Isolation and identification of bacterial endophytes from pharmaceutical agarwood-producing *Aquilaria* species

Subhash J. Bhore, Jagadesan Preveena, Kodi I. Kandasamy[1,2]

Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Bedong Semeling Road, Semeling 08100, Kedah, [1]Tissue Culture Laboratory, Forest Research Institute Malaysia, 52109 Kepong, Selangor, [2]Malaysian Biotechnology Corporation, Level 23, Menara Atlan, 161B, Jalan Ampany, 50450 Kuala Lumpur, Malaysia

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ABSTRACT

Background: Resins and gums are used in traditional medicine and do have potential applications in pharmacy and medicine. Agarwood is the fragrant resinous wood, which is an important commodity from *Aquilaria* species and has been used as a sedative, analgesic, and digestive in traditional medicine. Endophytic bacteria are potentially important in producing pharmaceutical compounds found in the plants. Hence, it was important to understand which types of endophytic bacteria are associated with pharmaceutical agarwood-producing *Aquilaria* species. **Objective:** This study was undertaken to isolate and identify endophytic bacteria associated with agarwood-producing seven (7) *Aquilaria* species from Malaysia. **Materials and Methods:** Botanical samples of seven *Aquilaria* species were collected, and endophytic bacteria were isolated from surface-sterilized-tissue samples. The 16S rRNA gene fragments were amplified using PCR method, and endophytic bacterial isolates (EBIs) were identified based on 16S rRNA gene sequence similarity based method. **Results:** Culturable, 77 EBIs were analyzed, and results of 16S rRNA gene sequences analysis suggest that 18 different types of endophytic bacteria are associated with (seven) *Aquilaria* species. From 77 EBIs, majority (36.4%) of the isolates were of *Bacillus pumilus*. **Conclusion:** These findings indicate that agarwood-producing *Aquilaria* species are harboring 18 different types of culturable endophytic bacteria.

Key words: 16S rRNA, biodiversity, endophytes, Malaysia, medicine, natural products, ribosomal DNA, traditional medicine

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INTRODUCTION

Naturally produced resins by plants has been used in traditional medicine around the world from the earliest times and have several potential applications in pharmacy and medicine.[1] *Aquilaria* species (family: Thymelaeaceae) are known to produce dark resinous heartwood, known as agarwood.[2] This agarwood is also known as Oud, Oodh, and Agar etc. Agarwood-producing *Aquilaria* species includes *Aquilaria beccariana* van Tiegh., *A. crassna* Pierre,[2,3] *A. hirta* Ridl., *A. malaccensis* Lamk.,[4] *A. microcarpa* Baill.,[5,6] *A. sinensis* (Lour) Gilg,[7] and *A. subintegra* Ding Hou. Currently, there is a huge demand for agarwood, and Indonesia, Malaysia, Thailand, and Vietnam are the major producers of agarwood.

Address for correspondence:
Dr. Subhash J. Bhore, Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Bedong-Semeling Road, Semeling 08100, Kedah, Malaysia.
E-mail: subhashbhore@gmail.com, subhash@aimst.edu.my

As stated by Lardos *et al.* (2011), agarwood is known to have many pharmacological functions that include analgesic, anti-inflammatory, anti-microbial, immunomodulatory, and wound healing properties.[1] Agarwood is also used as a digestive in traditional medicine, and its medico-pharmacological analysis using model mice indicated the laxative effect.[8,9] Furthermore, the essential oil obtained from agarwood is also known to have anti-microbial properties.[7]

Endophytes are microorganisms that live inside plants (inter or intra cellular in nature) without causing any plant disease. Although its true functions in the plants are poorly understood, reports suggest that bacterial endophytes can produce bioactive compounds found in their host[10] and have potential in providing new drugs,[11] plant hormones,[12] and novel natural products.[13] As a part of a broad study to explore bacterial endophytes for its various potential applications, research work on isolation, identification, and characterization of bacterial endophytes was initiated at AIMST University. The isolated and identified bacterial

endophytes of the economically and pharmaceutically important agarwood-producing (seven) *Aquilaria* species have been reported in this paper.

MATERIALS AND METHODS

Chemical materials

Nutrient medium, bacto-agar powder, and agarose-gel powder were purchased from Sisco Research Laboratories Pvt. Ltd. MEGAquick-spin™ PCR, and agarose-gel DNA Extraction System was procured from NHK Bioscience Solutions Sdn. Bhd. PCR components were purchased from Fermentas and SBS Genetech, and DNA markers were supplied by Dongsheng Biotechnology Pte. Ltd., China. Forward [Bak11W-F; 5'-AGT TTG ATC MTG GCT CAG-3'] and reverse [Bak-R; 5'-GGA CTA CHA GGG TAT CTA AT-3'] primers used in the study were supplied by First Base and SBS Genetech, and all other chemicals were procured from the Sigma-Aldrich Corporation (St. Louis, MO), USA.

Plant materials

Stem and leaf (along with their petiole) samples of *A. beccariana*, *A. crassna*, *A. hirta*, *A. malaccensis*, *A. microcarpa*, *A. sinensis*, and *A. subintegra* were collected. All seven *Aquilaria* species in sampling were from the plants collection of Forest Research Institute of Malaysia (FRIM), Malaysia.

Surface-sterilization of plant material samples

The surface-sterilization of the collected botanical samples was carried out as described elsewhere.[12] The surface sterilized stem, leaf, and petiole tissues were used in isolation of EBIs.

Isolation and identification of bacterial endophytes

The isolation of EBIs, amplification of 16S rRNA gene fragments, and identification of the EBIs was carried out as described by Bhore *et al.*[12]

RESULTS

As a result of botanical samples' incubation, culturable bacterial endophytes were able to grow on nutrient agar. Seventy-seven (77) EBIs were isolated from the seven *Aquilaria* species, and their pure cultures were examined. From the 77 EBIs, PCR-amplified 16S rRNA gene fragments were sequenced, and all isolates were identified as a result of nucleotide blast (megablast) hits analysis.

Seventy-seven EBI's annotated 16S rRNA gene fragments nucleotide sequences have been submitted to the GenBank/DDBJ/EMBL under accession numbers: JF819666-JF819685, and JF938917-JF938973.

Analysis of identified 77 EBIs revealed that agarwood-producing 7 *Aquilaria* species are harboring 18 different

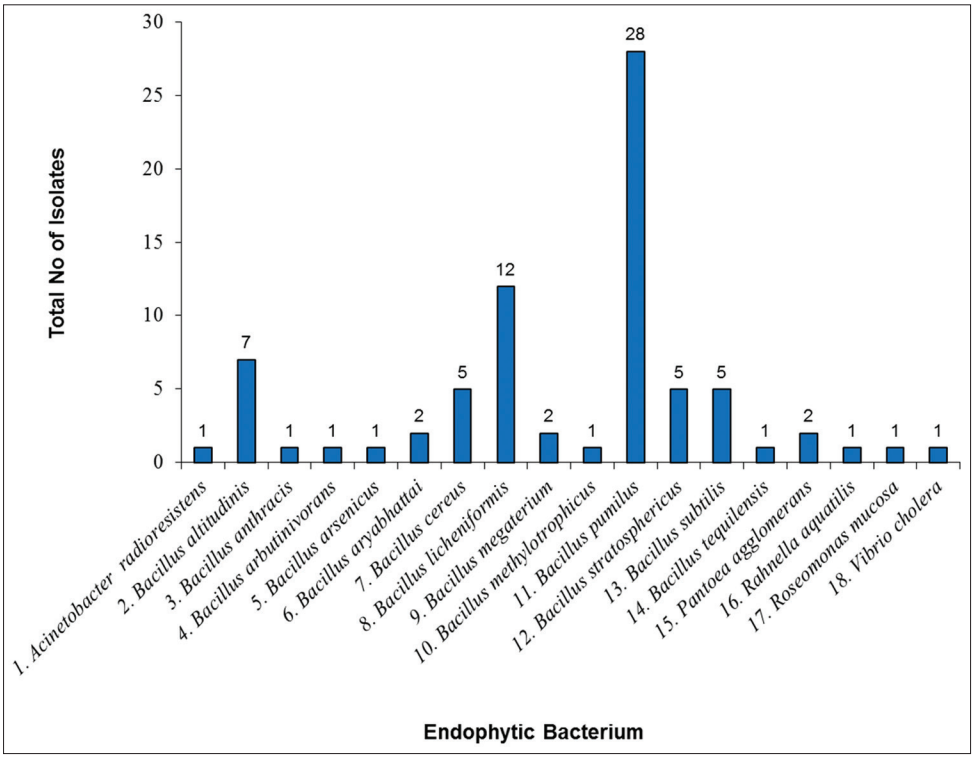

Figure 1: Eighteen (18) types of bacterial endophytes found in agarwood-producing *Aquilaria* species and total number of bacterial endophytes isolates representing each type.

types of bacterial endophytes. Figure 1 show the total number of isolates of identified 18 types of bacterial endophytes, and the identified bacterial endophytes and their respective host (*Aquilaria*) species are depicted in Table 1.

DISCUSSION

Almost every plant on the earth hosts endophytic bacteria that could serve as potential source of novel natural products, which are of a great potential not only in medicine but also in various other sectors of the biotechnology industry.[13,14] However, endophytes from medicinally important plants are of a great interest, especially in understanding their potential medicinal properties and to explore their potential applications.[10,15] In this study, we isolated and identified 77 strains of bacterial endophytes from seven agarwood-producing *Aquilaria* species. Likewise, bacterial endophytes have been reported from various medicinal plants; for examples, *Gynura procumbens*, *Piper nigrum*, *Strobilanthes crispa*, and *Vernonia amygdalina*.[12,16,17] However, this is the first study to elucidate diverse types of bacterial endophytes in (seven) agarwood-producing *Aquilaria* species.

The 16S rRNA gene sequence of each bacterium is species-specific, and hence can be used for accurate bacterial identification.[18] Thus, we amplified the 16S rRNA gene for rapid and precise identification of the isolated EBIs. The 16S rRNA gene fragment sequences comparison from the 77 isolated strains with the sequences from nucleotide sequence database of GenBank/DDBJ/EMBL using blast revealed the identity of these isolates. The 16S rRNA gene sequence similarity % was >95%, except for one isolate, where the similarity % was only 82%. This isolate (from *A. subintegra*) was putatively identified as *Pantoea agglomerans* (Accession no: JF819683) and need further verification.

Though 9 species of the bacterial endophytes are represented by only one isolate, it cannot be inferred that they are respective *Aquilaria* species-specific, because sample numbers used in the study were limited (in number) and samples were from only one plant of each *Aquilaria* species. The seasonal fluctuation of the endophytes has been reported in other plant species;[19,20] hence, it is possible that various other types of bacterial endophytes might be also associated with agarwood-producing *Aquilaria* species. In addition, it should be noted that soil type in which plants are growing can influence the diversity of bacterial endophytes in plants.[21] Therefore, if *Aquilaria* species are collected from other locations, then some other types of bacterial endophytes could also be detected.

Bacterial endophytes can produce novel natural products found in their host plant;[10] and therefore, bacterial endophytes are potential sources of the novel natural products including novel antibiotics. The anti-microbial (anti-bacterial, anti-fungal, and anti-viral) activities of some bacterial endophytes has also been reported by other researchers.[22-28] Similarly, it has been reported that the endophytic fungi associated with agarwood have potential anti-microbial and anti-tumor activity.[29] Therefore, further research is needed in order to explore the potential applications of the isolated bacterial endophytes. Furthermore, the in-depth understanding of

Table 1: Seven (7) agarwood-producing *Aquilaria* species and their bacterial endophytes as revealed by 16S rRNA gene sequence similarity based method of bacterial identification

No	Bacterial Endophyte	Host *Aquilaria* species*						
		Ab	Ac	Ah	Ama	Ami	Asi	Asu
1	*Acinetobacter radioresistens*						+	
2	*Bacillus altitudinis*			+		+	+	+
3	*Bacillus anthracis*		+					
4	*Bacillus arbutinivorans*				+			
5	*Bacillus arsenicus*	+						
6	*Bacillus aryabhattai*			+				
7	*Bacillus cereus*			+	+	+		
8	*Bacillus licheniformis*	+	+		+	+		+
9	*Bacillus megaterium*			+			+	
10	*Bacillus methylotrophicus*		+					
11	*Bacillus pumilus*	+		+	+	+	+	+
12	*Bacillus stratosphericus*			+	+			+
13	*Bacillus subtilis*			+	+	+		
14	*Bacillus tequilensis*			+				
15	*Pantoea agglomerans*[niv]							+
16	*Rahnella aquatilis*			+				
17	*Roseomonas mucosa*	+						
18	*Vibrio cholera*				+			

*Ab=*A. beccariana*; Ac=*A. crassna*; Ah=*A. hirta*; Ama=*A. malaccensis*; Ami=*A. microcarpa*; Asi=*A. sinensis*; Asu=*A. subintegra*; [niv]need identity verification; Sign '+' indicate presence of respective bacterial endophyte

symbiotic association between *Aquilaria* species and their bacterial (and fungal) endophytes could be helpful in the protection of threatened *Aquilaria'* species.[30]

On the basis of the results obtained, it could be concluded that agarwood-producing *Aquilaria* species are harboring diverse 18 types of culturable bacterial endophytes. However, the benefits of these 18 bacterial endophytes to *Aquilaria* species are not clearly understood. We hypothesize that isolated bacterial endophytes might be useful to its respective host *Aquilaria* species and might be producing economically and pharmaceutically important bioactive compounds. Nonetheless, our research findings could be useful, as a foundation for further research on both the agarwood-producing *Aquilaria* species, as well as its endophytic bacteria.

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