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Colletotrichum species on grape in Guizhou and Yunnan provinces, China

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ABSTRACT

Twenty-six strains representing three species of *Colletotrichum* were isolated from leaf and fruit lesions of vitaceous plants in Guizhou and Yunnan provinces, China. The strains were characterized by morphology and phylogenetic analyses of actin, β-tubulin, calmodulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase and rDNA internal transcribed spacer gene sequences. The combined dataset showed that 20 of 26 strains represented a novel species, the rest being *Colletotrichum fructicola* (four strains) and *Colletotrichum gloeosporioides* (two strains). The new species is described herein as *Colletotrichum viniferum*. Its conidia, compared with similar *Colletotrichum* species are cylindrical and 12–16 μm long. Based on pathogenicity tests, *C. viniferum* caused leaf spots and anthracnose of table grape but was not host-specific.

1. Introduction

Grape (*Vitis* spp.) is one of the most widely planted fruit trees worldwide (Sung et al. 2008) and the table grape (*Vitis vinifera*) is one of the most important grape vines in China. In recent years, there has been a rapid increase in areas planted with *V. vinifera* throughout China. In 2003, the total area with grape vines was 421,000 hm^2 with a yield of 517,600 tons. Due to the warm and rainy climate in southern China, yield losses of up to 50% have been reported due to disease and insects (Lu 2005). Ripe rot of grape caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and *Colletotrichum acutatum* J.H. Simmonds ex J.H. Simmonds were considered to be serious diseases occurring in most vineyards, and caused big loss and deterioration of grape vines (Sung et al. 2008).

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Southworth (1891) first reported grape ripe rot disease caused by *C. gloeosporioides* in the USA. *C . acutatum* was also reported as a pathogen of the disease in Australia, Japan, Korea and USA (Sung et al. 2008). The taxonomy and nomenclature of *Colletotrichum* remained confusing (Cai et al. 2009; Hyde et al. 2009) and a study of anthracnose of tropical fruits showed that *C. gloeosporioides* was not the causative agent as previously recorded (Phoulivong et al. 2010a). It was difficult to identify the species of *Colletotrichum* due to instability of its morphological characters, which depended upon experimental methods and conditions (Cai et al. 2009; Hyde et al. 2010). Many *Colletotrichum* strains have being successfully identified and epitypified by the application of morphology and multilocus phylogeny (Than et al. 2008; Moriwaki and Tsukiboshi 2009; Prihastuti et al. 2009; Shivas and Tan 2009; Yang et al. 2009; Phoulivong et al. 2010a, 2010b; Weir and Johnston 2010; Su et al. 2011; Wikee et al. 2011). That suggested molecular phylogeny could lead to better understanding and identification of *Colletotrichum* species (Cai et al. 2009; Hyde et al. 2010), which in turn was of great importance to correctly identify phytopathogen species for the sake of plant disease control (Hyde et al. 2010; Cai et al. 2011; Ko Ko et al. 2011; Udayanga et al. 2011).

The objective of the present was to identify *Colletotrichum* species causing disease on plants of Vitaceae in Guizhou and Yunnan provinces, China. Strains were collected from lesions in vineyards for lab experiment. Molecular and morphological data were used to identify the *Colletotrichum* species and revealed two known species and one new species.

2. Materials and methods

2.1. *Isolation of* Colletotrichum *species*

Colletotrichum samples were collected from anthracnose lesions on fruits or leaves of Vitaceae at locations in Guizhou and Yunnan provinces between June and September 2008 (Table 1). Single-spore isolations from infected leaves or fruits with sporulation were carried with the methods described by Choi et al. (1999) and Than et al. (2008). Spore masses were placed in sterilized water and streaked on to the surface of water agar (WA) plates which were then incubated overnight at 22–24°C. Single germinated spore was picked up with sterilized needles under the microscope and transferred onto potato dextrose agar plates (PDA). Pure cultures were stored at 4°C on PDA slants. Isolates were deposited in Agricultural College of Guizhou University, China. The ex-holotype and ex-paratype living culture were deposited at CBS, the Netherlands.

2.2. *Morphological and cultural characters*

Morphological and cultural characterization follow the methods of Cai et al. (2009). Mycelial discs (5 mm diam.) were taken from the growing edge of 5-day-old cultures and transferred onto PDA plates (Petri dishes diameter: 9 cm) and incubated in the dark at 25°C. Four replicate cultures of each isolate were investigated.

Colony diameter was measured daily for 8 days, growth rate (mm per day) was calculated and its colour, conidial masses and

Table 1 – Synopsis of characters of *Colletotrichum* isolates from Vitaceae.

Species	Specimen no.	Host	Symptom	Location
C. viniferum	[a]GZAAS5.08601	*V. vinifera* cv. shuijing	Fruit anthracnose	Kunming, Yunnan, China
	GZAAS5.08602	*V. vinifera* cv. meiguixiang	Fruit anthracnose	Kunming, Yunnan, China
	GZAAS5.08603	*V. vinifera* cv. hongti	Fruit anthracnose	Binchuan, Yunnan, China
	GZAAS5.08604	*V. vinifera* cv. jinya	Fruit anthracnose	Binchuan, Yunnan, China
	GZAAS5.08605	*V. vinifera* cv. shuijing	Fruit anthracnose	Pingba, Guizhou, China
	GZAAS5.08606	*V. vinifera* cv. baixiangjiao	Fruit anthracnose	Pingba, Guizhou, China
	GZAAS5.08607	*V. vinifera* cv. kyoho	Fruit anthracnose	Pingba, Guizhou, China
	GZAAS5.08608	*V. vinifera* cv. hongti	Fruit anthracnose	Guiyang, Guizhou, China
	GZAAS5.08609	*V. vinifera* cv. hongfushi	Fruit anthracnose	Guiyang, Guizhou, China
	GZAAS5.08611	*V. vinifera* cv. shuijing	Fruit anthracnose	Guiyang, Guizhou, China
	GZAAS5.08612	*V. vinifera* cv. baixiangjiao	Fruit anthracnose	Jinsha, Guizhou, China
	GZAAS5.08613	*V. vinifera* cv. shuijing	Fruit anthracnose	Jinsha, Guizhou, China
	GZAAS5.08614	*V. vinifera* cv. shuijing	Fruit anthracnose	Xifeng, Guizhou, China
	GZAAS5.08615	*V. vinifera* cv. heimeigui	Fruit anthracnose	Xifeng, Guizhou, China
	GZAAS5.08616	*V. vinifera* cv. shuijing	Fruit anthracnose	Zunyi, Guizhou, China
	GZAAS5.08617	*V. vinifera* cv. kyoho	Fruit anthracnose	Zunyi, Guizhou, China
	GZAAS5.08621	*C. japonica*	Leaf lesion	Libo, Guizhou, China
	GZAAS5.08626	*Ampelopsis* sp.	Leaf lesion	Libo, Guizhou, China
	GZAAS5.08622	*V. vinifera* cv. shuijing	Fruit anthracnose	Luodian, Guizhou, China
	GZAAS5.08623	*V. vinifera* cv. shuijing	Fruit anthracnose	Leishan, Guizhou, China
C. fructicola	GZAAS5.08610	*V. vinifera* cv. kyoho	Fruit anthracnose	Guiyang, Guizhou, China
	GZAAS5.08618	*V. vinifera* cv. shuijing	Fruit anthracnose	Dunyun, Guizhou, China
	GZAAS5.08627	*Ampelopsis* sp.	Leaf lesion	Dunyun, Guizhou, China
	GZAAS5.08628	*Ampelopsis* sp.	Leaf lesion	Dushan, Guizhou, China
C. gloeosporioides	GZAAS5.08624	*Ampelopsis* sp.	Leaf lesion	Zunyi, Guizhou, China
	GZAAS5.08620	*V. vinifera* cv. shuijing	Fruit anthracnose	Dushan, Guizhou, China

a GZAAS: Guizhou Academy of Agricultural Sciences, Guizhou Province, China.

zonation were recorded as well. Conidial appressoria were induced by inoculating conidia into two drops of distilled water on a glass slide, then placing onto Petri dishes containing cotton moistened with distilled sterile water and incubated in darkness at 25°C. After 24–48 h, conidial appressoria were measured, characterized and photographed.

2.3. *Pathogenicity testing and host range*

The isolates used for pathogenicity testing were GZAAS5.08620, GZAAS5.08601, GZAAS5.08608, and GZAAS5.08610.

Single-spore cultures of each isolate grew on PDA for 10–12 days at 25°C. The spores were harvested by adding

Table 2 – Sequences obtained from GenBank used in the analysis.

Species	Strain	Reference	GenBank accession number					
			ACT	TUB2	CAL	GPDH	GS	ITS
C. asianum	BPD-I4 holotype	Prihastuti *et al.* 2009	FJ907424	FJ907439	FJ917506	FJ972595	FJ972576	FJ972612
C. asianum	BML-I14	Prihastuti *et al.* 2009	FJ907421	FJ907436	FJ917503	FJ972598	FJ972573	FJ972615
C. cordylinicola	BCC 38872 holotype	Phoulivong *et al.* 2010b	HM470234	HM470249	HM470237	HM470240	HM470243	HM470246
C. cordylinicola	BCC 38864	Phoulivong *et al.* 2010b	HM470233	HM470248	HM470236	HM470239	HM470242	HM470245
C. crassipes	STE-U5302	Lubbe *et al.* 2004	–	–	–	–	–	AY376529
C. crassipes	STE-U4445	Lubbe *et al.* 2004	–	–	–	–	–	AY376530
C. fragariae	LC0220 = ICMP17927	Cai *et al.*, Pers comm.	–	–	–	–	–	–
C. fructicola	BPD-I12	Prihastuti *et al.* 2009	FJ907425	FJ907440	FJ917507	FJ972594	FJ972577	FJ972611
C. fructicola	BPD-I18	Prihastuti *et al.* 2009	FJ907427	FJ907442	FJ917509	FJ972592	FJ972579	FJ972602
C. fructicola	BPD-I16 holotype	Prihastuti *et al.* 2009	FJ907426	FJ907441	FJ917508	FJ972593	FJ972578	FJ972603
C. fructicola	**GZAAS5.08627**	**This paper**	**JN412746**	**JN412766**	–	**JN412753**	**JN412756**	**JN412762**
C. fructicola	**GZAAS5.08628**	**This paper**	**JN412747**	**JN412767**	**JN412751**	**JN412755**	**JN412759**	**JN412760**
C. fructicola	**GZAAS5.08618**	**This paper**	**JN412745**	**JN412764**	**JN412749**	**JN412752**	**JN412757**	**JN412763**
C. fructicola	**GZAAS5.08610**	**This paper**	**JN412748**	**JN412765**	**JN412750**	**JN412754**	**JN412758**	**JN412761**
C. gloeosporioides	CBS953.97 epitype	Prihastuti *et al.* 2009	FJ907430	FJ907445	FJ917512	FJ972589	FJ972582	FJ972609
C. gloeosporioides	CORCG5	Yang *et al.* 2009	HM034801	HM034811	HM034803	HM034807	–	HM034809
C. gloeosporioides	CORCG4	Yang *et al.* 2009	HM034800	HM034810	HM034802	HM034806	–	HM034808
C. gloeosporioides	**GZAAS5.08624**	**This paper**	**JN412777**	**JN412775**	**JN412769**	**JN412771**	**JN412773**	**JN412779**
C. gloeosporioides	**GZAAS5.08620**	**This paper**	**JN412776**	**JN412774**	**JN412768**	**JN412770**	**JN412772**	**JN412778**
C. horii	TSG001 = ICMP 10492 neotype	Wikee *et al.* 2011	GU133374	GU133375	GU133376	GQ329682	GU133377	AY787483
C. horii	TSG002	Wikee *et al.* 2011	GU133379	GU133380	GU133381	GQ329680	GU133382	AY791890
C. horii	LC0218 = ICMP12942	Cai *et al.* Pers comm.	–	–	–	–	–	–
C. hymenocallidis	CSSN2 holotype	Yang *et al.* 2009	GQ856775	GQ849438	GQ849463	GQ856757	–	GQ485600
C. hymenocallidis	CSSN3	Yang *et al.* 2009	GQ856776	GQ849439	GQ849451	GQ856759	–	GQ485601
C. ignotum	8395	Rojas *et al.* 2010	–	–	–	–	–	GU994370
C. ignotum	E183	Rojas *et al.* 2010	–	–	–	–	–	GU994367
C. jasmini-sambac	LLTA-01 ex-holotype	Wikee *et al.* 2011	HM131507	HM153768	HM131492	HM131497	HM131502	HM131511
C. jasmini-sambac	HLTX-01	Wikee *et al.* 2011	–	HM153769	HM131493	HM131498	HM131503	HM131512
C. jasmini-sambac	CLTA-01	Wikee *et al.* 2011	HM131510	HM153772	HM131496	–	HM131506	HM131515
C. kahawae	IMI319418 holotype	Prihastuti *et al.* 2009	FJ907432	FJ907446	FJ917514	FJ972588	FJ972583	FJ972608
C. kahawae	IMI 363578	Prihastuti *et al.* 2009	FJ907433	FJ907447	FJ917515	FJ972587	FJ972584	FJ972607
C. musae	CBS116870 epitype	Su *et al.* 2011	HQ596284	HQ596280	–	HQ596299	HQ596288	HQ596292
C. musae	MFLUCC 10-0977	Su *et al.* 2011	HQ596286	HQ596282	HQ596297	HQ596301	HQ596290	HQ596294
C. musae	MFLUCC 10-0978	Su *et al.* 2011	HQ596287	HQ596283	HQ596298	HQ596302	HQ596291	HQ596295
C. siamense	BML-I15	Prihastuti *et al.* 2009	FJ907422	FJ907437	FJ917504	FJ972597	FJ972574	FJ972614
C. siamense	BPD-I2 holotype	Prihastuti *et al.* 2009	FJ907423	FJ907438	FJ917505	FJ972596	FJ972575	FJ972613
C. simmondsii	BRIP28519	Shivas and Tan 2009	FJ907428	FJ907443	FJ917510	FJ972580	FJ972591	FJ972601
C. simmondsii	CBS 294.67	Shivas and Tan 2009	FJ907429	FJ907444	FJ917511	FJ972581	FJ972590	FJ972610
C. theobromicola	GJS0850	Rojas *et al.* 2010	–	–	–	–	–	GU994360
C. theobromicola	GJS0848	Rojas *et al.* 2010	–	–	–	–	–	GU994359
C. trichellum	HKUCC 10378	Yang *et al.* 2009	GQ856786	GQ849447	GQ849466	GQ856749	–	GQ485589
C. tropicale	3870	Rojas *et al.* 2010	–	–	–	–	–	GU994352
C. tropicale	E1533	Rojas *et al.* 2010	–	–	–	–	–	GU994351
C. viniferum	**GZAAS5.08622**	**This paper**	**JN412791**	**JN412812**	**JN412780**	**JN412796**	**JN412786**	**JN412806**
C. viniferum	**GZAAS5.08604**	**This paper**	**JN412792**	**JN412808**	**JN412781**	**JN412801**	**JN412788**	**JN412803**
C. viniferum	**GZAAS5.08614**	**This paper**	**JN412794**	**JN412810**	**JN412783**	**JN412797**	**JN412789**	**JN412805**
C. viniferum	**GZAAS5.08601 ex-holotype**	**This paper**	**JN412795**	**JN412813**	**JQ309639**	**JN412798**	**JN412787**	**JN412804**
C. viniferum	**GZAAS5.08608**	**This paper**	**JN412793**	**JN412811**	**JN412782**	**JN412800**	**JN412784**	**JN412802**
C. viniferum	**GZAAS5.08616**	**This paper**	**JN412790**	**JN412809**	**JQ309640**	**JN412799**	**JN412785**	**JN412807**

Note: ACT: actin; TUB-2: partial ß-tubulin; CAL: calmoudulin; GS: glutamine synthetase; GPDH: glyceraldehydes-3-phosphate dehydrogenase; ITS: complete rDNA-ITS region. The newly generated sequences in this study are shown in bold.

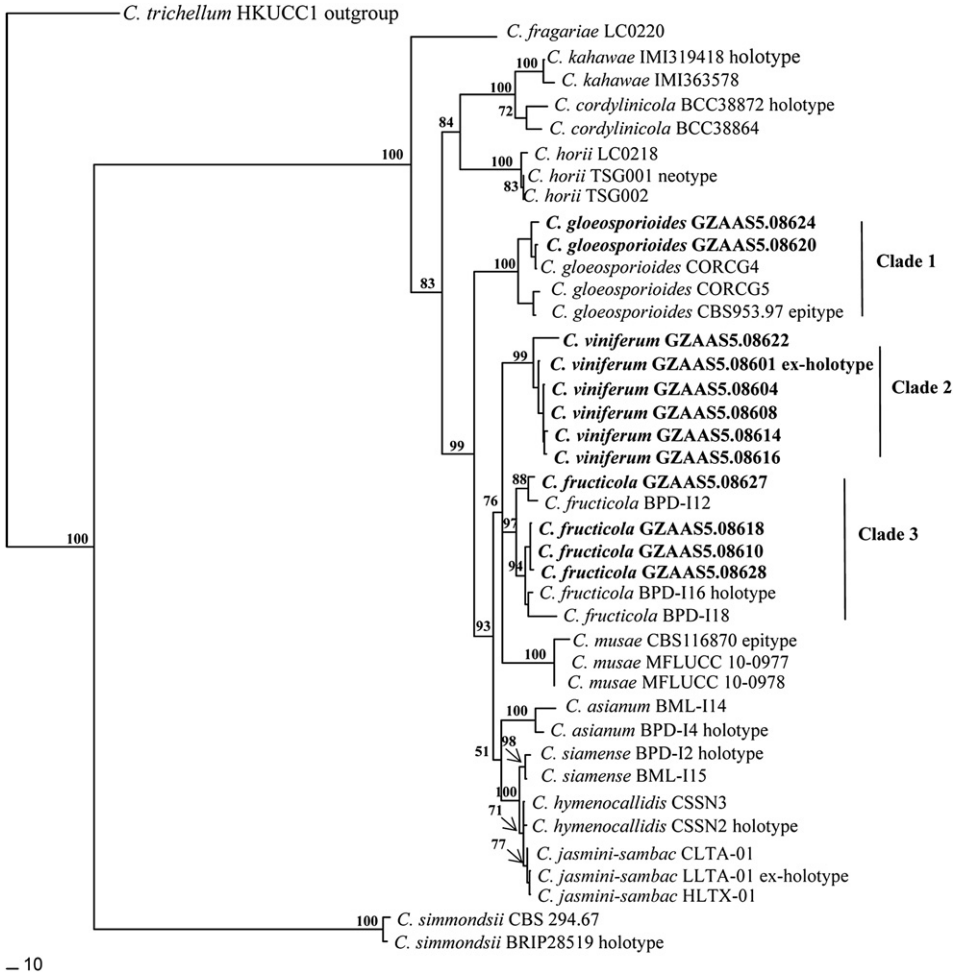

Fig. 1 – Phylogram generated from maximum parsimony analysis based on combined ACT, GPDH, ITS, TUB2, GS and CAL sequences, showing the phylogenetic relationships of *C. viniferum*. Values above the branches are parsimony bootstrap (>50%). The tree is rooted with *Colletotrichum trichellum*. Strains isolated in this study are shown in bold.

5 ml of sterilized distilled water onto the culture, which was then gently swirled to dislodge the conidia. Spore concentrations were adjusted to 10^6 conidia/ml using a haemocytometer.

The inoculation method that we applied has been described by Than et al. (2008) and Yang et al. (2009). The plants for pathogenicity testing were collected from the Experiment Farm of Guizhou University. Young leaves and

Fig. 2 – *C. viniferum* GZAAS5.08601 (holotype). Symptoms of ripe rot of grape on fruits, Yunnan Province.

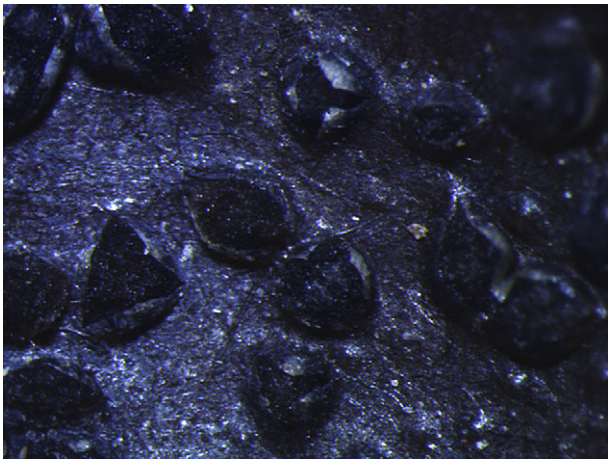

Fig. 3 – *C. viniferum* GZAAS5.08601 (holotype). Conidiomata on natural substrata.

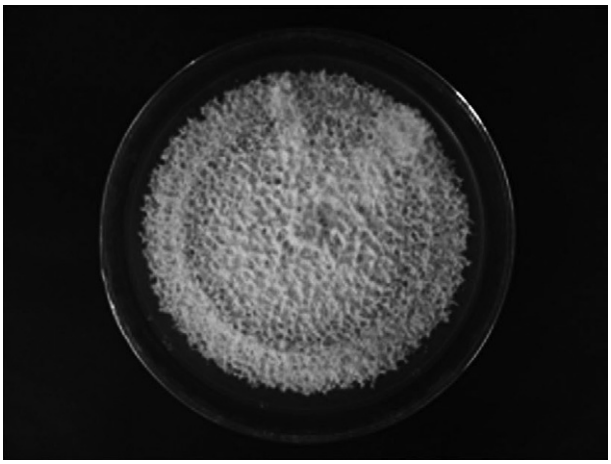

Fig. 4 – *C. viniferum* GZAAS5.08601 (holotype). Colony upper.

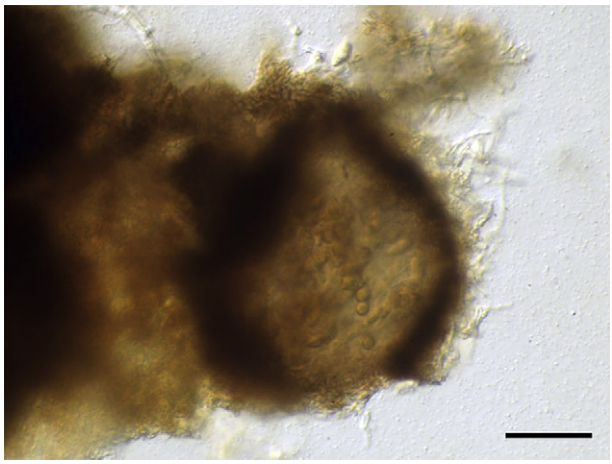

Fig. 6 – *C. viniferum* GZAAS5.08601 (holotype). Peridium produced on PDA (Bars: 6 = 100 μm, 7–9 = 10 μm).

fruits were chosen for pathogenicity testing. Healthy leaves or fruits were washed with tap water, and then disinfected in 1% sodium hypochlorite for 3–5 min. Disinfected leaves or fruits were washed three times with sterile distilled water, and then dried with sterilized filter paper. Leaves or fruits were kept individually in a 12 cm diameter Petri dish or tissue culture bottle containing distilled water to maintain humidity.

The wound/drop inoculation method was to prick the leaf or fruit wall with a pin to a 1 mm depth and then placing 6 μl of conidia suspension (10^6 conidia/ml) on the wound. The method of non-wound inoculation involved placing 6 μl conidia suspension on the fruits and leaves without pin pricks. Control fruits and leaves were inoculated with 6 μl of sterile distilled water onto the wound or non-wound. Each plant was inoculated with three leaves or fruits per selected strain and repeated four times. The test was carried out twice to confirm the results. The inoculated fruits and leaves were incubated at room temperature (22°C). After 4–5 days and 14–15 days of incubation leaves and fruits were checked for lesions with a microscope. The fungus was reisolated from lesions that developed and incubated at room temperature. The resultant cultures and spores were checked for morphological characters with Koch's postulates.

2.4. *DNA sequencing and PCR amplification*

Isolates grew on PDA for 8 days at 25°C and mycelia were scraped from the surface. A modified protocol of Chen et al. (2007) was used for DNA extraction. Partial actin (ACT), β-tubulin (TUB2), calmodulin (CAL), glutamine synthetase (GS), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS) were selected to amplify by PCR reaction. Primer pairs and PCR amplifications follow the method described by Prihastuti et al. (2009). The PCR products were examined with electrophoresis, to be stained with ethidium bromide on 1.2% agarose electrophoresis gels and were purified according to the manufacturer's instructions of a TIANgel Midi Purification Kit (TIANGEN, China). DNA sequencing of the six genes were conducted by SinoGenoMax, Beijing, China.

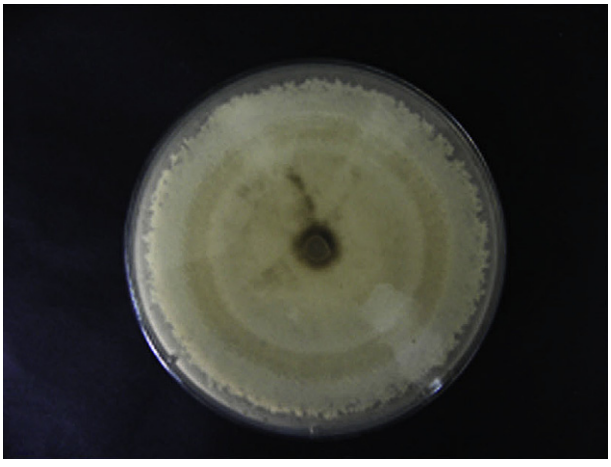

Fig. 5 – *C. viniferum* GZAAS5.08601 (holotype). Colony reverse.

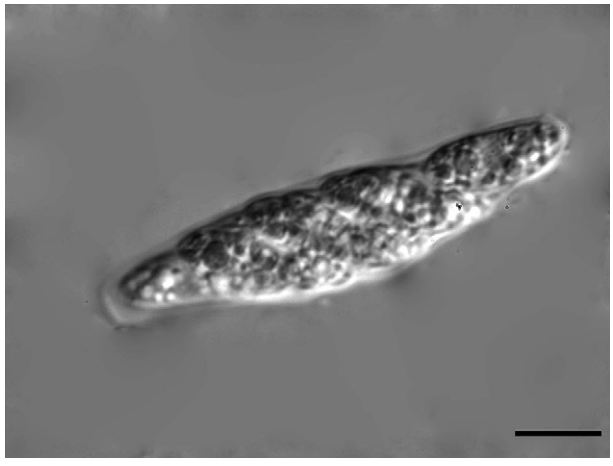

Fig. 7 – *C. viniferum* GZAAS5.08601 (holotype). Ascus produced on PDA (Bars: 6 = 100 μm, 7–9 = 10 μm).

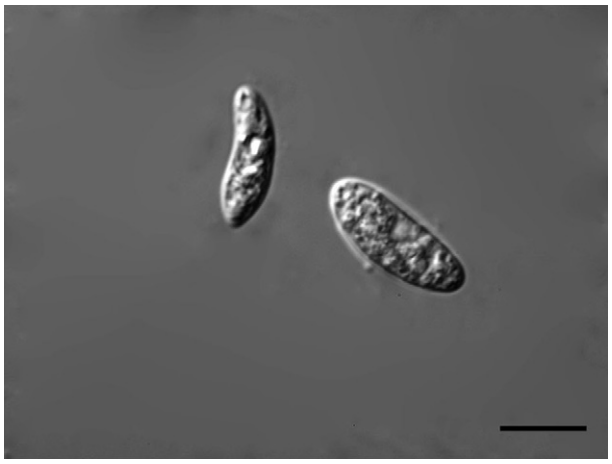

Fig. 8 – *C. viniferum* GZAAS5.08601 (holotype). Ascospore released from asci (Bars: 6 = 100 μm, 7–9 = 10 μm).

Fig. 10 – *C. viniferum*. Conidia GZAAS5.08601 (holotype) (Bars = 10 μm).

2.5. *Phylogenetic analysis*

In order to identify the strains in this study, fifteen *Colletotrichum* species including the new species were chosen for primarily molecular analysis using the ITS region (Fig. 25, Table 2). Phylogenetic analysis was performed with six gene regions. The GenBank accession numbers of all sequences are listed in Table 2. Multiple sequence alignments were generated with ClustalX 2.0.10 (Larkin et al. 2007). Gaps were treated as missing data. Ambiguously aligned regions were excluded from all analyses.

A partition homogeneity test (PHT) was performed with 1000 replicates in PAUP* 4b10 (Swofford 2002) to evaluate statistical congruence between six gene sequences.

The methods used for phylogenetic analysis were similar to Cai et al. (2006). ITS region and combined sequence alignments were analyzed with maximum parsimony (MP) in PAUP* 4b10. Trees were inferred with the heuristic search option with TBR branch swapping. Bootstrap values of MP were generated from 1000 replicates. Descriptive tree

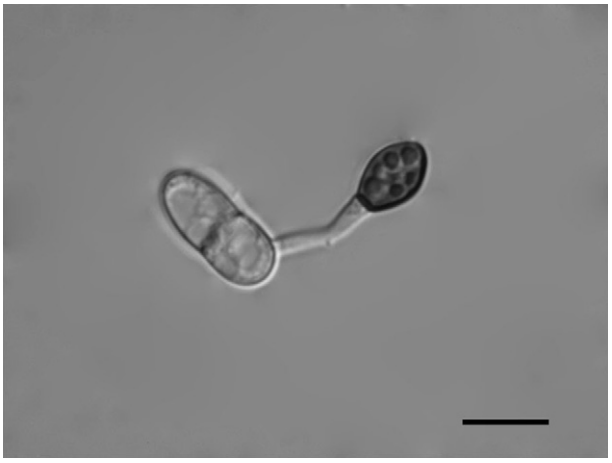

Fig. 11 – *C. viniferum*. Appressoria produced from conidia GZAAS5.08601 (Bars = 10 μm).

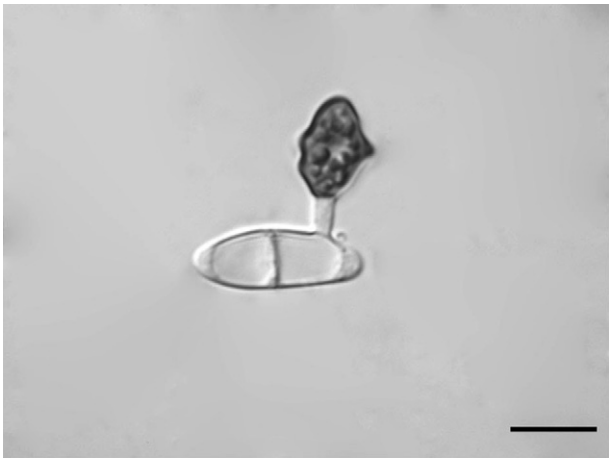

Fig. 9 – *C. viniferum* GZAAS5.08601 (holotype). Appressoria produced from ascospore in slide culture (Bars: 6 = 100 μm, 7–9 = 10 μm).

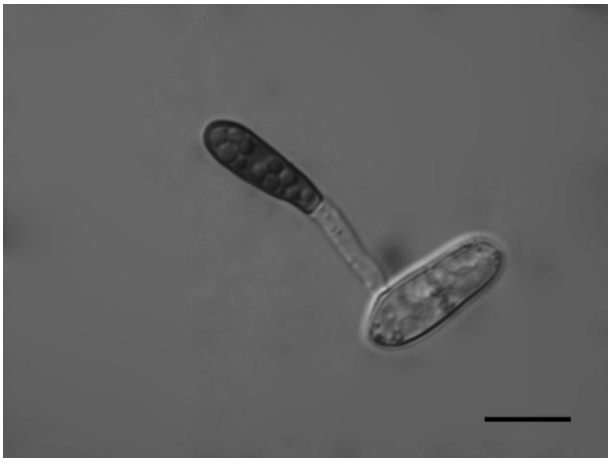

Fig. 12 – *C. viniferum*. Appressoria produced from conidia GZAAS5.08608 (Bars = 10 μm).

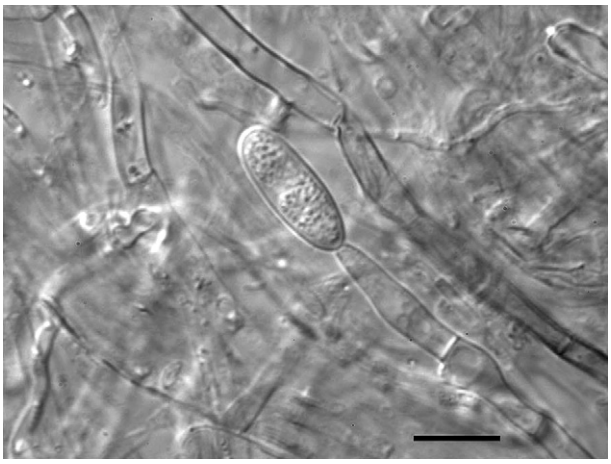

Fig. 13 – *C. viniferum*. Conidiophore and conidium GZAAS5.08601 (Bars = 10 μm).

statistics such as tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], and homoplasy index [HI] were calculated for trees resulted from different optimality criteria. The six genes sequence alignments had been deposited in TreeBASE. The submission ID is 11845.

3. Results

3.1. *Collection of* Colletotrichum *species*

Twenty-six strains of *Colletotrichum* were isolated from vitaceous plant from ten sites in Guizhou and Yunnan provinces, twenty-one strains from *V. vinifera* (table grape), four from *Ampelopsis* sp. and one from *Cayratia japonica*. A summary of all strains are illustrated in Table 1.

3.2. *Phylogenetic analysis*

The phylogeny generated from ITS sequences of fifteen *Colletotrichum* species delimited two strains (GZAAS5.08620 and GZAAS5.08624) together with *C. gloeosporioides*, six strains (GZAAS5.08601, GZAAS5.08604, GZAAS5.08608, GZAAS5.08614, GZAAS5.08616 and GZAAS5.08622) as a monophyletic lineage, while four strains (GZAAS5.08610, GZAAS5.08618, GZAAS5.08627 and GZAAS5.08628) could not be well-defined (see Fig. 25). The multilocus phylogeny analysis was needed to identify *Colletotrichum* species.

The partition homogeneity test ($P = 0.01$) indicated that the individual partitions were not highly incongruent (Farris et al. 1995).

The combined datasets of partial ACT, TUB2, CAL, GS, GPDH and the complete rDNA-ITS region comprised 3381 characters after alignment, of which 861 characters were parsimony informative. Four parsimonious trees were generated (TL = 1620, CI = 0.828, RI = 0.884, RC = 0.732, HI = 0.172). One of them was shown in Fig. 1. The phylogram based on the combined dataset showed that the isolates in the study fell into three categories namely clade 1, 2 and 3, two strains clustering with *C. gloeosporioides* (strain CBS953.97, epitype) in clade 1; six strains, into a monophyletic lineage in clade 2; and four strains with *C. fructicola* (strain BPD-I16, holotype) in clade 3 with bootstrap values 100%, 99% and 97% accordingly. The six strains in clade 2 were the new species we described as *Colletotrichum viniferum* L.J. Peng, L. Cai, K.D. Hyde & Z.Y. Liu, which was in different clades from the other species with similar morphological characters, e. g. *Colletotrichum asianum* Prihastuti, L. Cai & K.D. Hyde, *C. gloeosporioides* and *Colletotrichum siamense* Prihastuti, L. Cai & K.D. Hyde.

Table 3 – Summary of morphological data of *Colletotrichum* species on Vitaceae.

Species and isolate numbers	Conidia			Appresoria		Growth rate (mm/day)
	Length (μm)	Width (μm)	Shape	Length (μm)	Width (μm)	
C. gloeosporioides GZAAS5.08620	14.05 ± 0.79 B (12.09–16.60)	5.25 ± 0.32 C (4.29–6.09)	Cylindrical	8.45 ± 1.04 B (6.08–10.93)	6.08 ± 0.64 A (4.41–8.10)	9.22 ± 1.53 A
C. viniferum GZAAS5.08601	13.79 ± 1.03 BC (11.78–15.78)	5.45 ± 0.36 B (4.59–6.01)	Cylindrical	8.08 ± 0.82 C (6.32–10.31)	5.46 ± 0.47 C (4.78–6.30)	8.29 ± 1.01 C
C. viniferum GZAAS5.08614	12.27 ± 1.07 D (9.28–14.44)	5.56 ± 0.39 AB (4.63–6.77)	Cylindrical	7.165 ± 0.82 D (5.96–9.36)	5.35 ± 0.52 D (4.08–6.23)	8.52 ± 0.23 B
C. viniferum GZAAS5.08616	13.56 ± 0.71 BC (11.71–14.83)	5.56 ± 0.23 AB (5.17–6.03)	Cylindrical	8.61 ± 1.77 B (5.04–12.14)	5.45 ± 0.70 C (4.19–7.00)	8.02 ± 0.15 D
C. viniferum GZAAS5.08604	13.48 ± 0.79 BC (11.59–15.87)	5.35 ± 0.23 BC (4.81–5.87)	Cylindrical	8.09 ± 1.05 C (6.77–10.54)	5.61 ± 0.67 B (4.53–6.68)	8.52 ± 0.83 B
C. viniferum GZAAS5.08608	13.25 ± 0.92 C (11.00–15.41)	5.55 ± 0.27 AB (5.01–6.21)	Cylindrical	8.55 ± 1.55 B (6.47–12.59)	5.46 ± 0.61 C (4.17–7.11)	6.42 ± 0.65 E
C. viniferum GZAAS5.08622	17.44 ± 1.86 A (12.70–22.32)	5.74 ± 0.43 A (4.74–7.09)	Cylindrical	9.41 ± 1.11 A (6.68–11.68)	6.05 ± 0.65 A (7.31–5.22)	8.34 ± 0.84 C
C. fructicola GZAAS5.08610	12.56 ± 1.24 D (9.71–15.01)	5.38 ± 0.54 BC (4.02–6.35)	Cylindrical	7.99 ± 1.45 C (5.96–12.75)	4.99 ± 0.40 E (4.04–5.85)	6.74 ± 0.26 E
LSD (between group)	1.26	0.14		1.41	0.53	0.68

Least Significant Difference (LSD) means with the same letter in each column are not significantly different from each other based on DMRT test in Sirichai statistics version 6.

Table 4 – Pathogenicity testing and host range.

Strains	Plant											
	C. reticulata leaves		*C. sinensis* leaves		*V. viniferum* fruits		*A. sinica* leaves		*E. japonica* leaves		*C. frutescens* leaves	
	W	NW	W	NW	W	NW	W	NW	W	NW	W	NW
C. fructicola GZAAS5.08610	–	–	+	+	+	+	+	–	+	+	+	–
C. gloeosporioides GZAAS5.08620	–	–	+	–	+	+	+	–	–	–	–	–
C. viniferum GZAAS5.08601	–	–	+	+	+	+	+	–	+	–	–	–
C. viniferum GZAAS5.08608	+	–	+	–	+	+	+	–	+	–	–	–

Note: W, wound inoculation; NW, non-wound inoculation; +, symptom observed; –, no symptom.

In this study, we used six gene sequences, and this has provided a relatively robust support for identifying species, e. g. *C. asianum*, *C. fructicola*, *C. gloeosporioides*, *Colletotrichum horii* B. Weir & P.R. Johnst., *Colletotrichum kahawae* J.M. Waller & Bridge, *Colletotrichum musae* (Berk. & M.A. Curtis) Arx, *C. siamense*, *Colletotrichum simmondsii* R.G. Shivas & Y.P. Tan and *C. viniferum*.

3.3. *Taxonomy*

C. viniferum L.J. Peng, L. Cai, K.D. Hyde & Z.Y. Liu, **sp. nov.** (Figs. 3–13).

MycoBank: MB 563086

Coloniae albae vel griseo. Conidia 12–16 × 4.5–6 μm, unicellularae, laevae, hyalinae, cylindrici, ad apicem obtuse. Appressoria 6.5–10.5 × 4.8–6.3 μm, brunnea vel atro-brunnea, irregulariter ovoidea, clavatae.

Etymology: *Viniferum*, in reference to the host species, *V. vinifera* from which the species was isolated.

Description: Colonies on PDA at first white and becoming grey, reverse pale yellowish, reaching a maximum of 78 mm diam. in 7 days at 25°C, growth rate 3–13.5 mm/day ($\bar{x} = 8.29 \pm 1.01$, $n = 9$) (Figs. 4 and 5). Aerial mycelium greyish-white, dense, cottony, with visible conidial masses at the inoculum point. Sclerotia present in some cultures. Setae absent. Conidiomata dark. Conidiophores hyaline, one-celled, not branching, 16–25 × 3–4.5 μm ($\bar{x} = 20.6 \pm 3.2 \times 3.7 \pm 0.5$, $n = 20$) (Fig. 13). Conidia common in mycelium, 12–16 μm long ($\bar{x} = 13.84 \pm 0.98$, $n = 100$) × 4.5–6 μm wide ($\bar{x} = 5.43 \pm 0.38$, $n = 100$), one-celled, smooth-walled, guttulate, hyaline, cylindrical with obtuse to slightly rounded ends, sometimes oblong, when conidia germinate a septum may be formed (Figs. 10 and 11). Appressoria 6.5–10.5 × 4.8–6.3 μm ($\bar{x} = 8.13 \pm 1.80 \times 5.37 \pm 1.04$, $n = 30$) in slide cultures, brown, ovoid, clavate and sometimes irregularly lobed, usually containing guttules (Figs. 9 and 11).

Glomerella teleomorph produced on PDA.

Ascomata 248–464 × 220–423 μm ($\bar{x} = 340.7 \pm 61.54 \times 294.2 \pm 51.95$, $n = 10$), light brown to brown, globose to subglobose, semi-immersed or completely immersed in PDA. Peridium of textura angularis, thick-walled (Fig. 6). Asci 42–63 × 11–13.5 μm ($\bar{x} = 52.30 \pm 7.26 \times 12.25 \pm 0.76$, $n = 20$), unitunicate, thin-walled, 6–8 spored, clavate or cymbiform (Fig. 7). Ascospores 12–16 × 4.6–6 μm ($\bar{x} = 13.79 \pm 1.03 \times 5.45 \pm 0.37$, $n = 30$), one-celled, hyaline, with a large guttule at the centre and surrounded by small guttules, slightly curved to

Table 5 – Synopsis of morphological data of *Colletotrichum* species on grape.

Species	Conidia		Appressoria		Reference
	Size (μm)	Shape	Size (μm)	Shape	
C. acutatum	8.5–16.5 × 2.5–4 μm	Fusiform	8.5–10 × 4.5–6 μm	Ovate to obovate to slightly irregular with margins entire or slightly lobed	Sutton 1992
C. crassipes	14–28 × 5–7 μm	Cylindrical	10.5–14 × 7–9.5 μm	Long clavate to ovate, with crenate or deeply lobed margins	Sutton 1992
C. fructicola	9.7–14 × 3–4.3 μm	Cylindrical	4.3–9.7 × 3.7–7.3 μm	Ovoid, clavate and slightly irregular	Prihastuti et al. 2009
C. gloeosporioides	12–17 × 4.5–6 μm	Sub cylindrical	7.2–8.6 × 4.7–6.0 μm	Clavate, regular in outline or irregular and weakly lobed, the walls thickened	Cannon et al. 2008
C. viniferum	12–16 × 4.6–6.0 μm	Cylindrical	6.3–10.3 × 4.8–6.3 μm	Ovoid, clavate, long clavate and sometimes irregularly weakly lobed	This paper
C. vitis	21–25 × 2.5 μm	Cylindrical	–	–	Saccardo 1913

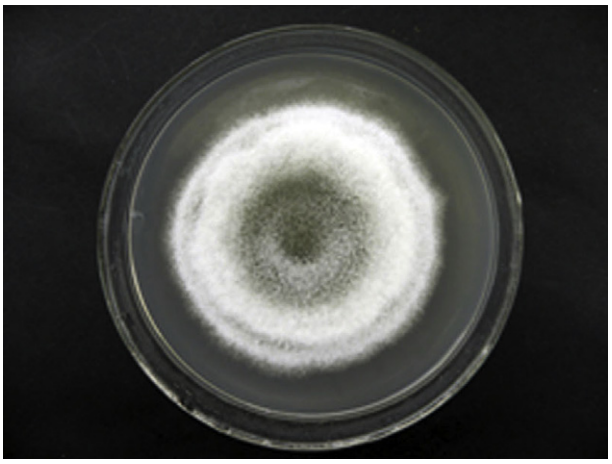

Fig. 14 – *C. fructicola* GZAAS5.08610. Colony upper (Bars = 10 μm).

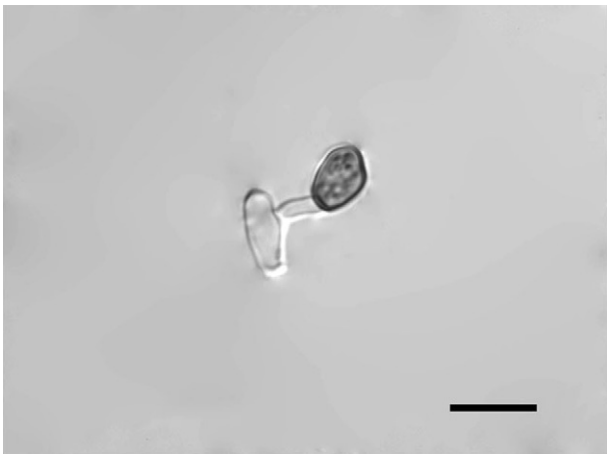

Fig. 17 – *C. fructicola* GZAAS5.08610. Appressoria produced from conidia (Bars = 10 μm).

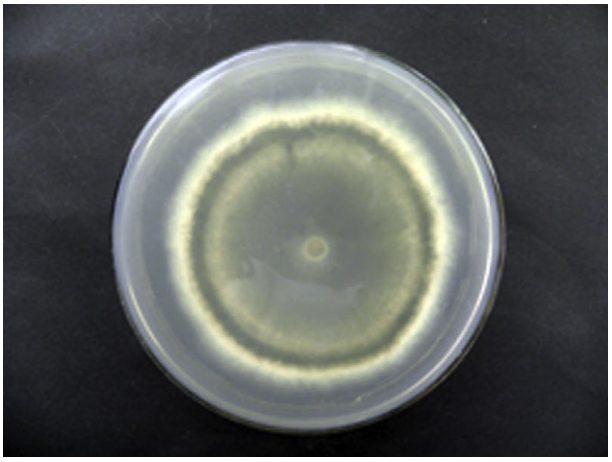

Fig. 15 – *C. fructicola* GZAAS5.08610. Colony reverse (Bars = 10 μm).

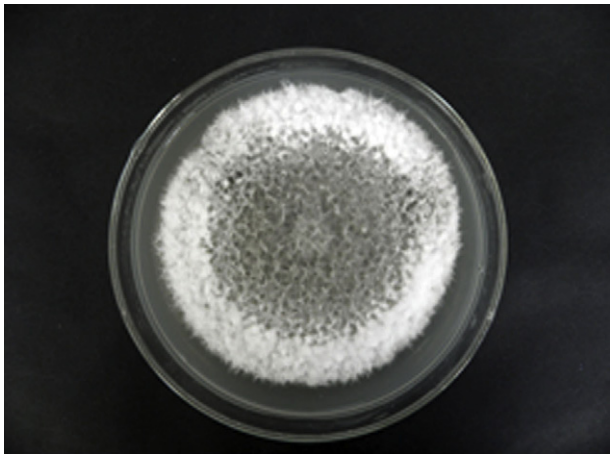

Fig. 18 – *C. gloeosporioides* GZAAS5.08620. Colony upper (Bars = 10 μm).

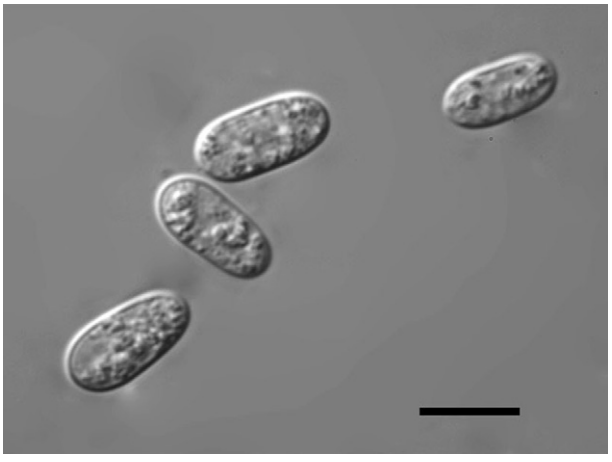

Fig. 16 – *C. fructicola* GZAAS5.08610. Conidia (Bars = 10 μm).

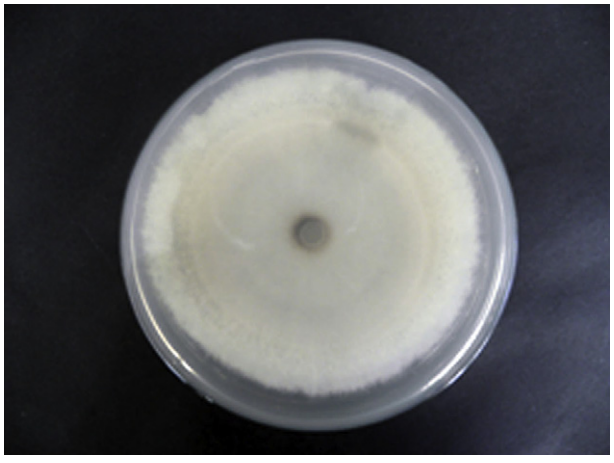

Fig. 19 – *C. gloeosporioides* GZAAS5.08620. Colony reverse (Bars = 10 μm).

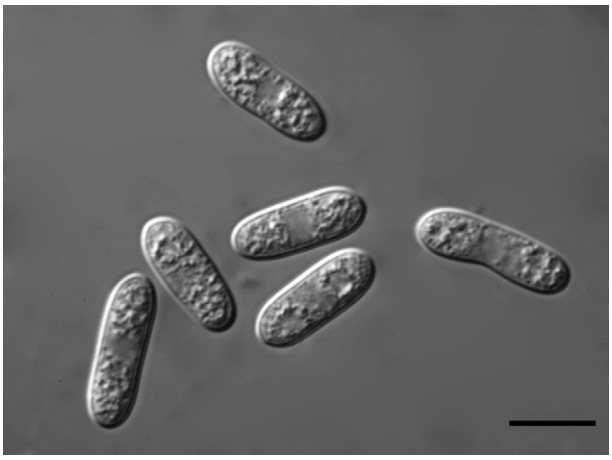

Fig. 20 – *C. gloeosporioides* GZAAS5.08620. Conidia (Bars = 10 μm).

curved with obtuse to slightly rounded ends (Figs. 8 and 9). Many ascomata formed within 2–3 months.

Holotype: CHINA, Yunnan Province, Kunming City, Ala Town, Gaopo Village, on fruits of *V. vinifera* cv. shuijing, 30 July 2008, L.J. Peng (GZAAS5H.08601; ex-holotype living culture GZAAS5.08601, CBS130643).

Known distribution: Yunnan and Guizhou provinces, China.

Additional specimens examined: CHINA, Guizhou Province, Guiyang City, Huaxi District, the Experiment Farm of Guizhou University, on fruits of *V. vinifera* cv. hongti, 3 August 2008, L.J. Peng (**Paratype** in GZAAS5H.08608; ex-paratype living culture GZAAS5.08608, CBS130644).

Notes: The conidia in *C. viniferum* are cylindrical. The species is characterized by its ovoid, clavate, long clavate and sometimes irregularly lobed appressoria (Figs. 11 and 12, Table 5) which is different from *C. gloeosporioides* which are aseptate, clavate, regular in outline or irregular and weakly lobed (Cannon et al. 2008).

C. fructicola Prihast., L. Cai & K.D. Hyde.

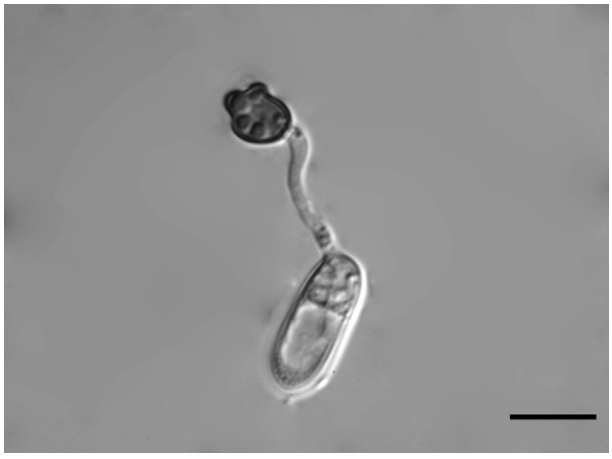

Fig. 21 – *C. gloeosporioides* GZAAS5.08620. Appressoria produced from conidia (Bars = 10 μm).

Fig. 22 – Symptoms on leaves of *C. sinensis* inoculation *C. viniferum* strain GZAAS5.08601, left wound, right non-wound inoculation.

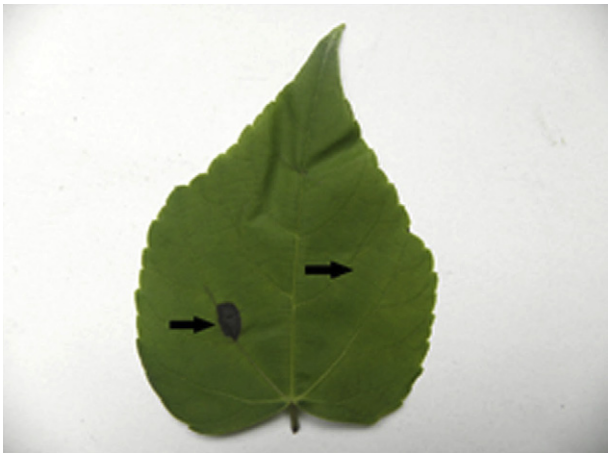

Fig. 23 – Symptoms on leaf of *A. sinica* inoculation *C. viniferum* strain GZAAS5.08608, left wound, right non-wound inoculation.

Fig. 24 – Symptoms on leaf of *A. sinica* inoculation *C. gloeosporioides* strain GZAAS5.08620, left wound, right non-wound inoculation.

Four strains of this species were isolated, two from leaves of *Ampelopsis* sp. caused brown irregular spots, one from fruits of *V. vinifera* cv. kyoho and one from fruits of *V. vinifera* cv. shuijing caused anthracnose appearing as black spots (Figs. 14–17, Tables 1 and 3).

C. *gloeosporioides* (Penz.) Penz. & Sacc.

Two strains were isolated, one from leaves of *Ampelopsis* sp. caused brown irregular spots, and one from fruits of *V. vinifera* cv. shuijing caused anthracnose appearing as black spots (Figs. 18–21, Tables 1 and 3).

3.4. *Pathogenicity testing and host range*

Six hosts were chosen to test the pathogenicity and host range of the *Colletotrichum* strains in this study using Koch's postulate (Table 4). *C. viniferum* (GZAAS5.08601, GZAAS5.08608) did not infect leaves of *Capsicum frutescens* (chili pepper) by non-wound/drop or wound/drop inoculation, but infected fruits of *V. vinifera* (grape) by both inoculation methods forming conidial masses. *C. viniferum* strains GZAAS5.08601 did not infect leaves of *Citrus reticulata* (citrus) by either inoculation method, but infected leaves of *Citrus sinensis* (orange) by both inoculation methods (Figs. 22 and 23). *C. gloeosporioides* strain GZAAS5.08620 did not infect leaves of *C. reticulata* (citrus), *Eriobotrya japonica* (loquat) and *C. frutescens* (chili pepper), but infected fruits of *V. vinifera* (grape) by both inoculation methods. This strain did not infect leaves of *C. sinensis* (orange) and *Ampelopsis sinica* (wild grape) by non-wound/drop method and infected leaves of *C. sinensis* (orange) and *A. sinica* (wild grape) by wound/drop method (Fig. 24).

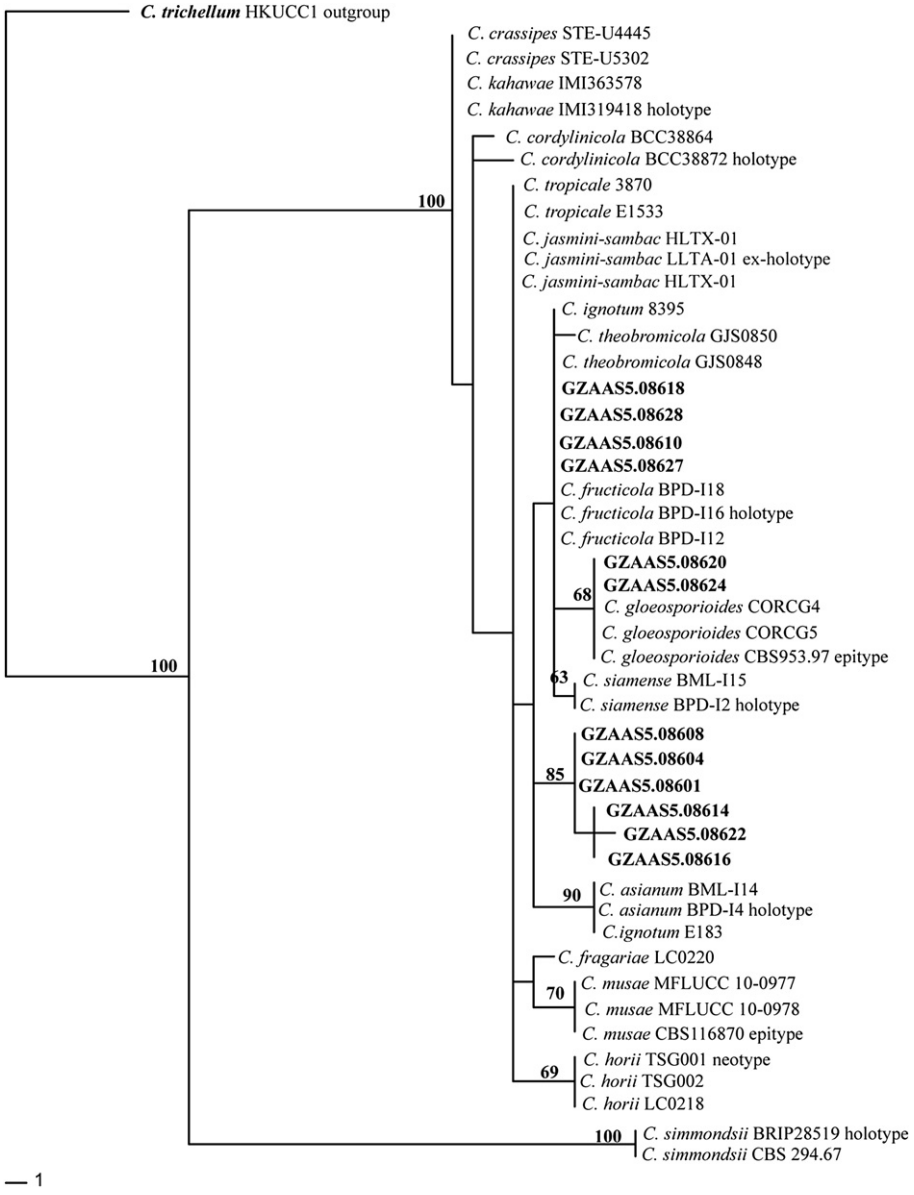

Fig. 25 – Phylogram generated from maximum parsimony analysis based on ITS sequences, showing the phylogenetic relationships of *C. viniferum*. Values above the branches are parsimony bootstrap (>50%). The tree is rooted with *C. trichellum*. Strains isolated in this study are shown in bold.

4. Discussion

Grape ripe rot is a disease effecting grapes at or near harvest. The fruits infected turn dark brown and then on the skin produced pink or orange spore masses. As infected fruits mature, tiny black fruiting bodies (acervuli) develop within the lesion in a circular arrangement (Figs. 2 and 3). The symptoms of disease on grape fruits caused by all three species isolated in this study could not be distinguished.

Several *Colletotrichum* species have been reported from *V. vinifera* (grape) including *C. acutatum* (Shiraishi et al. 2007; Whitelaw-Weckert et al. 2007; Greer et al. 2011), *Colletotrichum crassipes* (Speg.) Arx (Hyde et al. 2009), *C. gloeosporioides* (Sung et al. 2008; Greer et al. 2011) and *C. vitis* Istv. (Saccardo 1913) and *Colletotrichum* sp. (Gonzalez and Tello 2011). The morphological data of *Colletotrichum* species on grape are listed in Table 5. *C. crassipes* originally isolated from fruit of *V. vinifera* (grape) from Conegliano, Italy (Hyde et al. 2009) has wider, longer conidia and deeply lobed appressoria, which is different from *C. gloeosporioides* (Sutton 1992). Conidia in *C. crassipes* are 14–28 × 5–7 μm and larger than those of *C. viniferum* (12–16 × 4.5–6 μm). The average length of conidia in *C. viniferum* ($\bar{x}$ = 13.79 ± 0.98 μm) is shorter than that of *C. gloeosporioides* ($\bar{x}$ = 14.4 μm). Conidia in *C. vitis* are 21–25 × 2.5 μm, smaller than those of *C. viniferum* although the former species is hardly known. The name of *C. vitis* was not on the list of 66 *Colletotrichum* names in common use (Hyde et al. 2009).

Rojas et al. (2010) described two new species, *Colletotrichum tropicale* Rojas, Rehner & Samuels and *Colletotrichum ignotum* Rojas, Rehner & Samuels, that were frequent asymptomatic associates of cacao and other neotropical plant species, and epitypified *Colletotrichum theobromicola* Delacr. & Bull. Soc., which was associated with foliar and fruit anthracnose lesions of cacao in Panama among 77 *C. gloeosporioides* strains and different from *C. viniferum* in phylogeny with ITS gene (Fig. 25). The gene loci that Rojas et al. (2010) used were different from those used in this study. The ITS gene tree shows *C. viniferum*, the new species we identified is different from *C. ignotum*, *C. theobromicola* and *C. tropicale*.

Among the 26 strains isolated from vitaceous plants, five strains were isolated from leaf lesions with one strain of *C. gloeosporioides* from a leaf lesion of *Ampelopsis* sp., two of *C. fructicola* from the same lesions and the other two of *C. viniferum,* one from an *Ampelopsis* sp. leaf lesion and the other from a *Cayratis japonica* (Japanese cayratia herb) leaf lesion. The remaining 21 strains, eighteen of *C. viniferum*, two of *C. fructicola* and one of *C. gloeosporioides* were isolated from anthracnose lesions on *V. vinifera* (grape) fruits. This was the first report of *C. fructicola* from *Ampelopsis* sp. and *V. vinifera* (grape) that indicated the wide range host. *C. fructicola* had already been isolated from coffee berries in northern Thailand (Prihastuti et al. 2009) and fruits lesions of papaya and longan at Chiang Mai, Thailand (Phoulivong et al. 2010a). *C. gloeosporioides* was previously listed as infective of many fruits, especially in the tropics (Paull et al. 1997; Freeman et al. 1998; Afanador-Kafuri et al. 2003; Lubbe et al. 2004; Sangeetha and Rawal, 2008; Masyahit et al. 2009). It has been demonstrated however, that *C. gloeosporioides* (Cannon et al. 2008) rarely caused anthracnose of tropical fruits and is not a common pathogen in the tropics (Prihastuti et al. 2009; Phoulivong et al. 2010a; Rojas et al. 2010; Wikee et al. 2011).

The main objectives of this study were to identify the species of *Colletotrichum* from *Vitaceae* in Guizhou and Yunnan provinces with combined morphological and molecular data. The ITS region is the most widely used region in fungi identification (Moriwaki et al. 2002; Jeewon et al. 2003; Phillips et al. 2007; Yang et al. 2009). The ITS support for nodes was low and the current ITS tree could not well-defined certain *Colletotrichum* species (Moriwaki et al. 2002; Yang et al. 2009), such as *C. crassipes* and *C. kahawae*, *Colletotrichum jasmini-sambac* and *C. tropicale*, *C. fructicola* and *C. theobromicola* as this paper revealed (Fig. 25). While through multigene analyses, *C. fructicola*, *C. jasmini-sambac* and *C. kahawae* represented three clades with bootstrap value of 97%, 77% and 100% respectively (Fig. 1). The phylograms based on ITS and six genes showed that the isolates of *C. asianum*, *C. gloeosporioides*, *C. horii*, *C. musae*, *C. siamense*, *C. simmondsii* and *C. viniferum* were a monophyletic lineage in our study (Figs. 1 and 25).

Acknowledgements

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