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Assessing the genetic diversity of grape ripe rot pathogen *Colletotrichum* using SRAP markers

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Abstract

Grape ripe rot caused by species of *Colletotrichum* is one of the most serious grape diseases worldwide. Information on the genetic variation and population structure of grape ripe rot pathogens would help to develop the theoretical framework of disease occurrence and prevention, epidemic dynamics, and control technologies. In this study, twenty five *Colletotrichum gloeosporioides* strains isolated from grape diseased samples that were collected from 17 different locations in China were analyzed using six sequence-related amplified polymorphism (SRAP) primer pairs. In total, 164 discernible bands were obtained. Of which 156 showed high polymorphism at a rate of 95.12%. Cluster analysis revealed that the 25 *C. gloeosporioides* strains had a close genetic relationship and rich genetic diversity with similarity coefficients that ranged from 0.61 to 0.95. The data also demonstrated that no correlation was identified between the isolates or SRAP groups and their geographical distribution.

Key words – cluster analysis – *Colletotrichum gloeosporioides* – polymorphic richness – similarity coefficient

Introduction

Grapevine is one of the most economically important fruit crops in China because of the high demand for wine, juice, table grapes, and dried fruit (Cannon et al. 2012). Over the past few years, a rapid increase was observed in grape cultivation areas throughout China. In 2012, the total cultivation area was 552,000 hm^2 with a production of 843,000 tons (Zhang et al. 2013). During the final stages of grape ripening, the majority of grape cultivation areas in China receives high rainfall and has relatively high temperature, which results in yield losses due to pests and diseases (Lu 2005). Anthracnose is one of the most serious grapevine diseases that lead to yield losses due to fruit deterioration (Jang et al. 2011).

Grape anthracnose is caused by *Colletotrichum* spp., especially by the species belonging to gloeosporioides and acutatum species complexes (Greer et al. 2011). Yan et al. (2014) showed that *C. aenigma*, *C. fructicola*, *C. gloeosporioides*, *C. hebeiense* and *C. viniferum* were also associated with grapevine anthracnose in China using multi-gene analysis coupled with morphology. Of these species, *C. aenigma* rarely causes grape ripe rot, whereas *C. hebeiense* is a new species that was reported to be the ripe rot-causing microbe in Hebei and Shandong Provinces.

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C. gloeosporioides is considered as the major grape-ripe rot-causing pathogen in China (Lei et al. 2014). It is a heterogeneous and complex species, consisting of various host-specific populations (Kelemu et al. 1999). The 'gloeosporioides' complex arose because of the wide spore range (especially spore length) placed on *C. gloeosporioides* to overcome the instability of spore morphology under different conditions or from different hosts (von Arx 1957). Previous studies were focused on spore characters, and *Colletotrichum* strains with cylindrical conidia were identified as *C. gloeosporioides*. Molecular data that used to identify different strains in this complex were not accurate, since less than 86% of the internal transcribed spacer (ITS) sequences designated as *C. gloeosporioides* in GenBank are not conspecific to the *C. gloeosporioides* epitype (Cai et al. 2009, Hyde et al. 2009a). However, Cannon et al. (2008) epitypified *C. gloeosporioides* from an orange sample collected in Italy. Therefore, the living culture and sequence data of the epitype are available and could be used in molecular phylogeny studies, overcoming the limitations of traditional morphological data (Cai et al. 2009).

Sequence-related amplified polymorphism (SRAP) is a novel molecular marker first reported by Li & Quiros (2001). SRAP analysis is based on the preferential amplification of open reading frames (ORFs) using PCR. SRAP markers have been applied extensively in genetic linkage mapping, genetic diversity analysis, and comparative genetics (Li & Quiros 2001, Ferriol et al. 2003, Guo & Luo 2006). SRAP technology has some advantages, such as improving amplification efficiency, convenience operation, good repetition, and it is conducive to genetics diversity analysis for pathogens. In this study, we analyzed the genetic diversity and genetic structure of 25 *C. gloeosporioides* strains isolated from grape samples that were collected from 17 different locations in China using SRAP markers. Our objectives were to (1) evaluate the importance of grape anthracnose disease in China; (2) investigate the genetic diversity and distribution of *C. gloeosporioides*, and (3) identify any genetic relationships among the strains using cluster analysis.

Materials & Methods

Pathogen collection

Grape samples were collected from Liaoning, Heilongjiang, Beijing, Shanghai, Henan, Shanxi, and Guizhou Provinces. The pathogens were isolated using the tissue isolation method (Fan et al. 2013). Tissue samples (3 mm × 3 mm × 1 mm) were obtained from the border of healthy and diseased grape skins, sterilized with 70% ethanol for 30 s, transferred to 1% $HgCl_2$ for 1 min, and washed three times with sterile water. Next, they were placed on Potato Dextrose Agar medium and cultured in a biochemical incubator at 28 °C. Strains were purified using single spore isolation, cultured in a biochemical incubator at 28 °C for 5 d, and stored at 4 °C.

DNA extraction and pathogens identification

Total genomic DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Murray & Thompson 1980) with minor modifications. DNA integrity was evaluated by 1.2% agarose gel electrophoresis (AGE), whereas DNA quantity by spectroscopy at 260 nm and 280 nm. Species identification was performed by sequencing the ITS gene region. ITS4 and ITS5 (White et al. 1990) were selected as primer pairs and used for amplifying the corresponding gene regions. The PCR reaction mixture included 2.5 µl of 10× PCR buffer, 1.0 µl of 2.5 mmol L^{-1} dNTPs, 1.0 µl of each forward and reverse primer, 0.25 µl of 5 U μl^{-1} Taq DNA polymerase, 1.0 µl of genomic DNA, and 18.25 µl of ddH_2O. PCR amplification was carried out with a PTC-200 DNA Engine Cycler (MJ Research, Waltham, MA, USA). The reaction conditions were as follows: pre-denaturation at 95 °C for 5 min, 36 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were visualized by AGE, and the positive samples were sequenced by Sangon Biotech (Shanghai, China). The sequences were compared against the National Center for Biotechnology Information (NCBI) database using the BLAST tool.

Table 1 Primers used in this study.

Name	Forward primer	Name	Reverse primer
Me1	5′-TGAGTCCAAACCGGATA-3′	Em1	5′-GACTGCGTACGAATTAAT-3′
Me2	5′-TGAGTCCAAACCGGAGC-3′	Em2	5′-GACTGCGTACGAATTTGC-3′
Me3	5′-TGAGTCCAAACCGGAAT-3′	Em3	5′-GACTGCGTACGAATTGAC-3′
Me4	5′-TGAGTCCAAACCGGACC-3′	Em4	5′-GACTGCGTACGAATTTGA-3′
Me5	5′-TGAGTCCAAACCGGAAG-3′	Em5	5′-GACTGCGTACGAATTAAC-3′
Me6	5′-TGAGTCCAAACCGGACA-3′	Em6	5′-GACTGCGTACGAATTGCA-3′
Me7	5′-TGAGTCCAAACCGGACG-3′	Em7	5′-GACTGCGTACGAATTCAA-3′
Me8	5′-TGAGTCCAAACCGGACT-3′	Em8	5′-GACTGCGTACGAATTCAC-3′
Me9	5′-TGAGTCCAAACCGGAGG-3′	Em9	5′-GACTGCGTACGAATTCAT-3′
Me10	5′-TGAGTCCAAACCGGAAA-3′	Em10	5′-GACTGCGTACGAATTGTC-3′

Table 2 *Colletotrichum gloeosporioides* strains isolated from grape samples that collected from 17 different locations in China between August 26, 2010 and September 16, 2010.

Number	Code name	Location	Identification	Collection date
1	LJZ-14	Jinzhou	*C. gloeosporioides*	Sep 16, 2010
2	LJZ-2	Jinzhou	*C. gloeosporioides*	Aug 26, 2010
3	LHL-8	Huludao	*C. gloeosporioides*	Sep 16, 2010
4	LHL-8	Huludao	*C. gloeosporioides*	Sep 16, 2010
5	LFX-9	Fuxin	*C. gloeosporioides*	Sep 16, 2010
6	LSY-3	Shenyang	*C. gloeosporioides*	Aug 26, 2010
7	LFS-6	Fushun	*C. gloeosporioides*	Sep 1, 2010
8	LFS-7	Fushun	*C. gloeosporioides*	Sep 1, 2010
9	LFS-10	Fushun	*C. gloeosporioides*	Aug 26, 2010
10	LLY-1	Liaoyang	*C. gloeosporioides*	Aug 26, 2010
11	LLY-18	Liaoyang	*C. gloeosporioides*	Sep 16, 2010
12	KYK-15	Yingkou	*C. gloeosporioides*	Sep 16, 2010
13	LDL-16	Dalian	*C. gloeosporioides*	Sep 9, 2010
14	LAS-17	Anshan	*C. gloeosporioides*	Sep 9, 2010
15	LDD-4	Dandong	*C. gloeosporioides*	Sep 1, 2010
16	LDD-5	Dandong	*C. gloeosporioides*	Sep 1, 2010
17	HLJ-1	Heilongjiang	*C. gloeosporioides*	Sep 1, 2010
18	BJ-2	Beijing	*C. gloeosporioides*	Sep 2, 2010
19	HB-3	Hebei	*C. gloeosporioides*	Sep 2, 2010
20	XA-4	Shanxi	*C. gloeosporioides*	Sep 1, 2010
21	SH-5	Shanghai	*C. gloeosporioides*	Aug 30, 2010
22	SH-6	Shanghai	*C. gloeosporioides*	Aug 30, 2010
23	HN-7	Henan	*C. gloeosporioides*	Aug 30, 2010
24	GZ-8	Guizhou	*C. gloeosporioides*	Sep 9, 2010
25	GZ-9	Guizhou	*C. gloeosporioides*	Sep 9, 2010

Selection of SRAP primers

Of 100 SRAP primer pairs that are available (Li & Quiros 2001, Wang et al. 2010), we randomly selected ten forward and reverse primers that were synthesized by Sangon Biotech (Table 1). These ten primer pairs were used to perform a preliminary analysis of four strains, namely LSY-3, LDD-5, LJZ-14, and GZ-8, identified in four widely different locations. Six primer pairs were selected based on their relatively high allelic richness and stability of polymorphic bands and used for SRAP analysis.

SRAP analysis

The PCR reaction mixture included 2.5 μl of 10× PCR buffer, 2.0 μl of Mg^{2+}, 1.0 μl of 10 mmol L^{-1} dNTPs, 1.0 μl of 10 μmol L^{-1} of each forward and reverse primer, 0.25 μl of 5 U μl^{-1} Taq DNA polymerase, 1.0 μl of genomic DNA, and 16.25 μl of ddH_2O. PCR amplification was carried out with a PTC-200 DNA Engine Cycler (MJ Research). The reaction conditions were as follows: pre-denaturation at 95 °C for 5 min, four cycles of denaturation at 95 °C for 1 min, annealing at 35 °C for 45 s, and extension at 72 °C for 1 min; 34 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated by 6% native polyacrylamide gel electrophoresis. DNA bands

were visualized by 2% silver nitrate staining, developed with 3% NaOH and 0.8% CH_3OH for 5 min, and their sizes were recorded.

Data analysis

The presence and absence of alleles were scored as 1 and 0 for each marker, respectively, and data were used to construct a binary data matrix. The calculation of genetic similarity coefficients and cluster analysis using the un-weighted pair group method arithmetic average (UPGMA) method were carried out by NTSYSpc 2.1 (Exeter, Setauket, NY, USA).

Results

Pathogen collection and DNA extraction

Twenty-five *C. gloeosporioides* strains were isolated using the tissue isolation method and confirmed by sequence analysis of the ITS gene regions and BLAST search (Table 2). AGE showed that the product size of genomic DNA was more than 2,000 bp and had a standard quality (OD_{260}/OD_{280}, 1.88) appropriate for SRAP analysis (Wong et al. 2007).

Characteristics of SRAP markers

The preliminary screening results showed that 48 primer pairs out of 100 were amplified the genomic DNA, and of these, six primer pairs produced clear, stable, and highly polymorphic bands that could be used for SRAP analysis. The 25 *C. gloeosporioides* strains were analyzed using six highly polymorphic primer pairs, producing 164 discernible bands, of which 156 were highly polymorphic at a rate of 95.12% (Table 3). The number of unique bands amplified by the primer pair Me/Em8 is higher than that by the other primer pairs (Fig. 1).

SRAP analysis

Cluster analysis showed that the similarity coefficients of the 25 *C. gloeosporioides* strains ranged between 0.61 and 0.95. The strains were divided into four groups (I, II, III, and IV) with a similarity coefficient of 0.68 (Fig. 2). Group I was large and divided into five sub-groups with a similarity coefficient of 0.82. The first sub-group contained the strains LLY-1, LHL-8, LFX-9, and LFX-10 that isolated from grape samples that collected from three different locations in Liaoning Province; the second sub-group contained the strain LHL-12; the third sub-group the strains LJ-1 and LSY-3; the fourth sub-group the strains HLJ-1, XA-4, BJ-2, and LCY-18; and the fifth sub-group the strain LYK-15. Group II was also large and divided into five sub-groups with a similarity coefficient of 0.82. The first sub-group contained the strains LDD-4, LDD-5, LFS-6, LFS-7, and LJZ-14 that had a close relationship, whereas the second, third, fourth, and fifth sub-groups contained the strains LDL-16, LAS-17, SH-5, and GZ-9, respectively. Group III only contained the strain SH-6. Group IV was small and contained the strains HN-7 and GZ-8.

Discussion

C. gloeosporioides that causes grape rip rot has more than 600 synonyms, which reveals its heterogeneous genetic nature (von Arx 1970, Sutton 1980). It has been reported that there were 66 species were accepted in *Colletotrichum*, and the 19 recently used names which are regarded as doubtful (Hyde et al. 2009b). In this study, we isolated 25 *C. gloeosporioides* strains from grape samples that collected from 17 different locations in China. All the strains were analyzed with six highly polymorphic SRAP markers to explore their genetic diversity. SRAP markers are considered as well conserved among various species within a family. For instance, SRAP technology had been used to study the genetic diversity of *Sclerotinia sclerotiorum* (Li et al. 2009), *Puccinia striiformis* (Pasquali et al. 2010), and *Valsa mali* (Li et al. 2011). To our knowledge, this is the first report on the assessment of genetic diversity in *C. gloeosporioides* using SRAP markers.

Table 3 Number of bands of 25 *Colletotrichum gloeosporioides* strains isolated from grape samples that collected from 17 different locations in China and analyzed using six highly polymorphic sequence-related amplified polymorphism (SRAP) primer pairs.

Number	Isolate	Primer pairs					
		Me1/Em5	Me2/Em10	Me5/Em8	Me6/Em4	Me7/Em1	Me8/Em8
1	LLY-1	10	12	13	22	16	20
2	LJZ-2	1	8	10	21	13	19
3	LSY-3	3	10	11	21	19	21
4	LDD-4	3	8	10	16	19	17
5	LDD-5	3	6	10	15	19	14
6	LFS-6	4	10	10	13	18	14
7	LFS-7	6	9	11	14	16	16
8	LHL-8	12	7	11	21	16	20
9	LFX-9	10	10	10	20	20	18
10	LFS-10	10	7	12	21	16	20
11	LHL-12	6	6	8	20	16	18
12	LJZ-14	6	8	9	19	16	17
13	LYK-15	9	9	11	21	5	16
14	LDL-16	9	13	11	16	17	15
15	LAS-17	6	11	10	18	17	15
16	LCY-18	5	11	12	20	19	18
17	HLJ-1	5	13	10	21	16	16
18	BJ-2	8	11	11	20	15	20
19	HB-3	7	10	10	21	16	16
20	XA-4	4	9	12	19	16	17
21	SH-5	6	7	7	17	17	16
22	SH-6	4	4	6	16	12	17
23	HN-7	9	5	8	13	14	20
24	GZ-8	10	5	10	13	16	19
25	GZ-9	5	3	11	18	22	16
Total bands		20	23	27	31	28	35
Total polymorphic bands		20	23	27	27	28	31
Polymorphism rate		100%	100%	100%	87.10%	100%	88.57%

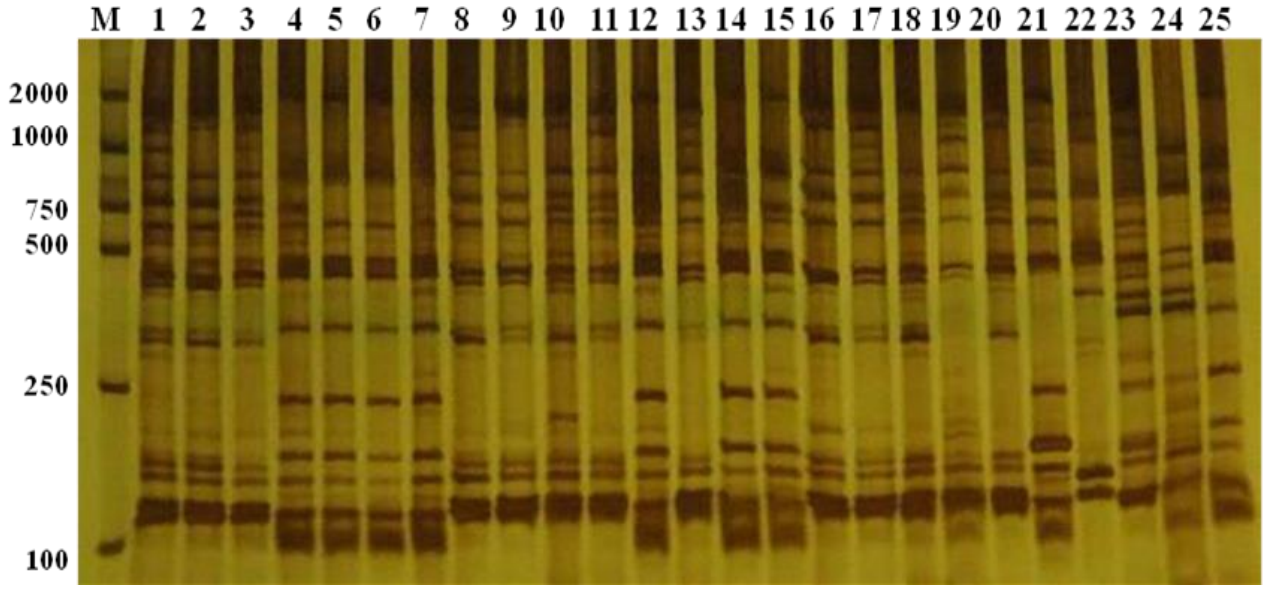

Fig. 1 – Amplification results of 25 *Colletotrichum gloeosporioides* strains isolated from grape samples that collected from 17 different locations in China and analyzed using the sequence-related amplified polymorphism (SRAP) primer pair Me/Em8. M means DNA marker, 1-25 means 25 strains respectively.

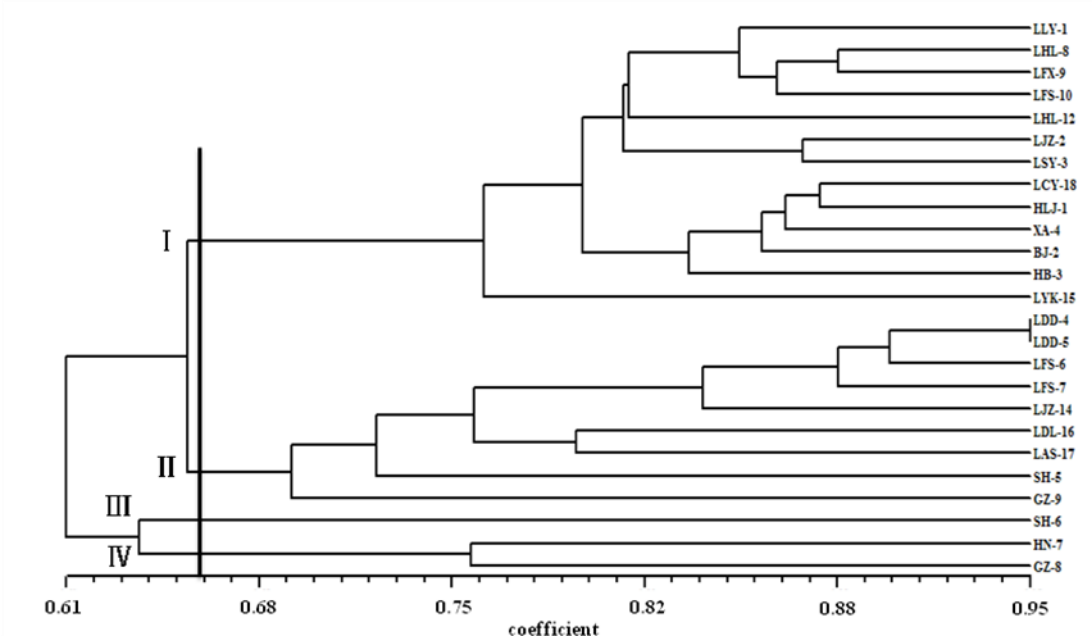

Fig. 2 – Dendrogram generated from the analysis of 25 *Colletotrichum gloeosporioides* strains isolated from grape samples that collected from 17 different locations in China using six high polymorphic sequence-related amplified polymorphism (SRAP) markers.

Cluster analysis indicated the close relationship among the 25 *C. gloeosporioides* strains; however, the genetic variation among the strains or the differences among the SRAP groups showed no significant correlation with their geographical distribution. Li et al. (2011) suggested that there is a relationship between the SRAP group and the geographic origin. However, previous studies reported the absence of significant correlation for *Colletotrichum* spp. isolated from Yunnan Province (Deng et al. 2015). These results could be attributed to the long-distance transportation of grape planting materials and the hybridization of different varieties.

The results of the study indicated that there were showed genetic diversity among the *C. gloeosporioides* from different locates. Maybe, it would be relevant to long distance transportation of grape plantlet and cross breeding between different cultivates. Overall, the number of strains analyzed in this study was limited, and thus, further studies are needed to more thoroughly assess the genetic diversity in *C. gloeosporioides* and would allow developing the theoretical framework of resistance breeding, occurrence, spread, prevention and control of grape anthracnose.

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