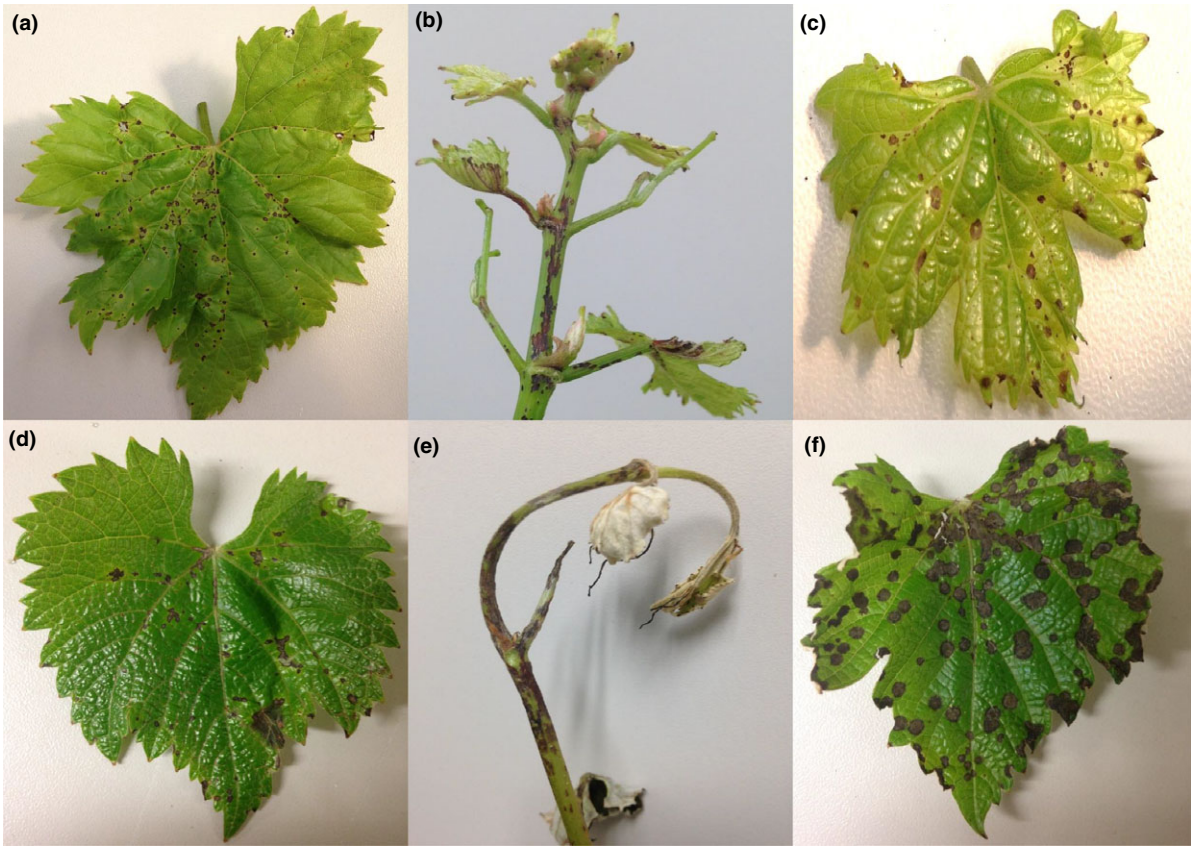

Figure 6 Anthracnose symptoms on grapevine 12 days after inoculation with *Elsinoë ampelina* (isolate AV47) in Brazil, 2015: (a–c) *Vitis vinifera* 'Moscato Giallo'; (a) leaf – environment 1 (relative humidity between 60% and 70%); (b) stem and leaves – environment 2 (relative humidity >95%); (c) leaf – environment 2. (d–f) *Vitis labrusca* 'Niagara Rosada': (d) leaf – environment 1; (e) stem and leaves – environment 2; and (f) leaf – environment 2. [Colour figure can be viewed at wileyonlinelibrary.com].

develop and were becoming less apparent as the leaf expanded. The highest severity of anthracnose in both cultivars was observed when relative humidity was higher than 95% ($P < 0.05$). Precipitation, relative humidity and temperature are the climatic factors that most influence the infection of *E. ampelina* (Thind *et al.*, 2004). In the present study, *E. ampelina* isolates caused reduction of shoot dry weight by up to 56% in Moscato Giallo and 80% in Niagara Rosada when compared to the control. In severe infections, when the lesions are numerous and deep, stems break, resulting in yield loss. Leaves and berries that are severely infected dry up and drop prematurely (Carisse & Lefebvre, 2011).

The results of the present study are in agreement with research in Australia and Japan that identified table grape cultivars as more susceptible to anthracnose than wine grape cultivars (Hart *et al.*, 1993; Kono *et al.*, 2013). Kono *et al.* (2013) attributed this result to the presence of resistance genes with a larger effect and/or more small-effect resistance genes within wine grape populations than within table grape populations. However, the potential sources and mechanisms of resistance to *E. ampelina* in grapevines are not clear.

The phylogenetic analysis of 13 *Colletotrichum* isolates obtained from anthracnose lesions resulted in four well-supported clades. The first clade contained six isolates belonging to the *C. gloeosporioides* species complex: *C. siamense*, *C. gloeosporioides*, *C. fructicola* and *C. viniferum*. *Colletotrichum viniferum* has been described in China as associated with ripe rot in *V. vinifera* (Yan *et al.*, 2015). The second largest group included four isolates clustered with *C. nymphaeae*, which was associated with anthracnose symptoms on stems of *V. vinifera* 'Red Globe' in China (Liu *et al.*, 2016). The third group included two isolates within the clade containing *C. truncatum* (synonym: *C. capsici*). *Colletotrichum capsici* has been reported to cause small dark brown spots on the leaves and petioles in table and wine grape in India (Sawant *et al.*, 2012b). Finally, a single isolate was clustered with *C. cliviae*. This species showed high genetic diversity compared to other species of *Colletotrichum* obtained in this study.

Analysis based only on ITS sequences allowed the classification of *Colletotrichum* isolates only at the level of species complexes. Multilocus concatenation alignment (ITS, *TUB2* and *GAPDH*) proved to be suitable for

Table 4 Number of lesions per leaf, leaf disease severity and shoot dry weight for different isolates of *Elsinoë ampelina* and *Colletotrichum* spp. inoculated on *Vitis vinifera* 'Moscato Giallo' in Brazil, 2015

		Environment 1[a]			Environment 2[b]		
Species	Isolate	No. of lesions[c]	Leaf disease severity (%)[d]	Shoot dry weight (g)	No. of lesions	Leaf disease severity (%)	Shoot dry weight (g)
Control	–	0.00 e	0.00 d	1.11 a	0.00 c	0.00 d	1.20 a
Elsinoë ampelina	AV20	0.75 d	0.40 c	0.67 b	1.63 b	2.06 c	0.99 b
	AV25	1.25 c	0.47 c	0.94 a	1.75 b	1.92 c	1.08 b
	AV34	1.50 c	0.53 c	0.49 b	1.88 b	2.51 c	0.62 d
	AV37	2.50 b	0.93 c	0.57 b	2.38 a	3.36 c	0.74 c
	AV47	2.00 b	2.13 a	0.79 a	2.00 b	3.20 c	0.80 c
	AV52	2.25 b	0.78 c	0.84 a	2.13 a	5.30 b	0.84 c
	AV53	1.25 c	0.64 c	0.63 b	1.63 b	4.92 b	0.82 c
	AV97	2.38 b	1.14 b	0.58 b	2.25 a	2.94 c	0.80 c
	AV113	1.50 c	2.36 a	0.86 a	1.75 b	7.11 a	0.72 c
	AV115	3.63 a	2.43 a	0.55 b	2.63 a	7.70 a	0.61 d
Colletotrichum cliviae	AV1	0.00 e	0.00 d	0.93 a	0.00 c	0.00 d	1.05 b
C. fructicola	AV24	0.00 e	0.00 d	0.78 a	0.00 c	0.00 d	0.99 b
C. gloeosporioides	AV4	0.00 e	0.00 d	0.80 a	0.00 c	0.00 d	1.08 b
C. nymphaeae	AV78	0.00 e	0.00 d	1.01 a	0.00 c	0.00 d	1.25 a
C. siamense	AV19	0.00 e	0.00 d	0.76 a	0.00 c	0.00 d	0.96 b
C. truncatum	AV6	0.00 e	0.00 d	0.97 a	0.00 c	0.00 d	1.18 a
C. viniferum	AV76	0.00 e	0.00 d	0.87 a	0.00 c	0.00 d	0.95 b

Means within column followed by different letters differ significantly ($P < 0.05$) according to Scott–Knott test.
[a]Environment 1: greenhouse with relative humidity 60–70%.
[b]Environment 2: growth room with relative humidity >95%.
[c]Number of lesions in 4 cm^2 leaf area were rated using the following scale: 0 = no lesions, 1 = 1–6 lesions, 2 = 7–25, 3 = 26–50; 4 = 51–100 and 5 = >100 lesions (adapted from Poolsawat *et al.*, 2012).
[d]Leaf disease severity was quantified based on the percentage of leaf area covered by lesions.

Table 5 Number of lesions per leaf, leaf disease severity and shoot dry weight for different *Elsinoë ampelina* and *Colletotrichum* spp. isolates inoculated on *Vitis labrusca* 'Niagara Rosada' in Brazil, 2015

		Environment 1[a]			Environment 2[b]		
Species	Isolate	No. of lesions[c]	Leaf disease severity (%)[d]	Shoot dry weight (g)	No. of lesions	Leaf disease severity (%)	Shoot dry weight (g)
Control	–	0.00 d	0.00 c	0.95 a	0.00 f	0.00 d	0.84 a
Elsinoë ampelina	AV20	2.13 c	1.32 b	0.77 a	1.75 e	14.29 c	0.40 b
	AV25	2.88 a	1.92 b	0.81 a	3.63 a	58.91 a	0.52 b
	AV34	2.50 c	3.64 a	0.61 b	2.75 c	26.39 b	0.36 b
	AV37	2.63 b	2.52 b	0.65 b	2.88 c	37.77 b	0.41 b
	AV47	3.00 a	3.44 a	0.72 b	3.25 b	72.16 a	0.21 c
	AV52	2.25 c	3.04 a	0.54 b	2.38 d	10.51 c	0.45 b
	AV53	3.13 a	3.78 a	0.64 b	3.75 a	60.20 a	0.17 c
	AV97	2.63 b	2.45 b	0.68 b	2.05 d	56.16 a	0.35 b
	AV113	3.00 a	1.89 b	0.85 a	3.75 a	62.11 a	0.55 b
	AV115	2.75 b	3.96 a	0.64 b	3.00 b	22.11 b	0.46 b
Colletotrichum cliviae	AV1	0.00 d	0.00 c	0.84 a	0.00 f	0.00 d	0.80 a
C. fructicola	AV24	0.00 d	0.00 c	0.92 a	0.00 f	0.00 d	0.87 a
C. gloeosporioides	AV4	0.00 d	0.00 c	0.84 a	0.00 f	0.00 d	0.81 a
C. nymphaeae	AV78	0.00 d	0.00 c	0.98 a	0.00 f	0.00 d	0.84 a
C. siamense	AV19	0.00 d	0.00 c	0.92 a	0.00 f	0.00 d	0.79 a
C. truncatum	AV6	0.00 d	0.00 c	1.00 a	0.00 f	0.00 d	0.81 a
C. viniferum	AV76	0.00 d	0.00 c	0.95 a	0.00 f	0.00 d	0.77 a

Means within column followed by different letters differ significantly ($P < 0.05$) according to Scott–Knott test.
[a]Environment 1: greenhouse with relative humidity 60–70%.
[b]Environment 2: growth room with relative humidity >95%.
[c]Number of lesions in 4 cm^2 leaf area were rated using the following scale: 0 = no lesions, 1 = 1–6 lesions, 2 = 7–25, 3 = 26–50; 4 = 51–100 and 5 = >100 lesions (adapted from Poolsawat *et al.*, 2012).
[d]Leaf disease severity was quantified based on the percentage of leaf area covered by lesions.

identification of the species belonging to different complexes, with clades being well supported. Similar results were observed in a study on identification of isolates belonging to *C. acutatum sensu lato* associated with anthracnose of *Carthamus tinctorius* (Baroncelli *et al.*, 2015). Although Sawant *et al.* (2012a,b) analysed hundreds of isolates of *C. gloeosporioides* and *C. capsici* associated with anthracnose of grapevine, there are only two ITS sequences of *C. gloeosporioides sensu lato* (accession nos. JN639880, JN639881) deposited in GenBank (Sawant *et al.*, 2012a). ITS sequences of *C. siamense* isolates obtained in this study (AV11 and AV19) showed 99% similarity to accession JN639881.

The wide range of cultural characteristics and conidial shape and size found in isolates of *Colletotrichum* spp. is due to the different species detected in this study. Similar conidial and cultural characteristics were reported in isolates of *C. gloeosporioides sensu lato* isolated from grapevines with anthracnose symptoms (Sawant *et al.*, 2012a) and ripe rot of grape (Yan *et al.*, 2015).

Colletotrichum viniferum (AV76) was the only species of *Colletotrichum* identified in this study that caused small necrotic spots on leaves of Moscato Giallo, resembling a hypersensitive reaction. Peng *et al.* (2013) reported that *C. viniferum* isolates obtained from grape berries with ripe rot symptoms showed nonspecific pathogenicity reactions on hosts when inoculated on leaves of *Citrus reticulata*, *Citrus sinensis*, *Ampelopsis sinica* and *Eriobotrya japonica*. Many species of *Colletotrichum* are associated with anthracnose in several crops and have demonstrated a lack of host specificity (Damm *et al.*, 2012; Weir *et al.*, 2012). *Colletotrichum* conidia can germinate on most surfaces, form an appressorium and remain attached to the surface as a viable propagule. *Colletotrichum* may also present latent infection and endophytic behaviour (Weir *et al.*, 2012). In grapevine, *C. acutatum* and *C. gloeosporioides* species complexes and *C. truncatum* are causal agents of ripe rot (Suzaki, 2011; Pan *et al.*, 2016). It is possible that isolates of *Colletotrichum* spp. obtained in this study survived in the plant endophytically or in the form of quiescent appressoria. Despite the isolation of multiple fungal species from grape tissues with anthracnose symptoms from the different grape-growing regions of Brazil, only *E. ampelina* was identified as the causal agent of the disease.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Isolation of *Elsinoë ampelina* 7 days after incubation on water agar (WA) medium: (a) small fragments of typical anthracnose lesions incubated on WA; and (b) initial mycelial growth of *E. ampelina* showing white to light coral colour. Scale bars: 1 cm for (a); and 1 mm for (b).