



Genetic variations of prevailing *Verticillium dahliae* isolates from cotton in China

Wenwei Zhang¹ · Yuhong Ren¹ · Huachong Zhang¹ · Ning Si¹ · Xueyan Zhu¹ · Fangjun Qi¹ · Guiliang Jian¹

Received: 24 January 2018 / Accepted: 29 December 2018 / Published online: 6 February 2019
© Società Italiana di Patologia Vegetale (S.I.Pa.V.) 2019

Abstract

Verticillium wilt caused by the soil-borne fungus *Verticillium dahliae* is a devastating disease of cotton, leading to serious loss of lint yield in China. It is important to characterize the prevailing populations of *V. dahliae* for understanding disease development. In this study, the representative 74 *V. dahliae* isolates from 28 different cotton regions in three major cotton-production areas in China were identified for their pathotypes, races, vegetative compatibility groups (VCGs) and genetic diversity by specific PCR, amplified fragment length polymorphism (AFLP) and inter simple sequence repeat (ISSR) analysis. Of 74 isolates, 60 isolates (81%) were defoliating (D) pathotype strains, especially in Yellow River/Yantze River Valley region. Seventy isolates belonged to race 2, whereas no race 1 specific fragment was amplified. These isolates were grouped into five VCGs: VCG1A (48), VCG2A/VCG4B (3), VCG2B⁸²⁴ (6), VCG6 (5). The D pathotype and VCG1A isolates have become the dominant strains, moderately and highly virulent isolates were widely distributed in three major cotton-growing areas in China. High level of polymorphic markers was obtained by AFLP and ISSR analyses. Through comparison of the obtained dendrograms by unweighted paired group method with arithmetic averages cluster analysis (UPGMA), the *V. dahliae* isolates were divided into two major groups and five subgroups. The obvious correlations with the geographic origin of the isolates between AFLP and ISSR were revealed, with significant diversity among *V. dahliae* isolates. In addition, AFLP dendrogram showed relationships with pathotypes. The results offered a better understanding of the epidemiology of Verticillium wilt in China.

Keywords *Verticillium dahliae* · Cotton · Pathotype · Races · Vegetative compatibility groups (VCGs) · Genetic diversity

Introduction

Verticillium wilt, a devastating disease of cotton, is caused by the soil-borne, destructive plant fungus *Verticillium dahliae* Kleb. This pathogenic fungus is widely distributed in agricultural soils with a broad host range of more than 400 plant species, including many economically important crops (Bell 1992). The disease was first reported on upland cotton in Virginia, USA and then widely spread to most of the cotton-growing countries worldwide. It was firstly introduced to

China via imported American cotton in 1935 (Ma 2007). Initially, the disease just occurred in limited areas in China. However, since 1990s, due to the widespread of *V. dahliae*, more than 70% of cotton-planting areas have been subjected to Verticillium wilt, resulting in a tremendous economic loss yearly (Ma 2007). Therefore, characterization of genetic variations among populations of the prevailing *V. dahliae* is an essential and important step to understand disease development and further to formulate the appropriate strategies for disease management.

Based on symptomatology, *V. dahliae* is generally classified into two pathotypes, namely defoliating pathotype (D) and non-defoliating pathotype (ND) (Schnathorst and Mathre 1966). The former causes defoliation of the cotton and is highly virulent while the latter causes mild wilt symptoms without defoliation (Schnathorst and Mathre 1966; Joaquim and Rowe 1990). Using random amplified polymorphic DNA (RAPD) and specific polymerase chain reaction (PCR) analyses, Perez-Artes et al. (2000) clearly differentiated D strains from ND ones. It is important to determine the

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s42161-018-00236-9>) contains supplementary material, which is available to authorized users.

✉ Wenwei Zhang
wwzhang@ipccaas.cn

¹ State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, People's Republic of China

V. dahliae pathotypes that infect cotton in order to manage the disease by planting *V. dahliae*-tolerant/resistant cotton cultivars to overcome this highly virulent D pathotype.

V. dahliae isolates have also been classified by vegetative compatibility groups (VCGs) (Leslie 1993). After surveys of a large collection of isolates, six VCGs from different hosts and geographic origins were identified (Daayf et al. 1995; Jimenez-Diaz et al. 2006). Due to the strong linkage disequilibrium in clonal populations, VCGs in *V. dahliae* are highly correlated with molecular markers and clonal lineages (Jimenez-Diaz et al. 2012; Jimenez-Gasco et al. 2014; Jimenez-Diaz et al. 2017). VCGs are informative, and isolates from different VCGs are thought to be genetically distinct populations which may vary in a number of ecological, physiological, virulence, and host-range traits (Angela et al. 2013).

Molecular markers are able to provide a direct measure of genetic diversity. Therefore, molecular markers, such as those based on amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), random amplification of polymorphic DNA (RAPD), simple sequence repeats (SSRs or microsatellites), the intergenic spacer region of the ribosomal DNA (IGS rDNA) and restriction fragment length polymorphism (RFLP) analyses, have been applied in the analysis of genetic diversity of *V. dahliae* (Atallah et al. 2010; Collado-Romero et al. 2006, 2009; ElSharawy et al. 2015; Erdogan et al. 2013; Gharbi et al. 2014, 2015; Pramateftaki et al. 2000). Among the molecular markers, AFLP (Vos et al. 1995) and ISSR (Zietkiewicz et al. 1994) are the highly polymorphic ones, which provide highly discriminating information with good reproducibility. In the present study, we applied both AFLP and ISSR markers to analyze the genetic diversity among *V. dahliae* populations in samples collected from three major cotton planting areas in China. We also tested the pathogenicity of all the isolates and used the specific PCR analysis to confirm the pathotypes, race and VCGs of *V. dahliae* in China (Perez-Artes et al. 2000; Collado-Romero et al. 2009; de Jonge et al. 2012; Short et al. 2014). The results obtained in this study will provide further information about the pathogenic diversity of *V. dahliae* in China, which can be helpful for forecasting disease development and formulating management measures for effective control and prevention of Verticillium wilt of cotton in China.

Materials and methods

Collection of isolates and culture

The 74 *V. dahliae* isolates used in this study were collected from infected cotton showing Verticillium wilt symptoms from 28 different cotton-planting regions in three traditional major cotton production areas across 11 provinces of China (Fig. 1) from 2014 to 2016. The three major cotton production

regions areas are: (a) Yellow River Valley region, which includes Hebei (Baoding, Langfang, Xingtai), Henan (Kaifeng, Xinxiang), Shandong (Heze), Shanxi (Yuncheng) provinces; (b) Yantze River Valley region, which includes Anhui (Anqing, Chizhou, Wuhu), Hubei (Qianjiang, Jingzhou, Tianmen, Xiantao, Zhijiang), Hunan (Yirang, Yueyang), Jiangsu (Dafeng, Nantong, Xuzhou), and Jiangxi (Jiujiang) provinces; and (c) inland of Northwest region, which includes Gansu (Dunhuang) province and Xinjiang Autonomous region (Akesu, Bole, Kashi, Kuitun, Manasi, Shawan). The *V. dahliae* isolates from these regions are described in Table 1 and Fig. 1.

To isolate *V. dahliae*, cotton stems showing wilt symptoms were cut into small pieces (0.5–2 cm) with a sterilized razor. The stem surface was sterilized with 5% sodium hypochlorite (12%) followed by rinsing twice in sterile distilled water. The washed pieces were dried on sterile filter paper, then placed onto potato dextrose agar (PDA) plates. After incubation at 25 °C for one week, the emerging fungi were transferred onto new PDA plates. Single spore was isolated and stored in a 20% glycerol solution at –80 °C.

Extraction of fungal DNA and specific PCR assay

Single spore isolates were grown on PDA plates at 25 °C for one week. Mycelia were collected and ground into powder in mortar with liquid nitrogen. Total fungal DNA was extracted from each isolate of *V. dahliae* essentially as described by Lee and Taylor (1990). The DNA integrity was assessed using agarose gel electrophoresis and NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., USA). All *V. dahliae* isolates in the study were characterized by PCR assays using eight primer pairs. Two pairs of primers, VDF/VDR and VNDF/VNDR, described by Perez-Artes et al. (2000) were used to determine the pathotype of the collected isolates. Primer pair VDF/VDR yields a specific PCR product (548 bp) related to the D pathotype, whereas primer pair VNDF/VNDR yields a specific PCR product (1410 bp) associated with the ND pathotype. Four pairs of primers were used to measure the VCGs of all *V. dahliae* isolates (Collado-Romero et al. 2009). Primer pair DB19/espdef01 (Mercado-Blanco et al. 2003) yields a specific 334 bp product which was amplified from VCG1A, VCG1B or VCG2B³³⁴ isolates. Primer pair INTND2F/INTND3R (Collado-Romero et al. 2009) yields a 688 bp marker which was amplified from VCG2A, VCG4B or VCG2B⁸²⁴ isolates. Primer pair INTND2F/INTND2R (Mercado-Blanco et al. 2001) yields a 824 bp marker which was amplified from VCG2A, VCG2B⁸²⁴, VCG4B isolates. Primer pair INTND2F/MCR2B (Collado-Romero et al. 2009) yields a 964 bp marker, which was amplified from VCG2B⁸²⁴ isolates.

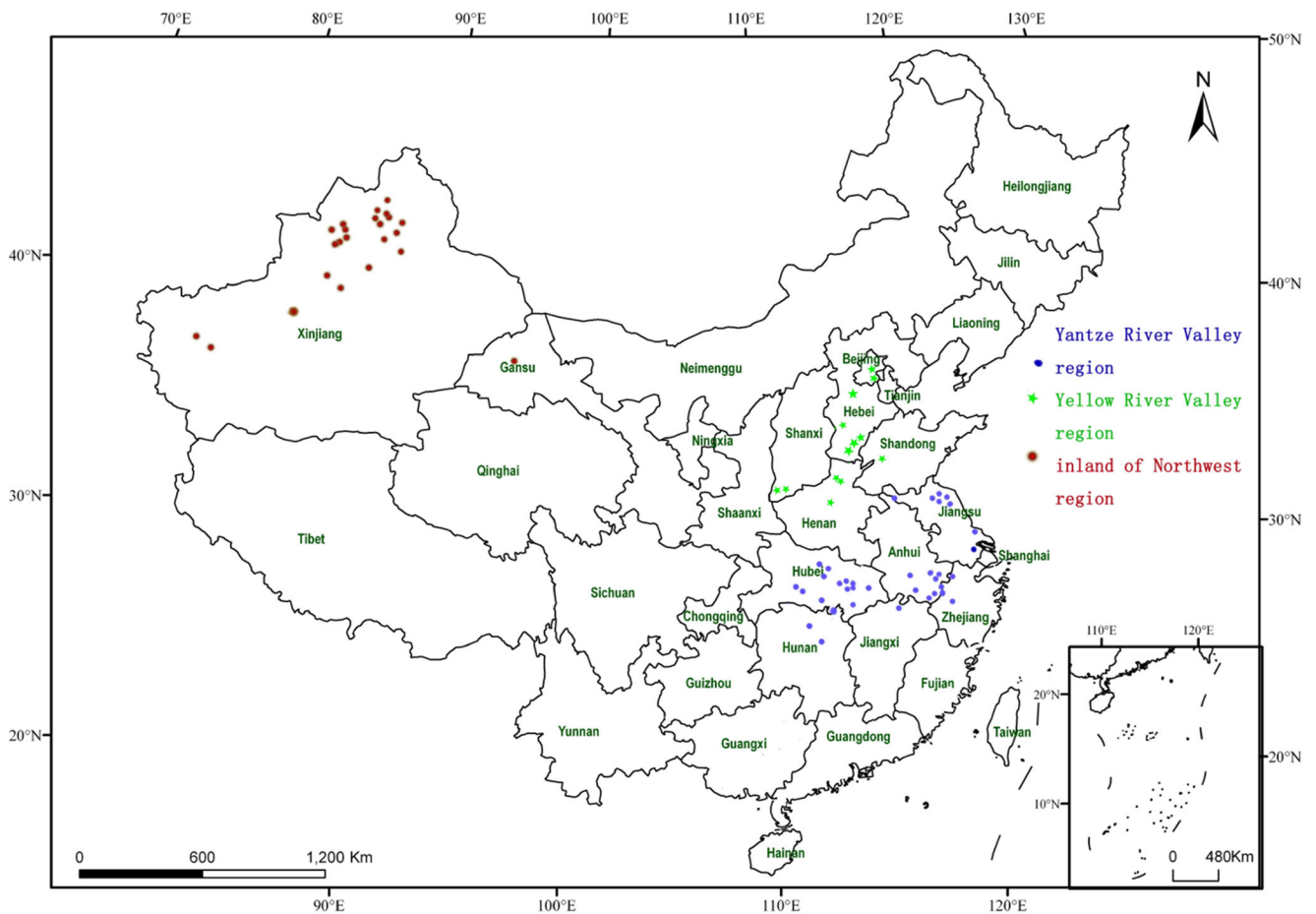


Fig. 1 Geographical distribution of the collected *V. dahliae* isolates

Primer pair VdAve1F/ VdAve1R was used to identify *V. dahliae* race 1 (de Jonge et al. 2012), primer pair VdR2F/VdR2R was used to identify *V. dahliae* race 2 (Short et al. 2014). All isolates were tested separately using these eight primer pairs.

AFLP and ISSR analysis

AFLP assays were performed according to the fluorescent procedures of the method described by Vos et al. (1995) with modifications. Adapters, pre-amplification primers, nine *Eco*RI: *Mse*I AFLP selective primer combinations were shown in Supplementary Table S1. The *Mse*I primer was labeled with FAM dye. The selective PCR amplification consisted of one cycle of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 80 s, followed by 11 cycles under the same conditions except that the annealing temperature was decreased by 0.7 °C per cycle. Then, the last 23 cycles were carried out at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 80 s. Nine primers (Supplementary Table S1) were used for ISSR analysis. PCR amplification was performed by initial denaturation at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s and extension at 72 °C

for 2 min, and a final extension of 10 min at 72 °C. The amplicons of AFLP and ISSR were separated by polyacrylamide gel electrophoresis in an automatic DNA sequencer (ABI337), respectively. The data were collected by GeneScan analysis software. Amplification products were scored as presence (1) or absence (0) based on the AFLP/ISSR pattern obtained for each isolate and the data were compiled as a binary matrix. Dendrograms were generated by unweighted pair-group mean arithmetic method analysis (UPGMA) and clustered with the Ntsys software (Rohlf 1997). The Mantel significance test was used to compare each pair of similarity matrices produced with the Ntsys 2.2 software.

Pathogenicity assay

Six different resistant varieties were used in virulence assays in a growth chamber under long-day condition (16 h photoperiod) with 26/20 °C day/night temperatures to determine the pathogenicity of all the isolates. Cotton cultivars included two resistant cultivars: Zhongzhimian No. 2 (Ren et al. 2018) and Jimian 958 (Geng et al. 2006); two tolerant cultivars: Luyanmian 28 (Ren et al. 2018) and Zhongmiansuo 35 (Zhu et al. 2012); two susceptible cultivars: 86–1 (Zhang et al.

Table 1 *Verticillium dahliae* isolates from cotton tested in this study

Geographic origin	Collection year	Isolate	VDF/VDR(548 bp) ^a	VNDF/VNDR (1410 bp) ^b	Pathotype	VdR2F/VdR2R (256 bp) ^d	DB19/espdef01 (334 bp) ^e	INTND2F/INTND2R (824 bp) ^f	INTND2F/MCR2B (964 bp) ^g	INTND2F/INTND3R (688) ^h	Predicted infecting VCG ⁱ	Vinulence ^j
Anhui, Anqing	2014	VD1	+	-	D	+	+	-	-	-	VCG1A	Moderate
Anhui, Anqing	2015	VD2	+	-	D	+	+	-	-	-	VCG1A	Moderate
Anhui, Anqing	2014	VD3	+	-	D	+	+	-	-	-	VCG1A	Moderate
Anhui, Wuhu	2014	VD4	+	-	D	+	+	-	-	-	VCG1A	Strong
Anhui, Wuhu	2015	VD5	+	-	D	+	+	-	-	-	VCG1A	Strong
Anhui, Wuhu	2015	VD6	+	-	D	+	+	-	-	-	VCG1A	Moderate
Anqing	2015	VD7	+	-	D	+	+	-	-	-	VCG1A	Strong
Anqing	2015	VD8	+	-	D	+	+	-	-	-	VCG1A	Moderate
Anhui, Anqing	2015	VD9	+	-	D	+	+	-	-	-	VCG1A	Moderate
Anhui, Chizhou	2015	VD10	+	-	D	+	+	-	-	-	VCG1A	Moderate
Anhui, Chizhou	2014	VD11	-	+	ND	+	-	+	+	+	VCG2B ⁸²⁴	Moderate
Wuhu	2014	VD12	+	-	D	+	+	-	-	-	VCG1A	Strong
Hebei, Baoding	2015	VD13	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hebei, Langfang	2016	VD14	+	-	D	+	+	-	-	-	VCG1A	Strong
Hebei, Langfang	2014	VD15	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hebei, Xingtai	2014	VD16	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hebei, Xingtai	2014	VD17	+	-	D	+	+	-	-	-	VCG1A	Strong
Hebei, Xingtai	2015	VD18	-	+	ND	+	-	+	+	+	VCG2B ⁸²⁴	Strong
Baoding	2014	VD19	+	-	D	+	+	-	-	-	VCG1A	Strong
Henan, Kaifeng	2014	VD20	+	-	D	+	+	-	-	-	VCG1A	Strong
Henan, Xinxiang	2015	VD21	+	-	D	+	+	-	-	-	VCG1A	Moderate
Henan, Xinxiang	2014	VD22	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hubei, Zhijiang	2015	VD23	+	-	D	-	+	-	-	-	VCG1A	weak

Table 1 (continued)

Geographic origin	Collection year	Isolate	VDF/ VDR(548 bp) ^a	VNDF/VNDR (1410 bp) ^b	Pathotype	VdR2F/ VdR2R (256 bp) ^d	DB19/ espedf01 (334 bp) ^e	INTND2F/ INTND2R (824 bp) ^f	INTND2F/ MCR2B (964 bp) ^g	INTND2F/ INTND3R (688) ^h	Predicted infecting VCG ⁱ	Vinulence ^d
Hubei, Zhijiang	2014	VD24	+	-	D	+	+	-	-	-	VCG1A	Strong
Hubei, Qianjiang	2014	VD25	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hubei, Qianjiang	2014	VD26	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hubei, Qianjiang	2015	VD27	+	-	D	+	+	-	-	-	VCG1A	Strong
Hubei, Qianjiang	2015	VD28	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hubei, Qianjiang	2014	VD29	+	-	D	+	+	-	-	-	VCG1A	Strong
Hubei, Jingzhou	2016	VD30	+	-	D	+	+	-	-	-	VCG1A	Strong
Hubei, Xiantao	2016	VD31	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hubei, Tianmen	2016	VD32	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hubei, Tianmen	2014	VD33	+	-	D	+	+	-	-	-	VCG1A	Moderate
Hunan, Tianmen	2014	VD34	+	-	D	+	-	-	-	-	-	Strong
Hunan, Yueyang	2014	VD35	+	-	D	+	-	-	-	-	-	Moderate
Jiangsu, Yirang	2014	VD36	+	-	D	+	+	-	-	-	VCG1A	Strong
Jiangsu, Dafeng	2014	VD37	+	-	D	+	+	-	-	-	VCG1A	Strong
Jiangsu, Dafeng	2015	VD38	+	-	D	+	+	-	-	-	VCG1A	Strong
Jiangsu, Dafeng	2015	VD39	+	-	D	+	+	-	-	-	VCG1A	Strong
Jiangsu, Dafeng	2016	VD40	+	-	D	+	+	-	-	-	VCG1A	Strong
Jiangsu, Dafeng	2016	VD41	+	-	D	+	-	-	-	-	-	Moderate
Jiangsu, Xuzhou	2015	VD42	+	-	D	+	+	-	-	-	VCG1A	Strong
Shandong, Jiujiang	2015	VD43	+	-	D	+	+	-	-	-	VCG1A	Moderate
Heze												
Shanxi, Heze	2014	VD44	+	-	D	+	+	-	-	-	VCG1A	Strong
Yuncheng	2014	VD45	+	-	D	+	+	-	-	-	VCG1A	Strong

Table 1 (continued)

Geographic origin	Collection year	Isolate	VDF/ VDR(548 bp) ^a	VNDF/VNDR (1410 bp) ^b	Pathotype	VdR2F/ VdR2R (256 bp) ^d	DB19/ espde101 (334 bp) ^e	INTND2F/ INTND2R (824 bp) ^f	INTND2F/ MCR2B (964 bp) ^g	INTND2F/ INTND3R (688) ^h	Predicted infecting VCG ⁱ	Vinulence ^j
Shanxi, Yuncheng	2014	VD46	+	-	D	+	+	-	-	-	VCG1A	weak
Xinjiang, Akesu	2014	VD47	-	+	ND	-	-	+	-	-	VCG6	weak
Xinjiang, Bole	2014	VD48	-	+	ND	+	+	+	-	+	VCG2A or VCG4B	Moderate
Xinjiang, Bole	2014	VD49	-	+	ND	+	+	+	-	-	VCG6	Moderate
Xinjiang, Bole	2016	VD50	-	+	ND	+	+	+	+	+	VCG2B ⁸²⁴	Strong
Xinjiang, Bole	2016	VD51	-	+	ND	+	+	+	+	+	VCG2B ⁸²⁴	Moderate
Xinjiang, Bole	2015	VD52	+	-	D	+	+	-	-	-	VCG1A	Moderate
Xinjiang, Kashi	2014	VD53	-	+	ND	-	+	+	-	+	VCG2B ⁸²⁴	Moderate
Xinjiang, Kashi	2015	VD54	-	+	ND	+	+	+	-	+	VCG2A or VCG4B	Moderate
Xinjiang, Kuitun	2014	VD55	-	+	ND	+	+	+	-	-	VCG6	Moderate
Xinjiang, Manasi	2015	VD56	+	-	D	+	+	-	-	-	-	Moderate
Xinjiang, Manasi	2015	VD57	+	-	D	+	+	-	-	-	-	Strong
Xinjiang, Shawan	2014	VD58	-	+	ND	+	+	+	-	+	VCG2A or VCG4B	Moderate
Xinjiang, Shawan	2015	VD59	+	-	D	+	+	-	-	-	-	Moderate
Xinjiang, Shawan	2015	VD60	+	-	D	+	+	-	-	-	-	weak
Anhui, Wuhu	2015	VD61	+	-	D	+	+	-	-	-	VCG1A	Moderate
Xinjiang, Shawan	2016	VD62	+	-	D	+	+	-	-	-	VCG1A	Moderate
Xinjiang, Shawan	2016	VD63	-	+	ND	+	+	+	-	-	VCG6	Moderate
Xinjiang, Shawan	2014	VD64	+	-	D	+	+	-	-	-	-	Moderate
Xinjiang, Shawan	2014	VD65	-	+	ND	+	+	+	-	-	VCG6	Moderate
Xinjiang, Shawan	2014	VD66	+	-	D	+	+	-	-	-	VCG1A	Strong
Xinjiang, Shawan	2015	VD67	+	-	D	+	+	-	-	-	-	Strong

Table 1 (continued)

Geographic origin	Collection year	Isolate	VDF/VDR(548 bp) ^a	VNDF/VNDR (1410 bp) ^b	Pathotype	VdR2F/VdR2R (256 bp) ^d	DB19/espdef01 (334 bp) ^e	INTND2F/INTND2R (824 bp) ^f	INTND2F/MCR2B (964 bp) ^g	INTND2F/INTND3R (688) ^h	Predicted infecting VCG ⁱ	Virulence ^j
Xinjiang, Shawan	2014	VD68	+	-	D	+	-	-	-	-	-	Strong
Xinjiang, Shawan	2015	VD69	+	-	D	+	-	-	-	-	-	Moderate
Jiangsu, Dafeng	2016	VD70	+	-	D	+	-	-	-	-	-	Strong
Hubei, Xiantao	2015	VD71	+	-	D	+	+	-	-	-	VCG1A	Moderate
Xinjiang, Akesu	2016	VD72	+	-	D	-	+	-	-	-	VCG1A	Strong
Gansu, Dunhuan- ^g	2014	VD73	-	+	ND	+	-	+	+	+	VCG2B ⁸²⁴	weak
Jiangsu, Nantong	2014	VD74	+	-	D	+	+	-	-	-	VCG1A	Strong

^a Sequence amplified with primer pairs VDF/VDR (548 bp) (Pérez-Artés et al. 2000)

^b Sequence amplified with primer pairs VNDF/VNDR (1410 bp) (Pérez-Artés et al. 2000)

^c pathotype, ND = non-defoliating, D = defoliating

^d Sequence amplified with primer pairs VdR2F/VdR2R (256 bp) (Short et al. 2014)

^e Sequence amplified with primer pairs DB19/espdef01 (334 bp) (Mercado-Blanco et al. 2003)

^f Sequence amplified with primer pairs INTND2F/INTND2R (824 bp) (Mercado-Blanco et al. 2001)

^g Sequence amplified with primer pairs INTND2F/MCR2B (964 bp) (Collado-Romero et al. 2009)

^h Sequence amplified with primer pairs INTND2F/INTND3R(688) (Collado-Romero et al. 2009)

ⁱ VCG = vegetative compatibility group verified as described in the main text (Collado-Romero et al. 2009)

^j virulence. Severity of *V. dahliae* was assessed on disease index (DI) values on six cotton varieties. Isolates with the mean DI value lower than 20.0 were designated as weakly virulent; isolates with the mean DI value between 20.01 to 35.0 were designated as moderately virulent; isolates with the mean DI value higher than 35.0 were designated as highly virulent (Zhang et al. 2012)

2013) and Xinluzao No. 7 (Ren et al. 2018). Cotton growth and inoculation with *V. dahliae* isolates by the root dipping method were as in Zhang et al. (2013). Seedlings dipped in sterile distilled water were used as non-inoculated controls. For each *V. dahliae* isolate, 10 plants were inoculated per experiment and the whole experiment was conducted three times. According to the percentage of affected plant tissues, such as chlorosis, necrosis, wilt and defoliation, a Verticillium wilt disease index (DI) scaling from 0 to 4 was introduced to evaluate the disease severity (Zhang et al. 2013). The DI of Verticillium wilt was determined using the following formula (Zhang et al. (2017):

$$DI = \frac{\sum(n \times \text{number of plants at level } n)}{4 \times \text{number of total plants}} \times 100$$

Then, the pathogenicity of the isolates was evaluated by the mean DI value on six cotton varieties. The mean DI value lower than 20.0 designated a weakly virulence isolate, the mean DI value between 20.01 to 35.0 designated a moderately virulent isolate, the mean DI value higher than 35.0 designated highly virulent isolates (Zhang et al. 2012). Statistical analyses were conducted by ANOVA using the SAS 9.4. The confidence level of all analyses was set at 95%, and $P < 0.05$ was considered as statistically significant.

Results

Pathotypes and race characterization

Seventy-four representative isolates were analyzed to detect pathotypes and races by specific PCR assay based on the presence of 548 bp (D pathotype), 1410 bp (ND pathotype), 900 bp (race 1), and 256 bp (race 2) amplicons (Fig. 2a, b, c). Most of the isolates produced the 548 bp specific fragment and they were thus, designated as D pathotype. Fourteen isolates produced the 1410 bp specific fragment and thus, were designated as ND pathotype (Table 1), whereas 60 isolates belonged to D pathotype, which accounted for 81% of all the isolates.

Seventy isolates produced the 256 bp specific fragment and were designated as race 2. Four isolates (VD23, VD47, VD53, VD72) could not be amplified with race 2-specific primer pair (Table 1). However, no isolate could be amplified with race 1-specific VdAve1F/VdAve1R primers. A previous study showed that defoliating pathotype is more virulent than ND pathotype (Joaquim and Rowe 1990). VD23 and VD72 were D isolates, while VD47 and VD53 were ND isolates. There is no obvious relationship between race 1 and race 2 with D and ND pathotypes.

VCGs characterization

Amplification of both the 334 bp and 548 bp specific fragments was obtained from 48 isolates, which were designated as VCG1A isolates. VD48, VD54 and VD58 amplified both the 688 bp and 824 bp specific fragments, indicating them as VCG2A or VCG4B isolates. VD11, VD18, VD50, VD51, VD53 and VD73 amplified three specific markers including 688 bp, 824 bp and 964 bp, which designated them as VCG2B⁸²⁴ isolates. VD47, VD49, VD55, VD63 and VD65 only produced 824 bp specific fragments, which designated them as VCG6 isolates. Moreover, 12 isolates only amplified the 548 bp specific fragment, but could not amplify the 334 bp specific fragment. The results are listed in Table 1 and Fig. 2a, d–g. It can be concluded that the VCG1A isolates are dominant across China cotton production areas.

Fingerprinting

In order to produce clear and reproducible polymorphic banding patterns from all *V. dahliae* isolates tested, nine primer pairs were used for AFLP and nine primers for ISSR analysis. The sizes of amplified markers were in the range of 70–500 bp for AFLP and 50–1000 bp for ISSR. These primers generated a large number of polymorphic amplification markers. In the case of AFLP, 881 unambiguous and easily discernible polymorphic markers were obtained from the 74 isolates, including 91 markers with *EcoRI*-AAC/*MseI*-CAA, 88 with *EcoRI*-AAC/*MseI*-CTA, 85 with *EcoRI*-AAG/*MseI*-CTC, 75 with *EcoRI*-AAG/*MseI*-CTT, 129 with *EcoRI*-ACA/*MseI*-CTC, 112 with *EcoRI*-ACG/*MseI*-CTC, 87 with *EcoRI*-AGC/*MseI*-CAG, 94 with *EcoRI*-AGG/*MseI*-CTC, and 120 with *EcoRI*-AGG/*MseI*-CTG. In the case of ISSR, 1676 markers were obtained from the 74 isolates, including 235 markers with ISSR807, 193 with ISSR808, 153 with ISSR809, 157 with ISSR811, 172 with ISSR835, 170 with ISSR836, 178 with ISSR840, 234 with ISSR881, and 184 with ISSR886.

Genetic diversity and comparison between AFLP and ISSR

To evaluate the effectiveness of the two different molecular markers (AFLP and ISSR) in studying genetic relationships between *V. dahliae* isolates, genetic similarity coefficient was calculated. The genetic similarity value for AFLP ranged from 0.26 (between VD32 and VD23, 55) to 0.61 (between VD37 and VD38). For ISSR, it ranged from 0.13 (between VD23 and VD55) to 0.56 (between VD39 and VD40).

The dendrogram generated from UPGMA clustering of two marker data divided all *V. dahliae* isolates into two major clusters (Fig. 3). Each of their dendrograms was unique with some evident similarities. The AFLP-based dendrogram divided the isolates into two major groups at a similarity index

of 0.26 (Fig. 3a), placing VD23, VD32, and VD55 in one cluster and the remaining 71 isolates in the other cluster. The latter cluster was further divided into five distinct subgroups. Isolates collected from Yellow River Valley region and Yantze River Valley region were mainly grouped together in subgroup A. The ISSR-based dendrogram was discriminated at a higher level of diversity, two major clusters were divided at a similarity index of 0.13 (Fig. 3b), placing VD23 in one cluster and the remaining 73 isolates in the other cluster. The larger group was further separated into five subgroups which were grouped mainly according to the geographical origin. There is an obvious correlation with the geographic origin of isolates between the dendrograms of AFLP and those of ISSR.

Furthermore, the results of AFLP dendrogram showed a relationship to pathotypes. In AFLP clusters, the ND isolates were separated from D isolates. Isolates of subgroup B and E were ND pathotype while the isolates of subgroups A, C and D belonged to D pathotype. However, in ISSR clustering patterns, ND pathotype showed a mixed distribution between them. In ISSR, ND isolates VD11 and VD18 collected from Anhui and Hebei were placed in subgroup B. Subgroup C included seven ND isolates from inland of Northwest region (VD47–51, VD53 and VD54), whereas four ND isolates from inland of Northwest region (VD58, VD63, VD65 and VD73) were placed in subgroup D. ND isolate VD55 constituted subgroup E by itself.

In addition, goodness of fit test was performed for DICE similarity distances by Mantel test in both AFLP and ISSR analyses. The strongest correlation coefficients (r) were found to correspond to the dendrograms produced by AFLP and ISSR at a threshold of $P < 0.001$. ISSR's correlation coefficient value was the highest with r being 0.94, followed by AFLP with r being 0.85.

Virulence of isolates

All the isolates showed variation in virulence on the six cotton varieties. Based on the results of mean DI values on six cotton varieties, these isolates were classified into three main virulence categories: weakly virulent, moderately virulent and highly virulent isolates. Five isolates were weakly virulent isolates, that means DI value lower than 20.0. Thirty-nine isolates were moderately virulent isolates, with DI value between 20.01 and 35.0. Thirty-nine isolates were highly virulent isolates, that means DI value higher than 35.0 (Table 1, Supplementary Table S2). Among them, highly virulent isolates were distributed in nine of 11 provinces, including Anhui (3), Hebei (4), Henan (2), Jiangsu (7), Jiangxi (1), Hubei (4), Hunan (1), Shanxi (2), Xinjiang (6). As for defoliating isolates, only VD23, VD46 and VD60 were weakly virulent isolates, whereas the other isolates were moderately or highly virulent isolates. Among ND isolates, VD47 and VD73 were weakly virulent isolates, VD18 and VD50 were highly virulent isolates, the other 10 isolates were moderately virulent. This indicated that

moderately and highly virulent isolates were widely distributed in three major cotton production areas of China.

Discussion

As the most important cotton pathogen, *V. dahliae* infecting cotton can result in substantial yield loss in China. Therefore, it is important to know the disease development in China. In this study, we selected 74 representative isolates from three major cotton production areas distributed across 11 provinces in China from 2014 to 2016, which represented more than 70% of cotton-planting areas in the country. The prevailing *V. dahliae* isolates were molecularly tested for their pathotype, races, vegetative compatibility, genetic diversity. These results, together with those from our experiment on virulence of isolates of *V. dahliae* on a group of six genotypes, some of them carrying genes for resistance against the disease, will allow a better understanding of Verticillium wilt of cotton in China.

The pathotypes of *V. dahliae* were classified as either D or ND based on disease symptomatology. In the last century, the ND pathotype of *V. dahliae* was widespread (Perez-Artes et al. 2000), while the D pathotype was found only from a few places (Bejarano-Alcazar et al. 1996; Xia et al. 1998). On the contrary, and according to our molecular results, the D pathotype dominates in China. In this study, 81% of all the isolates belonged to D pathotype. Especially in Yellow River Valley region and Yantze River Valley region, 47 isolates were D pathotypes, and only two isolates were ND pathotypes. In inland of Northwest region, D pathotype isolates accounted for 52%, while ND pathotype isolates accounted for 48%. It was reported that only small numbers of D pathotypes (about 8.6%) were scattered at inland of Northwest region (Zhang et al. 2006). The D pathotype seems to have expanded rapidly in inland of Northwest region, what can be due to the wide spread of infected cotton seeds.

Several pathogenic races of *V. dahliae* have been described on lettuce, pepper, strawberry, sunflower and tomato (Maruthachalam et al. 2010; Short et al. 2014; Martín-Sanz et al. 2018). Race 2 lacks the virulence gene (*Ave 1*) and is not recognized in plants by resistant *Ve* gene, resulting in disease (Jonge et al. 2012; Short et al. 2014). In previous studies, nearly 37% of the *V. dahliae* isolates from cotton were characterized as race 1/ND isolates, and 61% of the *V. dahliae* isolates from cotton were characterized as race 2/D isolates (Hu et al. 2015). But in this study, no race 1 bands were amplified. The *V. dahliae* isolates from cotton were only characterized as race 2 isolates. Moreover, D type had significant relationship with race 2 and ND type correlated with race 1 (Hu et al. 2015). Nearly 86% ND isolates were characterized as race 2, and 97% D isolates were characterized as race 2. In

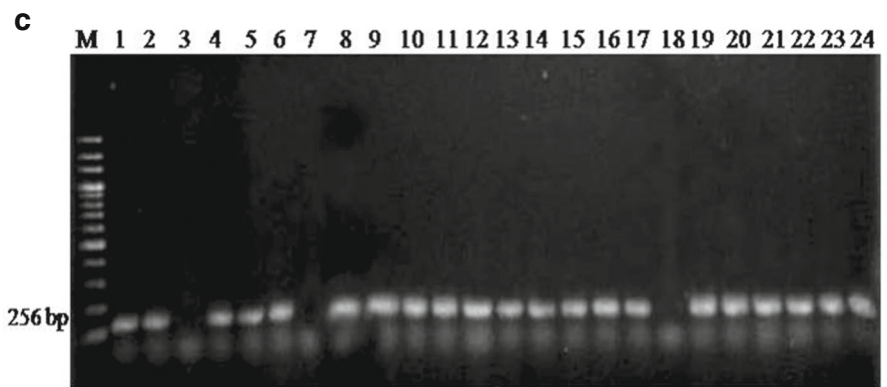
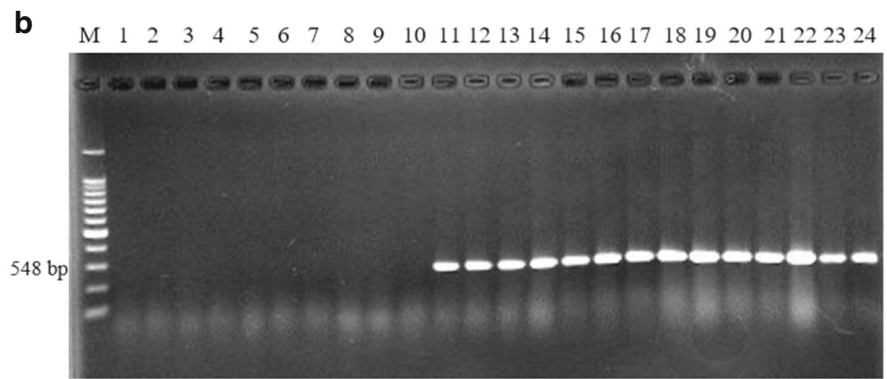
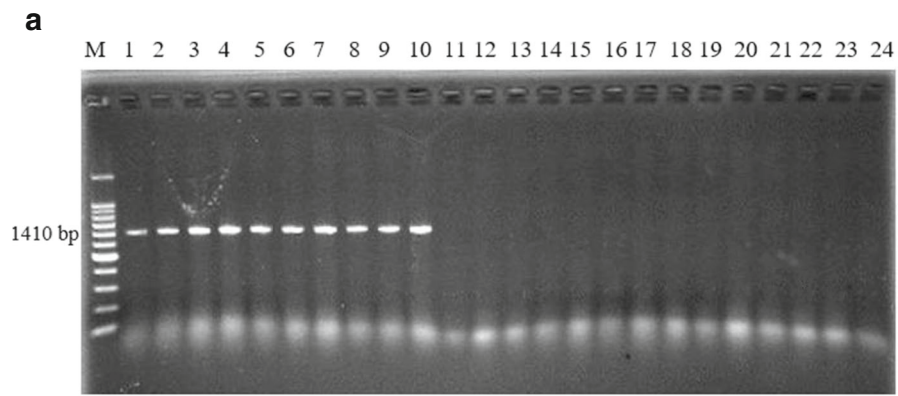


Fig. 2 PCR products obtained with the different primer pairs used in this study. **a** PCR products obtained with non-defoliating specific primers VNDF/VNDR (1410 bp). **b** defoliating specific primers VDF/VDR (548 bp). **c** race 2 specific primers VdR2F/VdR2R (256 bp). **d** primer pairs DB19/espdef01 (334 bp). **e** primer pairs INTND2F/INTND3R (688 bp). **f** primer pairs INTND2F/INTND2R (824 bp). **g** primer pairs INTND2F/MCR2B (964 bp). Lane M: 200 bp ladder. Lanes 1 to 24: VD11, VD18, VD47, VD48, VD50, VD51, VD53, VD55, VD65, VD73, VD1, VD4, VD6, VD9, VD10, VD19, VD22, VD23, VD25, VD31, VD38, VD40, VD62, VD71

disagreement with Hu et al. (2015), there is no significant relationship between race and pathotypes in cotton.

There are six main VCGs in *V. dahliae* (Jimenez-Diaz et al. 2006). In China, isolates from cotton were usually grouped into VCG1 and VCG2. Xia et al. (1998) found that 98 isolates from cotton belonged to VCG2, and 11 isolates belonged to VCG1. Xu et al. (2012) showed that 332 (97%) isolates from Yantze River Valley region (including Province Hubei, Hunan, Jiangxi, Anhui and Jiangsu) belonged to VCG1A and the remaining nine (3%) isolates belonged to VCG2. Consistently with previous results, VCG1A isolates were

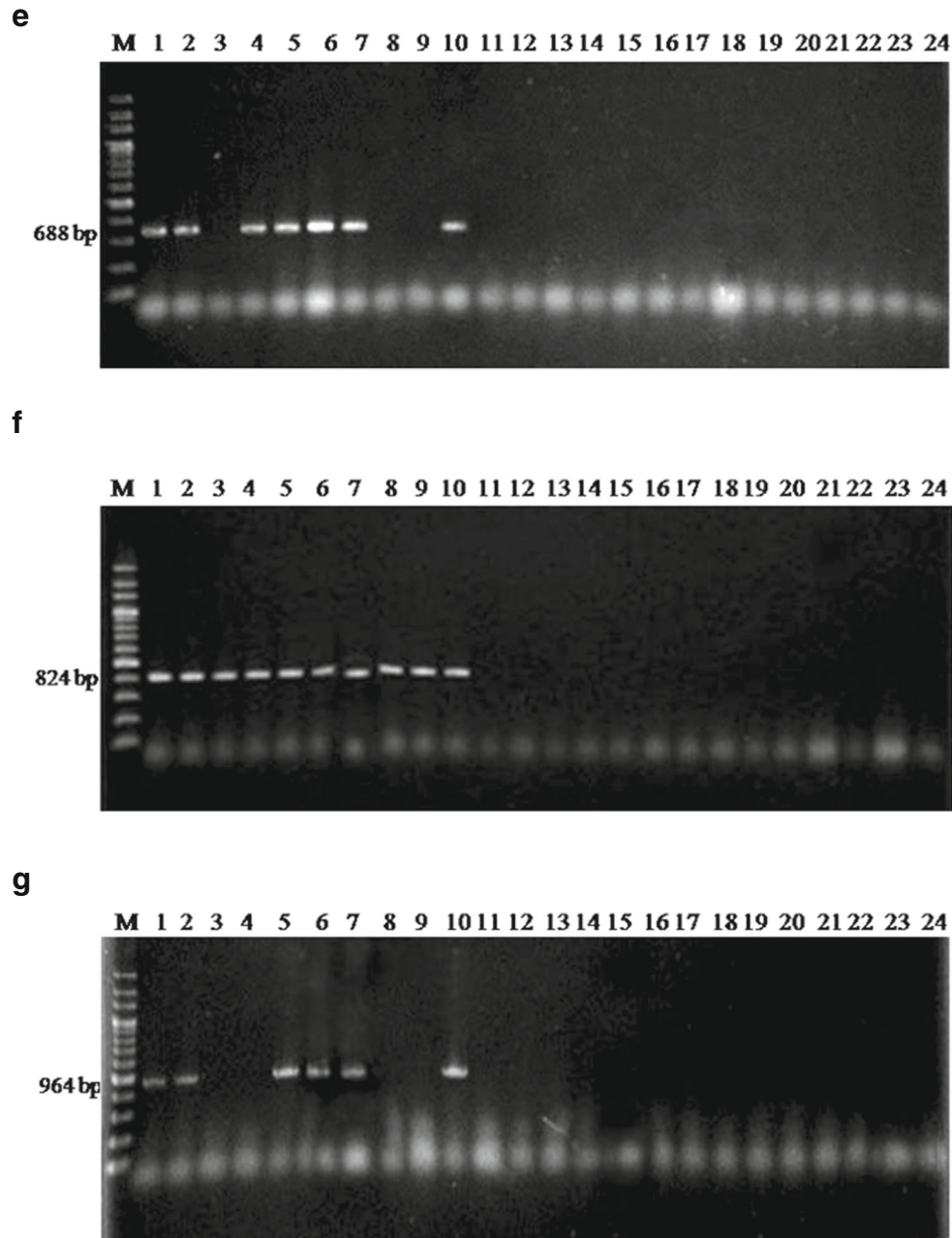


Fig. 2 (continued)

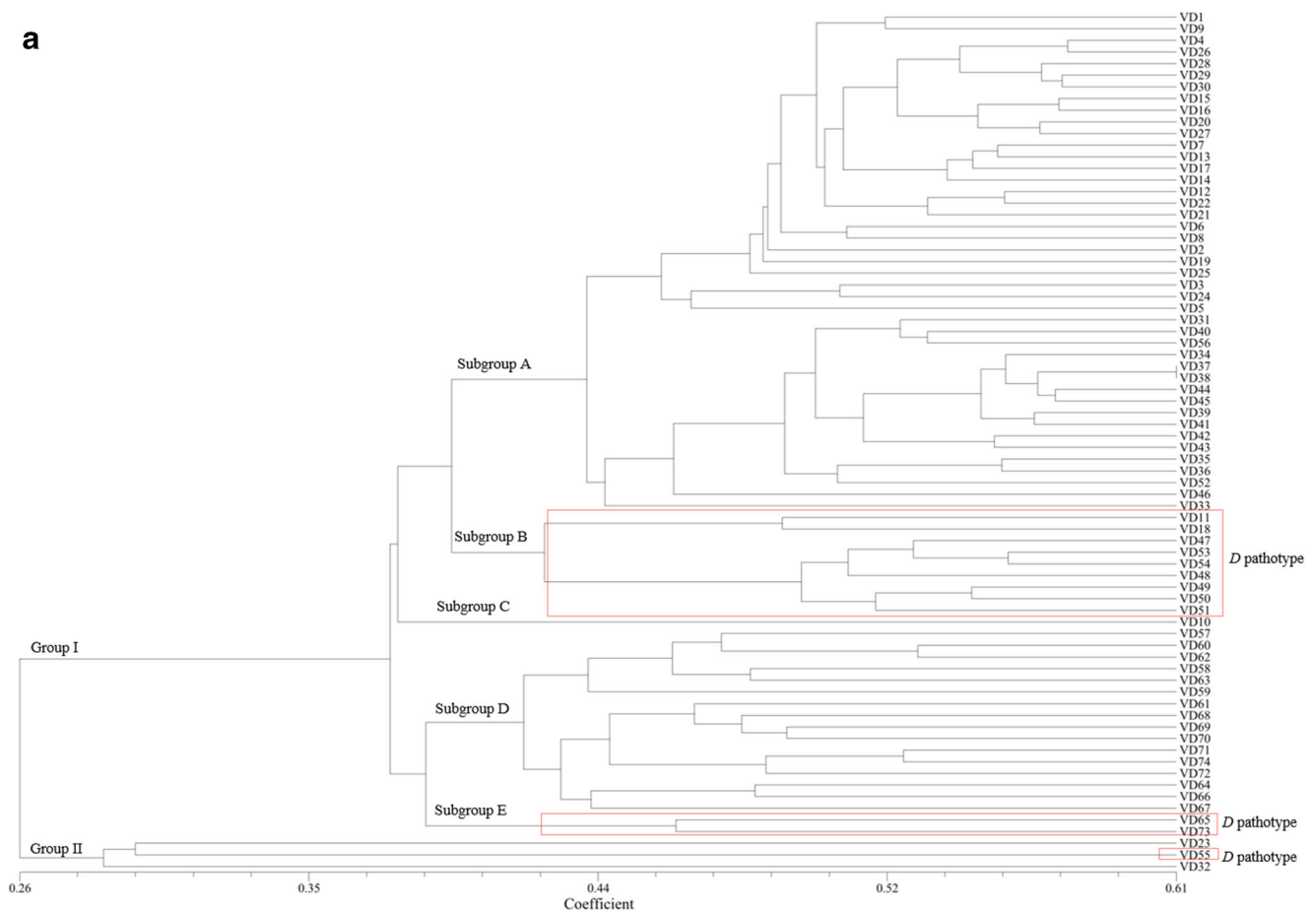


Fig. 3 Dendrograms of the 74 *V. dahliae* isolates generated from AFLP and ISSR. **a** Dendrograms generated from AFLP data of 74 *Verticillium dahliae* isolates from cotton in China. **b** Dendrograms generated from ISSR data. UPGMA was based on DICE similarity distance

dominant in Yellow River Valley region and Yantze River Valley region. However, a diversity of VCGs was molecularly detected in inland of Northwest region getting our attention. In inland of Northwest region, four isolates were identified as VCG1A, four isolates belonged to VCG2B⁸²⁴, three isolates belonged to VCG2A or VCG4B, and five isolates belonged to VCG6. These results suggest that the genetic exchange between different strains of *V. dahliae* (Jimenez-Diaz et al. 2006) might be more probable in inland of Northwest region than in Yellow River region and Yantze River Valleys region.

Molecular determination of VCGs provided useful information, but the overall genetic diversity among *V. dahliae* strains still needed further molecular analyses. In previous studies, several molecular-based techniques have been successfully used to detect genetic diversity among isolates of *V. dahliae* (Erdogan et al. 2013; Fahleson et al. 2003; Gharbi et al. 2014, 2015; ElSharawy et al. 2015; Collado-Romero et al. 2006, 2009). In the present study, the applications of both

AFLP and ISSR markers revealed a high level of polymorphism. The isolates were clustered using UPGMA method to determine their relationships in the dendrogram and a clear correlation between clusters was found. Within each cluster, individuals collected from the same geographical origin tended to be grouped together as shown in both AFLP and ISSR dendrograms. These results agreed well with those obtained in other studies conducted on *V. dahliae* using AFLP and ISSR methods (Erdogan et al. 2013; Gharbi et al. 2015; ElSharawy et al. 2015). Based on the dendrograms of AFLP and ISSR, isolates from Anhui, Hebei, Henan and Hubei were clustered to one subgroup while isolates from Hunan, Jiangsu, Jiangxi, Shanxi and Shandong were clustered to another subgroup. Such consistent results obtained from both AFLP and ISSR analyses showed the high reliability results of genetic diversity of isolates. Based on the dendrograms results, the minimum variations of AFLP and ISSR were 0.26 and 0.13, and the maximum variations were 0.61 and 0.56, respectively. The results showed that large genetic

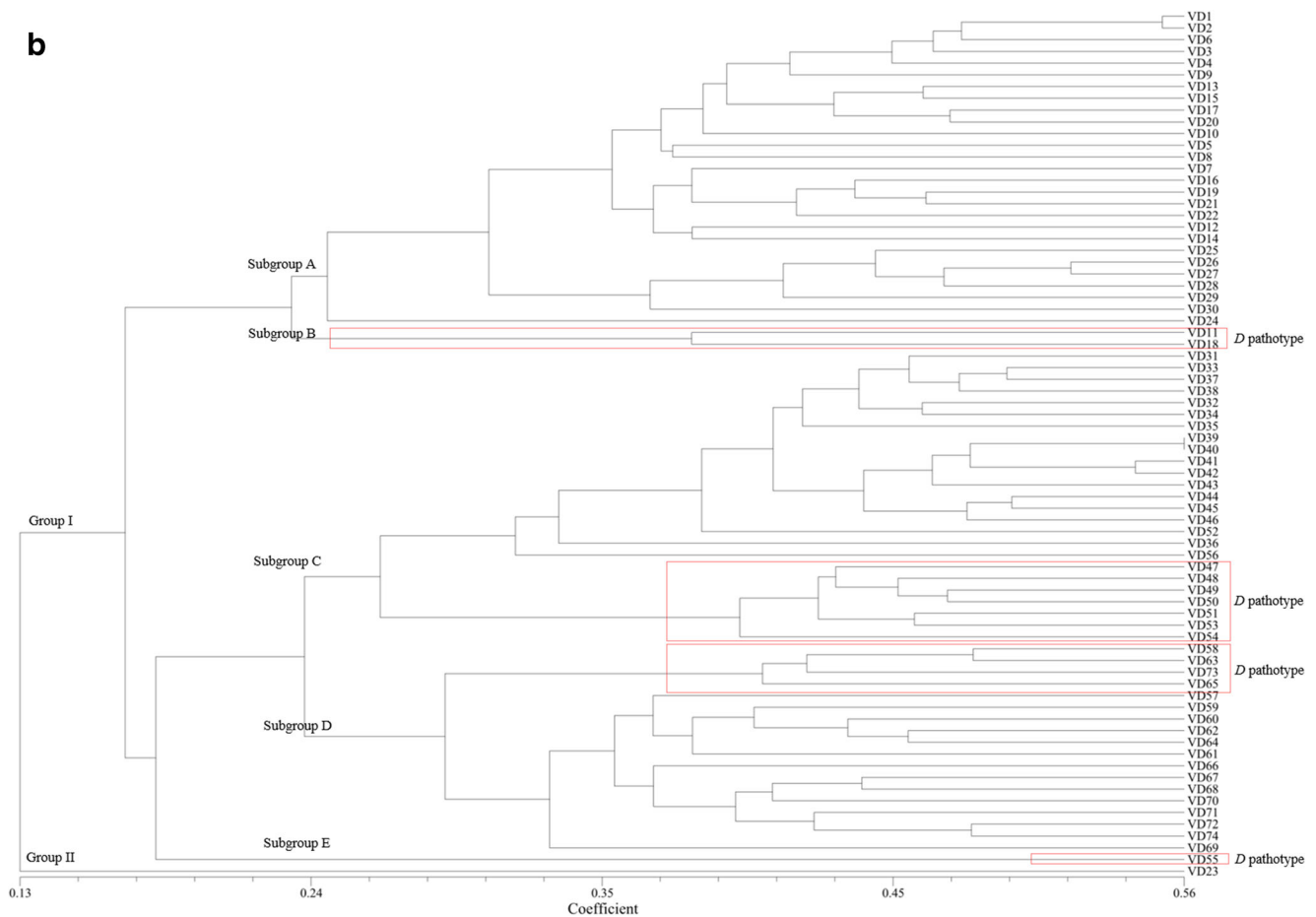


Fig. 3 (continued)

diversity existed among the isolates studied by both AFLP and ISSR assays. Erdogan et al. (2013) analyzed 33 *V. dahliae* isolates from cotton in western Turkey by AFLP, and the genetic distance of these isolates was between 0.24 to 0.79. This rate was also similar to the study of Collado-Romero et al. (2006) on *V. dahliae* diversity. The AFLP analyses also revealed some correlations with pathotypes, which can separate ND isolates from D isolates. Our previous study showed that primer pairs E64/M53 and E49/M65 could yield 433 bp and 110 bp fragments from ND isolates, respectively, which could differentiate ND pathotype from D pathotype (Zou et al. 2003). Collado-Romero et al. (2006) reported that AFLP analysis could cluster the isolates according to their assignment to VCG. Moreover, no association was found between the virulence phenotype, races characterization (data not shown) and AFLP/ISSR clustering of the isolates.

In summary, we tested the pathotypes, races, VCGs and genetic diversity of representative *V. dahliae* isolates from three major cotton-growing areas in China. The results offered a better understanding of the epidemiology of

Verticillium wilt in China and will help formulating the appropriate strategies for cotton wilt management in production practice.

Acknowledgements This study was supported by the Special Fund for Agro-scientific Research in the Public Interest (201503109), National Key R&D Program of China (No. 2017YFD0201900).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

- Angela M, Iglesias-Garcia MI et al (2013) Pathogenicity, virulence, and vegetative compatibility grouping of *Verticillium* isolates from spinach seed. *Plant Dis* 97(11):1457–1469
- Atallah ZK, Maruthachalam K, du Toit L et al (2010) Population analyses of the vascular plant pathogen *Verticillium dahliae* detect recombination and transcontinental gene flow. *Fungal Genet Biol* 47:416–422
- Bejarano-Alcazar J, Blanco-Lopez MA, Melero-Vara JM et al (1996) Etiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. *Plant Dis* 80:1233–1238

- Bell AA (1992) Verticillium wilt. In: Hillocks RJ (ed) Cotton diseases. C.A.B. International, Oxford, pp 87–126
- Collado-Romero M, Mercado-Blanco J, Olivares-García C et al (2006) Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. *Phytopathology* 96:485–495
- Collado-Romero M, Berbegal M, Jimenez-Diaz RM et al (2009) A PCR-based ‘molecular tool box’ for in planta differential detection of *Verticillium dahliae* vegetative compatibility groups infecting artichoke. *Plant Pathol* 58:515–526
- Daayf F, Nicole M, Geiger JP (1995) Differentiation of *Verticillium dahliae* populations on the basis of vegetative compatibility and pathogenicity on cotton. *Eur J Plant Pathol* 101:69–79
- ElSharawy AA, Yang G, Hu X et al (2015) Genetic relationships between virulence, vegetative compatibility and ISSR marker of *Verticillium dahliae* isolated from cotton. *Arch Phytopathol Plant Protect* 48(8): 646–663
- Erdogan O, Nemli S, Oncu T et al (2013) Genetic variation among pathotypes of *Verticillium dahliae* Kleb. from cotton in western Turkey revealed by AFLP. *Can J Plant Pathol* 35:354–352
- Fahleson J, Lagercrantz U, Hu Q et al (2003) Estimation of genetic variation among *Verticillium* isolates using AFLP analysis. *Eur J Plant Pathol* 109:361–371
- Geng JY, Zhang XY, Cui RM et al (2006) The development of high-yielding and insect resistant cotton cultivar Jimian 958. *J Hebei Agric Sci* 10(4):9–13
- Gharbi Y, Triki MA, Jolodar A et al (2014) Genetic diversity of *Verticillium dahliae* from olive trees in Tunisia based on RAMS and IGS-RFLP analyses. *Can J Plant Pathol* 36:491–500
- Gharbi Y, Triki MA, Trabelsi R et al (2015) Genetic structure of *Verticillium dahliae* isolates infecting Olive trees in Tunisia using AFLP, pathogenicity and PCR markers. *Plant Pathol* 64:871–879
- Hu XP, Gurung S, Short SPG et al (2015) Nondefoliating and defoliating strains from cotton correlated with races 1 and 2 of *Verticillium dahliae*. *Plant Dis* 99(12):1713–1720
- Jimenez-Diaz RM, Mercado-Blanco J, Olivares-García C et al (2006) Genetic and virulence diversity in *Verticillium dahliae* populations infecting artichoke in eastern-Central Spain. *Phytopathology* 96:288–298
- Jimenez-Diaz RM, Cirulli M, Bubic G (2012) Verticillium wilt, a major threat to olive production: current status and future prospects for its management. *Plant Dis* 96:304–329
- Jimenez-Diaz RM, Olivares-García C, Trapero-Casas JL et al (2017) Variation of pathotypes and races and their correlations with clonal lineages in *Verticillium dahliae*. *Plant Pathol* 66:651–666
- Jimenez-Gasco MM, Malcolm GM, Berbegal M et al (2014) Complex molecular relationship between vegetative compatibility groups (VCGs) in *Verticillium dahliae*: VCGs do not always align with clonal lineages. *Phytopathology* 104:650–659
- Joaquim TR, Rowe RC (1990) Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. *Phytopathology* 80:1160–1166
- de Jonge R, van Esse HP, Maruthachalam K, et al, 2012. Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *PNAS*, 109 (13): 5110–5115
- Lee SB, Taylor JW. 1990. Isolation of DNA from fungal mycelia and single spores. Pages 282–287 in: PCR protocols: A guide to methods and applications. eds. Innis, M.A., D.H. Gelfand, J.J. Sninsky, and T.J. White. Academic Press, Inc, New York
- Leslie JK (1993) Fungal vegetative compatibility. *Annu Rev Phytopathol* 31:127–150
- Ma C (2007) Verticillium wilt. In: Ma C (ed) Fusarium wilt and Verticillium wilt of cotton. China Agriculture Press, Beijing, pp 61–70
- Martín-Sanz A, Rueda S, García-Cameros Ana B et al (2018) Genetics, host range, and molecular and pathogenic characterization of *Verticillium dahliae* from sunflower reveal two differentiated groups in Europe. *Front Plant Sci* 9:288. <https://doi.org/10.3389/fpls.2018.00288>
- Maruthachalam K, Atallah Z, Vallad G et al (2010) Molecular variation among isolates of *Verticillium dahliae* and polymerase chain reaction-based differentiation of races. *Phytopathology* 100:1222–1230
- Mercado-Blanco J, Rodriguez-Jurado D, Perez-Artes E et al (2001) Detection of the nondefoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. *Plant Pathol* 50:609–619
- Mercado-Blanco J, Rodriguez-Jurado D, Parrilla-Araujo S et al (2003) Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. *Plant Dis* 87:1487–1494
- Perez-Artes E, Garcia-Pedrajas MD, Bejarano-Alcazar et al (2000) Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. *Eur J Plant Pathol* 106:507–517
- Pramateftaki PV, Antoniou PP, Typas MA (2000) The complete DNA sequence of the nuclear ribosomal RNA gene complex of *Verticillium dahliae*: intraspecific heterogeneity within the intergenic spacer region. *Fungal Genet Biol* 29:19–27
- Ren YH, Zhang WW, Si N et al (2018) Research on integrated management strategy for controlling cotton Verticillium wilt. *China Cotton* 45(2):28–30
- Rohlf FJ (1997) NTSYS-pc: numerical taxonomy and multivariate analysis system. Exeter Publishing, LTD, New York
- Schnathorst WC, Mathre DE (1966) Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. *Phytopathology* 56:1155–1161
- Short DP, Gurung S, Maruthachalam K et al (2014) *Verticillium dahliae* race 2-specific PCR reveals a high frequency of race 2 strains in commercial spinach seed lots and delineates race structure. *Phytopathology* 104:779–785
- Vos P, Hogers R, Bleeker M et al (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* 23:4407–4414
- Xia ZJ, Achar PN, Gu BK (1998) Vegetative compatibility groupings of *Verticillium dahliae* from cotton in mainland China. *Eur J Plant Pathol* 104:871–876
- Xu F, Yang L, Zhang J et al (2012) Prevalence of the defoliating pathotype of *Verticillium dahliae* on cotton in Central China and virulence on selected cotton cultivars. *J Phytopathol* 160:369–376
- Zhang L, Duan WJ, Li GY et al (2006) Population monitoring study of *Verticillium dahliae* in cotton in Xinjiang. *J of Northwest A & F University (Natural Science)* 34(11):189–193
- Zhang WW, Zhao L, Wang SZ et al (2012) Stability analysis on *Verticillium* wilt resistance of ten cotton cultivars in disease nursery. *China Cotton* 39(5):20–22
- Zhang WW, Jiang TF, Cui X et al (2013) Colonization in cotton plants by a green fluorescent protein labeled strain of *Verticillium dahliae*. *Eur J Plant Pathol* 135:867–876
- Zhang WW, Zhang HC, Liu K et al (2017) Large-scale identification of *Gossypium hirsutum* genes associated with *Verticillium dahliae* by comparative transcriptomic and reverse genetics analysis. *PLoS One* 12(8):e0181609
- Zhu HQ, Feng ZL, Yin ZX et al (2012) Pathogenicity differentiation and ISSR fingerprint analysis of cotton *Verticillium dahliae* in China. *Acta Phytopathol Sin* 42:225–235
- Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20(2):176–183
- Zou YF, Jian GL, Ma C et al (2003) AFLP analysis of pathotype of *Verticillium dahliae* of cotton. *Acta Phytopathol Sin* 33:135–141