available at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/funeco

Molecular phylogenetic identification of endophytic fungi isolated from resinous and healthy wood of *Aquilaria malaccensis*, a red listed and highly exploited medicinal tree

K. PREMALATHA*, A. KALRA

Department of Microbial Technology and Entomology, CSIR — Central Institute of Medicinal and Aromatic Plants, Lucknow 226 015, Uttar Pradesh, India

ARTICLE INFO

Article history:
Received 12 September 2012
Revision received 30 October 2012
Accepted 23 January 2013
Available online 19 March 2013
Corresponding editor:
Kevin Hyde

Keywords:
Agarwood
Aquilaria malaccensis
Endophytes
ITS rRNA gene
Phylogeny

ABSTRACT

Aquilaria malaccensis is a fast-growing, tropical tree belonging to the family Thymelaeaceae and is locally known as Agar. Agarwood formation takes place in the stem or main branches of the tree where an injury has occurred. It is believed that the tree is first attacked by a pathogenic fungus, which causes it to weaken. Most fungal diversity studies have previously been based on morphological examination and cultivation methods. In this study, we used both culture-dependent and culture-independent approaches (metagenomic) to study the endophytic fungi on wood chips of *A. malaccensis*. The culture-based approach revealed *Alternaria*, *Cladosporium*, *Curvularia*, *Fusarium*, *Phaeoacremonium* and *Trichoderma* as members of the agarwood community. Also analysis of ITS sequencing of these culture isolates provided further verification of the identity of the cultured groups. Analysis of community DNA (metagenome) extracted from both infected and healthy wood samples revealed that the majority of fungi present had highest sequence similarity to members of Dothideomycetes, followed by Sordariomycetes and Saccharomycetes. Thus, morphological and genetic characteristics showed that most isolates from agarwood belong to phylum Ascomycota. A neighbour-joining tree showed the relationships between the isolates sequence data and the closest identified relatives from GenBank.

Introduction

The genus *Aquilaria* of the Thymelaeaceae (Daphne family) consists of generally fast-growing trees found in lowland tropical forests. Natural populations of *Aquilaria malaccensis* are widely distributed in South and South East Asia. Oldfield *et al.* (1998) listed Bangladesh, Bhutan, India, Indonesia, Iran, Malaysia, Myanmar, Philippines, Singapore and Thailand as locations for this species. India is home to the following three *Aquilaria* spp., *Aquilaria khasiana*, *Aquilaria macrophylla* and *A. malaccensis*. While *A. macrophylla* is restricted to the Nicobar Islands (Anon 2003), *A. khasiana* is limited to the Khasi Hills of Meghalaya (Kanjilal *et al.* 1982). *A. malaccensis* occurs mostly in the foothills of the north eastern region (Assam, Arunachal Pradesh, Nagaland, Meghalaya, Mizoram, Manipur, Tripura and Sikkim) and West Bengal (Barden *et al.* 2000).

There are many names for the resinous, fragrant heartwood produced primarily by trees in the genus *Aquilaria*. The common names include agarwood, aloeswood, eaglewood, gaharu or oudh, and it is also mentioned in the Old Testament

* *Corresponding author.*
E-mail addresses: k.premalatha@cimap.res.in, premalatha1977@gmail.com (K. Premalatha).
1754-5048/$ — see front matter © 2013 Elsevier Ltd and The British Mycological Society. All rights reserved.
http://dx.doi.org/10.1016/j.funeco.2013.01.005

as 'aloe' or 'ahaloth'. The Agar tree produces a resin as a defence mechanism against infection or injury – this is valued in many cultures for its distinctive fragrance, and is used as a principal component in incense and perfumes as well as in traditional medicine (Saikia & Khan 2012). This valuable and highly fragrant wood has been used in many ways for over 2000 yr, especially as incense in Buddhist, Hindu and Islamic traditional ceremonies, and as a significant component of traditional Ayurvedic, Tibetan and Far Eastern medicine and Middle Eastern perfumes. For hundreds of years, agarwood has been harvested from forests, and this has resulted in decline in the number of *Aquilaria* trees.

Large-scale harvesting of *A. malaccensis* has resulted in rapid depletion of the species in its natural habitats. It has been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 1994), to bring international trade within sustainable levels. *A. malaccensis* has also been included in the World List of Threatened Trees (Oldfield *et al.* 1998), and is highly threatened in India due to exploitation of the species for commercial purposes (Chakrabarty *et al.* 1994). The species is 'vulnerable' globally, considered 'critically endangered' in India (IUCN 2009), and almost 'extinct in wild' in Assam (Anon 2003). One way to conserve this valuable tree taxon would be to produce agarwood in a sustainable manner by mass planting the trees and collecting agarwood in a non-destructive manner. This also includes inducing agarwood formation in trees using artificial methods with the help of microorganisms (Mohamed *et al.* 2010).

Plants serve as a repository of untold numbers of organisms known as endophytes (Hoffman & Arnold 2008; Botella & Diez 2011; Rivera-orduña *et al.* 2011; Sun *et al.* 2012). Endophytes are microorganisms that establish an endosymbiotic relationship with plants, whereby plants receive ecological benefits from the presence of the symbionts, such as the ability to increase tolerance to stresses or plant growth promotion and can be isolated from surface-sterilised plant parts or from the inner tissues of plants (Oses *et al.* 2008; Huang *et al.* 2009; Nimnoi & Pongsilp 2009; Saikkonen *et al.* 2010; Purahong & Hyde 2011; Vesterlund *et al.* 2011). Many endophytes have been demonstrated to improve and promote the growth of the host plants as well as reduce disease symptoms caused by plant pathogens or various environmental stresses (Hasegawa *et al.* 2006; Giordano *et al.* 2009; Nimnoi & Pongsilp 2009; Rocha *et al.* 2011; Hamilton & Bauerle 2012; Hamilton *et al.* 2012).

Endophytic fungi have previously been identified based on morphological characters on artificial media (Hyde & Soytong 2007, 2008). Despite the advances in identifying endophytes by enhanced cultural techniques, studies are still flawed by the fact that fast-growing fungi will be isolated preferentially while unculturable fungi and slow-growing fungi will escape detection (Duong *et al.* 2006). Culture-independent methods for screening fungal diversity from natural samples are, therefore, necessary (Guo *et al.* 2001; Lim *et al.* 2005). With this in mind, Guo *et al.* (2001) developed a technique using direct amplification of rDNA extracted from frond tissue of *Livistona chinensis* followed by cloning, sequencing and phylogenetic analysis to identify endophytic fungi. They successfully recovered some endophytic fungi that had not previously been isolated in cultural studies. Therefore, a combination of these two approaches may provide deeper insight into diversity assessment (Jebaraj *et al.* 2010; Purnima *et al.* 2012).

The present work was carried out to determine the fungal endophyte community in wounded and healthy wood of *A. malaccensis*, using both culture dependent and independent (metagenomic) approaches.

Materials and methods

Source of plant material

Samples of mature agarwood trees were collected from Jorhat, Assam, India. Two types of wood samples were taken: (1) infected wood containing resin (B) and (2) healthy wood (W). All the collected infected wood samples emitted fragrance when they were burnt, confirming production of resin. All samples were tagged, stored at 4 °C in a clean plastic bag, and taken to the laboratory for isolation of endophytic fungi.

Fungal isolation and identification

Isolation of the endophytic fungi was performed based on the procedures described by Xu *et al.* (2008). The cleaned samples were cut into about 5 mm × 5 mm × 5 mm cubes and then surface-disinfected by washing in 75 % ethanol for 1 min, sterile distilled water twice, 0.05 g ml^{-1} sodium hypochlorite solution for 3 min followed by several rinses in sterile distilled water. The surface-sterilised samples were placed on plates with potato dextrose agar medium (PDA, HiMedia), prepared according to the manufacturer's instruction. The antibiotics streptomycin sulphate and chloramphenicol, were added each to a final concentration of 50 μg ml^{-1}, to inhibit bacterial contamination. For each sample type five wood chips were plated. Plates were incubated at 28 °C for 7–14 d. When colonies appeared, they were subcultured onto fresh plates containing the same medium and allowed to grow for 14 d before they were subjected to morphological examination. To examine fungal structures, the culture was transferred into a drop of 0.01 % cotton blue in 60 % lactic acid on a microscope slide using a sterile needle and observed under a light microscope.

DNA extraction

Genomic DNA was extracted from mycelium collected from 7 to 14 d old cultures growing on PDA agar plates, using the DNeasy Plant Mini Kit (Qiagen, USA). A total of 0.2 mg fungal mycelium was disrupted using a mortar and pestle in liquid nitrogen. Powdered sample was transferred to a 1.5 ml microcentrifuge tube and processed according to the manufacturer's protocol. Isolated DNA from isolates was stored at −20 °C for further use.

Total DNA was extracted directly from wood chips using a modified CTAB procedure as outlined by Duong *et al.* (2006). Each type of wood sample (B, W) was separately ground into powder with liquid nitrogen. Fifty milligrammes of leaf powder was placed in a sterile 1.5 ml tube. 650 μl of pre-heated 2× CTAB was then added, followed by 1 hr incubation at 65 °C.