Front. For. China 2007, 2(1): 78–81
DOI 10.1007/s11461-007-0012-5

RESEARCH ARTICLE

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RAPD analysis of genetic relationships among *Sphaeropsis sapinea* isolates

Abstract Genetic relationships were studied among 23 isolates of *Sphaeropsis sapinea* collected from China, the United States, England, South Africa and Chile by using a random amplification of a polymorphic DNA (RAPD) analytical method. One hundred and 35 DNA fragments were amplified with 12 random primers by a polymerase chain reaction PCR technique and 96.3% were polymorphic. The genetic dendrogram based on RAPD analysis showed that the *S. sapinea* isolates could be divided into three types. Isolate CWS41 from Chile was separated genetically as the first type that was different from other isolates and isolates F2 and J2 from China comprised the second group. The third RAPD group accommodated other isolates including the B morphotype isolate CWS43 from the United States.

Keywords *Sphaeropsis sapinea*, random amplification of a polymorphic DNA, genetic relationship

1 Introduction

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton affects most coniferous species throughout the world and causes symptoms including shoot blight, stem canker and resin flow. The heavy occurrence of the disease has been associated with considerable damage to pine plantations (Wu, 1999). Because of the extensive host range and vast distribution area of this pathogenic fungus, genetic variation among native *S. sapinea* isolates has been investigated at the phenotypic or molecular level by researchers from the United States, China, South Africa and Canada (Palmer et al., 1987; Swart et al., 1991; Smith and Stanosz, 1995; Stanosz et al., 1996; Hausner et al., 1999; Wu, 2000a, 2000b, 2000c). However, little has been done on comparative studies of *S. sapinea* isolates collected from different countries and there are no reports about genetic relationships between Chinese and exotic isolates. By applying random amplifications of a polymorphic DNA (RAPD) technique, we compared *S. sapinea* isolates from Europe, America and Africa with Chinese isolates, with the objective to elucidate the genetic polymorphism and phylogenetic relationship among *S. sapinea* isolates of different geographical locations.

Translated from *Journal of Nanjing Forestry University*, 2006, 30(1): 13–16 [译自: 南京林业大学学报]

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2 Materials and methods

2.1 Fungal isolates

Chinese isolates of *S. sapinea* were selected from 55 isolates used in previous studies based on geographic location, morphology and pathogenicity (Wu, 2000b, 2000c). Six exotic isolates parasitizing five pine species were from the United States, England, South Africa and Chile. The geographic origins and hosts of the isolates are listed in Table 1.

The fungal isolates were cultured on PSA plates. After 96 h incubation at 25°C, the hyphae were scraped and stored at –30°C in a freezer.

2.2 Random amplification of a polymorphic DNA (RAPD) amplification

Genomic DNA was extracted by a CTAB-based procedure.

PCR reactions were carried out in a 20 μL volume containing a 2 μL 10 × amplification reaction buffer, 5 ng genomic DNA, 2 μL 10 pmol/L primer, 1 μL 25 μmol/L of each dNTP (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.), 1.5 U of Taq DNA polymerase (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.) and 5 μL diluent of Taq polymerase. Aseptic distilled water was added to a total volume of 20 μL.

DNA amplifications were performed in a Perkin Elmer 9600 DNA thermal circler programmed as follows: 3 min at 94°C; 38 cycles of 30 s at 94°C, 30 s at 40°C, 2 min at 72°C;

Table 1 *Sphaeropsis sapinea* isolates analyzed with RAPD

No.	Isolate	Host	Geographical location
13	J2	*Pinus pinaster* Ait	Lianyungang, Jiangsu Province
2	D2	*P. sylvestris* var. *sylvestriformis* Ch. et al.	Harbin, Heilongjiang Province
10	HN1	*P. taeda* L.	Queshan, Henan Province
16	SX2	*P. elliottii* Engelm.	Ankang, Shanxi Province
21	A1	*P. taeda* L.	Chuxian, Anhui Province
12	J21	*Cedrus deodara* (Roxb.) Loud	Nanjing, Jiangsu Province
15	H1	*P. elliottii* Engelm.	Jingshan, Hubei Province
23	ZJ2	*P. griffithii* McClelland	Hangzhou, Zhejiang Province
22	S6	*P. elliottii* Engelm.	Mingshan, Sichuan Province
17	Hu6	*P. elliottii* Engelm.	Yueyang, Hunan Province
3	JX1	*P. elliottii* Engelm.	Xinyu, Jiangxi Province
7	F9	*P. massoniana* Lamb.	Minhou, Fujian Province
20	F7	*P. taeda* L.	Qingliu, Fujian Province
19	F2	*P. elliottii* Engelm	Changle, Fujian Province
11	YN4	*P. caribaea* Morelet.	Kunming, Yunnan Province
14	GD1	*P. massoniana* Lamb.	Guangzhou, Guangdong Province
18	LZ1	*P. caribaea* Morelet	Leizhou, Guangdong Province
8	*CWS58	*P. sylvestris* L	England
9	*CWS60	*P. resinosa* Ait	United States
6	*CWS61	*P. nigra* Arn	United States
5	**CWS43	*P. banksiana* Lamb.	United States
1	*CWS41	*P. radiata* D. Don	Chile
4	*CWS1	*P. radiata* D. Don	South Africa

Isolates with * were kindly provided by Mr. Swart. ** a type B isolate.

followed by 7 min at 72°C; and then stored at 4°C. Primers were selected according to Wu (2000c). Seventeen primers selected from 96 primers in groups A, B, C, D, E, K and X (Operon, United States) were screened once again for appropriate primers for amplification.

A mixture of amplification products (12 μL) and a 2 μL TBE buffer were analyzed by electrophoresis on 1.2% agarose gels (5 V/cm, 3 h), detected by staining with ethidium bromide (EB, 1 μg/mL) and photographed on a UVP digital gel-documentation system.

2.3 Data analysis

Each isolate was scored for the presence or absence of each amplification product. Each RAPD amplification fragment was regarded as one genetic locus designated as 1. The absence of an RAPD fragment was designated as 0. The data were analyzed by POPEGENE (Francis C. Yeh, POPGENE Version 1.21) and TFPGA software package. Similarity coefficients and genetic distances were calculated. A dendrogram was constructed after cluster analysis of the similarity coefficients by an unweighted pair-group method using arithmetic averages (UPGMA).

3 Results and analysis

3.1 Selection of primers and analysis of RAPD profiles

Genomic DNA of four isolates (CWS7, CWS41, D2 and J2) was used as templates for re-selection in 17 primers. The results showed that 12 out of the 17 primers efficiently produced clear DNA fragments. The 12 primers were used for PCR amplification of 23 native and exotic *S. sapinea* isolates. The primers and the number of amplified DNA fragments are listed in Table 2. The 12 primers produced 135 fragments—11.25 RAPD markers per primer. OPA-20 produced the most fragments, 15, while OPD-20 the least, 5 fragments. The size of the amplified fragments ranged from 400 bp to 3.2 kb. One hundred fragments were polymorphic, accounting for 96.3% of the total. It revealed definite genetic variation among native and exotic *S. sapinea* isolates (Fig. 1).

Table 2 Ribonucleotide sequence of primers used for RAPD analysis of *Sphaeropsis sapinea* isolates and the number of amplified DNA bands

Primers	5′–3′ ribonucleotide sequence	Amplified bands	Polymorphic bands
OPA-02	TGCCGAGCTG	11	10
OPA-07	GAAACGGGTG	10	10
OPA-20	GTTGCGATCC	15	14
OPB-07	GGTGACGCAG	13	13
OPB-08	GTCCACACGG	11	10
OPB-11	GTAGACCCGT	14	14
OPC-04	CCGCATCTAC	11	10
OPC-05	GATGACCGCC	12	12
OPC-08	TGGACCGGTG	11	11
OPC-11	AAAGCTGCGG	10	9
OPC-14	TGCGTGCTTG	12	12
OPD-20	ACCCGGTCAC	5	5
Total		135	130

3.2 RAPD analysis of the genetic relationship among native and exotic isolates

A simple matching coefficient (S_{xy}) for each pair of isolates was calculated based on the number of RAPD fragments

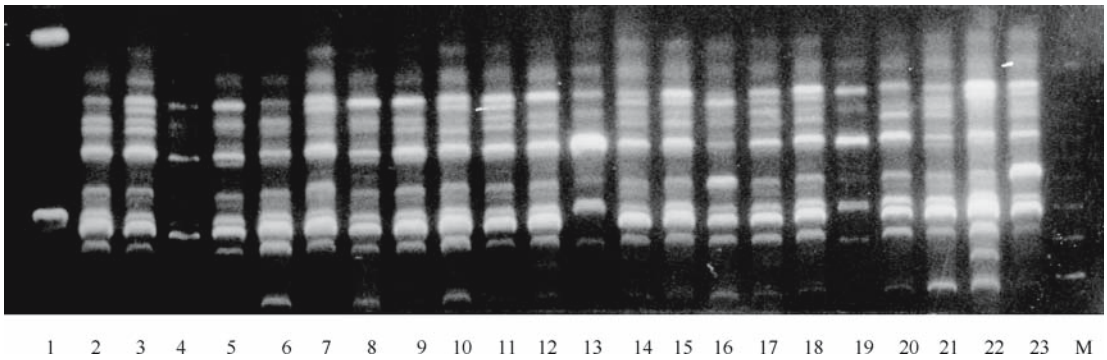

Fig. 1 RAPD gel profiles for 23 *S. sapinea* isolates (Primer OPA-20) (See Table 1 for No. of isolates)

found in common among isolates and unique to each isolate. The matching similarity coefficients indicate that H1 from *P. elliottii* in Hubei is more similar (0.95 similarity) to Hu6 from Hunan province than it was to other isolates, whereas CWS41 from *P. radiata* in Chile was genetically (0.39 similarity) different from YN4 from *P. caribaea* in Yunan province, China. The matching similarity coefficients were low between CWS41 and other isolates ($0.39 \leqslant S_{xy} \leqslant 0.57$) and between one group containing J2 (from *P. pinaster* in Jiangsu province) and F2 (from *P. elliottii* in Fujian province) and the other group containing other isolates ($0.50 \leqslant S_{xy} \leqslant 0.68$). As well, the similarity coefficients were high ($0.70 \leqslant S_{xy} \leqslant 0.95$) among other exotic isolates, among native isolates and among native and exotic isolates.

Cluster analysis divided the 23 native and exotic isolates into three types: (1) the Chilean isolate CWS41was genetically farthest from other isolates; (2) the two Chinese isolates J2 and F2 are relatively different from other isolates; (3) the rest of the 20 isolates (from the United States, South Africa and most of the Chinese isolates) were more similar to each other than to the other two types. The 20 isolates could be separated into two groups: the Chinese isolate SX2 and the other 19 isolates. The latter group was further separated into four sub-groups. The results are shown in Fig. 2.

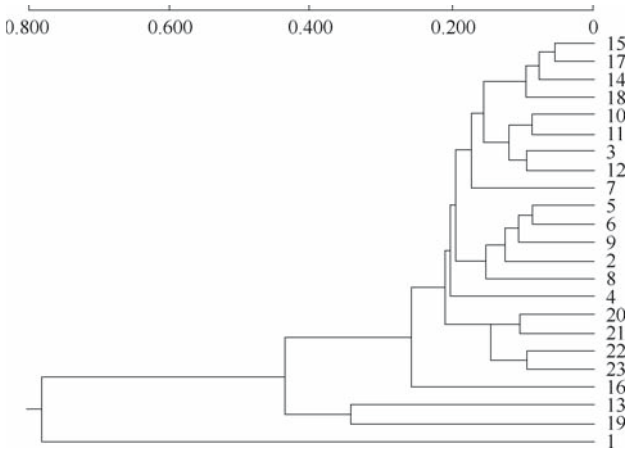

Fig. 2 Dendrogram generated from RAPD of *S. sapinea*

3.3 Correlation between RAPD cluster analysis and other physiological or phenotypic features

The dendrogram generated by UPGMA (Fig. 2) separated CWS41 from other isolates; this isolate secreted specific yellow pigment when cultured on PSA plates, apparently different from the dark green pigment secreted by other isolates. The Chinese isolates, J2 and F2, with their filamentous mycelium closely appressed to the agar surface of PSA plates, were clearly different from the fluffy aerial mycelium of most of the other isolates (Wu, 2000b). Therefore, J2 and F2 were distinguished from other isolates as a type. F7 and A1, belonging to the same vegetative compatibility group (Wu, 2000a), fell into the same type in the dendrogram. For some isolates, results from the cluster analysis seemed to be related to geographical locations and environmental conditions. For instance, CWS41, from South America, was geographically far away from most of the other isolates; J2 and F2 were both from the southeastern coastal areas in China. In the third type, American isolates were more similar to one another than to other isolates; isolate CWS1 (from South Africa) alone was placed into a subgroup. D2 and CWS58 were genetically close to each other; and native isolates from south-central and southern regions, H1, Hu6, GD1, and LZ1, clustered together. Overall, there was no apparent correlation between genetic relationship and host species.

4 Discussion

Genetic differentiation among *S. sapinea* isolates has been verified in many studies. In the United States, Palmer et al. (1987) for the first time discovered and verified that *S. sapinea* isolates fell into type A and type B. Thereafter Stannosz et al. (1996, 1999) compared *S. sapinea* isolates collected from Europe, North America, Oceania and Africa by RAPD and isozyme analysis. They also found type B isolates from outside Michigan, Minnesota and Wisconsin, and from hosts other than *P. banksiana* and *P. resinosa* (Stanosz et al., 1999). In South Africa, de Wet et al. (2000) compared *S. sapinea* isolates from South Africa, Indonesia and Mexico and suggested that a third type (isolates from

Indonesia and Mexico) might exist. Subsequently they analyzed four types of *S. sapinea* isolates (type A, B, C and I, classified on the basis of morphological characteristics and pathogenicity) by molecular techniques. The results showed that *S. sapinea* only consisted of type A and type C. Type B represented a new independent taxonomic unit and type I was *Botryosphaeria obtuse* (de Wet et al., 2001). In Canada, Hausner et al. (1999) divided native *S. sapinea* isolates into three types (type A, B, and I). We separated the Chinese isolates into three types (Wu, 2000b, 2000c). In the current study, RAPD cluster analysis also divided Chinese and exotic isolates into three types: the Chilean isolate CWS41 was a unique type not contained in previous studies; J2 and F2; other exotic isolates from Europe, America, and Africa clustered with Chinese isolates (except J2 and F2).

Investigators designated several types in previous studies on genetic relationships among *S. sapinea* isolates, but there were no uniform criteria to classify the isolates, due to differences in the origin of the fungal isolates and in experimental techniques. Nevertheless, the genetic variation among *S. sapinea* populations and diversity of type were obvious. The result also implied that although *S. sapinea* differentiated among species, isolates designated as type A predominated in populations from different continents.

Although CWS43 was the only type B isolate from the United States in our experiment, it was not placed into a separate type, but clustered with CWS61 (from *P. nigra* in United States), other exotic isolates and D2 (from *P. sylvestris* var. sylvestris formis in Helongjiang, China). Therefore, whether CWS43 is a typical type B isolate or not needs further investigation. Furthermore, although J2 and F2 were similar to CWS43 in some characteristics (such as the filamentous mycelium closely appressed to the agar surface) (Wang and Wu, 2005), the growth rate of the two Chinese isolates apparently differed from CWS43 (relatively slow) when cultured on various culture media and under different pH. Cluster analysis did not place F2, J2 and CWS43 into the same type, implying some genetic distance between F2, J2 and CWS43. It is to be noted that morphological characteristics can, to some extent, disclose genetic variation. Observation and identification of morphological characteristics should therefore not be ignored. For instance, CWS41 secreted specific yellow pigments, while RAPD analysis distinguished it from other isolates, revealing the farthest genetic distance from the other 22 isolates.

By using isolates from 16 pine species in Asia (China), Europe (England), Africa (South Africa), North America (the United States) and South America (Chile), our current experiments demonstrated, to a certain extent, intercontinental genetic differentiation among *S. sapinea* isolates.

Acknowledgements This study was supported by the National Natural Science Foundation of China (Grant No. 30070621).

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