Volatile Constituents of Endophytic Fungi Isolated from Aquilaria sinensis with Descriptions of Two New Species of Nemania

Saowaluck Tibpromma, Lu Zhang, Samantha C. Karunarathna, Tian-Ye Du, Chayanard Phukhamsakda, Munikishore Rachakunta, Nakarin Suwannarach, Jianchu Xu, Peter E. Mortimer

Abstract: Algae, bacteria, and fungi, as well as higher plants, produce a wide variety of secondary metabolites known as natural products. Natural products are well known as remarkable sources of many therapeutic agents. The genus Nemania is a wood-decaying fungus that belongs to family Xylariaceae. Nemania is often found as an endophyte in diverse hosts and some species are known to produce useful secondary metabolites. In this study, two Nemania species were isolated as an endophytic fungus from Aquilaria sinensis. Multi-gene phylogenetic studies showed that the newly described strains of Nemania are new to science, and this is the first report of Nemania from the host Aquilaria. One of the fermented species, Nemania aquilariae (KUMCC 20-0268), resulted in five sesquiterpenoids, which were previously reported from agarwood, and their structures were identified by gas chromatography-mass spectrometry (GC-MS). In addition, five different media were investigated in vitro to optimize conditions for growing the fungal biomass of Nemania aquilariae and N. yunnanensis.

Keywords: agarwood; chemical constituents; endophytic fungi; GC-MS analysis

1. Introduction

The genus Aquilaria Lam., belonging to the family Thymelaeaceae, consist of 31 accepted species according to the International Union for Conservation of Nature (IUCN) red list of threatened species [1], and 19 of them are recognized as agarwood-producing species [2–8]. Aquilaria subintegra Ding Hou, A. malaccensis Lam., A. crassa Pierre ex Lecomte, and A. sinensis (Lour.) Spreng. are major species capable of producing agarwood, which contains economically important essential oils [9]. At present, two native Aquilaria
species, viz., *A. sinensis* and *A. yunnanensis* S. C. Huang, have been widely cultivated in Southeast Asia, while *A. sinensis* is primarily planted in southern China. The resinous heartwood of *A. sinensis* is well known for its medicinal importance in traditional Chinese medicine (TCM), named ChenXiang [10–13].

Heartwood contains resin-impregnated fragrant wood that is extremely valuable and in high demand throughout the world [14,15]. Healthy *Aquilaria* trees can only produce agarwood after being subjected to damaging events [3,5,16–18]. In natural forests, agarwood formation occurs slowly and infrequently in old trees, and only 7–10% of *Aquilaria* trees contain agarwood. When compared with market demand, the supply of agarwood from wild sources is severely inadequate. Unfortunately, indiscriminate felling of trees and overharvesting in hopes of finding the treasured resin have led to the severe depletion of wild trees and other negative impacts on biodiversity [19]. As a result, eight *Aquilaria* species are now listed on the IUCN red list as endangered species [1,20].

Many artificial induction approaches, viz., chisel nails, burning, trunk breaking, and bark removal, for the development of agarwood via traditional methods have been developed but these methods are slow and produce poor-quality agarwood [3,21,22]. Several techniques inducing agarwood production are described in Tan et al. [23]. So far, more than 300 compounds have been isolated and reported from agarwood and *Aquilaria* trees [24,25]. Fungal inoculum development in *Aquilaria* trees first began in 1929 [26]. Later, several researchers isolated fungi from naturally occurring *Aquilaria* trees in the wild (using healthy or diseased parts) to investigate the role of fungi in agarwood formation, finding that most of the isolated fungi were endophytes [22]. Fungi are some of the organisms involved in inducing agarwood formation, and fungal culture for inoculum can be “pure” or “mixed” [22]. For instance, *Fusarium laseritum*, *Lasiodiplodia theobromae*, and *Menanotus flavolives* are able to promote agarwood formation [25–27]. Outcomes may vary between fungal strains and across sites when they are applied.

The genus *Nemania* is an endophytic fungal genus that has been reported on several hosts. Endophytic fungi of *Nemania* have shown interesting applications associated with its bioactive compounds on many hosts [28–32]. The pattern of *Nemania* geographic distribution from 1979–2020 is shown in Figure 1 [33]. This genus is distributed mostly in Europe (Denmark, Sweden, and the United Kingdom), Australia, and North America, while only few specimens have been recorded from Asia, Africa, and South America. This clearly shows that *Nemania* species are more diverse in temperate zones than tropical zones.

![Figure 1. Nemania collection and distribution. High, moderate, and low Nemania samples’ collection is indicated in red to yellow gradient hexagons.](image)

The aim of this study was to isolate two endophytic fungi from the resin of *Aquilaria sinensis* collected from Yunnan Province, China. Multi-gene phylogenetic analyses showed two endophytic fungi are new species of *Nemania*. Besides, one of the species *N. aquilariae* fungally ferments was investigated for how its volatile organic compounds are formed,
which is related to eventually forming agarwood, which was confirmed by GC-MS method. In addition, we optimized the best media for production of fungal biomass yields of the newly described strains’ isolates in vitro.

2. Materials and Methods

2.1. Sample Collection, Fungal Isolation, Preparation of Cultures, and Production of Fungal Biomass

Endophytic fungi species were isolated from dark resinous heartwoods of *Aquilaria sinensis* collected from Xishuangbanna Dai Autonomous Prefecture (N 21°44′38″, E 100°21′36″), Yunnan Province, China. Pieces of agarwood were burned to verify the presence of the agarwood fragrance before being stored in ice boxes and transported to the Kunming Institute Botany laboratory. Samples were cleaned under running tap water to remove dust and then air dried. Samples were cut into 0.5-cm, circular-shaped pieces. The surface of each sample was disinfected by being soaked in 75% ethanol for 1 min, 3% sodium hypochlorite solution for 2 min, and 75% ethanol for 30 s, followed by three rinses in sterile distilled water before finally being dried on sterile tissue papers [34]. All sections were placed in potato dextrose agar plates (PDA, Oxoid, Basingstoke, UK) and incubated at 28 °C for 1–3 days. Hyphal tips of fungal colonies appeared during incubation, so the colonies were transferred to new PDA plates and incubated to obtain pure cultures. New fungal taxa were examined in the pure culture, and photographs, morphological characteristics, and descriptions were completed.

For production of fungal biomass, fresh cultures of *Nemania aquilariae* and *N. yunnanensis* were inoculated into the following five liquid broth media (without agar): CzapekDox broth (CDB, oxoid), malt extract broth (MEB, oxoid), potato dextrose broth (PDB, oxoid), Richard broth (RB), and Sabouraud’s broth (SB). Broth media (100 mL) were prepared according to the manufacturer’s instructions, poured into clean, 150-mL flasks, covered with cotton lids and aluminum foil on the top, and sterilized via autoclaving at 121 °C for 30 minutes. The pure cultures (14 days old) on PDA were cut out near the margin by a 0.5-cm-diameter, sterilized cork borer. Five culture disks were transferred to each media flask (triplicate) under aseptic conditions. The flasks were inoculated at 28 °C on a rotary shaker at a speed of 120 rpm for seven days [35]. After seven days of incubation, mycelial masses were harvested via filtration through Grade 1 Whatman filter paper No. 1 (Madison, Walton-on-Thames, UK) (initial weight of the filter papers was recorded prior to use), dried at 40–45 °C for 24 h, weighed of biomass, and recorded. Three replicates of biomass in various liquid media were carried out for each treatment, and data are the average of these three assays. Statistical analyses were performed using one-way ANOVA and the Mann–Whitney ranks sum test. Graphs and statistical analyses used Sigmaplot version 12.5 (Systat, San Jose, CA, USA). Analysis of variance of \( p \leq 0.05 \) was used as the threshold for significance. Herbarium specimens were prepared from cultures that were dried in silica gel. The holotypes were deposited in Kunming Institute of Botany Academia Sinica (HKAS), Kunming, China. The ex-type cultures were deposited in the Kunming Institute of Botany culture collection (KMUCC). New taxa were registered in Facesoffungi (FoF) [36] and Index Fungorum [37]

2.2. Genomic DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was isolated from pure fungal cultures using the Biospin Fungus genomic DNA extraction kit-BSC14S1 (Bioflux, Kunming, China). Polymerase chain reaction (PCR) was used to amplify partial gene regions of Internal Transcribed Spacers (ITS), 28S ribosomal RNA (LSU), RNA polymerase II second largest subunit (RPB2), beta-tubulin (BT), and actin (ACT), using primers shown in Table 1. Total volume of PCR mixtures for amplifications was 25 μL [38]. Purification and sequencing of PCR products were performed by TsingKe Biotech, Kunming, Yunnan, China.
Table 1. Primer names, sequences, and references.

| Gene | Primer | Primer Sequence | References |
|------|--------|-----------------|------------|
| ITS  | ITS5   | 5′-DDAAGTAAAGAGTCGTAACAAGG-3′ | [39]       |
|      | ITS4   | 5′-TCTTCGCTTATCATATGC-3′       |            |
| LSU  | LROR   | 5′-ACCCGCTGAACCTAAC-3′         | [39]       |
|      | LR5    | 5′-TCTCGAGG-GAACTTC-3′         |            |
| RPB2 | RPB2-5F| 5′-GGGGWGYACAGAAGAGCC-3′       | [40]       |
|      | RPB2-7cR| 5′-CCCCATRGCTTGGYTRCCCAT-3′  | [41]       |
| BT   | T1     | 5′-AACATGCCGAGATTGIAAGT-3′     | [42]       |
|      | T22    | 5′-TCTGAGAATGTTGCAGAATC-3′     |            |
| ACT  | 512F   | 5′-ATGTCACAAGGGCCGGTTGCCG-3′   | [43]       |
|      | 783R   | 5′-TACGAGTCTTCTGGCCCAT-3′      |            |

2.3. Phylogenetic Analyses

Sequence data generated in this study were checked for the quality of chromatograms, and raw forward and reverse sequences were assembled using Geneious Pro.v4.8.5. Subjected to Basic Local Alignment Search Tool (BLAST) searches in the nucleotide database of GenBank (http://blast.ncbi.nlm.nih.gov, accessed on 16 April 2021) to determine their most probable closely related taxa. Sequence data were retrieved from GenBank based on BLAST searches and recent publications [44]. Sequence alignments were carried out through MAFFT v.6.864b [45] and the alignments were manually improved where necessary. Sequence data sets were combined using BioEdit [46].

Dissyanake et al. [47] were followed for construction of the combined phylogenetic trees using maximum likelihood (ML) and Bayesian Inference posterior probabilities (BYPP). The GTR+I+G model of nucleotide substitution and searches for model selected for ML were applied. Bootstrap supports were obtained by running 1000 pseudo-replicates. Bayesian Inference analysis was conducted when two parallel runs were performed using the default settings in addition to the following adjustment: Six Markov chains were run simultaneously for 5,000,000 generations, trees were sampled every 100th generation, and 20% of trees representing the burn-in phase were discarded. The remaining 80% of trees were used to calculate probability proportional to size (PPs). Bootstrap support values for ML and BYPP were given next to each node in the phylogenetic trees (Figure 2), which were configured in Fig Tree v1.4.0 [48] and edited using Microsoft Office PowerPoint 2019 and Adobe Photoshop CC 2019 (Adobe Systems, San Jose, CA, USA). Sequences of the new strains generated in this study were submitted to GenBank (Table 2).

Table 2. Names, isolate numbers, and GenBank accession numbers of the fungal taxa used for the phylogenetic analyses of this study.

| Species                        | Isolates | GenBank Accession Numbers |
|--------------------------------|----------|---------------------------|
| Amphirosellinia fushanensis    | HAST 91111209 | GU339496, N/A, GQ848339, GQ495950, GQ453260 |
| Amphirosellinia nigrospora     | HAST 91092308 | GU322457, N/A, GQ848340, GQ495951, GQ453260 |
| Astrocytis bambusae            | HAST 89021904 | GU322449, N/A, GQ84836, GQ495942, GQ449239 |
| Astrocytis mirabilis           | HAST 94070803 | GU322448, N/A, GQ84835, GQ495941, GQ449238 |
| Astrocytis sublimbata          | HAST 89032207 | GU322447, N/A, GQ84834, GQ495940, GQ449236 |
| Barrmaelia rhamnicola          | BR1       | MF488991, MF488991, MF488900, MF489019, N/A |
| Barrmaelia rhamnicola          | CBS 142772 | MF488990, MF488990, MF488999, MF489018, N/A |
| Brunneiperidium gracilentum    | MFLUCC 14-0011 | KP297400, KP340542, KP340528, KP406611, N/A |
| Brunneiperidium involucratum    | MFLUCC 14-0009 | KP297399, KP340541, KP340527, KP406610, N/A |
| Collodiscula bambusae          | GZU H0102  | KP054279, KP054280, KP276675, KP276674, N/A |
| Species                        | Isolates          | GenBank Accession Numbers |
|-------------------------------|-------------------|---------------------------|
|                               |                   | ITS | LSU | RPB2 | BT | ACT |
| Collodiscula fangjingshanensis| GZU H0109         | KR002590 | TR002591 | TR002592 | TR002589 | N/A |
| Collodiscula japonica         | CBS 124266        | N/A | M874889 | K624273 | K624316 | N/A |
| Dematophora buxi              | JDR 99           | GU00070 | N/A | G844780 | 70228 | N/A |
| Dematophora necatrix          | CBS 349.56       | MH855581 | K719204 | K624275 | K624310 | N/A |
| Entoleuca mammata             | JDR 100          | GU00072 | N/A | G844782 | 70230 | Q389230 |
| Euepityval sphaeriostomum     | JDR 261          | GU29821 | N/A | G844774 | 70224 | Q389696 |
| Kretzschmaria deusta          | CBS 163.93       | KC477237 | K610458 | K624227 | K71251 | N/A |
| Kretzschmaria guyanensis      | HAST 89062903    | GU00079 | N/A | G844792 | 78214 | Q408901 |
| Nemania abortiva              | CBS 263          | M164828 | N/A | G844727 | 70221 | N/A |
| Nemania aenea var. aenea      | ATCC 60818       | N/A | N/A | N/A | N/A | N/A |
| Nemania aenea var. aureolatum | N2A              | A199248 | N/A | N/A | N/A | N/A |
| Nemania afgi. abortiva        | GAB028           | KY250393 | N/A | N/A | N/A | N/A |
| Nemania aquilariae            | KUMCC 20-026     | MW729422 | MW792420 | MW718911 | MW81142 | MW71889 |
| Nemania beaumontii            | HAST 405         | GU29821 | N/A | G844772 | 70222 | Q389694 |
| Nemania beaumontii            | FL0980           | JQ605608 | N/A | K684243 | 84161 | K684065 |
| Nemania bipapillata           | HAST 90080610    | GU29821 | N/A | G844771 | 70221 | N/A |
| Nemania bipapillata           | GQ523-03-02      | N/A | N/A | K852275 | 852276 | K852274 |
| Nemania chestersii            | J040024          | N/A | DQ440072 | D631949 | D840889 | N/A |
| Nemania diffusa               | FR AT-113        | DQ658238 | D400073 | D631947 | D840888 | N/A |
| Nemania illita                | 236 (JDR)        | N/A | N/A | G844770 | N/A | N/A |
| Nemania macrocarpa            | CBS 205-026     | MW729423 | MW792421 | MW718921 | MW811411 | MW71890 |
| Nemania maritima              | HAST 89120401    | N/A | N/A | G844775 | 70225 | Q389697 |
| Nemania maritima              | MFLU 16-1236     | MN047122 | MN078866 | N/A | N/A | N/A |
| Nemania phetchaburensis       | MFLU 16-1185     | MN047124 | MF615402 | N/A | N/A | N/A |
| Nemania plumbea               | 6540             | JQ846087 | N/A | N/A | N/A | N/A |
| Nemania pouzarii              | ATCC 2612        | KC477228 | N/A | N/A | N/A | N/A |
| Nemania primolucum            | YMJ 91102001     | N/A | N/A | G844776 | 05607 | 05592 |
| Nemania serpens               | CBS 679.86       | KU683765 | N/A | K684284 | 84188 | K684088 |
| Nemania viridis               | MFLU 17-2600     | MN047123 | MN017887 | N/A | N/A | N/A |
| Nemania yunnanensis           | KUMCC 20-0267    | MW729423 | MW792421 | MW718921 | MW811411 | MW71890 |
| Neoxylaria arencae            | MFLUCC 15-0292   | MT496747 | N/A | MT502418 | N/A | N/A |
| Neoxylaria jurusinensis       | 2024501 (HAST)   | GU322439 | N/A | G844825 | 95932 | Q438753 |
| Rosellinia aquila             | MUCL 31703       | KY610392 | KY610460 | KY624285 | K271253 | N/A |
| Rosellinia corticium          | MUCL 51693       | KY610392 | KY610461 | KY624229 | K271254 | N/A |
| Rosellinia merrillii          | 89112601 (HAST)  | GU300071 | N/A | G844781 | 70229 | Q389229 |
| Stilbohypoxylon elaeicola     | YMJ 173          | EF026148 | N/A | G844826 | 056016 | 056016 |
| Stilbohypoxylon elaeicola     | MFLUCC 15-0295a  | GU322440 | N/A | G844827 | 95933 | Q438754 |
| Stilbohypoxylon elaeicola     | MFLUCC 15-0295b  | MF088896 | N/A | N/A | N/A | N/A |
| Stilbohypoxylon elaeidis      | MT496745         | MT496755 | MT502416 | MT502420 | N/A | N/A |
| Stilbohypoxylon quisquisiarum | EF026119         | N/A | N/A | G853020 | 05605 | 05590 |
| Stilbohypoxylon quisquisiarum | EF026120         | N/A | N/A | G853021 | 05606 | 05591 |
| Species                          | Isolates | GenBank Accession Numbers |
|---------------------------------|----------|---------------------------|
| Stilbohypoxylon quisquiliarum   | PR39     | ITS: N/A, LSU: N/A, RPB2: N/A, BT: N/A, ACT: N/A |
| Stilbohypoxylon quisquiliarum   | JDR 173  | ITS: N/A, LSU: GQ844826, RPB2: EF025616, BT: N/A, ACT: N/A |
| Stilbohypoxylon quisquiliarum   | YMJ 89091608 | ITS: N/A, LSU: GQ853021, RPB2: EF025606, BT: EF025591 |
| Xylaria arbuscula               | CBS 126415 | ITS: KY610394, LSU: KY610463, RPB2: KY624287, BT: N/A, ACT: KX271257 |
| Xylaria bambusicola             | WSP 205  | ITS: N/A, LSU: GQ844802, RPB2: AY951762, BT: N/A, ACT: N/A |
| Xylaria discolour               | HAST 131023 | ITS: N/A, LSU: JQ087411, RPB2: JQ087414, BT: N/A, ACT: N/A |
| Xylaria grammica                | 479 (HAST) | ITS: N/A, LSU: GQ844813, RPB2: GQ487704, BT: N/A, ACT: GQ427197 |
| Xylaria hypoxylon               | CBS 122620 | ITS: KY610407, LSU: KY610495, RPB2: KY624231, BT: N/A, ACT: KX271279 |

Table 2. Cont.

Figure 2. Phylogram generated from RAxML analysis based on combined ACT, ITS, LSU, BT, and RPB2 sequence data. Related sequences were obtained from Dayarathne et al. [44]. Bootstrap support values for ML equal to or greater than 60% and BYPP from MCMC analyses equal to or greater than 0.90 are given above/below the nodes. The ex-type strains are indicated in bold. Newly generated sequences are indicated in red bold.
2.4. Volatile Compound Analysis

Volatile organic compounds (VOCs) analysis was performed using a headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS). The GC-MS was equipped with a HS-SPME 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (PDMS/CAR/DVB) extraction head 57328-U (Sigma-Aldrich, St. Louis, MO, USA) connected with a headspace bottle (40 mL, 5190-4000, Agilent Technologies, Santa Clara, CA, USA). The GC-MS analysis was performed using the Agilent 5975C VL GC/MSD System with the 7890A GC System equipped with a DB-5ms capillary column (50 m × 0.25 mm, 0.25 µm, Agilent Technologies, Santa Clara, CA, USA).

2.5. Headspace Solid-Phase Microextraction (HS-SPME) Conditions

Fungal species determined in this study were recultivated on PDA and transferred to 40-mL headspace vials containing 5 mL of PDA solid medium (triplicate). VOCs were extracted by SPME, while uninoculated PDA headspace vial was set as the blank control. The inocula were incubated in a constant temperature incubator at 28 °C for 14 days for solid-phase microextraction. The silicon cap of the vial was closed and conditioned for 250 °C for 30 min by its insertion into the GC injection port under helium atmosphere. The extraction head was inserted into the headspace bottle and adsorbed for 30 min at room temperature (22 °C).

2.6. GC-MS Analysis Conditions for the Analyzation of VOC Emissions

The GC-MS conditions were set as follows: The initial temperature was set at 80 °C for 1 min and programmed to increase the temperature at a rate of 20 °C min⁻¹ to 180 °C and then increase by 4 °C min⁻¹ to 230 °C for 2 min. The inlet temperature was 250 °C, the connection port temperature was 290 °C, and desorption was performed for 5 min. Helium with a purity exceeding 99.999% was used as the carrier gas; the flow rate was set at 1.0 mL min⁻¹ (splitless mode). The condition of mass spectrometry sources was set as connection temperature 280 °C. The ionization mode was electron ionization (EI), and the ionization temperature was 230 °C. The MS, four-stage rod temperature was 150 °C. Analyses were performed by setting the electron energy at 70 eV in full-scan mode (m/z 50–600). All identified components were quantified using NIST (National Institute of Standards and Technology) mass spectral database search and GC/MSD ChemStation data analysis software (Agilent Technologies, Santa Clara, CA, USA) and summarized as a percentage of relative peak area, shown in Table 3.

| No. | Name of the Active Constituent | Retention Time (min) | Molecular Formula (MF) | Molecular Weight (MW) | Relative Peak Area (% ± SD) Blank | Relative Peak Area (% ± SD KUMCC 20-0268) | Components Having Biological Properties (Based on CAS Data Only) |
|-----|--------------------------------|----------------------|------------------------|-----------------------|----------------------------------|-----------------------------------------|-------------------------------------------------|
| 1.  | Bicyclo[3.1.1]hept-3-ene-2-acetaldehyde, 4,6,6-trimethyl-, (1R,2R,5S) rel- | 10.198 | C₁₂H₁₉O | 178.27 | - | 20.52 ± 0.01 | - |
| 2.  | Alloaromadendrene | 10.436 | C₁₅H₂₄ | 204.35 | - | 4.16 ± 0.01 | Antibacterial and antimicrobial |
| 3.  | Naphthalene, 1,2,3,4,4a,5,6,7-octahydro-4a,8-dimethyl-2(1-methylene)- | 10.852 | C₁₅H₂₄ | 204.35 | - | 4.09 ± 0.01 | - |
| 4.  | Valencen | 11.149 | C₁₅H₂₄ | 204.35 | - | 43.75 ± 0.05 | - |
| 5.  | α-Selinene | 11.206 | C₁₅H₂₄ | 204.35 | - | 13.38 ± 0.01 | Antibacterial |

* : All the compounds have matching quality ≥80%, when compared with NIST mass spectral database.
3. Results

3.1. Phylogenetic Analyses

The data set consisted of 58 strains included in the combined sequence analyses, comprising 5193 characters with gaps (349 bp ACT, 1938 bp BT, 848 bp LSU, 1217 bp RPB2). Barrmaelia rhamnicola strains BR1 and CBS 142772 were used as outgroup taxa. Tree topology of the ML analysis was similar to the BYPP. The best scoring ML tree with a final likelihood value of ~54284.74586 was presented. The matrix had 2725 distinct alignment patterns, with 50.86% undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.234252, C = 0.278693, G = 0.245890, and T = 0.241164; substitution rates AC = 1.243781, AG = 4.000858, AT = 0.997311, CG = 1.026916, CT = 5.673379, and GT = 1.000000; and gamma distribution shape parameter $\alpha = 0.922891$.

Phylogenetic analyses (Figure 2) showed newly described taxa group with Nemania species, and this genus separated into three clades: Clade I, N. phetchaburiensis (MFLU 16-1185), Clade II N. beaumontii (strains HAST 405 and FL0980), and most other Nemania species in clade III. Based on multi-gene phylogenetic analyses, newly described taxa grouped in clade III, N. aquilariae (KUMCC 20-0268) clustered with N. primolutea (MJ 91102001) with high support (100% in ML, 1.00 in BYPP) and N. yunnanensis (KUMCC 20-0267), were well separated from other species in Nemania with good support from ML (76% ML) but moderate support from BYPP (Figure 2).

3.2. Taxonomy

3.2.1. Nemania Gray 1821

Nemania was erected for a heterogeneous assemblage of taxa by Gray [49] that belongs to family Xylariaceae. This genus is known to contain saprobes or endophytes from terrestrial or marine environments worldwide [50,51]. There are 55 records of Nemania in Species Fungorum [52].

3.2.2. Nemania aquilariae Tibpromma & Lu, sp. nov.

Index Fungorum number: IF558188; Facesoffungi number: FoF 09704; Figure 3H–N.

Etymology: name referring to the host genus Aquilaria, on which the fungus was found.

Holotype: HKAS 111935

Culture characteristics: Colonies on PDA at room temperature (25 °C) reaching 9 cm in two week; circular, yellow-white with white margin; flossy, velvety, and raised; yellow-brown from below. Generative hyphae simple-septate, sub-hyaline, cells with guttules, thick-walled, 1.5–4 µm wide. Not sporulating in culture (Oatmeal agar (OMA) and PDA).

Material examined: CHINA, Yunnan Province, Xishuangbanna, on dark resinous wood of Aquilaria sinensis (Lour.) Gilg (Thymelaeaceae), 1 May 2019, Lu Z, No. 30 (HKAS 111935, holotype); ex-type living cultures, KUMCC 20-0268.

Notes: Based on BLASTn searches of ACT, ITS, LSU, BT and RPB2 sequence data, Nemania aquilariae showed a high similarity to N. primolutea (ACT=98.62%(EF025592); ITS=99.65%(MG881830); BT=98.52%(EF025607), and RPB2=95.41%(GQ844767) while LSU showed high similarity to N. beaumontii 98.10% (MFL161217). In the multi-gene phylogeny, N. aquilariae clustered sister to N. primolutea with 100% in ML and 1.00 in BYPP statistical support (Figure 2). The newly described strain is an endophytic fungus, which does not sporulate in culture so its morphological characteristics cannot be compared with N. primolutea. Sequence comparison results revealed 1.72% (ACT), 3.10% (RPB2), while other genes <1% base pair differences (without gaps) between N. aquilariae and N. primolutea (YMJ 91102001, holotype). Nemania primolutea has also been found in China (Taiwan region) on dead trunk of Artocarpus communis, which differs from N. chrysoconia and N. flavitextura in having carbonaceous tissue between perithecia and absence of perithecial mounds [53].

Based on significant statistical supports in molecular phylogenetic studies, N. aquilariae is introduced herein as a new species on Aquilaria sinensis from Yunnan Province, China. In addition, MEB, PDB, and RB media are most ideal for culturing fungal biomass, while CDB and SB use led to the lowest level for culturing N. aquilariae (Figure 4).
Figure 3. *Nemania yunnanensis* (KUMCC 20-0267, ex-type). (A, B) Colony on PDA at room temperature after seven days from above and below. (C–G) Mycelia masses. (O) Fermented in the various media. *Nemania aquilariae* (KUMCC 20-0268, ex-type). (H, I) Colony on PDA at room temperature after seven days from above and below. (K–N) Mycelia masses. (P) Fungal cultures growing in various media. Scale bars: (C, D, J, K) = 20 µm; (E–G, L–N) = 10 µm.
Figure 4. *Nemania yunnanensis* (KUMCC 20-0267, orange) and *N. aquilariae* (KUMCC 20-0268, purple) cultures fermented in different liquid media for seven days. All data are the averages of three measurements at 28 °C on a rotary shaker at 120 rpm. Letters indicate a significant difference ($p \leq 0.05$, Mann–Whitney rank sum test) between different media. Error bars show standard error of the arithmetic mean.

3.2.3. *Nemania yunnanensis* Tibpromma & Lu, sp. nov.

Index Fungorum number: IF558189; Facesoffungi number: FoF 09705; Figure 3A–G.

Etymology: named after Yunnan Province, the place where the fungus was first discovered.

Holotype: HKAS 111934

Culture characteristics: Colonies on PDA at room temperature (25 °C) reaching 9 cm in four weeks, circular, white, entire edge with raised on-media surface, smooth. Generative hyphae simple-septate, sub-hyaline, thin-walled, mycelium always packed together, 1.5–2 μm wide. Not sporulating in culture (OMA and PDA).

Material examined: CHINA, Yunnan Province, Xishuangbanna, on dark resinous heart wood of *Aquilaria sinensis* (Lour.) Gilg (Thymelaeaceae), 1 May 2019, Lu Z, No. 4 (HKAS 111934, holotype); ex-type living cultures, KUMCC 20-0267.

Notes: *Nemania yunnanensis* well separates from other species in *Nemania* with moderate statistical support in ML analysis (Figure 2). Based on BLASTn searches of ACT, ITS, LSU, BT and RPB2 sequence data, *Nemania yunnanensis* showed a high similarity to *N. serpens* (ACT = 93.13%(KU684031); ITS = 98.70%(MN844431); BT = 90.84%(KU684188) and RPB2 = 92.60%(GQ844773)), while LSU sequence data showed high similarity to *N. beau-montii* 98.29%(MF161217). As newly described, the strain is an endophytic fungus and does not sporulate in culture. We were not able to compare morphological characteristics with other species in the genus. Based on phylogenetic analyses, *N. yunnanensis* is introduced herein as a new species on *Aquilaria sinensis* from Yunnan Province, China. In addition, the best media to support fungal biomass are RB and MEB media, while CDB, PDB, and SB media led to the lowest level for cultivating *N. aquilariae* (Figure 4).

3.3. Screening Best Culture Media in Shake Flask Culture Method

Fresh and pure cultures of *Nemania yunnanensis* (KUMCC 20-0267) and *N. aquilariae* (KUMCC 20-0268) were used for fermentation in five different media. MEB, PDB, and RB media showed the highest dry weight for mycelium mass. For *Nemania yunnanensis* (KUMCC 20-0267), RB (38.22%) and MEB (26.22%) showed the highest mycelium mass followed by PDB (17.78%), CDB (14.67%), and SB (3.11%). For *Nemania aquilariae* (KUMCC 20-0268), MEB (28.76%), PDB (27.21%), and RB (23.01%) showed the highest mycelium mass followed by CDB (13.94%) and SB (7.08%) (Figure 4).
3.4. GC-MS Analyses

Five volatile components were found in *Nemania aquilariae*, accounting for 85.90% of total volatile components (Table 3 and Figure 4). All the five components’ structures were confirmed as sesquiterpenoids (Figure 5). However, no volatile components were detected in *Nemania yunnanensis* (data not shown). The dominant components of *Nemania aquilariae* were reported as compound 4 with 43.75%, compound 1 with 20.52%, and compound 5 with 13.38%, respectively (Figure 6).

![Figure 5](image-url)

**Figure 5.** Chemical structures of volatile constituents of *Nemania aquilariae* (KUMCC 20-0268) detected by GC-MS. (1) Bicyclo[3.1.1]hept-3-ene-2-acetaldehyde, 4,6,6-trimethyl-, (1R,2R,5S) rel-. (2) Alloaromadendrene. (3) Naphthalene, 1,2,3,4,4a,5,6,7-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-. (4) Valencen. (5) α-Selinene.

![Figure 6](image-url)

**Figure 6.** Typical gas chromatography–mass spectrometry (GC-MS) chromatogram (total iron current) of volatile constituents in fermented *Nemania aquilariae* (KUMCC 20-0268). The retention time (RT) refers to peak compounds and are listed in Table 3.

4. Discussion

Several endophytic fungi are known as potentially bioactive metabolite producers in *Aquilaria* trees, and this is used in agarwood-producing trees [54–56]. In this study, two endophytic fungi belonging to *Nemania* were isolated from the dark resinous wood of *Aquilaria sinensis*, collected from Xishuangbanna, and this is the first report of *Nemania* from the host genus *Aquilaria*. Multi-gene phylogenetic analyses (Figure 2) showed that new isolates are new species of *Nemania*. It was also shown that *Nemania* species separated into three clades when more genes were included (Figure 2). So, we suggest protein-coding genes are important for resolving the placement of *Nemania*. In phylogenetic analyses (Figure 2), new isolates grouped in *Nemania* Clade III, and most of species in this clade were found on decorticated rotten wood and as endophytic fungi but they are not host specific [29,31,57]. Moreover, *Euepixylon sphaeriostomum* clusters within *Nemania* which is in consistent with Dayarathne et al. [44].

In this study, *N. aquilariae* was able to induce the formation of agarwood in *Aquilaria sinensis* and was capable of producing certain agarwood compounds, such as guaiane-type (2), eudesmane-type (3 and 5), and eremophilane-type (4), and these types of sesquiterpenoids are related to chemical constituents of agarwood [58]. Thus, *N. aquilariae* can be used in biological fermentation to produce agarwood-related compounds and can also be
used to infect other *Aquilaria* plants in the production of agarwood. *Nemania aquilariae* is an endophytic fungus from *Aquilaria sinensis* and can be used as an alternative source for catalyzing the production of agarwood and its key natural ingredients. *Nemania aquilariae* was shown to investigate the relationship between the chemistry and fungal associates of agarwood formed. The species presented that agarwood formation significantly affects the chemical and fungal constituents of agarwood in *A. sinensis*. In the present study, we indicated that *N. aquilariae* was able to produce the volatile compounds closely related to a primary determinant of agarwood properties. Thus, only few fungi are being tested for promoting agarwood formation. This species could further influence agarwood formation by injecting the fungi into the trunk, branches, or punch holes and then to subsequently inject fungi into the *Aquilaria* tree. Those techniques avoid severe damage to *Aquilaria* trees and also allow for easy agarwood collection. MEB, PDB, and RB nutrient broths are recommended for the cultivation of *N. aquilariae* for high yield and good quality of biomass.

**Author Contributions:** Conceptualization, S.T., L.Z., J.X., P.E.M. and Y.-H.W.; Data curation, S.C.K.; Formal analysis, S.T. and L.Z.; Methodology, S.T., L.Z., S.C.K., T.-Y.D. and M.R.; Resources, S.T., S.C.K., N.S., J.X., P.E.M. and Y.-H.W.; Supervision, J.X., Y.-H.W.; Writing—original draft, S.T., S.C.K., T.-Y.D., C.P., N.S. and Y.-H.W.; Writing—review and editing, S.T., S.C.K., C.P., M.R., P.E.M. and Y.-H.W. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by Yunnan Provincial Science and Technology Department, Grant No. 202003AD150004. National Sciences Foundation—Thailand Research Fund (NSFC-TRF), China, NSFC-TRF, Grant No. 4176144055; National Sciences Foundation, China (NSFC), Grant No. 41761144055, 41771063, 31750110478; the International Postdoctoral Exchange Fellowship Program (number Y18082251); CAS President’s International Fellowship Initiative (PIFI) (number 2020PC0009); China Postdoctoral Science Foundation; and the Yunnan Human Resources and Social Security Department Foundation and Chiang Mai University.

**Acknowledgments:** Austin Smith at World Agroforestry (ICRAF), Kunming Institute of Botany, China, is thanked for English editing and Li Huili is thanked for her help on statistical analyses. 

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. The IUCN Red List of Threatened Species. Version 2017–3. Available online: www.iucnredlist.org (accessed on 17 December 2017).
2. Novriyanti, E.; Santosa, E.; Syafii, W.; Turjaman, M.; Sitepu, I.R. Anti fungal activity of wood extract of *Aquilaria crassna* Pierre ex Lecomte against agarwood-inducing fungi, *Fusarium solani*. *Indones. J. For. Res.* 2010, 7, 155–165. [CrossRef]
3. Liu, Y.; Chen, H.; Yang, Y.; Zhang, Z.; Wei, J.; Meng, H.; Chen, W.; Feng, J.; Gan, B.; Chen, X.; et al. Whole-tree agarwood-inducing technique: An efficient novel technique for producing high-quality agarwood in cultivated *Aquilaria sinensis* trees. *Molecules* 2013, 18, 3086–3106. [CrossRef] [PubMed]
4. Li, W.; Cai, C.-H.; Dong, W.-H.; Guo, Z.-K.; Wang, H.; Mei, W.-L.; Dai, H.-F. 2-(2-Phenylethyl)chromone derivatives from Chinese agarwood induced by artificial holing. *Fitoterapia* 2014, 98, 117–123. [CrossRef] [PubMed]
5. Mohamed, R.; Jong, P.L.; Kamziah, A.K. Fungal inoculation induces agarwood in young *Aquilaria malaccensis* trees in the nursery. *J. For. Res.* 2014, 25, 201–204. [CrossRef]
6. Kalita, J.; Bhattacharyya, P.R.; Dekaboruah, H.P.; Unni, B.G.; Lekhak, H.; Nath, S.C. Association of Zeuzeraconferta Walker on agarwood formation in *Aquilaria malaccensis* Lamk. *Asian J. Plant Sci. Res.* 2015, 5, 4–9.
7. Peng, C.S.; Osman, M.F.; Bahar, N.; Nuri, E.A.K.; Zakaria, R.; Rahim, K.A. Agarwood inducement technology: A method for producing oil grade agarwood in cultivated *Aquilaria malaccensis* Lamk. *J. Agrobiotechnol.* 2015, 6, 1–16.
8. Kalra, R.; Kaushik, N. A review of chemistry, quality and analysis of infected agarwood tree (*Aquilaria sp*). *Phytochem. Rev.* 2017, 16, 1045–1079. [CrossRef]
9. Hashim, Y.Z.H.-Y.; Ismail, N.I.; Abbas, P. Analysis of chemical compounds of agarwood oil from different species by gas chromatography mass spectrometry (GCMS). *IJUM Eng. J.* 2014, 15, 55–60. [CrossRef]
10. Cheng, J.; Yang, J.; Liu, P. *Atlas of Chinese Woods*; Chinese Forestry Publishing House: Beijing, China, 1992.
11. Editorial Board of Flora of China of Chinese Academy of Sciences. *Flora of China*; Science Press: Beijing, China, 1999; Volume 52.
12. Lee, S.Y.; Mohamed, R. The origin and domestication of *Aquilaria*, an important agarwood-producing genus. In *Agarwood*; Springer: Singapore, 2016; pp. 1–20.
13. Editorial Board of Chinese Pharmacopoeia. *Chinese Pharmacopoeia*; China Medical Science Press: Beijing, China, 2020; Volume 1, pp. 192–193.
14. Naziz, P.S.; Das, R.; Sen, S. The scent of stress: Evidence from the unique fragrance of agarwood. *Front. Plant Sci.* **2019**, *10*, 840. [CrossRef] [PubMed]

15. Antonopoulou, M.; Compton, J.; Perry, L.S.; Al-Mubarak, R. *The Trade and Use of Agarwood (Oudh) in the United Arab Emirates*; Selangor: Petaling Jaya, Malaysia, 2010.

16. Pojanagaroon, S.; Kaewtrak, C. Mechanical methods to stimulate aloe wood formation in *Aquilaria crassa* Pierre ex H. Lec. (Krithana) trees. *Acta Hortic.* **2005**, *676*, 161–166. [CrossRef]

17. Blanchette, R.; Heuveling, V.B.H. Cultivated Agarwood. U.S. Patent No 7638145, 29 December 2009.

18. Okudera, Y.; Ito, M. Production of agarwood fragrant constituents in *Aquilaria calli* and cell suspension cultures. *Plant Biotechnol.* **2009**, *26*, 307–315. [CrossRef]

19. Li, W.; Liao, G.; Dong, W.-H.; Kong, F.-D.; Wang, P.; Wang, H.; Mei, W.-L.; Dai, H.-F. Sesquiterpenoids from Chinese agarwood induced by artificial Holing. *Molecules* **2016**, *21*, 274. [CrossRef]

20. Yang, D.L.; Li, W.; Dong, W.H.; Wang, J.; Mei, W.L.; Dai, H.F. Five new 5,11-epoxyguaiacene sesquiterpenes in agarwood “qi-nan” from *Aquilaria sinensis*. *Fitoterapia* **2016**, *112*, 191–196. [CrossRef] [PubMed]

21. Mohamed, R.; Jong, P.L.; Zali, M.S. Fungal diversity in wounded stems of *Aquilaria malaccensis*. *Fungal Divers.* **2010**, *43*, 67–74. [CrossRef]

22. Azren, P.D.; Lee, S.Y.; Emang, D.; Mohamed, R. History and perspectives of induction technology for agarwood production from cultivated *Aquilaria* in Asia: A review. *J. For. Res.* **2018**, *30*, 1–11. [CrossRef]

23. Tan, C.S.; Isa, N.M.; Ismail, I.; Zainal, Z. Agarwood induction: Current developments and future perspectives. *Front. Plant Sci.* **2019**, *10*, 122. [CrossRef]

24. Wang, S.; Yu, Z.; Wang, C.; Wu, C.; Guo, P.; Wei, J. Chemical constituents and pharmacological activity of agarwood and *Aquilaria* plants. *Molecules* **2018**, *23*, 342. [CrossRef] [PubMed]

25. Qi, S.Y.; Lin, L.D.; Ye, Q.F. Benzylacetone in agarwood and its biotransformation by melanotus flavolivens. *Chin. J. Biotech.* **1998**, *14*, 464–467. (In Chinese)

26. Ueda, J.Y.; Fujino, H.; Attamimi, F.; Kadota, S. A field survey of agarwood in Indonesia. *J. Tradit. Med.* **2018**, *26*, 244–251.

27. Chen, X.Y.; Liu, Y.Y.; Liu, P.W.; Peng, D.Q.; Wei, J.H. Study on biological characteristics of two strains of *Lasiodiplodia theobromae* promoting agarwood formation. *Acta Agric. Jiangxi* **2005**, *676*, 25–67. [CrossRef]

28. Kumarihamy, M.; Ferreira, D.; Croom, E.M., Jr.; Sahu, R.; Tekwani, B.L.; Duke, S.O.; Khan, S.I.; Techen, N.; Nanayakkara, N.P.D. Antiplasmodial and cytotoxic cytochalasins from an endophytic fungus, *Aspergillus giganteus* submerged culture using citrus pectin and orange waste. *Molecules* **2019**, *24*, 777. [CrossRef] [PubMed]

29. Ibrahim, A.; Serensen, D.; Jenkins, H.A.; Ejim, L.; Capreta, A.; Sumarah, M.W. Epoxynemanione A, nemanifuranones A–F, and naminalactones A–C, from *Nemania serpens*, an endophytic fungus isolated from riesling grapevines. *Phytochemistry* **2017**, *140*, 16–26.

30. Kumarishamy, M.; Ferreira, D.; Croom, E.M., Jr.; Sahu, R.; Tekwani, B.L.; Duke, S.O.; Khan, S.I.; Techen, N.; Nanayakkara, N.P.D. Antiplasmodial and cytotoxic cytochalasins from an endophytic fungus, *Nemania* sp. UM10M, isolated from a diseased *Torreya taxifolia* leaf. *Molecules* **2019**, *24*, 777. [CrossRef] [PubMed]

31. Medina, R.P.; Araujo, A.R.; Batista, J.M.; Cardoso, C.L.; Seidl, C.; Vilela, A.F.; Domingos, H.V.; Costa-Lotufo, L.V.; Andersen, R.J.; Silva, D.H. Botryane terpenoids produced by *Nemania bipapillata*, an endophytic fungus isolated from red alga *Asparagopsis taxiformis-Falkenbergia stage*. *Sci. Rep.* **2019**, *9*, 1–11.

32. Farr, D.F.; Rossman, A.Y. Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. 2021. Available online: http://nt.ars-grin.gov/fungaldata (accessed on 5 January 2020).

33. Global Biodiversity Information Facility (GBIF). Available online: https://www.gbif.org (accessed on 10 January 2021).

34. Pedrolli, D.B.; Gomes, E.; Monti, R.; Carmona, E.C. Studies on productivity and characterization of polygalacturonate from *Aspergillus giganteus* submerged culture using citrus pectin and orange waste. *Appl. Biochem. Biotechnol.* **2008**, *144*, 191–200. [CrossRef]

35. Jayasiri, S.C.; Hyde, K.D.; Ariyawansa, H.A.; Bhat, J.D.; Buyck, B.; Cai, L.; Dai, Y.-C.; Abd-Elsalam, K.A.; Ertz, D.; Hidayat, I.; et al. The faces of fungi database: Fungal names linked with morphology, phylogeny and human impacts. *Fungal Divers.* **2015**, *74*, 3–18. [CrossRef]

36. Index Fungorum. Available online: http://www.indexfungorum.org (accessed on 25 June 2013).

37. Shibata, K.; Shibata, S. Phylogeny of Fungi. Available online: http://shibatafungi.com (accessed on 25 June 2013).

38. Shibata, K.; Shibata, S. Phylogeny of Fungi. Available online: http://shibatafungi.com (accessed on 25 June 2013).

39. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M., Gelfand, D., Shinsky, J., White, T., Eds.; Academic Press: New York, NY, USA, 1990; pp. 315–322.

40. Liu, Y.J.; Whelen, S.; Hall, B.D. Phylogenetic relationships among ascomycetes: Evidence from an RNA polymerase II subunit. *Mol. Biol. Evol.* **1999**, *16*, 1799–1808. [CrossRef]
41. Sung, G.-H.; Sung, J.-M.; Hywel-Jones, N.L.; Spatafora, J.W. A multi-gene phylogeny of Clavicipitaceae (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. *Mol. Phylogenet. Evol.* 2007, 44, 1204–1223. [CrossRef] [PubMed]

42. O’Donnell, K.; Cigelnik, E. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* 1997, 7, 103–116. [CrossRef]

43. Carbone, I.; Kohn, L.M. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 1999, 91, 553–556. [CrossRef]

44. Dayarathne, M.C.; Jones, E.B.G.; Maharachchikumbura, S.S.N.; Devadatha, B.; Sarma, V.V.; Khongphinitbunjong, K.; Chomnunti, P.; Hyde, K.D. Morpho-molecular characterization of microfungi associated with marine based habitats. *Mycosphere* 2020, 11, 1–188. [CrossRef]

45. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* 2013, 30, 772–780. [CrossRef] [PubMed]

46. Hall, T. Bioedit Version 6.0.7. 2004. Available online: http://www.mbio.ncsu.edu/bioedit/bioedit.html (accessed on 1 December 2020).

47. Dissanayake, A.J.; Bhunjun, C.S.; Maharachchikumbura, S.S.; Liu, J.K. Applied aspects of methods to infer phylogenetic relationships amongst fungi. *Mycosphere* 2020, 11, 2652–2676. [CrossRef]

48. Rambaut, A.; Drummond, A. *FigTree: Tree Figure Drawing Tool, Version 1.2.2*; Institute of Evolutionary Biology, University of Edinburgh: Edinburgh, UK, 2008.

49. Gray, S.F. *A Natural Arrangement of British Plants: According to Their Relations to Each Other as Pointed Out by Jussieu, De Candolle, Brown, &c*; Baldwin, Cradock, and Joy: London, UK, 1821; pp. 1–824.

50. Lumbsch, H.T.; Huhndorf, S.M. Myconet Volume 14. Part one. Outline of Ascomycota—2009. Part Two. Notes on Ascomycete systematics. Nos. 4751–5113. *Fieldiana Life Earth Sci.* 2010, 1, 1–64. [CrossRef]

51. Wijayawardene, N.N.; Hyde, K.D.; Rajeshkumar, K.C.; Hawksworth, D.L.; Madrid, H.; Kirk, P.M.; Braun, U.; Singh, R.V.; Crous, P.W.; Kukwa, M.; et al. Notes for genera: Ascomycota. *Fungal Divers.* 2017, 86, 1–594. [CrossRef]

52. Species Fungorum. 2021. Available online: http://www.speciesfungorum.org (accessed on 5 January 2020).

53. Ju, Y.M.; Rogers, J.D.; Hsieh, H.M. New *Hypoxylon* and *Nemania* species from Costa Rica and Taiwan. *Mycologia* 2005, 97, 562–5567. [CrossRef]

54. Naef, R. The volatile and semi-volatile constituents of agarwood, the infected heartwood of *Aquilaria* species: A review. *Flavour Fragr. J.* 2011, 26, 73–87. [CrossRef]