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AFLP ANALYSIS OF RESISTANCE TO *COLLETOTRICHUM GLOEOSPORIOIDES* IN *CAMELLIA OLEIFERA* (THEACEAE)

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Abstract

Anthracnose is a highly destructive fungal disease caused by *Colletotrichum gloeosporioides* that results in severe economic losses to *Camellia oleifera* production. Herein, we investigated whether genotypes identified as resistant or susceptible to anthracnose in China could be distinguished using amplified fragment length polymorphism (AFLP) markers. A total of 30 unrelated *C. oleifera* genotypes were selected from three ecotype regions (Huangshan, Shucheng and Fengyang) in Anhui Province. Resistance was assessed by disease severity in plants following inoculation of detached fruit. AFLP selective primer combinations were used to identify *C. oleifera* genotypes. We amplified 147 bands, of which 129 (87.76%) were polymorphic and used to analyse genetic diversity. Among resistant *C. oleifera*, genetic similarity coefficients ranged from 0.40 to 0.85, indicating high genetic diversity. The 30 genotypes grouped into two major clusters based on polymorphic bands. This study provides knowledge of genetic diversity that will be useful for future breeding of *C. oleifera* for anthracnose resistance.

Key words: *C. oleifera*; Anthracnose resistance; AFLP; Genetic diversity.

Introduction

Camellia oleifera, belonging to the family Theaceae, is an evergreen shrub that is used to produce edible oil in Southern China. This plant is indigenous to China and distributed mainly along southern parts of the Yangtze River basin, but also sporadically occurs in Japan and Southeast Asian countries (Zhuang, 1988). In China, this species has been cultivated for more than 2,000 years.

C. oleifera is used to produce oil, obtained from the seeds. The oil has an unsaturated fatty acid content of 85-90%, compared with 75-90% in olive oil. The content of vitamin E is also twice that in olive oil, hence *C. oleifera* oil is known as "Oriental olive oil" (Zhuang, 1988; Shu & Zhang, 2009). This oil is used in the cosmetics, pharmaceutical and healthcare industries; byproducts including fruit shells and tea seed cake are also useful as raw materials for industry and agriculture. Moreover, *C. oleifera* is a deep-rooted tree that is important in soil and water conservation. Thus, *C. oleifera* has important economic and ecological value, and there remains scope to increase production in China.

Anthracnose, a fungal disease of *C. oleifera* in which is the pathogen is *Colletotrichum gloeosporioides*, causes fruits to fall, withering of buds and leaves, canker of branches and trunks, and ultimately plant death, decreasing fruit production by 30-50% and resulting in huge economic losses (Cao *et al.*, 2011; Cao *et al.*, 2014).

C. oleifera is a entomophilous and outcrossing species possessing high genetic variation that displays varied biological and morphological traits including blooming time (late September, middle October, early November and late November), flower colour (red and white), fruit colour (red, green, and yellow), fruit shape (spherical, flat, pear-shaped) and fruit size. Agronomic traits of trees also vary in terms of quantity and quality of seed oil, and disease resistance (Li *et al.*, 2009; Wu *et al.*, 2015; Hu *et al.*, 2016).

Conventional breeding and cultivation of *C. oleifera* have received widespread attention since the 1950's in China, including research on the prevention and control of anthracnose (Ji & Guo, 1992; Jin *et al.*, 2009; Lu *et al.*, 2009; Zhou *et al.*, 2010). The mechanisms of resistance to anthracnose in different cultivars of *C. oleifera* have also been studied at the morphological, physiological and biochemical level (Yang *et al.*, 2004; Duan *et al.*, 2005; Yang *et al.*, 2007). In recent years, genetic variation in *C. oleifera* has been investigated using molecular techniques (Chen *et al.*, 2005; Zhang *et al.*, 2007; Wang *et al.*, 2008; Wen *et al.*, 2008). However, relationships between genetic variation and agronomic traits, especially the oil content of seeds and disease resistance, have not been reported for *C. oleifera*, unlike some other plant species (Mignouna & Dixon, 1997; Fregene *et al.*, 2000; Chatterjee *et al.*, 2004; Lokko *et al.*, 2005; Mcharo *et al.*, 2005; Miano *et al.*, 2008; Pagnotta *et al.*, 2009).

This work aimed to investigate genetic relationships and diversity among *Camellia* spp. accessions that are resistant and susceptible to *Colletotrichum gloeosporioides*, to provide important information for resource conservation and tree breeding, and thereby contribute to the sustainable development of this economically important species.

Materials and Methods

Materials: Thirty cultivars of *C. oleifera* were selected for anthracnose resistance screening and genetic analysis, comprising accessions primarily from three regions in Anhui province (seven from Huangshan, six from Shucheng, and 17 from Fengyang; Table 1), and *C. yuhsiensis* Hu (No.5) originating from Hunan province. New leaves were collected from sample trees, dirt and dust were removed, and samples were numbered stored in plastic bags with silica gel at -70°C.

Table 1. *Camellia oleifera* plant materials and resistance testing.

No.	Latin name	Abbr.	Resistance	Origin	Location
1.	*C. oleifera* Abel cv. Huizhou-dahong	Cole-HZDH_1	Resistant	Anhui	Huangshan (SA)*
2.	*C. oleifera* Abel cv. Huizhou-dahong	Cole-HZDH_2	Resistant	Anhui	Huangshan (SA)
3.	*C. oleifera* Abel cv.Huizhou-daqing	Cole-HZDQ	Susceptible	Anhui	Huangshan (SA)
4.	*C. oleifera* Abel cv. Huizhou-xiaohong	Cole-HZXH	Resistant	Anhui	Huangshan (SA)
5.	*C. yuhsiensis* Hu	*C-yuhsiensis*	Resistant	Hunan	Huangshan (SA)
6.	*C. oleifera* Abel cv. Huizhou-xiaoqing	Cole-HZXQ	Susceptible	Anhui	Huangshan (SA)
7.	*C. oleifera* Abel cv. Luohanguo	Cole-HZLHG	Susceptible	Anhui	Huangshan (SA)
8.	*C. oleifera* Abel cv.Shucheng-xiaohong	Cole-SC_1	Susceptible	Hunan	Shucheng (WA)*
9.	*C. oleifera* Abel cv. Shucheng-xiaoqing	Cole-SC_2	Susceptible	Anhui	Shucheng (WA)
10.	*C. oleifera* Abel cv. Shucheng-daqing	Cole-SC_3	Susceptible	Jiangxi	Shucheng (WA)
11.	*C. oleifera* Abel cv. Shucheng-xiaoqing	Cole-SC_4	Susceptible	Anhui	Shucheng (WA)
12.	*C. oleifera* Abel cv. Shucheng-dahong	Cole-SC_5	Resistant	Anhui	Shucheng (WA)
13.	*C. oleifera* Abel cv. Shucheng-dahong	Cole-SC_6	Resistant	Anhui	Shucheng (WA)
14.	*C. oleifera* Abel cv. Caodianensis No.1	Cole-CD_1	Resistant	Anhui	Fengyang (EA)*
15.	*C. oleifera* Abel cv. Caodianensis No.2	Cole-CD_2	Resistant	Anhui	Fengyang (EA)
16.	*C. oleifera* Abel cv. Caodianensis No.6	Cole-CD_6	Resistant	Anhui	Fengyang (EA)
17.	*C. oleifera* Abel cv. Caodianensis No.3	Cole-CD_3	Susceptible	Anhui	Fengyang (EA)
18.	*C. oleifera* Abel cv. Caodianensis	Cole-CD_4	Resistant	Anhui	Fengyang (EA)
19.	*C. oleifera* Abel cv. Caodianensis	Cole-CD_5	Resistant	Anhui	Fengyang (EA)
20.	*C. oleifera* Abel cv. Caodianensis	Cole-CD_7	Susceptible	Anhui	Fengyang (EA)
21.	*C. oleifera* Abel cv. Caodianensis	Cole-CD_8	Susceptible	Anhui	Fengyang (EA)
22.	*C. oleifera* Abel cv. Hongxinensis No.2	Cole-HX_2	Resistant	Hunan	Fengyang (EA)
23.	*C. oleifera* Abel cv. Hongxinensis No.4	Cole-HX_4	Susceptible	Hunan	Fengyang (EA)
24.	*C. oleifera* Abel cv. Hongxinensis No.6	Cole-HX_6	Resistant	Hunan	Fengyang (EA)
25.	*C. oleifera* Abel cv. Hongxinensis No.7	Cole-HX_7	Susceptible	Hunan	Fengyang (EA)
26.	*C. oleifera* Abel cv. Hongxinensis No.8	Cole-HX_8	Resistant	Hunan	Fengyang (EA)
27.	*C. oleifera* Abel cv. Hongxinensis No.10	Cole-HX_10	Susceptible	Hunan	Fengyang (EA)
28.	*C. oleifera* Abel cv. Hongxinensis No.1	Cole-HX_1	Susceptible	Hunan	Fengyang (EA)
29.	*C. oleifera* Abel cv. Hongxinensis No.3	Cole-HX_3	Susceptible	Hunan	Fengyang (EA)
30.	*C. oleifera* Abel cv. Hongxinensis No.6	Cole-HX_9	Susceptible	Hunan	Fengyang (EA)

Note: (SA)* = Southern Anhui; (WA)* = Western Anhui; (EA)* = Eastern Anhui

Methods

Resistance test: Anthracnose resistance was tested using the detached fruit inoculation method with three experimental materials (Montri *et al.*, 2009; Kanchana-udomkan *et al.*, 2004). Two *C. gloeosporioides* (Penz.) strains (AAH48 and AAH69), both isolated from *C. oleifera* in China and previously characterised as virulent, were used as the inocula. Inoculated fruit was incubated under 95% relative humidity at 25°C with a light: dark cycle of 12h: 12h. Symptoms of anthracnose at the inoculation sites were evaluated 7 days after inoculation. Fruit areas showing necrotic lesion ≤15% were considered resistant, while fruit areas showing necrotic lesion >15% were considered susceptible (Montri *et al*., 2009).

Extraction of DNA: Total genomic DNA was extracted from a ~2 cm^2 piece of silica gel-dried leaf tissue using a DNeasy 50 Plant Kit (Qiagen, Germany). DNA quantity and quality were assessed by electrophoresis.

Amplified fragment length polymorphism (AFLP) analysis: Sangon Biotech Co. Ltd. (Shanghai, China) synthesized primers and adapters. *Taq* DNA polymerase was obtained from Transgen Biotechnology Co. (Shanghai). AFLP was performed as described by Vos *et al*., (1995) using 300 ng of genomic DNA digested with *Eco*RI and *Mse*I (TaKaRa Biotechnology Co. Ltd. Dalian, China) and ligated using T4 DNA ligase (TaKaRa) to specific adapters digested with the same restriction enzymes. AFLP-selective PCR products were resolved on denaturing 6% polyacrylamide gels that were stained with 0.1% $AgNO_3$ (Bassam *et al.*, 1991). The band pattern was manually analysed (Jiang *et al.*, 2005).

Data analysis

AFLP markers were generated using four selective primer combinations. Each clear marker was treated as a separate character and scored as present (1) or absent (0) in the 30 cultivars. Data were recorded in a binary data matrix. Genetic similarity between cultivar pairs was estimated as described by Nei & Li (1979):

$$Gs = 2Nxy/(Nx + Ny)$$

where Gs is the genetic similarity (GS) coefficient between the x^{th} and y^{th} accessions, Nxy is the number of bands shared by x and y, and Nx and Ny are the total number of bands in x and y, respectively (Jiang *et al.,* 2005).

The resultant similarity matrix was subjected to unweighted pair group method of averages (UPGMA) cluster analysis (Gottwald *et al.,* 1966). Analyses used NTSYS-pc v. 2.0 (Rohlf, 1997) and POPGENE v. 1.31 (Yeh *et al.*, 1999) software.

Results

Following optimisation using the AFLP-PCR amplification system, a single polymorphic primer combination that yielded the highest polymorphism rate and generated sufficient clearly detectable total fragments was selected from 50 primers (Table 2).

Table 2. Amplified fragment length polymorphism (AFLP) primer combinations for *C. oleifera.*

Primer ID	Sequence (5'-3')
E+A	GACTGCGTACCAATTCA
M+G	GATGAGTCCTGAGTAAG
E+ACA	GACTGCGTACCAATTCACA
M+GAA	GATGAGTCCTGAGTAAGAA

AFLP profiling and genetic diversity: AFLP analysis was performed on the 30 accessions of *C. oleifera* using the optimal polymorphic selective primer combination, E00 + ACA/M00 + GAA. In all, 147 clear bands were obtained with these primers, of which 129 bands were polymorphic (87.76% polymorphism level, fragments of 87 to 402 bp). *C. oleifera* possessed significant genetic diversity between populations, and particularly high higher genetic variation at the species level (PPLs = 87.76%, n_{as} = 1.8776, n_{es} = 1.3631, H_{es} =0.2296, and I_s = 0.3625.

UPGMA cluster analysis: Genetic similarity between cultivars (Gs) varied from 0.40 to 0.85 (mean 0.63) (Fig. 1), and was highest between varieties CD_1 and CD_4 and lowest between HZXQ and CD_1.

A dendrogram (Fig. 1) for the 30 varieties was constructed using the UPGMA method, and six clusters were defined at a GS of 62.0%. Cluster a contained only variety HZXQ, which was susceptible to both *Colletotrichum gloeosporioides* strains. Clusters C1 and C2 contained varieties HZXH and HZDH_2, respectively, which were resistant to both strains, and these were clustered together at 57% GS (B1). Cluster D1 contained only the susceptible variety SC_3. Cluster D2 contained 25 varieties; this cluster could be further divided into two clusters at 65% GS; E1 contained four resistant and 10 susceptible varieties, and could be subdivided into four clusters at 70% GS; F1 included only the resistant variety CD_2, while F2 included two resistant (HX_9 and HX_2) and two susceptible (SC_1 and HX_6) varieties; F3 contained three susceptible (CD_6, HX_10 and CD_3) and one resistant varieties; F3 contained the three susceptible varieties CD_6, HX_10 and CD_3, and the resistant variety HX_8; the five varieties in cluster F4 (HZLHG, SC_2, HX_1, HX_4, and HX_7) were all susceptible; F4, F3, F2 and F1 were clustered into E1. E2 contains 11 varieties and could be subdivided into two clusters at 66% GS; F5 included three resistant (SC_5, SC_6 and CD_8) and one susceptible (CD_8) varieties; F6 contained three resistant (HZDH_1, CD_1 and CD_4) and four susceptible (CD_7, HX_3, HZDQ and SC_4) varieties.

Discussion

China lies at the centre of the natural distribution of *C. oleifera*, and Anhui Province is the major production region. Our results showed that parameters of genetic diversity (*PPL* = 87.07%, He = 0.2289, I = 0.3606) are lower for *C. oleifera* than for *Hagenia abyssinica* in Ethiopia (*PPL* = 80.70%, He = 0.30, I = 0.43) (Tileye *et al.,* 2007), but higher than *Triticum dicoccon* in Italy (*PPL* = 52.0%, He = 0.20) (Mario *et al.*, 2009), grass pea (*Lathyrus sativus* L.) in Italy (PPL = 50.0%, He = 0.16 (Lucia *et al.*, 2011), and *Ulva pertusa* on the China coast (PPL = 68.57%, He = 0.21, I = 0.32 (Zhao *et al.*, 2010). Thus, AFLP analysis efficiently generated quantitative estimates of genetic similarity among *C. oleifera* varieties. It yielded 147 bands per variety and per primer combination, considerably exceeding the values in the ISSR and RAPD analyses reported by Chen *et al.,* (2005), Zhang *et al.,* (2007) and Akhtar *et al.*, (2014). Although the varieties of *C. oleifera* used in different studies may differ, the polymorphism rate from AFLP analysis (0.8707) exceeded that determined by ISSR (0.8444) (Zhang *et al.*, 2007) and RAPD (0.6453) (Chen *et al.*, 2005; Ali *et al.*, 2012; Jatoi *et al.*, 2013). Our experiments were very reproducible. We conclude that AFLP is excellent for studies of genetic diversity of *C. oleifera*.

Our data suggest that *C. oleifera* cultivars show high levels of both polymorphisms and genetic diversity. *C. oleifera* has been cross-pollinated by insects over a long evolutionary period. Moreover, plant material was collected from three ecotypes in Anhui province, resulting in high levels of genetic variation and genetic diversity.

Based on reliable AFLP genetic markers, a dendrogram was constructed for the 30 *C. oleifera* varieties using the UPGMA method. At 62% GS, the 30 varieties were divided into six groups; resistant varieties were distributed randomly among the groups. Anthracnose resistance in *C. oleifera* may be polygenic and inherited in an additive manner. However, varieties collected from the same place did not always cluster in the same sub-groups, with the exception of varieties from Eastern Anhui (Hongxin).

The resistant varieties HZDH_2, HZXH, HZXQ, and *C. yuhsiensis* that is native to Hunan province are the most distantly related from the other varieties, with a GSC value of 57%. Interesting, HZXQ fruits are small and pure green in colour, whereas those of HZXH and HZDH_2 are red, fruits of *C. yuhsiensis* are tiny and brown in colour. Although the origins of varieties may differ, genetic relationships are close between these varieties, presumably because they have been naturalised and cultivated together for many years.

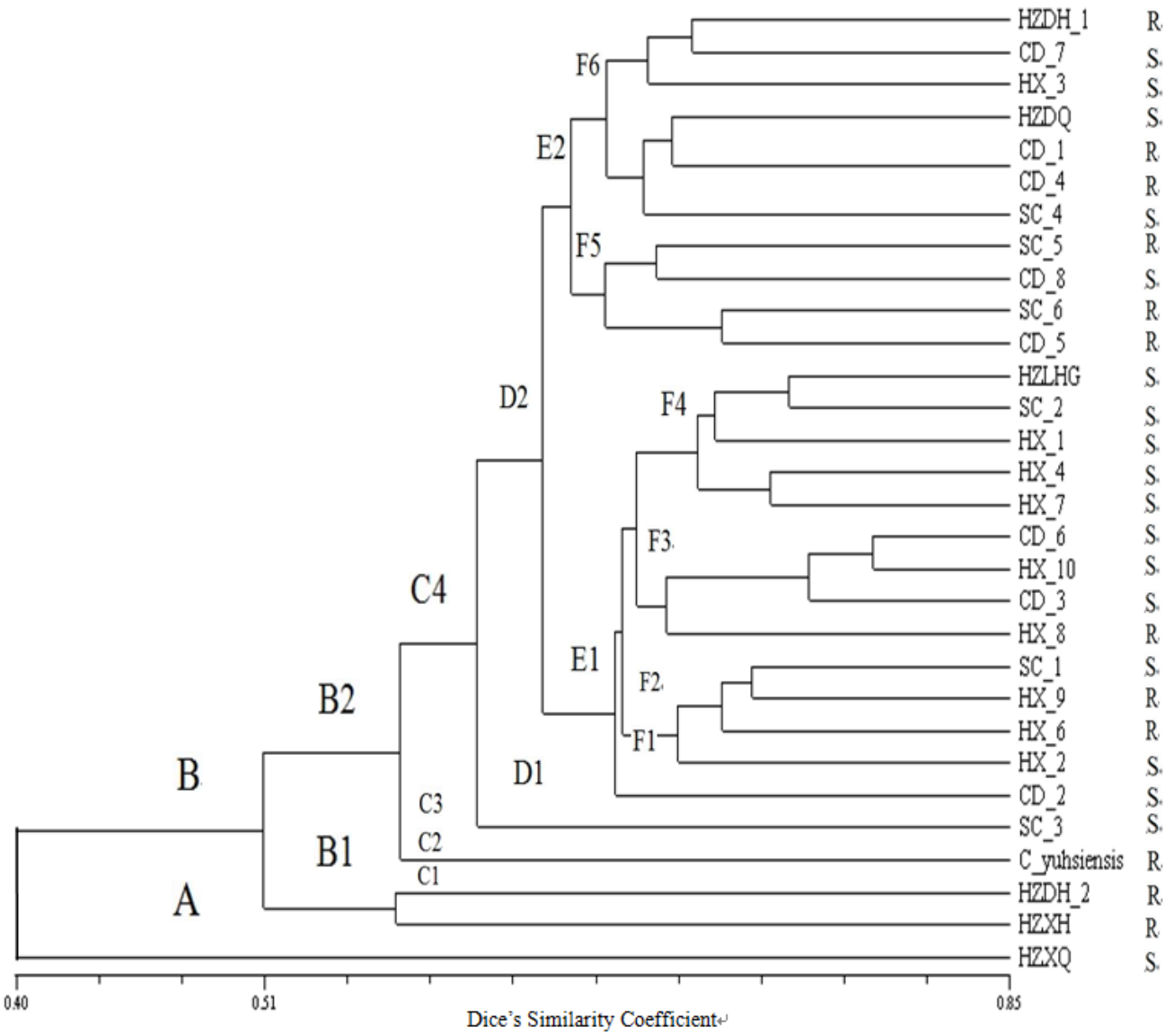

Fig. 1. Unweighted pair group method of averages (UPGMA) dendrogram of *Camellia oleifera* varieties based on amplified fragment length polymorphisms (AFLPs) generated using NTSYSpc version 2.0.

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