Front. For. China 2009, 4(4): 478–483
DOI 10.1007/s11461-009-0063-x

RESEARCH ARTICLE

Tailin LIAO, Jianren YE, Jiandong CHEN

AFLP analysis of *Fusarium circinatum* and relative species

Abstract AFLP fingerprinting analysis of *Fusarium circinatum*, Nirenberg and O'Donnell and relative species was carried out. Ten primer-pairs that could generate abundant polymorphism fragments were screened. A total of 298 nucleotide acid fragments were amplified with the primers from the template of the 17 strains of *Fusarium* spp., among which 283 fragments were polymorphic. Percentage of polymorphic loci produced by each pair of AFLP primer-pair was 94.97% in average and varied from 89.29% to 100%. All these data indicated that considerable genetic variation existed among *F. circinatum* and relative species at DNA level. Molecular genetic distances among *Fusarium* spp. were calculated, and the relationship among them was described quantitatively. Compared with biological species, the result of cluster analysis was basically similar to the phenotypic classification of species. Genetic diversity of E-AT/M-CAA AFLP fingerprinting of *Fusarium* spp. was analyzed, and specific and difference bands for each species and all *Fusarium* section *Liseola* tested were identified based on the E-AT/M-CAA AFLP fingerprinting.

Keywords *Fusarium circinatum*, AFLP, genetic relationship, identification

1 Introduction

Fusarium circinatum is the newly added pathogenic fungi released in the list of entry quarantine pests by Ministry of Agriculture, and this fungi was first discovered on *Pinus virginiana* in North Carolina of the USA in 1946 (Hepting, 1946). In recent years, *F. circinatum* has shown quick spreading trend all over the world and it has been founded successively in the places like Japan in 1989, Mexico in 1991, South Africa in 1994 and Chile in 2001. Especially, *P. elliottii* in seed orchards of South America, *P. radiata*, *P. muricata* and *P. attenuata* in California and *P. patula* in South Africa are most seriously affected by *F. circinatum*, which causes resin and ulcer on trunks, necrosis of female flowers and cones and seed deterioration and death of seedlings (Viljoen et al., 1994; Gordon et al., 2001; Liao, 2004).

F. circinatum was initially considered as *F. lateritium* because culture process of this fungi did not produce chlamydospore and chain-like small-sized conidia (Snyder et al., 1949). With the introduction of phialide as an important classification feature of *Fusarium* by Booth, *F. circinatum* is considered as *F. moniliforme* var. *subglutinans* due to its ability of producing single phialide and complex phialide (Kuhlman et al., 1978). The research is done about pathogenicity of isolates between pines and other hosts concerning *F. subglutinans* and the results show that the isolate on pines has obvious host specificity. In addition, RFLP study of mtDNA also shows that *F. circinatum* is a kind of specialized form of *F. subglutinans* on pines, namely *F. subglutinans* f. sp. *pini* (Correll et al., 1991). Nirenberg and O'Donnell (1998) combined the studies of morphology of *Fusarium* and molecular biology and reported that *F. circinatum* is a dependent species and is named *F. circinatum*, which is one species of *Fusarium* section. The perfect stage of *F. circinatum* is H mating population of *Gibberella fujikuroi* (sawada) Ito & Kimura (Steenkamp et al., 1999). Apart from the above mating population, *G. fujikuroi* includes at least seven mating populations with total difference in genetics, which are named A, B, C, D, E, F and G, respectively (Britz et al., 1999). In this paper, AFLP fingerprinting technology is used to analyze the polymorphism concerning *F. circinatum* and its relative species. The purpose is to find out the genetic relationship between H mating population and other mating populations and to determine the unique and different bands of each species, which provide basic

Translated from *Scientia Silvae Sinicae*, 2008, 44(9): 82–86 [译自: 林业科学]

Tailin LIAO, Jianren YE (✉)
College of Forest Resources and Environment, Nanjing Forestry University, Nanjing 210037, China
E-mail: jrye@njfu.edu.cn

Tailin LIAO
Kunshan Exit-Entry Inspection and Quarantine Bureau of Jiangsu Province, Kunshan 215301, China

Jiandong CHEN
Exit-Entry Inspection and Quarantine Bureau of Jiangsu Province, Nanjing 210009, China

theoretical foundation for the classification of *F. circinatum*.

2 Materials and methods

2.1 Strains tested

F. circinatum was provided by NUACR and all *Fusarium* section *Liseola* tested and other control strain were provided by NJAU (Table 1).

2.2 Test methods

2.2.1 Strain culture and mycelium collection

The method was the same as the one of Liao et al. (2007).

2.2.2 Genome DNA of mycelium extraction

The method took the one of Ausubel et al. (1998) as reference.

2.2.3 Testing of concentration and purity of template DNA

DNA solution of 10 μL was taken and diluted to 2 mL with the solution of 1× TE (pH 8.0). Absorbance values (OD) of wavelength 260 nm and 280 nm were measured with the ultraviolet spectrophotometer. Based on the formula: concentration of sample DNA (ng/μL) = 50 × OD_{260} × dilution multiple, DNA concentration was calculated. Then, DNA purity was reported according to the ratio of OD_{260}/OD_{280}.

2.2.4 AFLP analysis

The process of AFLP analysis was as follows: digestion of genome DNA, joint connection, preamplification, and selective amplification according to the operating instruction on the kit II of AFLP. The kit was produced by Invitrogen.

2.2.5 Electrophoresis

After degeneration of the product of selective amplification, the product was separated through electrophoresis on degenerated polyacrylamide sequence analysis glue, with the condition of 55°C, 1200 V, 100 W and about 4.5 h. After electrophoresis, silver dyeing detection method by Tixier et al. (1997) was used to do the AFLP fingerprinting color reaction.

2.2.6 Result records

The results were recorded as follows: "1"—with bands, "0"—without bands and "2"—data missing or fuzzy bands.

2.3 Data statistical analysis

The method by Zhou (2005) was taken as reference to do data statistical analysis.

Polymorphism: polymorphism (%) = number of amplified polymorphism fragments / number of total amplified fragments × 100% = $(N_i + N_j - 2N_{ij})$ / $(N_i + N_j - N_{ij})$, where N_{ij} represents the public bands of sample i and j and N_i and N_j are the bands number of sample i and j.

Table 1 Strains tested and their origins

mating type	organism	no. of strains	host	resource
H	*F. circinatum*	NFUH-1	*P. caribaea*	NCAUR (North Carolina, USA)
H	*F. circinatum*	NFUH-2	*P. lucheensis*	NCAUR (Japan)
H	*F. circinatum*	NFUH-3	*P. radiata*	NCAUR (Georgia, USA)
H	*F. circinatum*	NFUH-4	*P. patula*	NCAUR (South Africa)
A	*F. moniliforme*	NAUA-1		NJAU
A	*F. moniliforme*	NAUA-2		NJAU
B	*F. subglutinans*	NAUB-1		NJAU
C	*F. moniliforme*	NAUC-1		NJAU
C	*F. moniliforme*	NAUC-2		NJAU
D	*F. proliferatum*	NAUD-1		NJAU
D	*F. proliferatum*	NAUD-2		NJAU
E	*F. subglutinans*	NAUE-1		NJAU
E	*F. subglutinans*	NAUE-2		NJAU
E	*F. subglutinans*	NAUE-3		NJAU
F	*F. moniliforme*	NAUF-1		NJAU
–	*F. graminearum*	NAUG		NJAU
–	*F. solani*	NAUS		NJAU

Similarity coefficient: simple matching coefficient (S_{sm}) was used to calculate the similarity coefficient, and its formula is as follows: $S_{sm} = (N_{ij} + N_0) / (N_i + N_j + N_{ij} + N_0)$, where N_i, N_j and N_{ij} are the same as the former formula and N_0 represents the bands that neither sample i nor j has.

Clustering analysis: NTSYSpc analysis software is used to cluster by non-weighed pairing arithmetic mean method (UPGMA).

3 Results and analysis

3.1 Screening of polymorphism primer-pairs

Ten primer-pair combinations of *Fusarium* with high polymorphism (Table 2) were screened in the given 64 pairs of the kit. The amplified bands of these screened pairs distributed evenly on the gel with relative brightness among each band.

3.2 Polymorphism analysis of AFLP

Ten primer-pair combinations were used for analysis of testing samples and 298 marks were tested with 283 polymorphisms and the percentage of polymorphic loci was 94.97%; each primer-pair combination produces different numbers of polymorphism marks, with least 24 bands and most 33 bands, and the average amplified bands were 28.3 with polymorphic loci between 89.29% and 100% (Table 3). The marks produced by amplification of each *Fusarium* on combinations of E-TG/M-CTA and E-TG/M-CTC can be seen in Fig. 1.

3.3 *Fusarium* types and intraspecific genetic variation

According to the amplification results of 10 primer-pairs, similarity coefficient among testing strains was calculated. Then similarity coefficient amplitude and their means among each *Fusarium* strain and *F. circinatum* were estimated. The similarity coefficient among strains was 0.4771–0.9847 with average of 0.6242; the ones among *Fusarium* strains was 0.5208–0.9847 with average of 0.6467. The similarity coefficient among intraspecific populations was larger, for example, the one between two populations of mating population A was 0.9847 and 0.9804 between two of mating population C, 0.9479 between two of D, 0.8996–0.9615 among three of E and 0.7972–0.9024 among four of H.

3.4 Clustering analysis

Genetic similarities among each strain were used to do clustering analysis with UPGMA (Fig. 2). The results showed that similarity 0.58 can differentiate *Fusarium* and *F. graminearum*, *F. solani* of 17 testing strains, and each

Table 2 Screened primer-pairs suitable for AFLP analysis in *Fusarium*

primer	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AA	+	+				+		
E-AC								+
E-AG							+	
E-AT	+							
E-TA	+			+				
E-TC								
E-TG					+	+		
E-TT								

Table 3 Effect of different primer combinations on AFLP analysis

primer combination	number of bands	number of polymorphic bands	percent of polymorphic loci/%
E-AA/M-CAA	32	31	96.88
E-AA/M-CAC	29	26	89.66
E-AA/M-CTC	29	28	96.55
E-AC/M-CTT	28	28	100
E-AG/M-CTG	34	33	97.06
E-AT/M-CAA	31	29	93.55
E-TA/M-CAA	26	24	92.31
E-TA/M-CAT	28	25	89.29
E-TG/M-CTA	30	29	96.67
E-TG/M-CTC	31	30	96.77

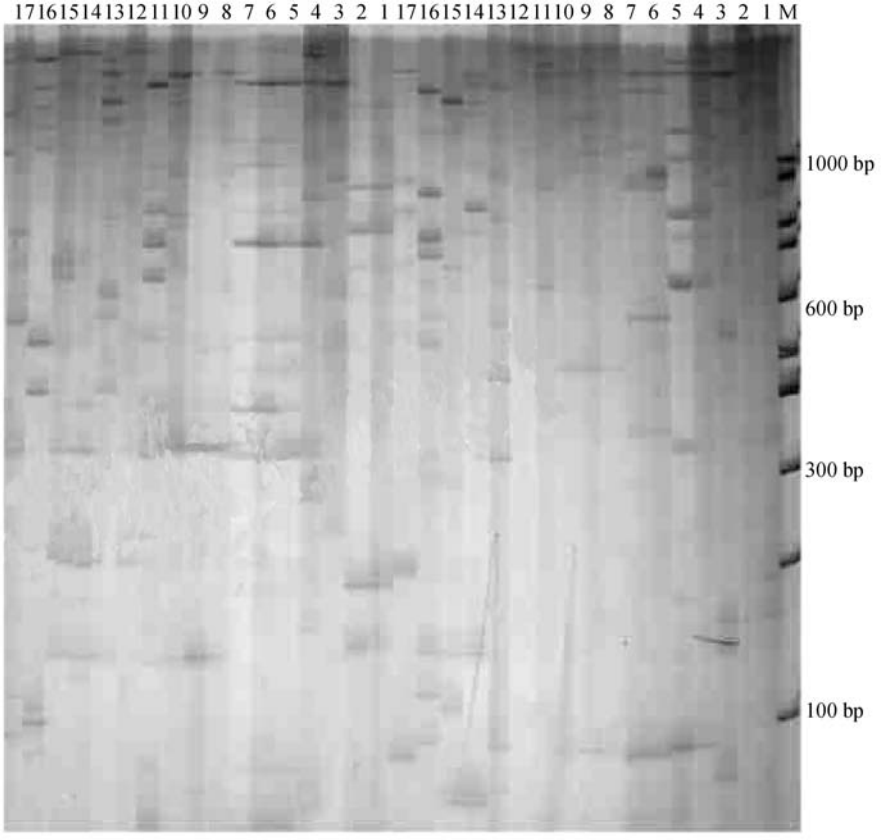

Fig. 1 Marker loci generated by AFLP primers combination of E-TGPM-CTC (left) and E-TGPM-CTA (right). 1: NAUA-1; 2: NAUA-2; 3: NAUB-1; 4: NAUC-1; 5: NAUC-2; 6: NAUD-1; 7: NAUD-2; 8: NAUE-1; 9: NAUE-2; 10: NAUE-3; 11: NAUF-1; 12: NFUH-1; 13: NFUH-2; 14: NFUH-3; 15: NFUH-4, 1; 16: NAUG; 17: NAUS; M: DL 1500 marker.

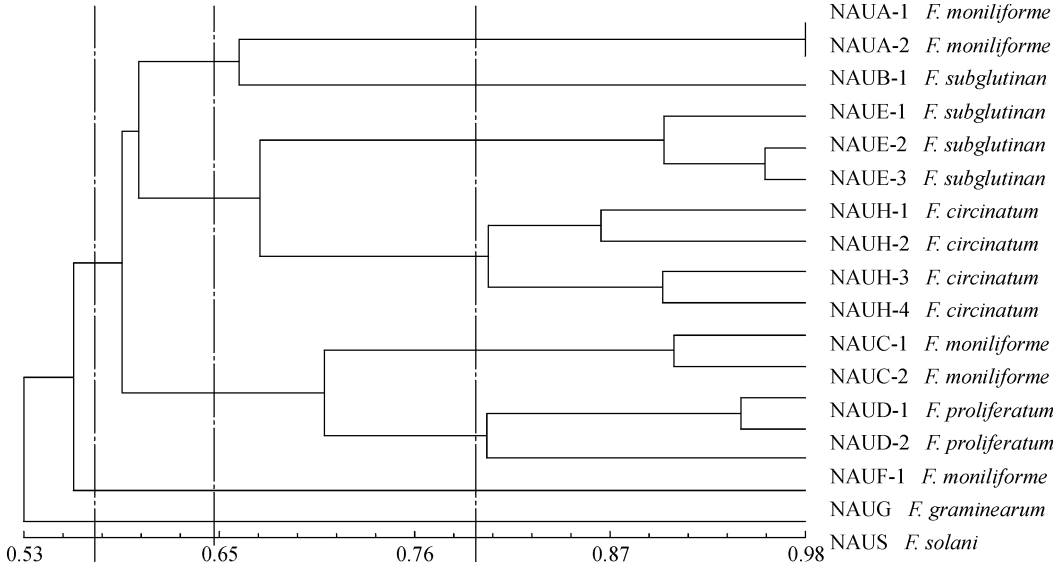

Fig. 2 Phylogenetic tree of *Fusarium* strains

mating type of *Fusarium* has high genetic similarity. The similarity 0.65 can put *Fusarium* into three genetic similarity groups, with mating populations A and B into one group, C, D and F into one and E and H into one as well. The similarity 0.79 can further differentiate totally different mating types of *Fusarium*. The above clustering analysis result of strains accorded with the classification of biological species.

3.5 Identification of difference bands

In the AFLP fingerprinting map of *Fusarium* constructed by primer E-AT/M-CAA, the testing mating populations A (340 bp), E (700 bp) and H (380 bp) all had specific bands. Others testing *Fusarium* could use dichotomy classification to determine each difference band in the AFLP map. According to each specific band and difference bands, seven mating populations of the testing *Fusarium* could be differentiated. Once the above bands were determined to have the specific of strains, characteristic fingerprinting can be used to do the quick identification of *Fusarium*, which will greatly improve the identification efficiency.

4 Conclusions and discussion

Ten primer-pair combinations were used to do the analysis of the mating populations A, B, C, D, E, F and H of *Fusarium*, 17 strains of nine species of *F. graminearum* as well as *F. solani*. Polymorphic loci were tested of 94.47%, indicating that testing strains had wide variation in the molecular level. The results of clustering analysis showed that in *Fusarium* the mating populations C, D and F have closer genetic relationship and E and H have closer relationship as well. The result accorded with the clustering results concerning base sequence of beta-tubulin gene and mitochondrial DNA of O'Donnell et al. (1998) as well as base sequence coded by histone H3 (Steenkamp et al., 1999). With no home report of mating population G and difficulty of introduction from abroad, the testing materials in this study lacked mating population G, leading to non-completed description of genetic relationship among each population of *Fusarium*.

F. circinatum was early divided into mating population B; however, from AFLP clustering analysis results of this study, genetic similarity coefficient was small between B and H (<0.60) (Swofford, 1998). *F. circinatum* is an independent mating population in *Fusarium* and the study makes a further confirmation in the study viewpoints of O'Donnell (1998) and Britz (1999). Four types of *F. circinatum* with different geographic origins had great genetic variance, demonstrating that there forms relatively independent groups in Georgia State, North Carolina in the USA, South Africa and Japan.

As the identification technology for strains, molecular marker technique should be applied to production practice, meeting the demands of good repetition, high resolution, intensified polymorphism and high efficiency. The results of this study showed that AFLP technology was stable and reliable. The analysis was done concerning primer E-AT/M-CAA on *F. circinatum* and its relative species, testing 31 markers with 29 polymorphism markers, and the testing loci had 93.55% difference between species. In the constructed *Fusarium* fingerprinting map, the mating populations A, E and H all had specific bands, and other testing *Fusarium* could use dichotomy classification to determine the difference bands in the map. Based on the specific bands and difference bands, relative strains could be identified.

Acknowledgements This study was financially supported by the State Quality Detection Bureau (No. 2004IK075).

References

Ausubel F, Brent R, Kingstone R E (1998). Current protocols in molecular biology. Yan Z Y, Wang H L, trans. Beijing: Science Press

Britz H T A, Coutinho M J, Wingfield W F O, Marasas T R, Leslie J F (1999). *Fusarium subglutinans* f . sp. *pini* represents a distinct mating population in the *Gibberella fujikuroi* species complex. Appl Environ Microbiol, 65: 1198–1201

Correll J C, Gordon T R, McCain A H, Fox C S, Koehler D L, Schultz M E (1991). Pitch canker disease in California: pathogenicity, distribution and canker development on Monterey pine (*Pinus radiata*). Plant Dis, 75: 676–682

Gordon T R, Storer A J, Wood D L (2001). The pitch canker epidemic in California. Plant Dis, 85(11): 1128–1139

Hepting G T, Roth E R (1946). Pitch canker, a new disease of some southern pine. J For, 44: 742–744

Kuhlman E G, Dwinell L D, Nelson P E, Ciegler A, Delucca A J, Dunn J J (1978). Characterization of the *Fusarium* causing pitch canker of southern pines. Mycologia, 70: 1131–1143

Liao T L, Li B S, Ye J R, Ji R, Wu C P, An Y L, Chen J D (2007). Molecular detection of *Fusarium circinatum*, the causal agent of pine pitch canker. Sci Silv Sin, 43(1): 111–115

Liao T L, Li B S, Ye J R, Zheng G H, Shen Y C, Zhou R (2004). Risk assessment of the *Fusarium circinatum* caused by importation of wooden materials. J Nanjing For Univ, 28(6): 10–14

Nirenberg H I, O'Donnell K (1998). New *Fusarium* species and combinations with the *Gibberella fujikuroi* species complex. Mycologia, 90(3): 434–458

O'Donnell K E, Cigelnik E, Nirenberg H I (1998). Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia, 90: 465–493

Snyder W C, Toole E R, Hepting G H (1949). Fusaria associated with mimosa wilt, sumac wilt, and pine pitch canker of southern pine. J Agric Res, 78: 365–378

Steenkamp E T, Wingfield B D, Coutinho T A, Wingfield M J, Marasas W F U (1999). Differentiation of *Fusarium subglutinans* f. sp. *pini* by histone gene sequence data. Appl Environ Microbiol, 65(8): 3401–3406

Swofford D L (1998). PAUP phylogenetic analysis using parsimony (version 4.0). Sunderland: Sinauer Associates

Tixier M H, Sourdille R M, Leroy P (1997). Detection of wheat microsatellites using a non-radioactive silver-nitrate staining method. J Genet Breed, 51: 175–177

Viljoen A, Wingfield M J, Marasas W H O (1994). First report of *Fusarium subglutinans* f. sp. *pini* on pine seedlings in South Africa. Plant Dis, 1994, 78: 309–312

Zhou Y Q (2005). The application of DNA molecular marker technique in plant study. Beijing: Chemical Industry Press