



## Identification and characterization of *Colletotrichum* species causing grape ripe rot in southern China

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### Abstract

Grape ripe rot is known to be causing serious losses in grapes in many countries. In order to clarify the species of pathogen causing grape ripe rot in southern China, 62 samples with ripe rot symptoms were collected from Fujian and Guizhou Provinces. Single spore isolation techniques resulted in 50 isolates of *Colletotrichum* species. Based on multigene data and morphology, 48 isolates were identified as *C. viniferum* and one isolate was identified as *C. citri*. A *Colletotrichum* species, sister to *C. cliviae*, was identified to be causing grape ripe rot. However, until further collections are made, this species will remain as *Colletotrichum* sp. This is the first time that *C. citri* and the undetermined *Colletotrichum* species sister to *C. cliviae* have been reported to be associated with grape ripe rot in the world.

**Key words** – China – *Colletotrichum citri* – Grape ripe rot – Identification – Pathogenicity

### Introduction

As one of the first fruits cultivated worldwide, grapes play a key role in winemaking as well as processed grape products, such as jam and raisins (Hong et al. 2008). Since 1990s, with the breakthrough in cultivation techniques and the changes in the structure of agricultural policy, the grape industry in Fujian Province has grown rapidly, evidenced by a rapid increase of cultivated acreage and production (Lei et al. 2011). By the end of 2014, the total area of grape production was 8,527 hm<sup>2</sup>, resulting in with more than 153,300 t of grapes (Fujian Provincial Bureau of Statistics 2015). In Guizhou Province, grapes are cultivated in nine cities, especially in the central and southeastern regions. The main variety is ‘Crystal’ with a cultivation area of approximately 17,000 hm<sup>2</sup>, which accounts for 60% of the total area in Guizhou Province (Tang 2015). However, Fujian Province and Guizhou Province both have warm summer conditions because of the humid subtropical seasonal and windy climate, resulting in the occurrence of a variety of pests in mature grapes (Lu 2005).

*Colletotrichum* is a phytopathogenic genus that is widespread in the world, causing disease in various crops and fruits, such as banana, citrus, grape, mango and strawberry. It often causes huge economic losses especially in tropical and subtropical regions (Phoulivong et al. 2010). *Colletotrichum* species can invade multiple parts of the plant (leaf, flower, stem and fruit), causing a variety of symptoms such as fruit rot, dry shoot, and withering. Fruits are often infected in the period of pre- and post-harvest seasons (Bailey & Jeger 1992). Grape ripe rot is one of the most important diseases in the mature stage of grape development. It was first reported in 1891 in the United States (Southworth 1891). *Colletotrichum gloeosporioides* s.l and *C. acutatum* were previously reported as the disease pathogens resulting in ripe rot of grapes (Daykin & Miholland 1984, Yamamoto et al. 1999, Melksham et al. 2002). Identification of *Colletotrichum* species based solely on morphology is prone to errors because there are no definite morphotaxonomic characters and these characters may suffer from inconsistency when subjected to different experimental methods and conditions (Cai et al. 2009, Hyde et al. 2009). In recent years, a large number of species of *Colletotrichum* have been identified using morphological characteristics with the combination of multi-gene analysis (Moriwaki & Tsukiboshi 2009, Weir & Johnston 2010, Wikee et al. 2011, Peng et al. 2013). Peng et al. (2013) satisfactorily classified 26 isolates of *Colletotrichum* from Guizhou and Yunnan provinces and also described a new species, *C. viniferum*. Yan et al. (2015) isolated and identified 34 isolates from six provinces and reported the grape ripe rot caused by *C. aenigma* for the first time from China. Therefore, the results of molecular approaches are more stable and reliable, and would also be helpful in understanding the evolutionary relationships of *Colletotrichum* species (Cai et al. 2009, Hyde et al. 2009).

To determine the species of grape ripe rot and their pathogenicity differences, we collected 62 samples from Fujian and Guizhou Provinces. Based on morphology and multi-gene phylogeny, the species were identified.

## Materials & Methods

### *Sample collection and isolation of fungi*

Fungi were isolated from grapevine fruits samples with typical symptoms of ripe rot from Fujian Province and Guizhou Province in July 2015 (Table 1). Grapevine varieties used in this study included *V. vinifera* cv. Kyoho, *V. Labruscana* cv. Summer Black and others listed in Table 1. Pathogens were isolated via purifying a single spore according to “Plant Pathology Research Methods” (Fang 1998). The grape variety used for the pathogenicity test was *V. vinifera* cv. Kyoho and was available from local farmer’s market in Beijing, China.

### *Morphological characterization*

Morphological and cultural characterization was carried out according to the method of Cai et al. (2009). The diseased fruits were disinfected with 0.1% Sodium hypochlorite and 70% ethanol for 1 min. A tissue block of approximately 0.3 mm × 0.3 mm × 0.3 mm was cut off from the junction of the healthy part and the lesion part and was re-disinfected with 70% ethanol for 2–3 min, and then cultivated at 25 °C on a potato dextrose agar (PDA) medium. After the colonies were visible clearly, a block was taken from the edge of the mycelium with a diameter of 5 mm punches, and then transferred to a new PDA plate for 7–10 d cultivation. Afterwards, purification was performed by a single spore separation method. Mycelial discs (5 mm diameter) were cut off from the edge of five-day old colonies then transferred to a new PDA plate and incubated in the dark at 28 °C. Each strain was repeated in triplicate. Colony diameter was measured after 7 days, and the average of growth rate (mm/ day) was calculated. The morphology of both colonies and conidia was observed, the size (length and width) of 10 pycnidia and 50 conidia was measured under a microscope (Nikon SMZ1500 and AXIO Imager. Z2) (Weir et al. 2012).

**Table 1** 50 isolates of *Colletotrichum* from this study.

<b>Isolate no.</b>	<b>Host</b>	<b>Origin</b>	<b>Date of collection</b>
FJ001	<i>V. vinifera</i> cv. Jingyu	Xingyuan, Zhengfang, Shunchang, Fujian	2015.07.07
FJ002	<i>V. vinifera</i> cv. Beauty Finger	Xingyuan, Zhengfang, Shunchang, Fujian	2015.07.07
FJ005	<i>V. labruscana</i> cv. Summer Black	Xinjie, Yangxi, Meilie, Sanming, Fujian	2015.07.07
FJ008	<i>V. labruscana</i> cv. Summer Black	Xinjie, Yangxi, Meilie, Sanming, Fujian	2015.07.07
FJ010	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.13
FJ011	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.13
FJ012	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.13
FJ014	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.13
FJ015	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.13
FJ016	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.13
FJ017	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.13
FJ018	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.13
FJ037	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.10
FJ038	<i>V. vinifera</i> cv. Kyoho	Beishan, Xibing, Fu'an, Fujian	2015.07.10
FJ041	<i>V. vinifera</i> cv. Kyoho	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ043	<i>V. vinifera</i> cv. Kyoho	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ044	<i>V. vinifera</i> cv. Kyoho	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ045	<i>V. vinifera</i> cv. Kyoho	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ047	<i>V. vinifera</i> cv. Kyoho	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ050	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ051	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ053	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ055	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ056	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ058	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ059	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ060	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ061	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ062	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ063	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ064	<i>V. labruscana</i> cv. Summer Black	Xianghuan, Saiqi, Fu'an, Fujian	2015.07.10
FJ067	<i>V. labruscana</i> cv. Summer Black	Houfang, Mayangxi, Changtai, Fujian	2015.07.11
FJ068	<i>V. labruscana</i> cv. Summer Black	Houfang, Mayangxi, Changtai, Fujian	2015.07.11
FJ069	<i>V. labruscana</i> cv. Summer Black	Houfang, Mayangxi, Changtai, Fujian	2015.07.11

Isolate no.	Host	Origin	Date of collection
FJ071	<i>V. vinifera</i> 'Rose red'	Houfang, Mayangxi, Changtai, Fujian	2015.07.11
FJ072	<i>V. vinifera</i> 'Shennong gold queen'	Pudang, Xindian, Jin'an, Fuzhou, Fujian	2015.07.13
FJ073	<i>V. vinifera</i> 'Shennong gold queen'	Pudang, Xindian, Jin'an, Fuzhou, Fujian	2015.07.13
FJ074	<i>V. vinifera</i> 'Shennong gold queen'	Pudang, Xindian, Jin'an, Fuzhou, Fujian	2015.07.13
FJ080	<i>V. vinifera</i> 'Shennong gold queen'	Pudang, Xindian, Jin'an, Fuzhou, Fujian	2015.07.13
FJ083	<i>V. vinifera</i> 'Shennong gold queen'	Pudang, Xindian, Jin'an, Fuzhou, Fujian	2015.07.13
GZ012	<i>V. vinifera</i> 'Crystal'	Xinlian, Jiaoli, Sandu, Guizhou	2015.07.19
GZ014	<i>V. vinifera</i> 'Crystal'	Xinlian, Jiaoli, Sandu, Guizhou	2015.07.19
GZ019	<i>V. vinifera</i> 'Crystal'	Wenquan, Hailong, Honghuagang, Zunyi, Guizhou	2015.07.19
GZ020	<i>V. vinifera</i> 'Crystal'	Wenquan, Hailong, Honghuagang, Zunyi, Guizhou	2015.07.20
GZ024	<i>V. vinifera</i> 'Crystal'	Wenquan, Hailong, Honghuagang, Zunyi, Guizhou	2015.07.20
GZ025	<i>V. vinifera</i> 'Crystal'	Grape base of Guizhou University, Huaxi, Guiyang, Guizhou	2015.07.21
GZ026	<i>V. vinifera</i> 'Crystal'	Grape base of Guizhou University, Huaxi, Guiyang, Guizhou	2015.07.21
GZ029	<i>V. vinifera</i> 'Crystal'	Grape base of Guizhou University, Huaxi, Guiyang, Guizhou	2015.07.21
GZ030	<i>V. vinifera</i> 'Crystal'	Grape base of Guizhou University, Huaxi, Guiyang, Guizhou	2015.07.21
GZ033	<i>V. vinifera</i> 'Crystal'	Grape base of Guizhou University, Huaxi, Guiyang, Guizhou	2015.07.21

### ***Molecular characterization***

Total genomic DNA was extracted from 5–7-day-old mycelia according to the method of Damm et al. (2012), and then used as a template after the detection by the electrophoresis (Bio-Rad gel imaging GelDoc XR + system, Bio-Rad, Hercules, CA, USA). Specific primers were used for PCR amplification of the 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS), a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), partial sequences of the chitin synthase 1 (CHS-1), actin (ACT) and  $\beta$ -tubulin (TUB2) genes were amplified using primer pairs ITS1/ITS4 (White et al. 1990), GDF/GDR (Templeton et al. 1992), CHS79F/CHS345R (Carbone & Kohn 1999), ACT512F/ACT783R (Carbone & Kohn 1999) and BT1/BT2 (O'Donnell & Cigelnik 1997) respectively. PCR was performed on a Bio-Rad PCR machine (Applied Bio-systems, VERITI Thermal Cycler) in a total volume of 25  $\mu$ L: 2  $\times$  Taq PCR Master Mix 12.5  $\mu$ L, DNA template 1  $\mu$ L, each primer 1  $\mu$ L and sterile double distilled water 9.5  $\mu$ L. The PCR amplification procedure was performed as follows: 95  $^{\circ}$ C denaturation for 4 min, 95  $^{\circ}$ C denaturation 30 s, 56  $^{\circ}$ C (ACT and TUB), 59  $^{\circ}$ C (CHS and GAPDH), 57  $^{\circ}$ C (ITS) annealing 30 s, 72  $^{\circ}$ C extension of 1 min, 35 amplification cycles, and 72  $^{\circ}$ C extension for 7 min. The PCR products were purified by 1.2 % agarose gel electrophoresis and then sequenced by Biomed Company, Beijing, China. After analysis by NCBI BLAST, the results were submitted to the GenBank database and the corresponding accession number was obtained (Table 2).

**Table 2** Strains of *Colletotrichum* spp. studied, with collection details and GenBank accessions.

Species	Strain no.	Genbank accession Numbers				
		ITS	BUT	ACT	CHS	GAPDH
<i>C. aenigma</i>	JZB330035	KF156870	KF288980	KF377502	KF289014	KF377502
<i>C. aeshynomenes</i>	ICMP17673	JX010176	JX010392	JX009483	JX009799	JX009930
<i>C. alatae</i>	ICMP12071	JX010190	JX010383	JX009471	JX009837	JX009990
<i>C. alienum</i>	ICMP12071	JX010251	JX010411	JX009572	JX009882	JX010028
<i>C. aotearoa</i>	ICMP18537	JX010205	JX010420	JX009564	JX009853	JX010005
<i>C. asianum</i>	ICMP18580	FJ972612	JX010406	JX009584	JX009867	JX010053
<i>C. boninense</i>	CBS123755	JQ005153	JQ005588	JQ005327	JQ005414	JQ005327
<i>C. citri</i>	GZ019	KX670382	KX756974	KX756972	KX687652	KX687654
<i>C. clidemiae</i>	ICMP18658	JX010265	JX010438	JX009537	JX009877	JX009989
<i>C. cordylinicola</i>	ICMP18579	JX010226	JX010440	HM470235	JX009864	JX009975
<i>C. dianesei</i>	CMM4083	KC329779	KC517254	KC517298	-	KC329779
<i>C. endophytica</i>	LC0324	KC633854	-	KF306258	-	KC832854
<i>C. fructicola</i>	ICMP18581	JX010165	JX010405	FJ907426	JX009866	JX010033
<i>C. fructivorum</i>	Coll414	JX145145	JX145196	-	-	-
<i>C. grevilleae</i>	CBS132879	KC297078	KC297102	KC296941	KC297056	KC297010
<i>C. gloeosporioides</i>	CBS112999	JQ005152	JQ005587	JQ005500	JQ005326	JQ005239
<i>C. hebeiense</i>	JZB330028	KF156863	KF288975	KF377532	KF289008	KF377495
<i>C. horri</i>	ICMP10492	GQ329690	JX010450	JX009438	JX009752	GQ329681
<i>C. kahawae</i>	ICMP17816	JX010231	JX010444	JX009452	JX009813	JX010012
<i>C. murrayae</i>	GZAAS5.09506	JQ247633	JQ247644	JQ247657	-	JQ247657
<i>C. musae</i>	ICMP19119	JX010146	HQ596280	JX009433	JX009896	JX010050
<i>C. nupharicola</i>	CBS470.96	JX010187	JX010398	JX009437	JX009835	JX009972
<i>Colletotrichum</i> sp.	FJ074	KX670383	KX756975	KX756973	KX687653	KX687655
<i>C. proteae</i>	CBS132882	KC297079	KC297101	KC296940	KC296986	KC297009
<i>C. psidii</i>	CBS145.29	JX010219	JX010443	JX009515	JX009901	JX009967
<i>C. queenslandicum</i>	ICMP1778	JX010276	JX010414	JX009447	JX009899	JX009934
<i>C. rhexiae</i>	Coll 1026	JX145128	JX145179	-	-	-
<i>C. salsolae</i>	ICMP19051	JX010242	JX010403	JX009562	JX009863	JX009916
<i>C. siamense</i>	ICMP18578	JX010171	JX010404	FJ907423	JX009865	JX009924
<i>C. syzygicola</i>	DNCL021	KF242094	KF254880	KF157801	-	KF242156
<i>C. temperatum</i>	Coll 883	JX145159	JX145211	-	-	-
<i>C. theobromicola</i>	ICMP18649	JX010294	JX010447	JX009444	JX009869	JX010006
<i>C. ti</i>	ICMP 4832	JX010269	JX010442	JX009520	JX009898	JX009952
<i>C. tropicale</i>	ICMP 18653	JX010264	JX010407	JX01407	JX009870	JX010007
<i>C. viniferum</i>	JZB330001	KF156840	KF288965	KF377506	KF288982	KF377469
<i>C. viniferum</i>	FJ001	KX594332	KX621321	KX621369	KX687604	KX687656
<i>C. viniferum</i>	FJ002	KX594333	KX621322	KX761398	KX687605	KX687657
<i>C. viniferum</i>	FJ005	KX594334	KX621323	KX621370	KX687606	KX687658
<i>C. viniferum</i>	FJ008	KX594335	KX621324	KX621371	KX687607	KX687659
<i>C. viniferum</i>	FJ010	KX594336	KX621325	KX621372	KX687608	KX687660
<i>C. viniferum</i>	FJ011	KX594337	KX621326	KX621373	KX687609	KX687661

Species	Strain no.	Genbank accession Numbers				
		ITS	BUT	ACT	CHS	GAPDH
<i>C. viniferum</i>	FJ012	KX594338	KX621327	KX621374	KX687610	KX687662
<i>C. viniferum</i>	FJ014	KX594339	KX621328	KX621375	KX687611	KX687663
<i>C. viniferum</i>	FJ015	KX594340	KX621329	KX621376	KX687612	KX687664
<i>C. viniferum</i>	FJ016	KX594341	KX621330	KX621377	KX687613	KX687665
<i>C. viniferum</i>	FJ017	KX594342	KX621331	KX621378	KX687614	KX687666
<i>C. viniferum</i>	FJ018	KX594343	KX621332	KX621379	KX687615	KX687667
<i>C. viniferum</i>	FJ037	KX594344	KX621333	KX621380	KX687616	KX687668
<i>C. viniferum</i>	FJ038	KX594345	KX621334	KX621381	KX687617	KX687669
<i>C. viniferum</i>	FJ041	KX594346	KX621335	KX621382	KX687618	KX687670
<i>C. viniferum</i>	FJ043	KX594347	KX621336	KX621383	KX687619	KX687671
<i>C. viniferum</i>	FJ044	KX594348	KX621337	KX621384	KX687620	KX687672
<i>C. viniferum</i>	FJ045	KX594349	KX621338	KX621385	KX687621	KX687673
<i>C. viniferum</i>	FJ047	KX594350	KX621339	KX621386	KX687622	KX687674
<i>C. viniferum</i>	FJ050	KX594351	KX621340	KX621387	KX687623	KX687675
<i>C. viniferum</i>	FJ051	KX594352	KX621341	KX621388	KX687624	KX687676
<i>C. viniferum</i>	FJ053	KX594353	KX621342	KX621389	KX687625	KX687677
<i>C. viniferum</i>	FJ055	KX594354	KX621343	KX621390	KX687626	KX687678
<i>C. viniferum</i>	FJ056	KX594355	KX621344	KX621391	KX687627	KX687679
<i>C. viniferum</i>	FJ058	KX594356	KX621345	KX621392	KX687628	KX687680
<i>C. viniferum</i>	FJ059	KX594357	KX621346	KX621393	KX687629	KX687681
<i>C. viniferum</i>	FJ060	KX594358	KX621347	KX621394	KX687630	KX687682
<i>C. viniferum</i>	FJ061	KX594359	KX621348	KX621395	KX687631	KX687683
<i>C. viniferum</i>	FJ062	KX594360	KX621349	KX621396	KX687632	KX687684
<i>C. viniferum</i>	FJ063	KX594361	KX621350	KX621397	KX687633	KX687685
<i>C. viniferum</i>	FJ064	KX594362	KX621351	KX621398	KX687634	KX687686
<i>C. viniferum</i>	FJ067	KX594363	KX621352	KX621399	KX687635	KX687687
<i>C. viniferum</i>	FJ068	KX594364	KX621353	KX621400	KX687636	KX687688
<i>C. viniferum</i>	FJ069	KX594365	KX621354	KX621401	KX687637	KX687689
<i>C. viniferum</i>	FJ071	KX594366	KX621355	KX621402	KX687638	KX687690
<i>C. viniferum</i>	FJ072	KX594367	KX621356	KX621403	KX687639	KX687691
<i>C. viniferum</i>	FJ073	KX594368	KX621357	KX621404	KX687640	KX687692
<i>C. viniferum</i>	FJ080	KX594369	KX621358	KX621405	KX687641	KX687693
<i>C. viniferum</i>	FJ083	KX594370	KX621359	KX621406	KX687642	KX687694
<i>C. viniferum</i>	GZ012	KX594371	KX621360	KX621407	KX687643	KX687695
<i>C. viniferum</i>	GZ014	KX594372	KX621361	KX621408	KX687644	KX687696
<i>C. viniferum</i>	GZ020	KX594373	KX621362	KX621409	KX687645	KX687697
<i>C. viniferum</i>	GZ024	KX594374	KX621363	KX621410	KX687646	KX687698
<i>C. viniferum</i>	GZ025	KX594375	KX621364	KX621411	KX687647	KX687699
<i>C. viniferum</i>	GZ026	KX594376	KX621365	KX621412	KX687648	KX687700
<i>C. viniferum</i>	GZ029	KX594377	KX621366	KX621413	KX687649	KX687701
<i>C. viniferum</i>	GZ030	KX594378	KX621367	KX621414	KX687650	KX687702
<i>C. viniferum</i>	GZ033	KX594379	KX621368	KX621415	KX687651	KX687703

### ***Phylogenetics analysis***

Single gene regions were aligned using Clustal X1.81 (Thompson et al. 1997) and combined using BioEdit v.7.0.9.0 (Hall 1999). Further alignment of the sequences was done using the default settings of MAFFT v.7 (Katoh & Toh 2008; <http://mafft.cbrc.jp/alignment/server/>) and manual adjustment was conducted using BioEdit, where necessary. Maximum parsimony analysis was performed using phylogenetic analysis using parsimony v. 4.0b10 (Swofford 2002) to obtain the most parsimonious trees. Gaps were treated as missing data and ambiguously aligned regions were excluded. Trees were inferred using the heuristic search option with tree-bisection-reconnection branch swapping and 1,000 random sequence additions. Maxtrees were set up to 5,000, branches of zero length were collapsed, and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony (tree length, consistency index, retention index, rescaled consistency index, and homoplasy index) were calculated for trees generated under different optimality criteria. The robustness of the most parsimonious trees was evaluated by 1,000 bootstrap replications resulting from the maximum parsimony analysis (Hillis & Bull 1993). Kishino-Hasegawa tests (Kishino & Hasegawa 1989) were performed in order to determine whether trees were significantly different.

Bayesian inference was used in addition to construct the phylogenies using Mr. Bayes v.3.1.2 (Ronquist & Huelsenbeck 2003). MrModeltest v. 2.3 (Nylander 2004) was used to carry out statistical selection of the best-fit model of nucleotide substitution and was incorporated into the analysis. Six simultaneous Markov chains were run for 1,000,000 generations, and trees were sampled every 100<sup>th</sup> generation. The 2,000 trees representing the burn-in phase of the analyses were discarded, and the remaining 8,000 trees were used for calculating posterior probabilities in the majority rule consensus tree.

### ***Pathogenicity testing***

In order to verify the source and pathogenicity of the strains, the grapevine surface was disinfected with 70% alcohol for 1 min, washed three times with sterile water. Every seven grapes were placed in a 9-cm petri dish, and every five dishes were considered as a group. A total of four groups were used in this study, namely CK (control), *C. citri*, *C. viniferum* and *Colletotrichum* sp. strain FJ074. Superficial wounds in the grape epidermis were carried out with a sterilized insect pin. Four-mm-diameter disks of PDA were removed with a puncher and then placed on the wound. The grape surface with sterile PDA was used as a negative control. Each petri dish was incubated at 25 °C with 80% humidity. After 48 hours, the PDA disks were removed, and the pathogenesis situation of grapevine was observed after 7–10 days incubation.

## **Results**

### ***Pathogen morphology***

The 50 strains used in this study were isolated from diseased grapevine fruits with typical ripe rot symptoms (Fig. 1) in Nanping, Sanming, Ningde, Zhangzhou, and Zunyi, and then separated into three types according to the morphology, color, and other characteristics. The three types were: Type A: the appearance of colonies was round or nearly round, mycelia appeared light gray, while the bottom was orange and there was no black generation, aerial mycelia were white and fluffy but no obvious stripes were seen (FJ074) (Fig. 3); Type B: colonies were also circular or nearly round, mycelia were off-white and distributed as stripes, melanin can be seen clearly in the center of the bottom, while the growth was slow and the white aerial mycelium were arranged densely (GZ019) (Fig. 4); Type C: colonies were round or nearly round, mycelia were brown and melanin was obvious in the bottom, the gray aerial mycelium were represented as flocculence and were arranged irregularly (FJ017) (Fig. 5). As shown in Table 3, the pycnidia and conidia were significantly different among the three types, the largest of which was FJ074, with a length and width of 191.301 µm and 237.728 µm, respectively.

**Table 3** Synopsis of morphological data of *Colletotrichum* species in grape from this study.

Species and isolate number	Conidioma		Conidia	
	length (µm)	Width (µm)	length (µm)	width(µm)
FJ017	180.953 (±9.364)	165.865 (±15.144)	14.677 (±1.282)	4.954 (±0.430)
FJ074	238.728 (±28.835)	191.301 (±39.516)	15.166 (±2.485)	5.232 (±0.673)
GZ019	162.328 (±23.490)	163.359 (±20.552)	9.045 (±1.153)	4.135 (±0.475)



**Fig. 1** – Symptoms of the ripe rot disease found in vineyards **a–b** Grape fruits in Fujian. **c–d** Grape fruits in Guizhou.

### Phylogenetic analysis

Phylogenetic analyses were carried out using combined ACT, GADPH, CHS, TUB2, and ITS sequences from 121 *Colletotrichum* strains with *Moniochaetes infuscans* as the outgroup taxa. As Fig. 2 shows, with the exception of strains FJ074 and GZ019, the other 48 strains were all identified as *C. viniferum*. Strain GZ019 was clustered with *C. citri* with a 100% bootstrap value. The strain of FJ074 created a separate branch with a 100% bootstrap value and is a sister taxon to *C. cliviae*. However, until further collections are made this species will be kept as *Colletotrichum* sp.

### Pathogenicity testing

The pathogenicity of the three species of ripe rot showed that all of them had the ability to infect *V. vinifera* cv. Kyoho and cause the typical symptoms of ripe rot (Fig. 6). The species that we re-isolated from the infection site of grapes were identical with the test strain, which was in full compliance with Koch's postulates.

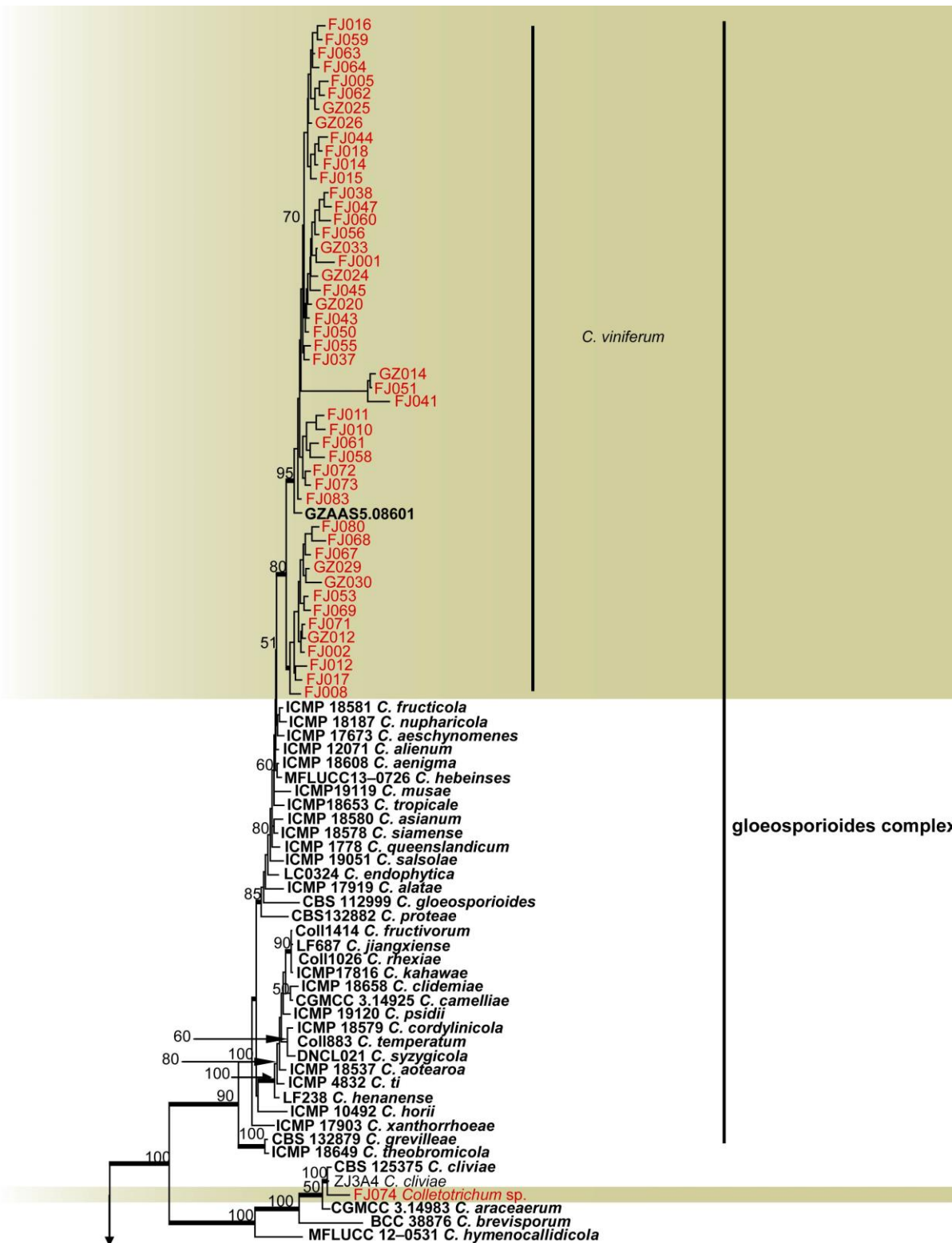
### Taxonomy

*Colletotrichum citri* F. Huang, L. Cai, K.D. Hyde & H.Y. Li

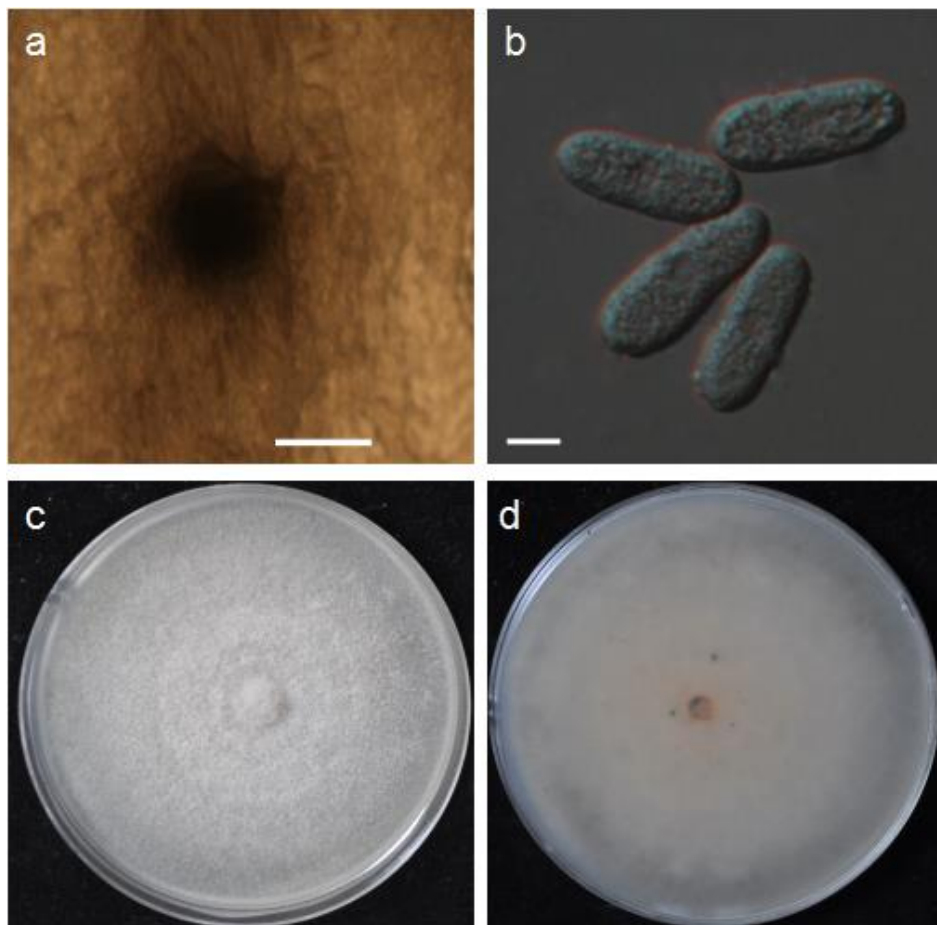
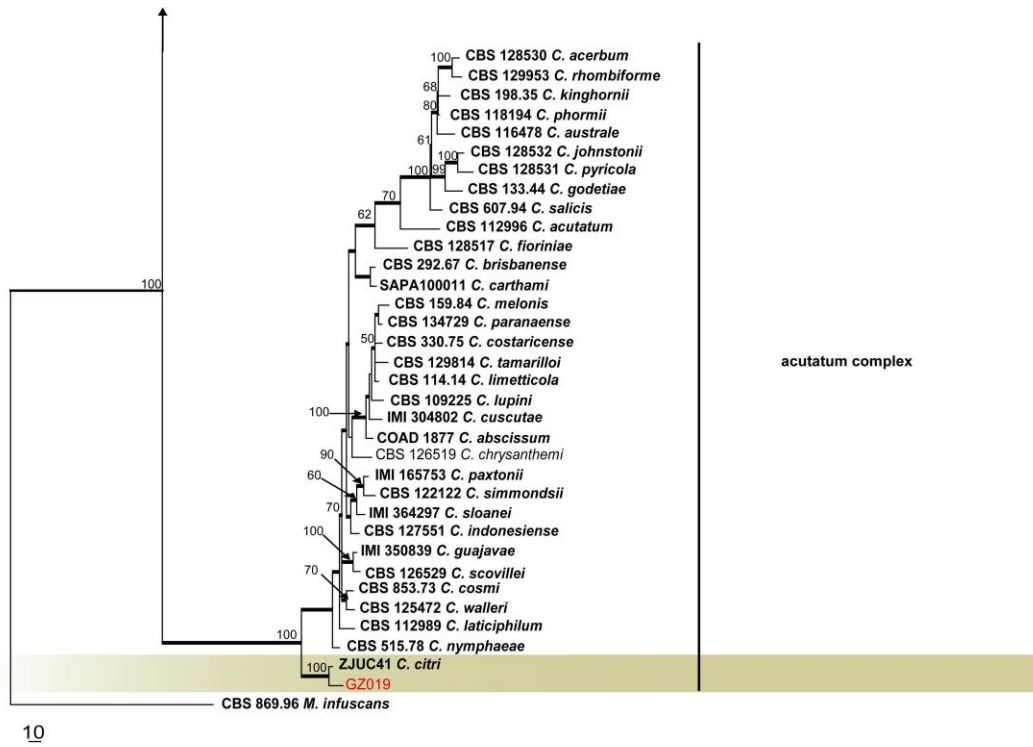
The species was isolated from *V. vinifera* 'Crystal' collected from Guizhou. This is the first report of this species causing grape ripe rot in the world. After incubated at 25 °C for 6 days, the diameter of colony was 6.50 cm. The round colony appeared to be gray on the positive, and gray to black on the back, melanin was visible. The size of the transparent conidia was  $9.05 \times 4.14 \mu\text{m}$ , both ends were blunt round.

*Colletotrichum* sp. strain FJ074

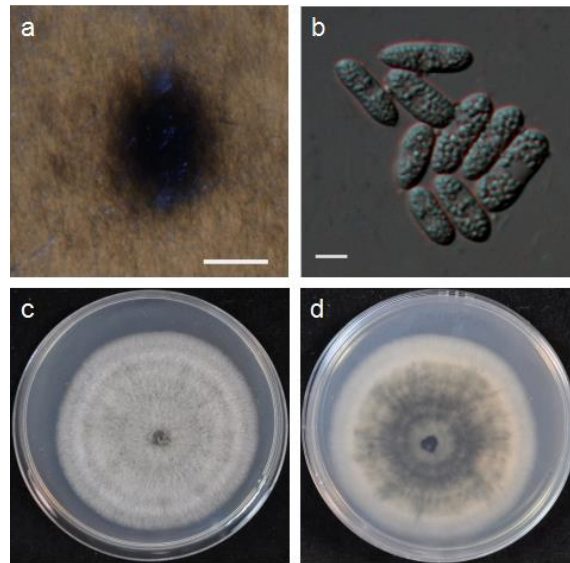
*Pathogen* on *V. vinifera*. Colonies growing from single conidia on PDA grey, reverse orange-red in centre, off-white towards the edge, reaching a maximum of 80.0 mm diameter in 7 days at 28 °C, growth rate 11.4 mm/day. Aerial mycelium was white, rarefaction, flocculence. *Asexual morph* developed on PDA. *Conidiomata* 210–267 µm diameter, abundant, black, oval, solitary to aggregated, with orange spore masses. *Conidia*  $13.7\text{--}17.7 \times 4.5\text{--}5.9$  ( $\bar{x} = 15.2 \times 5.2$ ,  $n = 10$ ) hyaline, smooth-walled, aseptate, both ends were sharp to cylindrical or clavate with rounded apices, contents granular and mostly present at the polar ends leaving a opaque region in the centre. *Appressoria* not observed. *Sexual morph* not observed.



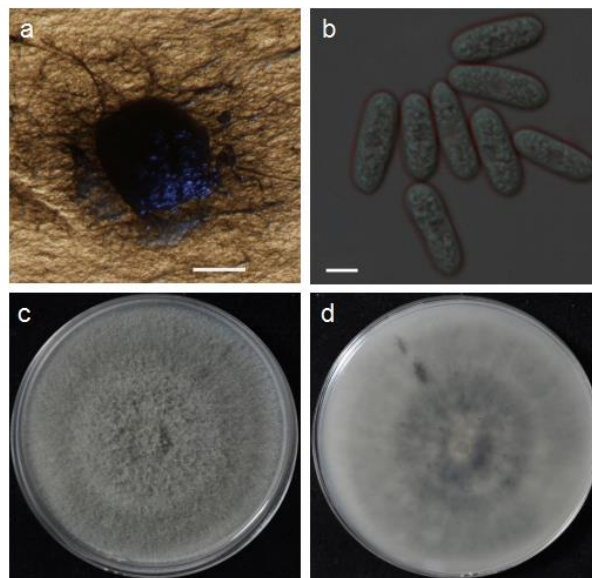
**Fig. 2** – One of the 100 most parsimonious trees obtained from a heuristic search of combined ACT,  $\beta$ -tubulin, CHS-1, GAPDH and ITS sequenced data of *Colletotrichum* species. Parsimony bootstrap support values greater than 70% are indicated above the nodes and branches with Bayesian posterior probabilities above 0.90 are given in bold. The ex-type strains are in bold; isolates of this study are in red. The scale bar indicates ten changes. The tree is rooted with *M. infuscans* CBS 869.96.



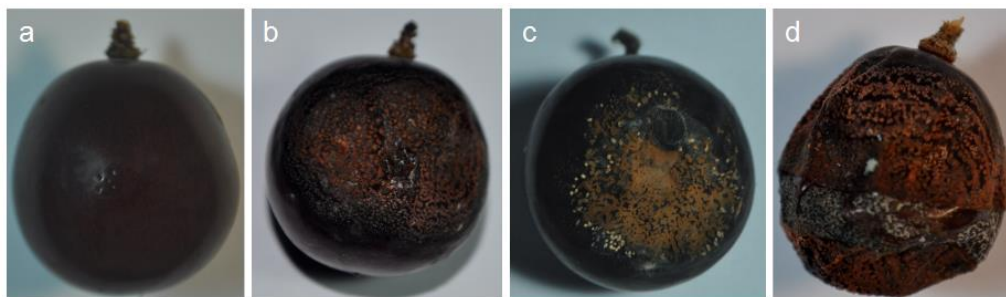
**Fig. 3** – *Colletotrichum* sp. strain FJ074 **a** Conidioma on PDA. **b** Conidia. **c** Upper view of colony (7 days old). **d** Reverse view of colony (7 days old). Scale bars: a=200  $\mu$ m , b=5  $\mu$ m.



**Fig. 4** – *Colletotrichum citri* **a** Conidioma on PDA. **b** Conidia. **c** Upper view of colony (7 days old). **d** Reverse view of colony (7 days old). Scale bars: a=200  $\mu\text{m}$  , b=5  $\mu\text{m}$ .



**Fig. 5** – *Colletotrichum viniferum* **a** Conidioma on PDA. **b** Conidia. **c** Upper view of colony (7 days old). **d** Reverse view of colony (7 days old). Scale bars: a=200  $\mu\text{m}$  , b=5  $\mu\text{m}$ .



**Fig. 6** – Pathogenesis situation of the grapevine **a** CK. **b** *Colletotrichum* sp.. **c** *C. viniferum*. **d** *C. citri*.

Material examined: CHINA, Fujian, Fuzhou, Jin'an, Xindian Pudang, from *V. vinifera* 'Shennong gold queen', July 13, 2015, Lei yan.

Notes: This species was isolated from *V. vinifera* 'Shennong gold queen' in Fujian. After incubated at 25 °C for 6 days, the colony was 8 cm in diameter. The appearance of colony was round and gray on the positive, off-white on the back. Hyphae were closely, and the orange conidiophores can be found on the bottom. The transparent conidia was  $15.17 \times 5.23 \mu\text{m}$  in dimension, both ends were blunt round. Based on multi-locus sequence data, this species is phylogenetically closely related to *C. cliviae* and *C. araceaerum*. However, until further collections are made we intend to keep this species as *Colletotrichum* sp.

*Colletotrichum viniferum* L.J. Peng, L. Cai, K.D. Hyde & Zi Y. Ying

This species was isolated from 48 diseased grape samples with irregular lesions, including one *V. vinifera* cv. Jingyu, one *V. vinifera* cv. Beauty Finger, one *V. vinifera* 'Rose red', four *V. vinifera* 'Shennong gold queen', fifteen *V. vinifera* cv. Kyoho and seventeen *V. Labruscana* cv. Summer Black from Fujian, as well as nine *V. vinifera* 'Crystal' from Guizhou. Peng et al. (2013) first reported *C. viniferum* in China and described the morphological characteristics in detail. Phylogenetic analysis showed that 48 isolates of *C. viniferum* were clustered together, which is consistent with their similar morphological characteristics.

## Discussion

Grape ripe rot is a serious disease that occurs on mature fruits or during the harvest season which damage to the yield and quality of grapes. The infected fruits turn dark brown and recessed, small black particles are distributed in a ring on the surface of the disease spots. Under high humidity conditions, a pink or orange conidia heap can be found on the surface, but it is difficult to distinguish the ripe rot species according to symptoms on leaves and fruits with visual observation. In a previous report, *Colletotrichum gloeosporioides* (Penz) was treated as the only ripe rot causing agent (Whitelaw et al. 2007), while with the progress of research, *C. acutatum* J.H. was identified to be the causal agent of grape ripe rot in Australia, Japan, South Korea and the United States (Hong et al. 2008). In fact, *C. viniferum* (Peng et al. 2013) as well as *C. aenigma* and *C. hebeiense* (Yan et al. 2015) have also been reported to be ripe rot pathogens.

In our study, 50 *Colletotrichum* isolates were isolated from diseased grape samples from Fujian and Guizhou Provinces. The strain FJ074 which isolated from Pudang, Xindian, Jin'an, Fuzhou, Fujian, is a *Colletotrichum* sp., whose colony morphology was significantly different with the other strains (Figs. 3–5), and pycnidia as well as conidia were obviously larger than *C. viniferum* (Table 3). Multi-gene analysis showed that *Colletotrichum* sp. and *C. cliviae* were in the same cluster. Chowdappa et al. (2014) has shown that *C. cliviae* led to anthracnose on *Cymbidium dendulum* in India. The colonies appeared olive gray on the front and dark green on the back and had cylindrical conidia harboring two blunt ends, but were a little bit bent. The size was 17.2 and 5.5  $\mu\text{m}$ , respectively, with the appressorium cracked and shaped irregularly. The strain *C. citri* (GZ019), isolated from Wenquan, Hailong, Honghuagang, Zunyi, Guizhou, was a new *Colletotrichum* sp. found in the citrus (Huang et al. 2013), our study is the first time to show the ripe rot on grapevine caused by *C. citri*, indicating that this species has a wide host range. Multi-gene analysis showed that except for the strains of *Colletotrichum* sp. and *C. citri*, all the other strains belonged to *C. viniferum*, while the genotypes were different. Yan et al. (2015) mentioned that *C. viniferum* has a divergence in phylogeny. Silva et al. (2012) mentioned that the method of 'powerful genes' to identify anthracnose species is useful, but until now, mycologists have not reached a consensus on which marker genes should be used for the classification and identification of *Colletotrichum* species (Doyle et al. 2013).

The purpose of our study is to identify the pathogens of grape ripe rot in Fujian and Guizhou by both morphological and molecular biology methods, which will be helpful in further study of species dynamics and the distribution of grape ripe rot in China, and provide a theoretical

basis for the prevention of diseases in the field. The results of our study show that grape ripe rot in Fujian Province and Guizhou Province is mainly due to *C. viniferum*, but not to *C. gloeosporioides* or *C. acutatum*, which consistent with Yan et al. (2015). More importantly, *Colletotrichum* sp. strain FJ074 is a new strain of causing grape ripe rot in Fujian Province, and *C. citri* is a new record for causing grape ripe rot.

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