Molecular identification of *Aquilaria* species with distribution records in China using DNA barcode technology

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**ABSTRACTS**

*Aquilaria* species is one of the main plant resources that produce agarwood, which containing black resin with important economic and medicinal values. There are about 15 species known to the genus around the world, but only two can be found in China, i.e. *A. sinensis* and *A. yunnanensis*. In this study, *A. sinensis* and *A. yunnanensis* that endemic respectively to Hainan and Yunnan were sampled, on the basis of the investigation and observation of their main morphological features in plantation. Five primers, i.e. ITS2, *matK*, *trnL-trnF1*, *trnL-trnF2*, and *trnH-psbA*, were eventually selected for DNA barcoding. The results showed that the seed surface of *A. sinensis* is smooth or sparsely pubescent, and the seed appendages were long. While the seed surface of *A. yunnanensis* is densely covered with yellow hairs and the seed appendages are short. The *trnL-trnF1* sequence fragment has significant intraspecific and interspecific genetic distances. However, the species identification success rate of ITS2+*matK* combination was finally screened to be the highest, which was verified by the BBA method of TaxonDNA. The phylogenetic trees cluster analysis revealed that the classification of *A. sinensis* and *A. yunnanensis* is significant, and there is geographic isolation between the two species. Therefore, on the premise of accurate identification of plant morphological characters, ITS2+*matK* combination can be used to accurately identify the *Aquilaria* species in China.

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**Introduction**

*Aquilaria* species of Thymelaeeaeceae, the tropical and subtropical evergreen trees, that are mainly distributed in tropical or subtropical regions of the Southeast Asia, are the most important plant resources for the production of rare agarwood (FOC Eco 1999). Agarwood is a precious traditional medicinal ingredient and natural perfume in China, and it has been used widely for cultural, religious, and medicinal purposes around the world. In recent years, wild resources of *Aquilaria* species are found depleted due to the serious human logging and the destruction of their natural environments. *Aquilaria crassa* is listed as a critically endangered species by International Union for Conservation of Nature (IUCN), and *A. malaccensis*, as well as *A. sinensis*, are also regarded as vulnerable species (Hashim et al. 2016). In addition, all *Aquilaria* species have been included in the Convention on International Trade in Endangered Species (CITES) ((CITES) 2004).

Because of the high level of similarity in morphological features of *Aquilaria* species, previous identification methods are only dependent on the classifications of the different morphological features of flower, seed, and fruit. In addition, the flowering and fruiting period of *Aquilaria* species is unstable, and its wild resources are on the verge of extinction. Therefore, it is extremely difficult to identify the *Aquilaria* species by only collecting their fruits and flowers through field sampling, not to mention the high error generated (Lee and Mohamed 2016). In conclusion, this is also one of the factors that have not been clear about the taxonomic study of *Aquilaria* species. Several studies have shown that more than 20 *Aquilaria* species are distributed in the tropical regions of Southeast Asia (Lee et al. 2016; Gao et al. 2017). The Flora of Malaysia is one of the earliest Flora to describe the morphological characteristics of *Aquilaria* species (Hou 1960). And *Aquilaria* species are divided into 12 taxons in this flora, i.e. *A. malaccensis*, *A. microcarpa*, *A. brachyantha*, *A. urdanetensis*, *A. citrinaecarpa*, *A. apiculata*, *A. filaria*, *A. parvifolia*, *A. hirta*, *A. rostrata*, *A. beccariana*, and *A. cunningiana*. Over the past few years, *Aquilaria* species in Asian mainland were indicated that can be divided into 13 taxons (Santisuk 2007), i.e. *A. baillonii*, *A. banaensis*, *A. beccariana*, *A. crassa*, *A. hirta*, *A. khasiana*, *A. malaccensis*, *A. microcarpa*, *A. rostrata*, *A. rugosa*, *A. sinensis*, *A. subintegra*, and *A. yunnanensis*. 

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However, about 15 *Aquilaria* species that discovered across the world were recorded in Flora of China (FOC Eco 1999), including *A. sinensis* and *A. yunnanensis*.

DNA barcoding has been proved the quick and accurate approach to identify different species based on selection of standard DNA segments (Hebert et al. 2003). This technology are also used widely for the identification of *Aquilaria* species. For example, trnL-trnF sequence was found that can provide new molecular framework for the identification of *Aquilaria* species (Eurlings and Gravendeel 2005). Similarly, phylogenetic trees constructed by trnL-trnF+ITS2 combination was demonstrated that is useful for identifying the *Aquilaria* species (Lee et al. 2016). Meanwhile, ITS sequence was applied in first time to analyze *A. malaccensis* from different sources (Lee et al. 2018). In addition, phylogenetic tree constructed by trnL-trnF+ITS1 was found that could aggregate the DNA sequence of *A. sinensis* in GenBank (Jiao et al. 2014). When comparing *A. sinensis*, *A. yunnanensis* and *A. crassna*, Li et al. found that the combinations of ITS+matK and ITS+trnL-trnF are suitable for molecular identification of these three species (Li et al. 2018). In the previous research, our group also found that matK fragments play an important role in *Aquilaria* species from multiple sources (Kang et al. 2019).

Although DNA barcoding is crucial in the identifications of various *Aquilaria* species, the selection of barcode fragments or combinations may be different for different materials. Moreover, DNA barcode technology combined with traditional classification features can obtain the best identification results on the basis of accurate collection of samples. Hence, 18 samples of *A. sinensis* and seven of *A. yunnanensis* were collected respectively from six plantations in Hainan and two plantations in Yunnan. Both *Aquilaria* species were studied on site during their flowering and fruit-bearing periods, and their morphological features were summarized. Five primers were selected for DNA barcodes study. On the premise of determining the morphological characteristics, it is planned to screen out the barcode fragments or combinations suitable for the identification and the construction of phylogenetic trees of both *Aquilaria* species by analyzing the sequence characteristics, genetic distances and species identification rates of different primers.

### Materials and methods

#### Materials

A total of 25 samples of *A. sinensis* and *A. yunnanensis* that collected from 9 plantations in Hainan and Yunnan of China were used as the experimental materials. Fresh leaves were dried and preserved with silica gel, then extracted the total plant DNA. The voucher specimens were also deposited in the Herbarium of Traditional Chinese Medicine, Hainan Branch of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. Localities of all sampled accessions are shown in Table 1.

#### Methods

**Observation of the main morphological features of *Aquilaria* species**

The six plantations in Hainan and two plantations in Yunnan were investigated. Description of the main reproductive organ characteristics, such as fruits and seeds of *A. sinensis* and *A. yunnanensis* by collecting samples of the *Aquilaria* species. And it was made into wax leaf specimens for preservation.

**DNA extraction, amplification and sequencing**

The total DNA extraction kit for plants that acquired from Tiangen Biotech (Beijing) Co., Ltd was used to extract the DNA, and five primers (i.e. ITS2, matK, trnL-trnF1, trnL-trnF2 and trnH-psbA) were used for PCR amplification (Table 2). Optimization and adjustment were made based on the previously reported PCR reaction system (Group et al. 2009). The sequencing of all amplification products was completed by Guangzhou IGE Biotechnology Ltd. Bioedit (Hall 1999), SequenceMatix (Vaidya et al. 2011), Mega X (Sudhir et al. 2018), MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) and PAUP 4 b (http://paup.phylosolutions.com) were used to edit and compare the sequences, match the barcodes, calculate the genetic distances and build the phylogenetic trees, while TaxonDNA (Meier et al. 2006) was used to calculate species identification rates, and R 4.0.0 (https://www.r-project.org) and Figtree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) were used to beautify the phylogenetic trees.

#### Data analysis

The rate of PCR amplification can be defined as the ratio in the percentage of the number of successful individuals to the total number of individuals while sequencing success rate is the percentage of the number of high-quality sequences obtained to the total number of sequences (Kress et al. 2009). MEGA X was used to compare multiple sequences, calculate sequence length, variable sites and conserved sites. Intraspecific and interspecific genetic distances were calculated by K2P model of MEGA X. The ‘Best match’, ‘Best close match’ and ‘All species barcodes’ (BBA method) in TaxonDNA software were used to evaluate the success rates of species identification and to screen for the best DNA fragments or combinations. Moreover, the phylogenetic trees were generated using the neighbor-joining (NJ) and Unweighted Pair-group Method with Arithmetic Mean (UPGMA) methods in MEGA X, with individual node support calculated based on 1000 bootstrap re-samplings. In addition, Bayesian inference (BI) and Maximum Likelihood (ML) approaches were also chosen for the construction of phylogenetic trees in MrBayes 3.2.6 and PAUP 4 b, respectively.

#### Results

**Main morphological features of *A. sinensis* and *A. yunnanensis***

Fruit of *A. sinensis* is oblong, with a long beak, moderate calyx without wrapping the fruit, and its seed surface is...
### Table 1. Sample collection information and GenBank accession numbers of the *Aquilaria* species generated through this study.

| Species       | Collection number | Location                                      | Region of origin (number of samples) | GenBank accession numbers |
|---------------|-------------------|-----------------------------------------------|--------------------------------------|---------------------------|
| *A. sinensis* | HH0001            | Tropical Medicinal Plant Garden, Haikou, IMPLAD | Hainan, China (3)                    | MW118060                  |
|               |                    |                                               |                                      | MW118085                  |
|               |                    |                                               |                                      | MW124309                  |
|               |                    |                                               |                                      | MW124359                  |
|               |                    |                                               |                                      | MW124334                  |
| *A. sinensis* | HH0002            |                                               |                                      | MW118061                  |
|               |                    |                                               |                                      | MW118086                  |
|               |                    |                                               |                                      | MW124310                  |
|               |                    |                                               |                                      | MW124360                  |
|               |                    |                                               |                                      | MW124335                  |
| *A. sinensis* | HH0003            |                                               |                                      | MW118062                  |
|               |                    |                                               |                                      | MW118087                  |
|               |                    |                                               |                                      | MW124311                  |
|               |                    |                                               |                                      | MW124361                  |
|               |                    |                                               |                                      | MW124336                  |
| *A. sinensis* | HX0001            | Tropical Medicinal Plant Garden, Xinglong, IMPLAD | Hainan, China (3)                    | MW118063                  |
|               |                    |                                               |                                      | MW118088                  |
|               |                    |                                               |                                      | MW124312                  |
|               |                    |                                               |                                      | MW124362                  |
|               |                    |                                               |                                      | MW124337                  |
| *A. sinensis* | HX0002            |                                               |                                      | MW118064                  |
|               |                    |                                               |                                      | MW118089                  |
|               |                    |                                               |                                      | MW124313                  |
|               |                    |                                               |                                      | MW124363                  |
|               |                    |                                               |                                      | MW124338                  |
| *A. sinensis* | HX0003            |                                               |                                      | MW118065                  |
|               |                    |                                               |                                      | MW118090                  |
|               |                    |                                               |                                      | MW124314                  |
|               |                    |                                               |                                      | MW124364                  |
|               |                    |                                               |                                      | MW124339                  |
| *A. sinensis* | HC0001            | Plantation, Fushan, Chengmai                   | Hainan, China (3)                    | MW118066                  |
|               |                    |                                               |                                      | MW118091                  |
|               |                    |                                               |                                      | MW124315                  |
|               |                    |                                               |                                      | MW124365                  |
|               |                    |                                               |                                      | MW124340                  |
| *A. sinensis* | HC0002            |                                               |                                      | MW118067                  |
|               |                    |                                               |                                      | MW118092                  |
|               |                    |                                               |                                      | MW124316                  |
|               |                    |                                               |                                      | MW124366                  |
|               |                    |                                               |                                      | MW124341                  |
| *A. sinensis* | HC0003            |                                               |                                      | MW118068                  |
|               |                    |                                               |                                      | MW118093                  |
|               |                    |                                               |                                      | MW124317                  |
|               |                    |                                               |                                      | MW124367                  |
|               |                    |                                               |                                      | MW124342                  |
| *A. sinensis* | HD0001            | Plantation, Longlu, Dingan                    | Hainan, China (3)                    | MW118069                  |
|               |                    |                                               |                                      | MW118094                  |
|               |                    |                                               |                                      | MW124318                  |
|               |                    |                                               |                                      | MW124368                  |
|               |                    |                                               |                                      | MW124343                  |
| *A. sinensis* | HD0002            |                                               |                                      | MW118070                  |
|               |                    |                                               |                                      | MW118095                  |
|               |                    |                                               |                                      | MW124319                  |
|               |                    |                                               |                                      | MW124369                  |
|               |                    |                                               |                                      | MW124344                  |
| *A. sinensis* | HD0003            |                                               |                                      | MW118071                  |
|               |                    |                                               |                                      | MW118096                  |
|               |                    |                                               |                                      | MW124320                  |
|               |                    |                                               |                                      | MW124370                  |
|               |                    |                                               |                                      | MW124345                  |
| *A. sinensis* | HW0001            | Plantation, Maoyang, Wuzhishan                | Hainan, China (3)                    | MW118072                  |
|               |                    |                                               |                                      | MW118097                  |
|               |                    |                                               |                                      | MW124321                  |
|               |                    |                                               |                                      | MW124371                  |
|               |                    |                                               |                                      | MW124346                  |
| *A. sinensis* | HW0002            |                                               |                                      | MW118073                  |
|               |                    |                                               |                                      | MW118098                  |
|               |                    |                                               |                                      | MW124322                  |
|               |                    |                                               |                                      | MW124372                  |
|               |                    |                                               |                                      | MW124347                  |
| *A. sinensis* | HW0003            |                                               |                                      | MW118074                  |
|               |                    |                                               |                                      | MW118099                  |
|               |                    |                                               |                                      | MW124323                  |
|               |                    |                                               |                                      | MW124373                  |
|               |                    |                                               |                                      | MW124348                  |
| *A. sinensis* | HY0001            | Plantation, Yanfeng, Haikou                   | Hainan, China (3)                    | MW118075                  |
|               |                    |                                               |                                      | MW118100                  |
|               |                    |                                               |                                      | MW124324                  |
|               |                    |                                               |                                      | MW124374                  |
|               |                    |                                               |                                      | MW124349                  |
| *A. sinensis* | HY0002            |                                               |                                      | MW118076                  |
|               |                    |                                               |                                      | MW118101                  |
|               |                    |                                               |                                      | MW124325                  |
|               |                    |                                               |                                      | MW124375                  |
|               |                    |                                               |                                      | MW124350                  |
| *A. sinensis* | HY0003            |                                               |                                      | MW118077                  |
|               |                    |                                               |                                      | MW118102                  |
|               |                    |                                               |                                      | MW124326                  |
|               |                    |                                               |                                      | MW124376                  |
|               |                    |                                               |                                      | MW124351                  |
| *A. yunnanensis* | YML0001     | Baihua, Mengia, Xishuangbanna                | Yunnan, China (3)                    | MW118078                  |
|               |                    |                                               |                                      | MW118103                  |
|               |                    |                                               |                                      | MW124327                  |
|               |                    |                                               |                                      | MW124377                  |
|               |                    |                                               |                                      | MW124352                  |
| *A. yunnanensis* | YML0002     |                                               |                                      | MW118079                  |
|               |                    |                                               |                                      | MW118104                  |
|               |                    |                                               |                                      | MW124328                  |
|               |                    |                                               |                                      | MW124378                  |
|               |                    |                                               |                                      | MW124353                  |
| *A. yunnanensis* | YML0003     |                                               |                                      | MW118080                  |
|               |                    |                                               |                                      | MW118105                  |
|               |                    |                                               |                                      | MW124329                  |
|               |                    |                                               |                                      | MW124379                  |
|               |                    |                                               |                                      | MW124354                  |
| *A. yunnanensis* | YMY0001     | Nabanhe, Mengyang, Xishuangbanna              | Yunnan, China (3)                    | MW118081                  |
|               |                    |                                               |                                      | MW118106                  |
|               |                    |                                               |                                      | MW124330                  |
|               |                    |                                               |                                      | MW124380                  |
|               |                    |                                               |                                      | MW124355                  |
| *A. yunnanensis* | YMY0002     |                                               |                                      | MW118082                  |
|               |                    |                                               |                                      | MW118107                  |
|               |                    |                                               |                                      | MW124331                  |
|               |                    |                                               |                                      | MW124381                  |
|               |                    |                                               |                                      | MW124356                  |
| *A. yunnanensis* | YMY0003     |                                               |                                      | MW118083                  |
|               |                    |                                               |                                      | MW118108                  |
|               |                    |                                               |                                      | MW124332                  |
|               |                    |                                               |                                      | MW124382                  |
|               |                    |                                               |                                      | MW124357                  |
| *A. yunnanensis* | YB0001     | Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences | Yunnan, China (1) | MW118084                  |
|               |                    |                                               |                                      | MW118109                  |
|               |                    |                                               |                                      | MW124333                  |
|               |                    |                                               |                                      | MW124383                  |
|               |                    |                                               |                                      | MW124358                  |
smooth or sparsely covered with white pubescence, with long appendages. However, fruit of *A. yunnanensis* is oval, with small and scattered calyx, short seed appendages, and the seed surface is densely covered with yellow pubescence.

In conclusion, the main distinguishing characteristics between *A. sinensis* and *A. yunnanensis* are whether the seed surface is densely covered with yellow pubescence and the length of seed appendages (Figures 1 and 2).

### Table 2. Details on the PCR primers used in this study.

| DNA barcode | Primer | Primer sequence (5'-3') | PCR reaction conditions |
|-------------|--------|-------------------------|------------------------|
| ITS2        | ITS-S2F| ATGGGATTCCTCTGGTAAAT    | 94 °C 5 min; 94 °C 30 s, 56 °C 30 s, 72 °C 45 s, 40 cycles; 72 °C 10 min; 4 °C save. |
| (Chen et al. 2010) | ITS-S3R | GAGGCTTCTCCGACTAACAT   |
| matK        | 3F_KIM | CGTACATTCTTGTGTACAGAG  | 94 °C 1 min; 94 °C 30 s, 52 °C 20 s, 72 °C 50 s, 35 cycles; 72 °C 5 min; 4 °C save. |
| (Kim, unpublished) | 1R_KIM | ACCGATCCATCTGGAATCTTGGGTC |
| trnL-trnF1  | e      | GGTGCAAGTCCTCTGATACC   | 94 °C 5 min; (94 °C 45 s, 50 °C 45 s, 72 °C 90 s, 30 cycles); 72 °C 10 min; 4 °C save. |
| (Lee et al. 2016) | f     | ATTTGAACTGGTGACAGAG     |
| trnL-trnF2  | F-fow-2| CAAATACCATTTTGTAGAATGGAA | 94 °C 5 min; (94 °C 20 s, 52–55 °C 20 s, 72 °C 45 s, 35 cycles); 72 °C 5 min; 4 °C save. |
| (Lee et al. 2016) | E-Aq-rev-1 | CGAACGGGAATTGACAGAAT |
| trnH-psbA   | trnHf_05| CGGCGATGGGATATTCAATCC   | 94 °C 5 min; (94 °C 1 min, 55 °C 1 min, 72 °C 90 s, 30 cycles); 72 °C 7 min; 4 °C save. |
| (Sang et al. 1997; Tate and Simpson 2003) | psbA3-f | GTTATGCAAGCTAAGGTC |

**Figure 1.** Fruits and seeds of *A. sinensis*.

**Figure 2.** Fruits and seeds of *A. yunnanensis*.
### Table 3. Evaluation of the five DNA barcode loci and their combinations.

| DNA barcode | PCR success (%) | Sequencing success (%) | Sequence length | No. of variable/Conserved sites | No. of parsimony informative sites | No. of singleton sites |
|-------------|-----------------|------------------------|-----------------|---------------------------------|------------------------------------|-----------------------|
| ITS2        | 100             | 100                    | 456             | 2/450                           | 2                                  | 0                     |
| matK        | 100             | 100                    | 775             | 1/769                           | 1                                  | 0                     |
| trnL-trnF1  | 100             | 100                    | 456             | 11/439                          | 7                                  | 4                     |
| trnL-trnF2  | 100             | 100                    | 120             | 0/115                           | 0                                  | 0                     |
| trnH-psbA   | 100             | 100                    | 408             | 0/407                           | 0                                  | 0                     |
| ITS2 + matK | –               | –                      | 1231            | 3/1219                          | 3                                  | 0                     |
| ITS2 + trnL-trnF1 | – | – | 912 | 13/889 | 9 | 4 |
| ITS2 + trnL-trnF2 | – | – | 576 | 2/565 | 2 | 0 |
| matK + trnL-trnF1 | – | – | 864 | 2/857 | 2 | 0 |
| matK + trnL-trnF2 | – | – | 1231 | 12/1208 | 8 | 4 |
| matK + trnH-psbA | – | – | 895 | 1/884 | 1 | 0 |
| trnL-trnF1 + trnL-trnF2 | – | – | 1183 | 1/1176 | 1 | 0 |
| trnL-trnF1 + trnH-psbA | – | – | 576 | 11/554 | 7 | 4 |
| trnL-trnF2 + trnH-psbA | – | – | 864 | 11/846 | 7 | 4 |
| ITS2 + matK + trnL-trnF1 | – | – | 528 | 0/522 | 0 | 0 |
| ITS2 + matK + trnL-trnF2 | – | – | 1687 | 14/1658 | 10 | 4 |
| ITS2 + matK + trnH-psbA | – | – | 1351 | 3/1334 | 3 | 0 |
| matK + trnL-trnF1 + trnL-trnF2 | – | – | 1639 | 3/1626 | 3 | 0 |
| matK + trnL-trnF1 + trnH-psbA | – | – | 1351 | 12/1323 | 8 | 4 |
| matK + trnL-trnF2 + trnH-psbA | – | – | 1639 | 12/1615 | 8 | 4 |
| ITS2 + matK + trnL-trnF1 + trnL-trnF2 | – | – | 984 | 11/961 | 7 | 4 |
| ITS2 + matK + trnL-trnF1 + trnH-psbA | – | – | 1032 | 13/1004 | 9 | 4 |
| ITS2 + matK + trnL-trnF2 + trnH-psbA | – | – | 1320 | 13/1296 | 9 | 4 |
| ITS2 + matK + trnL-trnF2 + trnH-psbA | – | – | 984 | 2/972 | 2 | 0 |
| matK + trnL-trnF2 + trnH-psbA | – | – | 1303 | 1/1291 | 1 | 0 |
| ITS2 + matK + trnL-trnF1 + trnL-trnF2 | – | – | 1807 | 14/1773 | 10 | 4 |
| ITS2 + matK + trnL-trnF1 + trnH-psbA | – | – | 2095 | 14/2065 | 10 | 4 |
| matK + trnL-trnF1 + trnL-trnF2 + trnH-psbA | – | – | 1759 | 12/1730 | 8 | 4 |
| ITS2 + matK + trnL-trnF2 + trnH-psbA | – | – | 1759 | 3/1741 | 3 | 0 |
| ITS2 + trnL-trnF1 + trnL-trnF2 + trnH-psbA | – | – | 1440 | 13/1411 | 9 | 4 |
| ITS2 + matK + trnL-trnF1 + trnL-trnF2 + trnH-psbA | – | – | 2215 | 14/2180 | 10 | 4 |
Distinguishing both Aquilaria species using DNA barcoding

Sequence characteristics of the DNA barcodes

PCR amplification and sequencing were implemented for ITS2, matK, trnL-trnF1, trnL-trnF2 and trnH-psbA sequences of all samples to obtain the corresponding rates of PCR amplification, sequencing rates, and variable sites data (Table 3). It could be observed from the Table3 that the rates of PCR amplification and sequencing rates of above 5 sequences were 100.00%. Ordering their lengths in a descending order, matK (775) > ITS2 (456) > trnH-psbA (408) > trnL-trnF2 (115). The number of variant sites from highest to lowest is trnL-trnF1 (11) > ITS2 (2) > matK (1) > trnL-trnF2 (0) = trnH-psbA (0), while the number of conserved sites from highest to lowest is matK (769) > ITS2 (450) > trnL-trnF1 (439) > trnH-psbA (407) > trnL-trnF2 (115). The number of singleton sites in trnL-trnF1 was 4, and the rest of the fragments were 0.

Genetic distance

The K2P distance model was selected by MEGA X software to calculate the interspecific and intraspecific genetic distances among five fragments and their combinations. The results are as shown in Supplementary Table 1. For A. sinensis, the range of intraspecific genetic distances among the five primers and their combinations was 0.000–0.016%, while the range of average intraspecific genetic distances was 0.000–0.007%. For A. yunnanensis, the range of intraspecific genetic distances among the five primers and their combinations was 0.000–0.016%, while the range of average intraspecific genetic distances was 0.000–0.006%. Moreover, the range of interspecific genetic distances between two species was 0.000–0.016%, while the range of average intraspecific genetic distances was 0.000–0.006%. To summarize, this study found that trnL-trnF1 has the maximum average intraspecific and interspecific genetic distances, followed by ITS2.

Species identification

This paper used the ‘BBA’ method in TaxonDNA to verify and analyze the identification rates for the Aquilaria species, and the results are as shown in Supplementary Table 2. The best performing single fragment in ‘best match’ and ‘best close match’ was matK, with an accurate identification rate of 81.00%. Some of the best performing multi-fragment combinations in ‘best match,’ ‘best close match,’ and ‘all species’ include ITS2+matK (96.00%), matK+trnL-trnF2 (81.00%),
matK+trnH-psbA (81.00%), ITS2+matK+trnL-trnF1 (84.00%), ITS2+matK+trnL-trnF2 (96.00%), ITS2+matK+trnH-psbA (96.00%), matK+trnL-trnF2+trnH-psbA (81.00%), ITS2+matK+trnL-trnF1+trnL-trnF2 (84.00%), ITS2+matK+trnL-trnF1+trnH-psbA (84.00%), ITS2+matK+trnL-trnF2+trnH-psbA (96.00%), and trnL-trnF1+trnL-trnF2+trnH-psbA (84.00%).

Phylogenetic tree
Because ITS2+matK, ITS2+matK+trnL-trnF2, ITS2+matK+trnH-psbA and ITS2+matK+trnL-trnF2+trnH-psbA have the highest success rates of species identification (96%) and the 2 fragment combinations are more convenient in the process of constructing phylogenetic tree, with the sequencing cost was low, so this study chose ITS2+matK combination to construct NJ, UPGMA, BI and ML phylogenetic trees respectively (Figures 3–6). The results showed that the phylogenetic trees constructed by the four methods can clearly divide A. sinensis from Hainan and A. yunnanensis from Yunnan, with significant geographic isolation between the two species.

Discussion
DNA barcoding evaluation of Aquilaria species in China
This study used five primers (i.e. ITS2, matK, trnL-trnF1, trnL-trnF2 and trnH-psbA) to analyze the DNA barcodes of A. sinensis and A. yunnanensis (Table 2). In 2009, rbcL and matK were proposed officially as universal barcodes for terrestrial plants by CBOL research team. Then, ITS and trnH-psbA, which demonstrate faster evolutionary rates, were suggested as candidate barcodes by all participants in the 3rd International Academic Conference of DNA Barcode (Group et al. 2009). That is, ITS, matK, rbcL and trnH-psbA are considered as common DNA barcodes. However, the coding sequence of rbcL is highly conserved, leading its variation mainly exists at the level of genus or above and is usually small at the species level (Newmaster et al. 2006; Kress and Erickson 2007; Lahaye et al. 2008). By filtering DNA barcodes from medical biology and analyzing 6600 ITS2 sequences of 4800 algae, fungus and higher plants under 753 genera,
Chen Shilin’s team found that the resolution success rate of ITS2 at the species level is 92.7%. They proposed that ITS2 could be used as a new DNA barcode for fungi and green plants (Chen et al. 2010). Furthermore, ITS2 has a shorter sequence length than ITS, and its high rates of PCR amplification or sequencing (Wang et al. 2016). Meanwhile, trnL-trnF was also found that plays an important role in molecular identification for *Aquilaria* species (Eurlings and Gravendeel 2005). Therefore, ITS2, matK, trnH-psbA, trnL-trnF1 and trnL-trnF2 were selected for the identification of *Aquilaria* species in this study.

By comparing the sequence characteristics of different primers and analyzing their genetic distances and species identification rates, we found that the ITS2+matK barcode combination has the highest species identification success rate (96%), which could be apply to identification and phylogenetic tree construction of *A. sinensis* and *A. yunnanensis* (Figures 3–6). DNA barcoding has been widely for identifying *Aquilaria* species in nowadays. For example, Jiao et al. found that the significant clustering effect of phylogenetic tree construction by trnL-trnF and ITS1 for *A. sinensis* (Jiao et al. 2014). When analyzing *Aquilaria* species, Lee et al. concluded that the phylogenetic tree constructed with combination ITS2+trnL-trnF was applicable for *Aquilaria* species (Lee et al. 2016). Li et al. found that the phylogenetic tree constructed by ITS+matK and ITS+trnL-trnF is beneficial to the identification of three *Aquilaria* species (Li et al. 2018). In this study, our results are different from those previous studies due to different fragments or combinations tend to have different identification effects for various *Aquilaria* species. Although the trnL-trnF1 sequence showed significant intraspecific and interspecific genetic distances, the fragment and its combination displayed low species identification rates (Table S2). This may result from some repeated sequences in trnL-trnF1 sequencing results, which is not useful for constructing the phylogenetic tree. Moreover, trnL-trnF2 is not applicable for phylogenetic tree construction in this study due to the short sequence length, insignificant genetic distance, and low species identification rate. By analyzing the *Aquilaria* species in different countries, our the research team found that the

![Figure 5. Construction of Bayes trees of *A. sinensis* and *A. yunnanensis* using ITS2+matK.](image)
The *matK* fragment plays an important role in identifying *Aquilaria* species (Kang et al. 2019). And *matK* (81%) also showed the highest species identification rate in this study. However, the combination of two fragments is more convenient to operate and reduce the cost of sequencing, and the *trnH-psbA* variable sites is low. (Kress et al. 2005). For these reasons, *ITS2* + *matK* was finally selected from the four combinations (i.e. *ITS2* + *matK* (96.00%), *ITS2* + *matK* + *trnL-trnF2* (96.00%), *ITS2* + *matK* + *trnH-psbA* (96.00%), *ITS2* + *matK* + *trnL-trnF2* + *trnH-psbA* (96.00%)) and used for the clustering analysis of the phylogenetic tree for the *Aquilaria* species in this study.

**Morphological difference and study progress of *A. sinensis* and *A. yunnanensis***

This study found that *A. sinensis* has either smooth surface or is grown with sparse hair and long appendages on its seeds through field investigation, while *A. yunnanensis* has dense yellow pubescence and short appendages on its seeds, which are consistent with the main identification features as recorded in Flora of China. For example, the texture of the capsule of *A. sinensis* is slightly thin, the skin does not shrink when it is dry, the seeds are white silky or glabrous, the apex has a long beak, the base appendage is longer, about 1.5 cm, longer than the seed. However, *A. yunnanensis* has thick capsules, shriveled dry pericarps, yellow pubescence on the seeds, short floral organs on top, and short base appendages that are almost equivalent with that of the seeds, with a length of about 0.8–1 cm (FOC Eco 1999). In addition, *A. sinensis* is mainly distributed in Guangdong (including HK and Macao), Hainan and Guangxi, while *A. yunnanensis* is mainly distributed in Xishuangbanna, Yunnan (Huang 1985).

The phylogenetic tree that constructed by *ITS2* + *matK* combination has clearly branched out *A. sinensis* and *A. yunnanensis* (Figures 3–6), showing their distant genetic relationship, which is speculated to have resulted from geographical isolation. At present, the research of *A. sinensis* has involved...
Aquilaria species. Moreover, the investigation and collection of Aquilaria species is the key to identify the original species of agarwood, and this work can promote the stability of agarwood market.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are openly available in NCBI GenBank database at (https://www.ncbi.nlm.nih.gov), and the reference numbers [MW118060-MW118109, MW124309-MW124383] are shown in Table 1.

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