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Molecular identification of endophytic fungi from *Aquilaria sinensis* and artificial agarwood induced by pinholes-infusion technique

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Agarwood, the resinous portions of *Aquilaria* plants, have been used as medicines and incenses. *Aquilaria sinensis* is the major producer of agarwood in China. Agarwood are generally viewed as pathological products formed as defense symptom against fungal infection. In this study, microbial communities inhabiting the leaves of non-resinous and agarwood-producing wounded *A. sinensis* tree were investigated by cultivation-independent approaches, such as PCR, restriction fragment length polymorphism (RFLP) analysis and sequencing of rDNA internal transcribed spacer (ITS) library. Molecular phylogenetic analysis demonstrated that *Botryosphaeria*, *Colletotrichum gloeosporioides*, *Phomopsis* and *Cylindrocladium* species are members of the agarwood-producing wounded tree, while *Phoma*, *Mycosphaerella*, *Sagenomella*, *Alternaria* and *Ramichloridium* species is able to colonize the non-resinous tree internally. *C. gloeosporioides* was the only fungus shared by the two rDNA ITS libraries. *C. gloeosporoides*, *Botryosphaeria*, and *Cylindrocladium* were considered to be related to agarwood formation. A pinholes-infusion method to induce the generation of agarwood by chemically stimulated and/or inoculate combined method was established. One to two years after the artificial inoculation, resinous wood were collected and the inoculating effects were detected by ethanol extraction content, thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) techniques. The results reveal that chemically stimulated with formic acid and infected by *Botryosphaeria dothidea* produced high yield and high quality artificial agarwood in a relatively short time.

Key words: Agarwood, endophytic fungi, *Aquilaria sinensis*, molecular identification, artificial induce of agarwood.

INTRODUCTION

Agarwood or Chinese eaglewood, the fragrant wood of *Aquilaria* trees, has been used as incense in social ceremonies and traditional antiemetic, analgesic, and digestive medicine for hundreds of years in Asia. *Aquilaria sinensis* (Lour) Gilg. (Thymeleaceae), distributed in Fujian, Guangdong, Guangxi and Hainan provinces of China, is the major producer of agarwood in China. The main active compounds in agarwood have been revealed to be

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sesquiterpenes and 2-(2-phenylethyl) chromanone derivatives (Chen et al., 2011). Recent researches have demonstrated that agarwood also has anti-inflammatory (Zhou et al., 2008), antianaphylaxis (Kim et al., 1997) and anticancer (Gunasekera et al., 1981) properties. With increased consumption of agarwood in recent years, over-exploitation of agarwood in forest caused depletion of the natural resources. The International Union for the Conservation of Nature (IUCN) in 2002 classified all species of *Aquilaria* including *A. sinensis* as "vulnerable" (IUCN 2002).

In addition, *A. sinensis* had been recommended to be the grade II protected plant in China (1999). However, the efforts in mass planting agarwood-producing trees seem to have not yet resulted in successful production of agarwood. In order to conserve these valuable tree resources, it is important to clarify the mechanism of the resin formation in agarwood and produce agarwood in a sustainable manner using artificial methods. For agarwood to be produced in a natural environment, the *Aquilaria* species need to be exposed to damage or attacked by pathogens or insects. Black and fragrant resin produced under occasional stimulation is deposited in the trucks or main branches of the trees. It is generally considered that the more the resin, the better the quality of agarwood. The formation of agarwood has long been mysterious and associated with arcane forces. In 1952, the fungus *Epicoccum granulatum* was isolated from infected *Aquilaria agallocha* and associated with the plant ability to produce resinous wood (Bhattacharyya, 1952). In the past decades, the origin of resin deposits has been investigated with varying and sometimes conflicting results.

South China Botanical Garden isolated *Menanotus flavoliven* from infected *A. sinensis*, another agarwood-promoting endophyte (Guangdong Institute of Botany, 1976). Others concluded that it was unlikely that there was specific fungal species responsible for the production of agarwood (REF). The confusion lasted until this century as more fungi were isolated from *Aquilaria* spp. and their effects on stimulating the resin deposits were displayed. Successful induction of agarwood by *Fusarium oxysporum* and *Chaetum globosum* was reported in 2000 (Tamuli et al., 2000). The important roles of *Fusarium* spp. on agarwood formation were again affirmed in the studies of Tabata (Tabata et al., 2003) in 2003 and Subehan (Subehan et al., 2005) in 2005. The majority agreed that several fungi played roles in the formation of agarwood while difference in their inoculating effects existed. Endophytes are micro-organisms that live asymptomatically within plant tissues (Petrini, 1991). The colonization and propagation of endophytes may in some ways offer significant benefits to their host plants, by improving and promoting plant growth and helping to reduce disease symptoms caused by pathogens or environmental stress (Strobel et al., 2004). Agarwood are pathological products formed as defense symptom against fungal infection. Therefore, it is important to study the endophytic fungal communities isolated from *Aquilaria* trees. We had reported on the endophytes in leaves, stems, roots, white wood, and resinous wood of *A. sinensis* using a traditional culture method (Wang et al., 2009). In this paper, leaf tissues were collected from non-resinous *A. sinensis* tree and agarwood-producing one, respectively. Different composition of endophytic fungi were first directly detected and identified within the leaf tissues mentioned earlier through sequencing of the rDNA internal transcribed spacer (ITS) regions. Differences in endophytic fungal species identified could be candidate pathogens that are responsible for agarwood formation in *A. sinensis*.

To further study these endophytes actual role in agarwood formation and produce artificial agarwood, a pinholes-infusion method to induce the generation of agarwood was established. After the inoculation, resinous wood were collected and the inoculating effects were detected by ethanol extraction content, thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) techniques. This would be a new system to study the mechanism of agarwood formation and produce agarwood in plantation-grown *Aquilaria* trees.

MATERIALS AND METHODS

Molecular identification and phylogenetic analysis of endophytic fungi in *Aquilaria sinensis*

Source of samples

A. sinensis located in the courtyard of a farmer in Xinyi of Guangdong province, China were chosen in this study. All the trees were identified as *A. sinensis* by the school of Traditional Chinese Medicine of Guangdong Pharmaceutical University. Leaf tissues were collected respectively from both a 35-year-old *A. sinensis* and a five-year-old tree on the 10th of December, 2008. The former tree was wounded or injured by the native people cutting with axe in their attempt to gather agarwood in 2003. Five years later, the wounded site wood colors changed from white-cream to dark brown or black. The 35-year-old *A. sinensis* tree was considered agarwood-producing wounded tree, while the latter one was considered as the non-resinous tree because no resin was observed on the tree.

Surface sterilization

To study the fungi within plant tissues, samples were rigorously surface sterilized by a procedure modified from Rodrigues (Rodrigues, 1994). Initially, dust on the surface of samples was removed with detergent, washed with sterilized water, and then dried with sterilized filter paper. Subsequently, they were submerged in 70% ethanol (v/v) for 1 min, 10% hydrogen peroxide (v/v) for 1 min, 70% ethanol for 1 min again, and for several minutes in sterile water. Leaves were then dried with sterilized filter paper for DNA extraction. The sterile water used at the last step of surface sterilization was collected to form a control sample to examine whether foreign DNA contamination was removed completely.

DNA extraction and PCR amplification

Total genomic DNA was extracted from fresh surface-sterilized leaf tissues of *A. sinensis* with a modified cetyltrimethylammonium bromide (CTAB) method (Bachellerie et al., 1993). Approximately 0.1 g fresh sterilized leaf tissues were ground with mortar and pestle for about 30 to 60 min under liquid nitrogen. Fine powder was transferred into a 1.5 ml Eppendorf microcentrifuge tube containing 700 µl of pre-warmed (65°C) 2 × CTAB buffer and 0.2% β-mercaptoethanol. The sample was incubated in a 60°C water bath for 1 h with occasional gentle swirling. The supernatant was transferred into a new 1.5 ml tube. Equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) was added and mixed to form an emulsion. The aqueous phase containing total DNA was transferred to a new tube after spun at 10,000 rpm for 10 min. The residue phenol was removed with chloroform:isoamyl alcohol (24:1) at least twice until no interface was visible. 1/10 volume of 3 M sodium acetate and 2.5 volume of ethanol were added to precipitate DNA. The tube was inverted gently to mix and stored at -20°C overnight. The DNA floccules was washed twice with 70% ethanol and resuspended in 60 µl Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). A pair of eukaryotic universal primers LH2 and SM73 designed by Qu (Pearson et al., 1998) and Gao (Gao et al., 2005) were used to amplify the ITS regions of eukaryotic organisms. The basic PCR reaction (26 µl each) contained 20 mM Tris-HCl, 100 mM KCl, 3.0 mM $MgCl_2$, 500 µM dNTPs and 2.6 U Taq DNA polymerase (Heda Technology, China). The PCRs were run for 35 cycles of 94°C denaturation for 1 min, 56°C annealing for 1 min, 72°C extension for 2 min after a 4 min-pre-denaturation at 94°C and followed by a final elongation step at 72°C for 10 min. The annealing temperature was tested ranging from 55 to 65°C and finally determined to be 56°C. Two negative controls were designed in the PCR amplification. In negative control I, water was used to replace DNA templates to detect any primer-dimmer product. The solution collected from the last step of sterilization was run parallel to DNA samples starting from the precipitation process. This control sample was amplified to examine the existence of any foreign DNA contamination. PCR products of different fragment sizes were separated by electrophoresis in a 2.0% (W/V) agarose gel at 100 V for 1 h and purified with the QIA quick gel extraction kits (Qiagen, Germany).

DNA cloning and restriction fragment length polymorphism (RFLP) analysis

The purified products of the fungal ITS regions were ligated into the pMD-18T vector (TaKaRa, China). Recombinants were then transferred into *Escherichia coli* TG1 competent cells in the presence of ampicillin. HY library and IY library were separately established representing the fungal communities within the *A. sinensis*. Positive clones of both libraries were selected by PCR amplification of inserts using the vectors primers P47/P48. Inserts with the correct size were subjected to a RFLP analysis, which involved digestion of the amplified inserts using restriction enzyme Msp I (Sangon, China) for 6 to12 h at 37°C. Plasmid DNA from recombination colonies were extracted and purified. The purified plasmids were sequenced by the Beijing Genomics institute.

Phylogenetic analysis

The entire ITS region (ITS1-5.8S-ITS2) was compared with the available sequences in GenBank searches by using the basic local alignment search tool (BLAST) programs (Benson et al., 2000; Hall, 1999). The rDNA ITS sequences were aligned using the Lasergene software 7.0 (Dnastars, Madison, WI, USA). Pre-aligned sequences were checked manually and edited by the BioEdit 5.0.9 program (Hall, 1999). Phylogenetic trees were constructed from the maximum parsimony (MP) and neighbor-joining (NJ) methods in PAUP versions 4.0 b8 (Swofford, 2000). Statistical support for the internal branches was estimated by bootstrapping 1000 replications. In addition to the MP and NJ analyses, Bayesian (BA) method was carried out as instructed in the computer program MARBAYES 3.0. The Bayesian analysis was run for 3,000,000 generations and sampled in every 500 generations.

Nucleotide sequences accession number

The ITS sequences was deposited in the EMBL nucleotide sequence database under accession number FN667926 to FN667945.

Artificial agarwood induced by chemically stimulated and/or inoculate combined method

The pinholes-infusion technique to induce the generation of agarwood

Five year-old matured trees of plantation-grown *A. sinensis* were collected randomly from Xinyi city, Guangdong province, China. The trees were approximately with height of about 3 to 4 m, and distances between every two trees were 50 to 70 cm. A drill was employed to make 0.5 cm width and 4 to 5 cm deep holes in the trunks of trees on the height of 1 m which were then stimulated with formic acid. 500 ml of formic acid (pH 2.0) was injected into the holes to loosen the wood for the better absorption of the fungal liquid, taking non-resinous tree stimulated by the H_2O as the negative control. Hyphae of fungi were smashed and diluted to appropriate concentration with H_2O and then filtered with cotton gauze to serve as fungi solutions that can be infused after formic acid infusion. The speed of infusion was controlled at one drop/10 s. The inoculation holes were then covered by tape.

Source of fungi

Several fungal species from *A. sinensis* were isolated and identified (Wang et al., 2009). *Pestalotiopsis* sp. (EU781662), *Colletotrichum gloeosporioides* (EU781669), *Xylaria* sp. (EU781660), *Fusarium* sp. (EU781659), Chaetomium sp. (EU781676), *Tichodema* sp. (EU781674), *Penicillium* sp. (EU781668), *Botrysphaeria rhodina* (EU781670) and unidentified fungi (EU781665) were selected to infect the non-resinous *A. sinensis* tree.

Wood samples collection

Wood chips around the inoculation holes were collected by the increment borer at regular intervals.

Quality identification

Content of the ethanol extraction

Content of ethanol extraction, color reaction and TLC were performed following the method of Pharmacopoeia of the People's Republic of China (Chinese Pharmacopoeia Commission, 2010). 2 to 4 g of sample was exactly weighted in a 250 ml conical flask and added 50 to 100 ml ethanol. Solution was standing for 1 h and weighted before the 1 h circumfluence distillation. After distillation, the lost weight was complemented with ethanol and filtrated by the dry filter. 25 ml of successive filtrate was precisely added to evaporation dishes which were dried to constant weight. After the solution was vaporized, dishes were oven dried for 3 h under the temperature at 105°C, and then cooled in the desiccators and weighted quickly. The weight of the ethanol extraction divided by the weight of the samples was the content of the ethanol extraction of eaglewood.

Color reaction

Ethanol extraction prepared according to content of the ethanol extraction was micro sublimated to oil, after which one drop of hydrochloric acid, little vanillin and one to two drops of ethanol were added. Deeper cherry-red color was required at a time.

TLC identification

10 ml of the ethanol extraction prepared according to content of the ethanol extraction was precisely condensed to 1 ml. 5 µl of the solution was spotted onto silica gel G plate, using chloroform: acetone (95 : 5) as the developing agent, developed for 8 cm, and 10% alcoholic solution of sulfuric acid was used as the coloration, and detected under the reflected wavelength of 365 nm and the transmission peak wavelength of 300 nm.

GC-MS analysis

1 to 2 g of samples was exactly weighted in the Erlenmeyer with cover, 30 ml of chloroform was precisely added, and the solution was shaken and weighted. Compounds were extracted by the 24 h standing method; the extraction was complemented with the lost weight and filtrated before it was then dried with water bath. Residue was dissolved with chloroform as the sample solution.

Capillary column VF-5 MS (30 m × 0.25 mm × 0.25 µm) was used, with the injector temperature of 250°C, high-purity of N_2 was used as the carrier gas in a flow rate of 1 ml/min, and the sample size was 1 µl. The programmed temperature used was that of the initial temperature of 90°C maintained for 4 min, after which it went up by 1°C/min to 145°C and was maintained for 30 min. It later increased to 180°C by 2°C/min and was maintained for 1 min, before it finally went up to 230°C by 2°C/min and was maintained for 100 min.

The following parameters were included in the analysis: ion source (EI), ionizing voltage (1788 mv), alereon energy (70 ev), solvent delay (4 min), and scan range (50 to 500 m/z). Compounds identification was done by comparing the Wiley library data of the peaks with those reported in the literature and mass spectra of the peaks with literature data. Percentage composition was computed from GC peak areas on with VF-5 MS column without applying correction factors.

RESULTS

Molecular identification and phylogenetic analysis of endophyte in *A. sinensis*

Two DNA fragment of different size were obtained from the PCR products of each DNA sample (Figure 1). The 700 bp band was the typical rDNA ITS size for angiosperms (Baldwin et al., 1995). The other was approximately 600 bp, corresponding to a typical rDNA ITS size for fungi (Skouboe et al., 1999). The target 600 bp fragments from the agarwood-producing and non-resinous trees were gel purified and used to construct rDNA ITS libraries, which were named Library IY and Library HY respectively. Two negative controls were designed in the PCR amplification process. No fragment was obtained from the negative control I which demon-strated the specificity of the PCR amplification. Absence of PCR products in the negative control II indicated that foreign DNA was successfully excluded.

RFLP patterns of the positive inserts from two libraries revealed different degrees of the fungal endophyte diversities within the agarwood-producing and non-resinous *A. sinensis* and 120 single clones of each library were selected for re-amplification using the vector primer pair P47/P48. Positive rate of the IY and HY libraries were calculated to be 72.5 and 76.2%, respectively. The inserts of correct size were subjected to a restriction using Msp I. The RFLP analysis based on the restriction map resulted in 14 and six different RFLP types in the IY and HY libraries, respectively. One to three clones representing each RFLP type from two libraries were selected for DNA sequencing. A total of 20 rDNA ITS representative sequences (14 of the IY library and six of the HY library) were determined after seven chimerical sequences were excluded (five of the IY library and two of the HY library). All the rDNA ITS sequences from the clones could be grouped within the fungi domain by BLAST in GenBank. 13 sequences had significant identities (>99.0% similarity) to listed species in GenBank, that is, IY1, IY4, IY7, IY37, IY38, IY40, IY41, IY42, IY49, HY6, HY8, HY10 and HY30. Among them, IY4, IY42 and HY10 had 100% similarity to their closest relatives. HY106 and HY 27 respectively had the similarities of 97.3 and 91.2% with their corresponding closest relatives in GenBank. Five sequences including IY22, IY27, IY43, IY103 and IY147 had low levels of similarity (<75%) with their closest counterparts in the database. A NJ tree, containing all the sequences, rooted with *Saccharomyces cerevisiae*, *Saccharomyces castellii* and *Candida glabrata* grouped the total 20 sequences into six clades (Figure 2). Further phylogenetic analysis was performed based on maximum parsimony and Bayesian method. MP tree and BA tree gave similar results, therefore, only the BA trees were shown in this study.

14 sequences of the IY library were identified as four genera, among them IY1, IY4, IY7 and IY37 were identified as species and the others as genera. Four sequences, that is, IY1, IY7, IY40 and IY41, accounting for 40.5% of the IY library, were phylogenetically analyzed with nine *Botryosphaeria* spp., three different *Dothioraceae* spp., three different *Pleosporales* spp. *S. cerevisiae*, *S. castellii* and *C. glabrata* were used as out-group. The four sequences were grouped with nine *Botryosphaeria* spp. with a 98% bootstrap support and the 0.52 posterior probabilities (PP) (Figure 3). Within this monophyletic group, IY1 and *Botryosphaeria dothidea* formed a terminal cluster with a 99 bootstrap percentage and the 1.0 PP. IY1 was believed to be *B. dothidea*. IY7, IY40 and IY41 were formed as paraphyletic clade with *Botryosphaeria ribis*, *Botryosphaeria parvum*, *Botryosphaeria parva* and *Botryosphaeria eucalyptorum* with 81% bootstrap support and 0.66 PP. They were relatives belonging to *Botryosphaeria* spp. This *Botryosphaeria* community was dominant within the infected *A. sinensis*. Five sequences accounting for 28.6% of the IY library, that is, IY22, IY27, IY43, IY103 and IY147, had less than 75% sequence

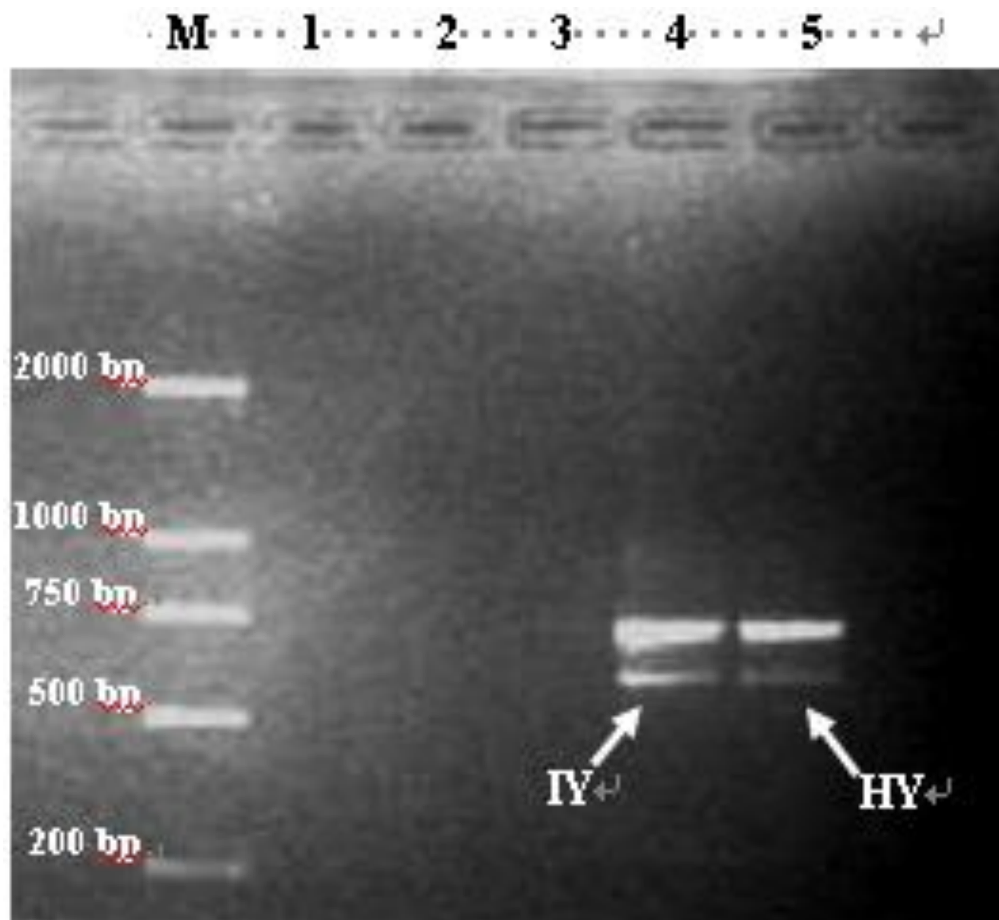

Figure 1. PCR products of rDNA ITS regions from the genomic DNA extracted from the leaves of *A. sinensis*. Lane 1, negative control; lane 2, negative control of sample IY; lane 3, negative control of sample HY; lane 4, sample IY; lane 5, sample HY. The DNA were resolved on 2.0% agarose gel electrophoresis in 1×TAE buffer, and visualized by ethidium bromide staining. Size markers are indicated by M in all gels.

identities to any sequences in GenBank. They were phylogenetically analyzed within the 5.8S regions. Alignment of the 5.8S gene sequences of the 15 taxa showed a data matrix of 161 base sits, of which 129 (80.1%) were parsimony informative. Results of the phylogenetic analysis showed the more closely relation between the five sequences and *Cylindrocladium* species (Figure 4). Reference sequences of *Cylindrocladium* spp. and its teleomorph *Calonectria* sequences were very few in GenBank. Therefore, all these reference sequences in GenBank were collected for further phylogenetic analysis (Figure 5). None of the sequence was clustered with the reported sequences in GenBank. The 5 sequences which consist of the second dominant population were probably from new species of *Cylindrocladium*. IY4, IY37 and IY49, accounting for the 26.2% of the IY library were ITS highly similar to the *Collectotrichum* species. Five *Collectotrichum* species and five other species sequences were collected for the phylogenetic analysis. *S. cerevisiae, S. castellii* and *C. glabrata* were used as the roots of the tree (Figure 6). Three sequences were clustered with five *Collectotrichum* sequences with 100% bootstrap percentage and 1.0 PP and formed terminal cluster with three *C. gloeosporiodes* sequences. They were all considered to be *C. gloeosporiodes*. The other sequences of the IY library, that is, IY38 and IY42, accounting for the 4.76% of the IY library, had 99.8 and 100% similarities, respectively with the referenced *Phomopsis* spp. With *Glomus geosporum* and *Glomus coronatum* as out-group, nine *Phomopsis* sequences and another 10 sequences of other genera were used as relative taxa in the phylogenetic analysis of these two sequences (Figure 7). They were finally identified as *Phomopsis* sp. Although, with less RFLP patterns to the IY library (14 RFLP patterns of the IY library), six sequences representing six different RFLP types of the HY library were phylogenetically associated with six different genera.

HY6 and HY30, accounting for 40 and 10% respectively, of the HY library separately, both have the uncultured environmental sequences as their closest relatives

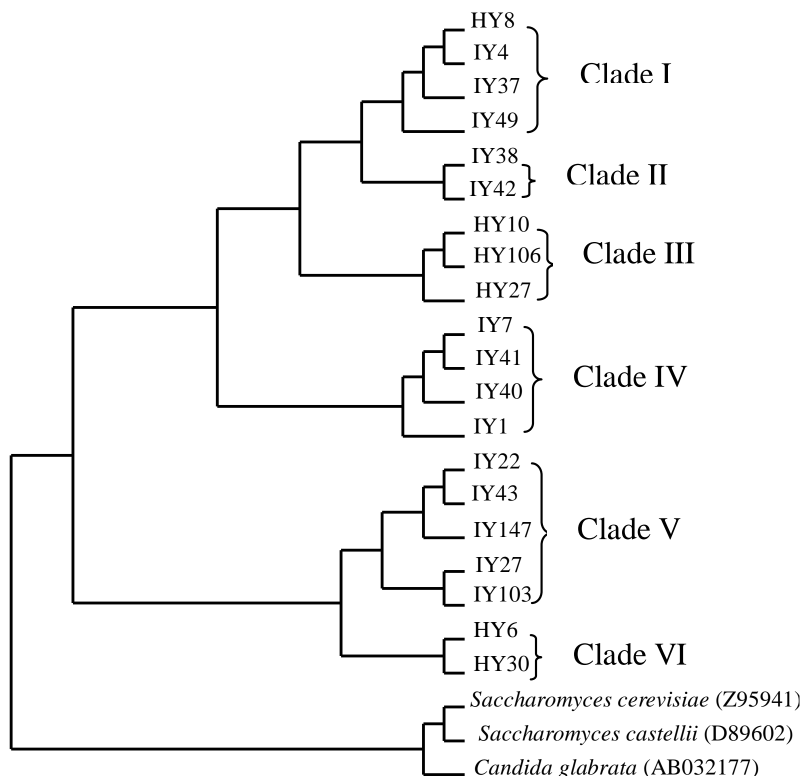

Figure 2. Neighbor-joining tree obtained from analysis of the rDNA ITS shows the relationships of the 20 representative sequences in the IY and HY libraries. Reference sequences of *Saccharomyces cerevisiae*, *Saccharomyces castellii* and *Candida glabrata* were used as out-group.

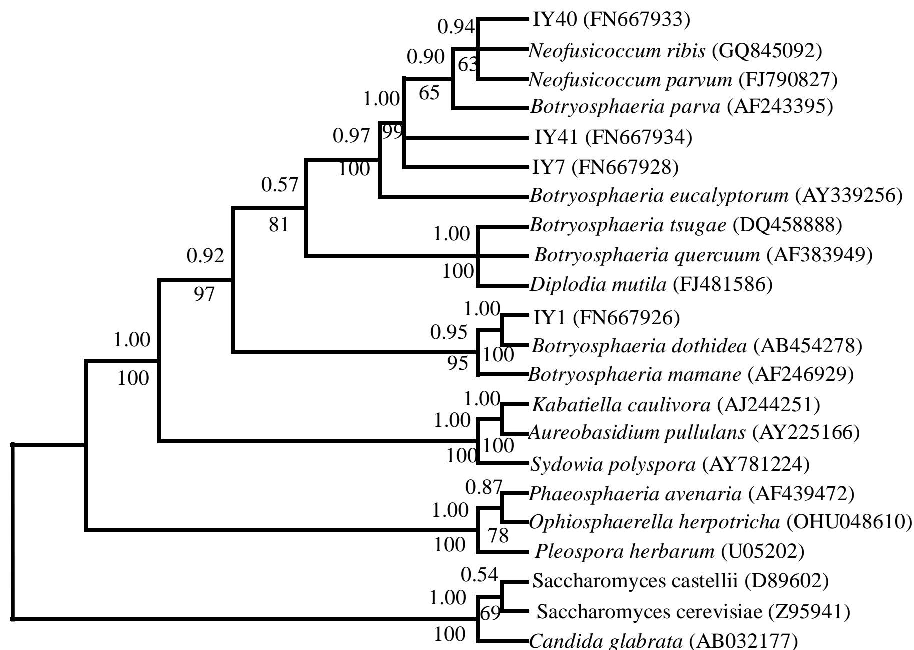

Figure 3. Phylogenetic analysis based on the ITS sequence data shows the relationships of four sequences with reference taxa. The tree shown was derived by Bayesian analysis. Bayesian posterior probabilities (PP) and maximum parsimony bootstrap values (MP) (≥ 50%) are shown above and below the lines, respectively. *Saccharomyces castellii*, *Saccharomyces cerevisiae* and *Candida glabrata* were used as out-group.

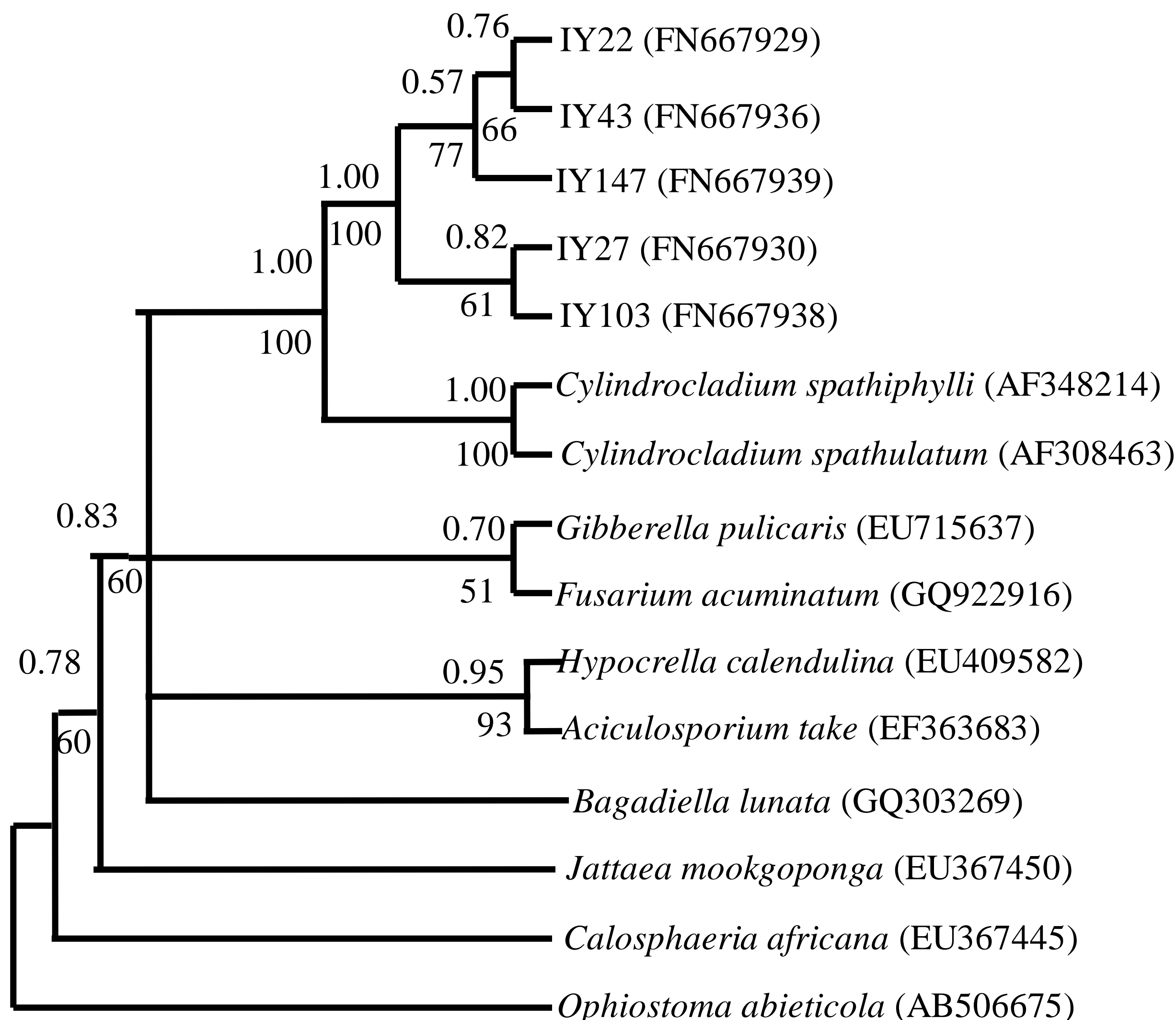

Figure 4. Phylogenetic analysis based on the 5.8S sequence data shows the relationships of five sequence types with reference taxa. The tree shown was derived by Bayesian analysis. Bayesian posterior probabilities (PP) and maximum parsimony bootstrap values (MP) (≥50%) are shown above and below the lines, respectively. *Saccharomyces castellii*, *Saccharomyces cerevisiae* and *Candida glabrata* were used as out-group.

and were grouped together from the NJ analysis of the total 20 endophytic sequences. Phylogenetic analysis of these two sequences and the other 16 different sequences was carried out (Figure 8). HY6 and HY30 formed a monophyletic clade with another *Pleosporale* sp., that is, *Ophiosphaerella herpotricha* with 100% bootstrap support and 0.99 PP. Within this clade, HY30 formed the subclade with three *Pleosporaceae* spp. with 100% bootstrap percentage and 1.00 PP. The sequence terminal clustered with HY30 was the uncultured soil fungal sequence. Therefore, HY30 was finally inferred as belonging to *Alternaria* sp. from the paraphyletic clade formed by HY30 and two different *Alternaria* sequences. HY6 was related to *Phoma* sp. from the result of the phylogenetic trees. The NJ tree showed that the relationship of the total 20 sequences indicated the close relations of HY10, HY27 and HY106 (accounting for 20, 10 and 10%, respectively, of the HY library). While, results both from the search for similar ITS sequences and the phylogenetic analysis divided them into three different genera (Figure 9). HY10, HY27 and HY106 respectively formed terminal cluster with *Mycosphaerella* sp., *Sagenomella* sp. and *Ramichloridium* sp. HY8, accounting for 10% of the HY library was clustered with IY7, IY39 and IY41 and identified as *C. gloeosporiodes* based on the closest relative reference and phylogenetic analysis (Figure 6).

Successfully artificial induction of the generation of agarwood

Based on the pinholes-infusion technique, fungal isolates

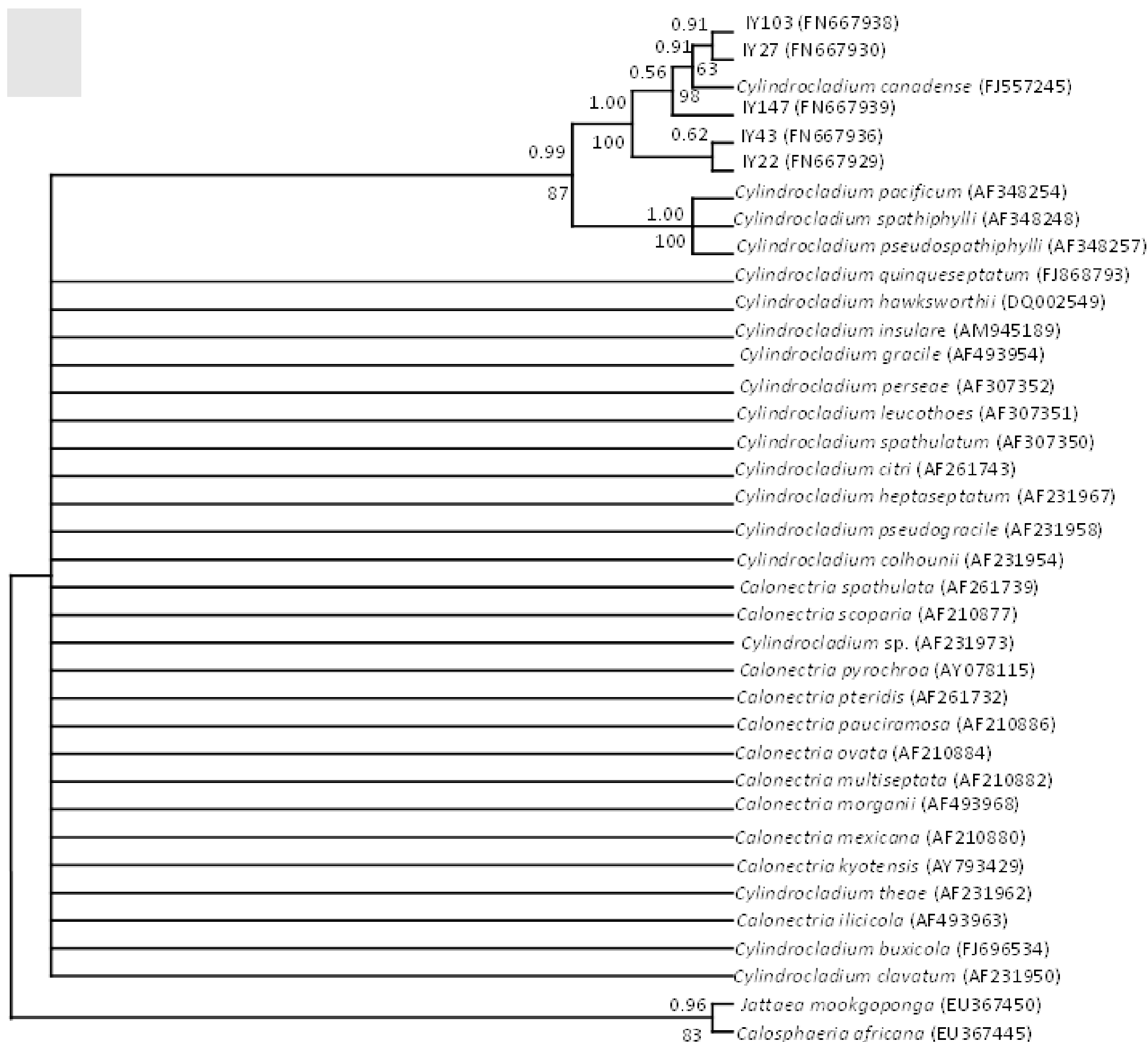

Figure 5. Phylogenetic analysis based on the 5.8S sequence data shows the relationships between five sequence types and reference taxa. Bayesian posterior probabilities (PP) and maximum parsimony bootstrap values (MP) (≥ 50%) are shown above and below the lines, respectively. *Calosphaeria africana* and *Jattaea mookgoponga* were used as out-group.

including Penicillium, Trichoderma, *Fusarium*, *Xylaria*, *Nodulisporium*, *Pestalotiopsis*, *Colletotrichum*, *Botrysphaeria*, *Trichoferma*, and *Chaetomium* were selected to induce agarwood production. A total of 232 dark-brown wood chips were obtained, which were resinous wood. The quality analysis of these wood chips was based on the content of ethanol extraction, color reaction (Table 1). High yield of artificial agarwood occurs in the trunks or branches of trees that have been stimu-lated with formic acid and infected by *Fusarium* sp., *Botryosphaeria* sp. (Figures 10A and D) and uncultured fungus clone (Figure 10C) cultivated from *A. sinensis*. Based on the comprehensive results of the content of ethanol extraction, color reaction and resin collection, *B. rhodina* was considered the best inoculator. Agarwood originated from No. A9 tree (eight and 12 months) stimulated with formic acid and infected *Botryosphaeria* sp., were further measured by TLC and GC-MS, taking two commercial wild agarwood (1st grade Hainan and 3rd grade Guangdong agarwood) as controls (Figure 11, Table 2). A total of 53 compounds were identified from the aforementioned four samples (Table 2). 31 and 32 components were identified in 1st and 3rd grade agarwood, representing 53.78 and 42.04% of the total volatility components, with the major constituents

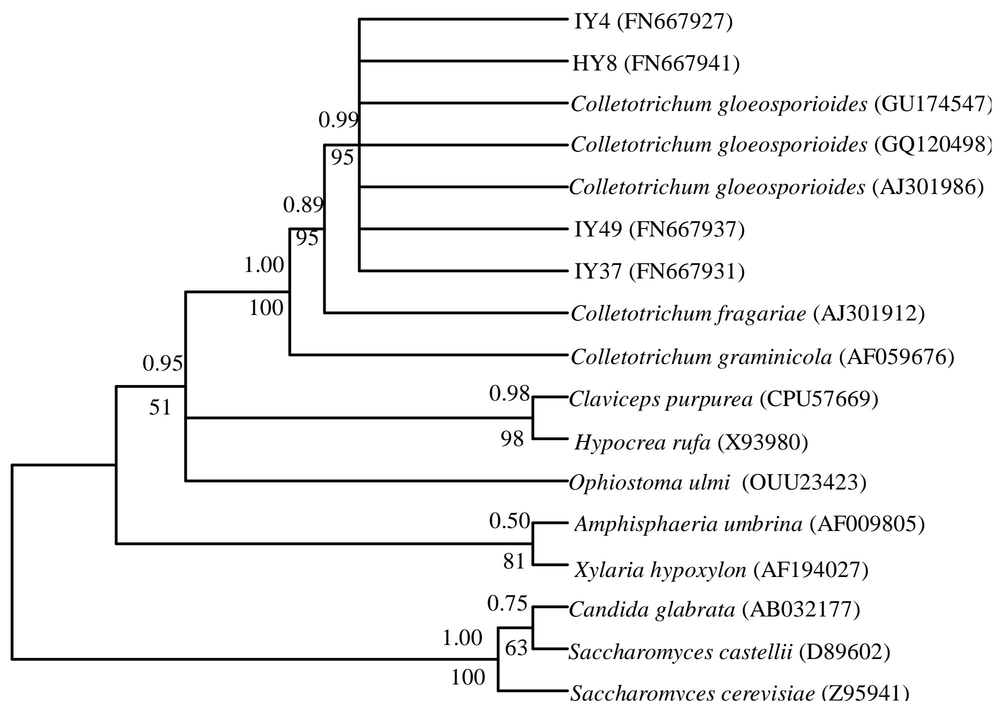

Figure 6. Phylogenetic analysis based on the ITS sequence data shows the relationships of four sequence types with reference taxa. The tree shown was derived by Bayesian analysis. Bayesian posterior probabilities (PP) and maximum parsimony bootstrap values (MP) (≥50%) are shown above and below the lines, respectively. *Saccharomyces castellii*, *Saccharomyces cerevisiae* and *Candida glabrata* were used as out-group.

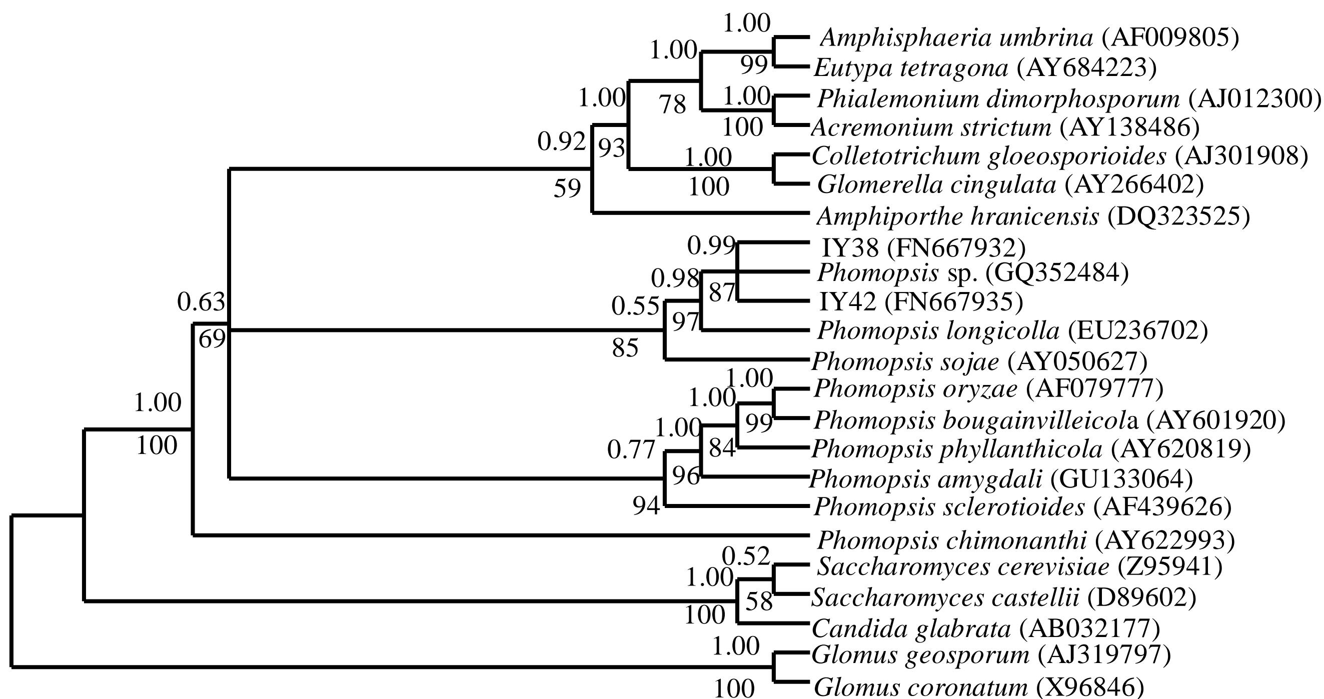

Figure 7. Phylogenetic analysis based on the ITS sequence data shows the relationships of two sequence types with reference taxa. The tree shown was derived by Bayesian analysis. Bayesian posterior probabilities (PP) and maximum parsimony bootstrap values (MP) (≥50%) are shown above and below the lines, respectively. *Glomus geosporum* and *Glomus coronatum* were used as out-group.

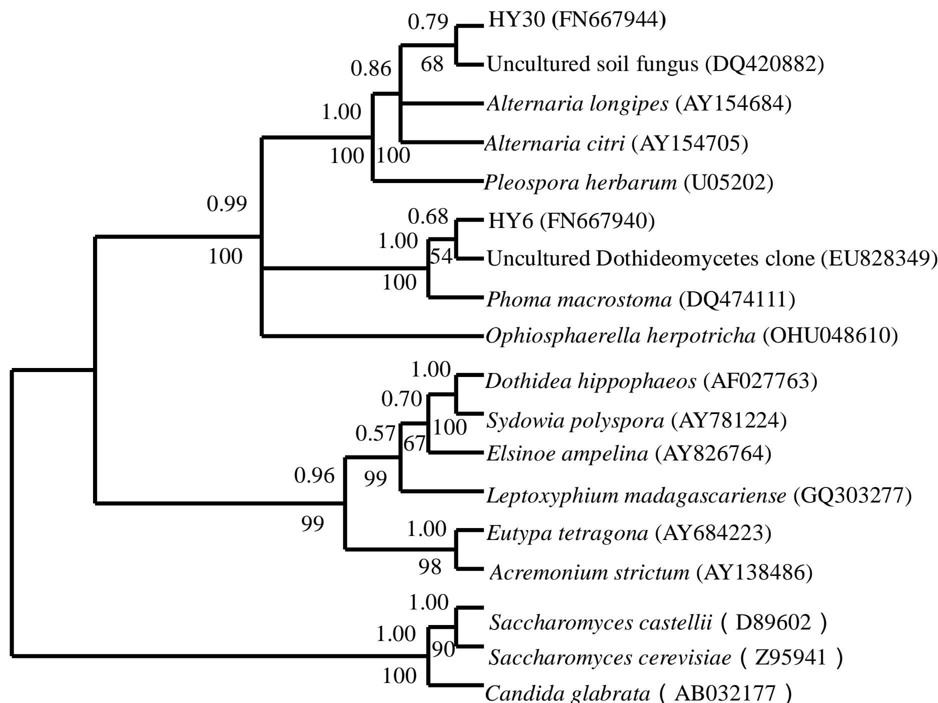

Figure 8. Phylogenetic analysis based on the ITS sequence data shows the relationships of two sequence types with reference taxa. The tree shown was derived by Bayesian analysis. Bayesian posterior probabilities (PP) and maximum parsimony bootstrap values (MP) (≥50%) are shown above and below the lines, respectively. *Saccharomyces castellii*, *Saccharomyces cerevisiae* and *Candida glabrata* were used as out-group.

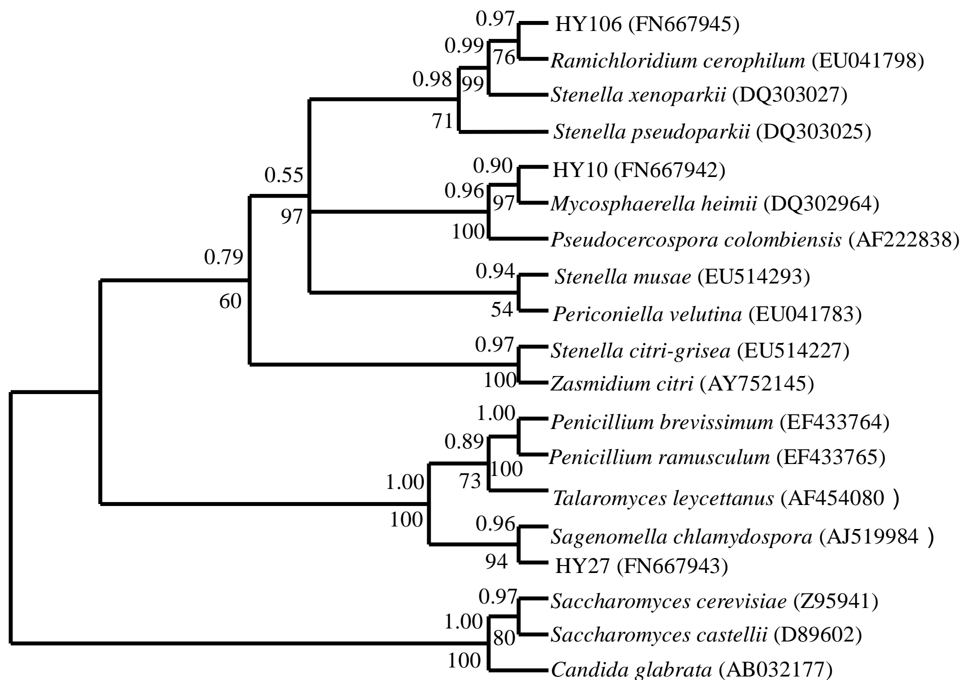

Figure 9. Phylogenetic analysis based on the ITS sequence data shows the relationships of three sequence types with reference taxa. The tree shown was derived by Bayesian analysis. Bayesian posterior probabilities (PP) and maximum parsimony bootstrap values (MP) (≥50%) are shown above and below the lines, respectively. *Saccharomyces castellii*, *Saccharomyces cerevisiae* and *Candida glabrata* were used as out-group.

Table 1. Quality analysis of resinous wood chips induced by pinholes-infusion method.

Artificial inducement technology	Number	Tree Number	Collection time	Color-reaction	Ethanol-soluble extraction content (%)	Resins formation time / month
White wood	1	29	01-05-2008	Pale yellow	3.3	6
	2		01-09-2008	Pale yellow	3.4	8
	3		05-01-2009	Brown-red	4.6	12
	4	95	20-02-2011	Brown-red	4.3	8
	5		20-06-2011	Pale yellow	8.0	12
						3
Only holes	6	11	11-09-2009	Pale yellow	3.3	
	7		11-11-2009	Green	1.7	5
	8		10-03-2010	Pale yellow	6.5	9
	9	24	11-09-2009	Pale yellow	5.0	3
	10		11-11-2009	Pale yellow	2.3	5
	11		10-03-2010	Green	7.4	9
	12		11-09-2009	Pale yellow	8.7	3
	13	29	11-11-2009	Pale yellow	3.2	5
	14		10-03-2010	Pale yellow	3.7	9
	15		01-01-2011	Pale yellow	6.5	17
	16		13-09-2011	Pale yellow	7.5	24
H_2O	17	B5	05-01-2009	Pale yellow	3.2	8
	18		02-06-2009	Pale yellow	1.9	12
	19		25-09-2009	Pale yellow	4.9	15
	20		11-11-2009	Pale yellow	3.6	17
	21		10-03-2010	Pale yellow	6.1	21
	22	80	09-02-2011	Pale yellow	5.8	8
	23		20-06-2011	Pale yellow	8.6	12
Formic acid + Uncultured fungus clone RFLP50	51	54	11-10-2010	Pale yellow	4.0	7
	52		09-02-2011	Pale yellow	7.2	13
	53		20-06-2011	Pale yellow	7.5	17
	54		22-12-2011	Red	15.3	21
	55	87	09-01-2011	Pale yellow	6.2	7
	56		20-06-2011	Pale yellow	7.9	12
	57		05-10-2011	Red	19.2	16
	58	92	09-05-2011	Pale yellow	5.7	8
	59		29-09-2011	Red	26.8	12

Table 1. Contd.

Colletotrichum gloeosporioides	60	14	25-09-2009	Pale yellow	5.9	3
	61		11-11-2009	Pale yellow	2.7	5
	62		10-03-2010	Green	4.5	9
Formic acid +*Colletotrichum gloeosporioides*	63	B3	05-02-2009	Pale yellow	5.8	7
	64		02-06-2009	Dark green	4.3	12
	65	N7	10-02-2010	Yellow	4.3	9
	66		11-06-2010	Pale yellow	4.2	12
	67		09-01-2011	Pale yellow	15.8	19
Formic acid + *Botryosphaeria dothidea*	68	A9	05-01-2009	Pale yellow	4.0	7
	69		02-06-2009	Cherry-red	11.8	12
	70	89	09-02-2011	Pale yellow	5.4	7
	71		20-06-2011	Cherry-red	10.2	12
	72	90	09-01-2011	Pale yellow	4.9	7
	73		20-06-2011	cherry-red	10.2	12
Formic acid + Uncultured fungus clone RFLP50 + *Colletotrichum gloeosporioides*	74	22	25-09-2009	Pale yellow	6.2	3
	75		11-11-2009	Yellow green	2.5	5
	76		10-03-2010	Yellow green	5.9	9
	77		09-01-2011	Red	5.3	19
	78	20	25-09-2009	Pale yellow	2.6	3
	79		11-11-2009	Pale yellow	2.6	5
	80		10-03-2010	Green	3.9	9
	81		11-06-2010	Pale yellow	2.9	12
	82		09-01-2011	Pale yellow	5.2	17

being aromatics and 2-(2-phenyl-ethyl) chromanone, respectively. 16 components were identified in the sample from the 12-month A9 tree, representing 35.09% of the total volatility components, with the major constituents being 2-(2-phenylethyl) chromanone, aromatics and sesquiterpenes. The results demonstrate that the volatility components of No. 9 tree (12-month) had similar components to those of commercial wild agarwood.

DISCUSSION

Compared with the traditional cultivation-based method, the DNA-based methods may have the advantage to identify micro-organisms that are difficult to culture *in vitro*. Culture-independent molecular technique has been widely used in several other studies, which has also proved useful for evaluating endophytes communities of plants (for example, Guo et al., 2001, 2005; Duong et al., 2006; Seena et al., 2008). In this work, we extracted DNA directly from non-resinous and agarwood-producing *A. sinensis* leaves by PCR and cloning to obtain endophytic fungi rDNA ITS sequence data. The sequence results demonstrated that DNA-based method can be used to identify fungal species directly and rapidly from the plant tissues without having first to

Table 2. Chemical compositions and relative amounts of volatility components from artificial and commercial wild agarwood.

Name of the component	Relative amount (%)			
	3rd grade Guangdong	1th grade Hainan	Number of A9 tree	
			8 months	12 months
Sesquiterpenes	3.22%/ (5)	4.16%/ (5)	0%/ (0)	6.31%/ (4)
χ-Selinene	—	—	—	0.493
α-Costol	—	—	—	0.340
Aristolone	0.290	0.374	—	1.274
Agaruspinol	0.274	0.354	—	—
Aromadendrene	0.143	0.185	—	—
Guaiol	0.399	0.515	—	—
Bamuxinal	2.115	2.731	—	4.204
2-(2-Phenylethyl) chromanone	**11.60%/ (3)**	**14.98%/ (3)**	**30.22%/ (4)**	**19.88%/ (3)**
5,6,7,8-Tetrahydro-2,2-dimethyl-5-Chromanone	—	—	1.493	—
4H- 1-Benzopyran- 4- one, 2- methyl-	0.467	0.603	0.370	0.462
2- (2-Phenylethyl) chromen-4-one	4.874	6.295	13.995	6.421
8-Methoxy-2- (2-phenylethyl) chromen-4-one	6.257	8.082	14.366	13.00
Aromatics	**19.66%/ (12)**	**25.40%/ (12)**	**16.06%/ (12)**	**7.86%/ (6)**
2-Butanone, 4-phenyl-	1.000	1.292	2.737	2.625
Benzenepropanoic acid	1.021	1.318	0.618	0.602
Ethyl 3-phenylpropionate	—	—	—	1.208
Phenol, 2,6-dimethoxy-	—	—	0.469	—
2-Butanone, 4-(4-methoxyphenyl)-	0.567	0.732	0.409	0.886
Phenol, 2,4-bis(1,1-dimethylethyl)-	—	—	1.498	—
Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	—	—	0.235	—
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.548	0.708	3.007	2.268
1,2-Benzenedicarboxylic acid, dibutyl ester	1.253	1.619	3.989	—
3-Pentanone, 1,5-diphenyl-	—	—	0.302	—
2-Naphthalenamine, N-phenyl-	—	—	0.165	—
(+)-Threo-1,5-Diphenylpentane-1,3-diol	—	—	1.966	—
1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	—	—	0.660	0.273
Benzene, (1, 2-dimethoxyethyl)-	1.279	1.652	—	—
N, 3-Diphenyl-1- isoquinolinamine	1.132	1.462	—	—
Anisole, o-pentyl-	1.077	1.391	—	—
Benzene, 1- methoxy- 4- propyl-	0.786	1.016	—	—

Table 2. Contd.

Naphthalene, 1, 1'-(1, 10- decanediyl) bis[decahydro-	0.933	1.205	—	—
Benzene, 1,1'-(1, 2-ethanediyl) bis [4-methoxy-	1.711	2.211	—	—
11-Phenyl-11H- indolo[3, 2-c] quinoline- 6(5H) -one	8.353	10.789	—	—
Alkanes/fatty acid/ester of fatty acid	**5.11%/ (9)**	**6.08%/ (8)**	**3.85%/ (7)**	**0.73%/ (2)**
Decane, 2-methyl-	—	—	0.292	—
1,2,3-Propanetricarboxylic acid, 2-hydroxy-, triethyl ester	—	—	0.434	—
3-(4-methoxyphenyl) Propionic acid ethyl ester	—	—	—	0.411
Tetradecane	0.562	0.725	—	—
Pentadecane	0.818	1.057	—	—
Hexadecane	0.216	0.279	—	—
Tetradecane, 2-methyl	0.310	0.401	—	—
Hexadecane, 7,9-dimethyl	0.274	0.354	—	—
Hexadecanoic acid	0.946	1.222	1.580	—
Tetradecanoic acid	1.186	1.533	—	—
Pentadecanoic acid	0.405	—	—	—
Octadecanoic acid	0.392	0.506	0.327	—
Hexadecanoic acid, ethyl ester	—	—	0.316	—
Octadecanoic acid, ethyl ester	—	—	0.165	—
Cyclohexadecane	—	—	—	—
9-Octadecenoic acid (Z)-, ethyl ester	—	—	—	0.315
Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	—	—	0.738	—
Others	**2.45%/ (3)**	**3.16%/ (3)**	**2.54%/ (2)**	**0.31%/ (1)**
Tricyclo [3. decane, 3. 1. 1(3, 7)] 1, 3- dimethyl	0.344	0.444	—	—
Capnellane-8-one	0.591	0.763	—	—
2, 2, 4- Trimethylfuro[6,7- c]-1, 3, 8H- azulene	1.511	1.952	—	—
1-penten-3-one, 1,5-diphenyl	—	—	1.227	0.309
Stigmast-5-en-3-ol, (3.beta.,24S)-	—	—	1.315	—
Total	42.04%/ (32)	53.78%/ (31)	53.0%/ (25)	35.09%/ (16)

culture the organism. Fungal communities within non-resinous and agarwood-producing *A. sinensis* leaf tissues were totally different. *C. gloeosporoides* was the only fungal species detected both in the two types of *A. sinensis* with different levels of abundance (26.2% of the agarwood-producing tree and 10% of the non-resinous one). *Collectotrichum* and its anamorph *Glomerella* species are responsible for huge losses in many tropical, subtropical and temperate crops. *C. gloeosporoides* are the biggest family in this genus causing anthracnose (for example, Bussaban et al., 2001; Taylor et al.,

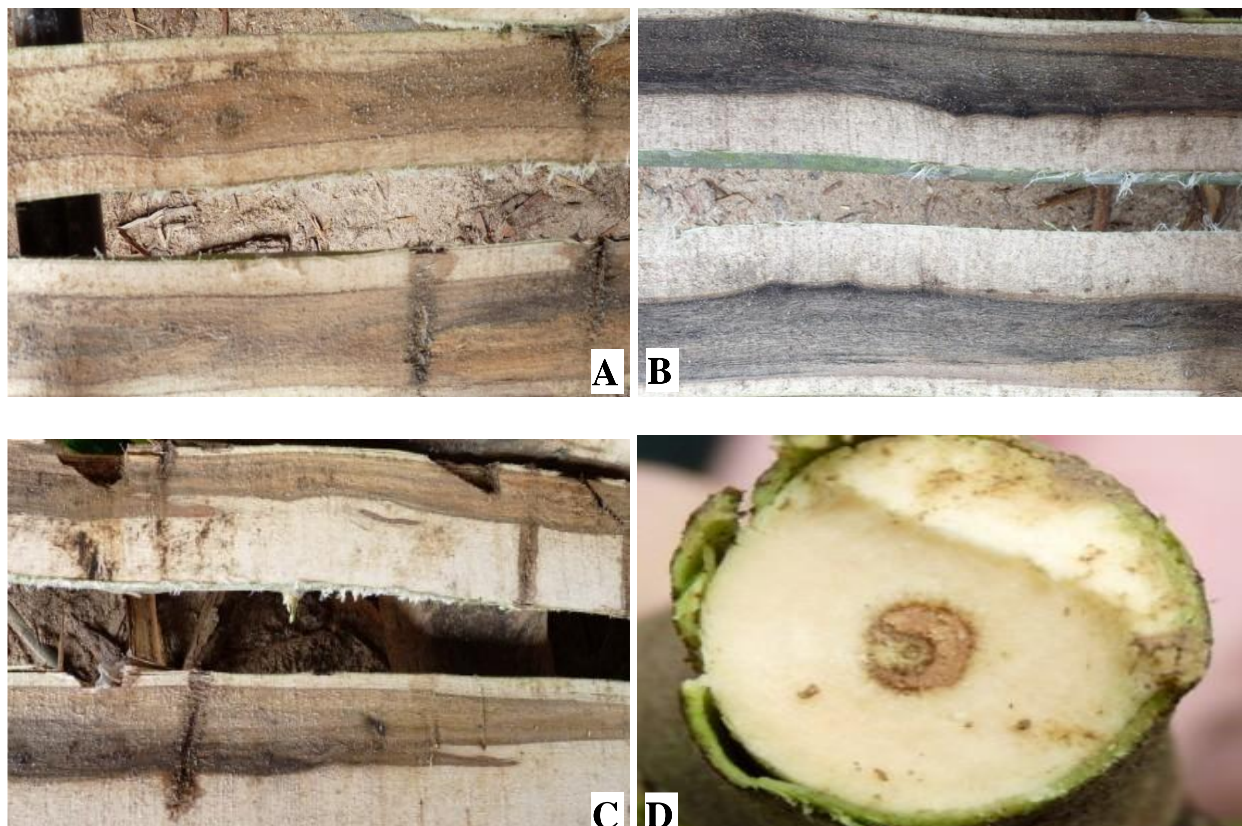

Figure 10. The longitudinal sections cut from the truck and branch of *Aquilaria sinensis* trees induced by formic acid and infected by *Botryosphaeria dothidea* (panel A and panel D), *Trichoderma harzianum* (panel B), uncultured fungus clone RFLP50 (panel C).

1999; Wang, et al., 2008). It is noteworthy that they were much more abundant within agarwood-producing *A. sinensis* than within non-resinous one. *Botryosphaeria* sp. and *Cyindrocladium* sp. were found only within the agarwood-producing tree. *Botryosphaeria*, a genus with a cosmopolitan distribution, is widely isolated from mono-cotyledonous, dicotyledonous and gymnosperm hosts, causing die-back and canker diseases of numerous woody hosts. This genus is generally identified with their anamorphs based on morphological features because it is difficult to find the teleomorph *Botryosphaeria* either from nature or at laboratories due to non-sporulation in the presence of current media (Perotto et al., 2000; Zhao et al., 2007). Direct detection within leaf tissues of agarwood-producing *A. sinensis* showed high abundance of the teleomorph *Botryosphaeria* (40.5% of the IY library). It is noteworthy that high abundance of these rare founded teleomorph *Botryosphaeria* within the non-resinous *A. sinensis* may contribute to certain extent to the formation of agarwood. In addition, members of the genus *Calonectria* and their *Cylindrocladium* anamorphs are one of the most important pathogens associated with a wide range of plant diseases (for example, Wang et al., 2003; Blum and Dianese, 1993). Compared to other genera, *Cylindrocladium* spp. was discovered late and many new species were reported in the recent years (Crous, 2004). *Botryosphaeria* spp., *Cylindrocladium* spp. and *C. gloeosporoides* were considered to be involved in agarwood formation for their extensive distribution within the agarwood-producing trees (Gong and Guo, 2009).

Several fungal species from diverse tissues of *A. sinensis* were isolated and identified. The culture-based results showed that the distribution of endophytic fungi in different tissues of *A. sinensis* was not distinct. The frequency of isolates revealed that *C. gloeosporoides* was the dominant species within the non-resinous wood, while *Fusarium* sp. was an absolute advantageous species in resinous wood. Remarkably, it is less likely to find *Cyindrocladium* sp. in different tissues of *A. sinensis* based on culture-based methodology.

To further explore the endophytic fungi actual role in agarwood formation, pinholes-infusion method was conducted by fungal isolates. Previous researches on agarwood inoculating experiments were all based on the traditional large wound method. This method involves cutting a large wound and inoculating fungi. Several years later, resin form on the surface of the wounded wood. The only way to improve the yield is to enlarge the area of wound as much as possible. It is thought that the formation of agarwood may be the result of interaction of

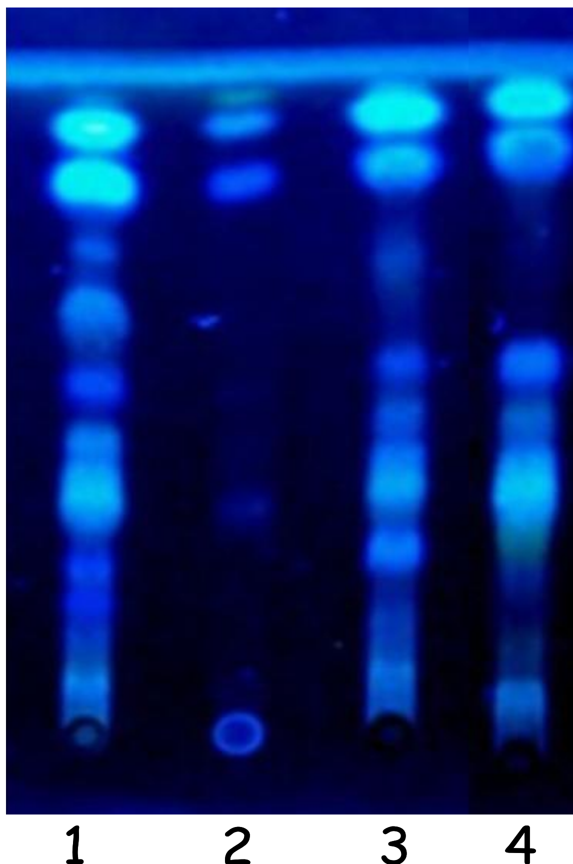

Figure 11. TLC spectrum of artificial agarwood induced by formic acid and infected by *Botryosphaeria dothidea*. Lane 1, A9 tree after inoculation 12 months; lane 2, A9 tree after inoculation eight months; lane 3, 1st grade Hainan agrawood; lane 4, 3rd grade Guangdong agarwood.

several fungi rather than the effect of a single fungus (Susann et al., 1997), though the mechanism of agarwood formation cannot be clearly defined through this large wound method. Therefore, we employed pinholes - infusion method to overcome aforementioned limitations. One to two years after the inoculation, resin was seen around the hole and branches of the trees. The results reveal that chemically stimulated with formic acid and infected by *B. dothidea* produced high yield artificial agarwood (10.2 to 11.8% ethanol-soluble extraction content) in a relatively short time. This indicates that acid and *Botryosphaeria* spp. might play an important role in resin formation, or perhaps have a role at different times.

Research on the mechanism of agarwood formation focused on fungal invasion, wounding and elicitor induction, and other aspects (Gong and Guo, 2009; Mohamed et al., 2010; Kumeta and Ito, 2010). In this study, the role of the chemical and fungal elicitor in agarwood formation was revealed based on the investigation of the variation of endophytic fungi community, combined with the dynamic regularity of chemical composition. The different production and quality of artificial agarwood were collected from the *A. sinensis* trees by different chemical and fungal elicitor induced. Our results suggest that the mechanism of agarwood formation based on the critical chemical and fungal elicitor-induced needs to be further studied and a more efficient artificial technique to increase the production and improve the quality of agarwood needs to be developed.

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