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Characterization of morphological, micromorphological, anatomical structures and *matK* gene-based identification of aromatic litsea (*Litsea cubeba*)

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Abstract. *Aini IN, Wisanti. 2023. Characterization of morphological, micromorphological, anatomical structures and matK gene-based identification of aromatic litsea (*Litsea cubeba*). Biodiversitas 24: 4557-4567.* Aromatic litsea or krangean (*Litsea cubeba* (Lour.) Persoon) has potential as a drug. *L. cubeba*'s status based on the IUCN red list is the least concern. This research aimed to (i) characterize morphology, micromorphology, and anatomy; (ii) indetify *L. cubeba* species based on DNA barcodes. The research samples were collected in the Mt. Anjasmoro, East Java, Indonesia at three different altitudes. Data in the form of various characters were analyzed using the one-way ANOVA method to determine the significance of the difference. The statistical test analysis of the quantitative character of *L. cubeba* showed a significant difference between heights ($\alpha = 0.05$). The results showed that the characterization of *L. cubeba* includes (i) morphology: structure of lenticels, color, and substance that coats the lower surface of leaves, type of flower, fruit taste, and aroma; (ii) micromorphology: glands lining the abaxial surface of the lamina, trichomes on both surfaces of the lamina, structure of abaxial epidermal cells, the shape of pollen, shape and distribution of exin (iii) anatomy: cuticle thickness, epidermal cell shape, and presence of secretory cells in the lamina. The molecular identification in the form of *L. cubeba* barcode DNA based on the *matK* gene marker matched the *Litsea cubeba* sequence in NCBI (AB259073.1).

Keywords: Anatomy, DNA barcode, *Litsea cubeba*, micromorphology, morphology

INTRODUCTION

Indonesia is a country with the second-highest biodiversity richness in the world (von Rintelen et al. 2017). *Litsea cubeba*, with the local name *krangean* or aromatic litsea, is a species from the Lauraceae family; it has potential as a medicine; almost all of its parts have aroma and contain essential oils (Thielmann and Muranyi 2019). *Litsea cubeba*, a plant native to Indonesia, thrives in forest habitats and typically grows in clusters on mountain slopes at elevations ranging from 700 to 2,300 meters above sea level. This species can be found scattered throughout the forests of Sumatra, Kalimantan, and Java Island (Putri 2015).

The parts of *L. cubeba* commonly used as medicine are roots, bark, leaves, fruit, and seeds. *L. cubeba* leaves can improve blood circulation, heal wounds, and treat insect and snake bites (Kamle et al. 2019). *L. cubeba* fruit has the potential as a pain reliever, improve blood circulation, and relieve gastric distention, asthma, dementia, diarrhea, and injuries. The *L. cubeba* essential oil is widely used in cosmetics and perfume (Huang et al. 2013).

The morphological characteristics of *L. cubeba* are the presence of large and prominent lenticels on the stem so that the surface of the stem becomes rough. The lower surface of the lamina is dull-slightly hairy, while the upper and lower surfaces are covered with whitish or bluish wax, and when the leaves are squeezed, a pungent lemon odor appears. *Litsea cubeba* flowers include light yellow unisexual flowers (Chen et al. 2013).

Morphology is the main character used to characterize plants. Characterization can be done by observing the morphological characters of stems, leaves, flowers, fruit, and other parts of a species. Studies on morphological characterization are critical to identifying a species and obtaining data sources for kinship analysis (Sulassih 2013). The results related to the morphological characterization of *L. cubeba* include the structure of the lenticels, the structure of the underside of the leaves, and the aroma of *L. cubeba* bark, leaves, and fruit. In addition to morphology, micromorphology can also characterize plant species. Several micromorphological characterization studies have been carried out on close-range species, including stomata shape, stomatal margin shape, and stomatal opening shape in 41 *Ocotea* species (Trofimov and Rohwer 2018); epidermal cell shape, periclinal wall shape, and epidermal cell anticlinal wall in 48 *Cinnamomum* species (Gang et al. 2021).

Characterization can also be based on the micro-morphological character. The science of micromorphology is essential sometimes for the definition and classification of taxa and their relation to specific groups studies on plants enable micro-level analysis of pollen, leaves, fruit, epidermal patterns, tissues, seeds, etc (Asadi et al. 2019). Pollen providing essential taxonomic information and evidence (Othman and Hamad 2022). Merwe et al. (1990) researched pollen micromorphology in nine genera belonging to Lauraceae, and *Litsea* was among those

researched. The results of this study describe that *L. cubeba* pollen units are single pollen grains (monad), do not have an aperture, and the exine differentiates into a regularly distributed, cone-shaped spinulate sexin. Other characterization research has been carried out on leaf anatomy. Anatomical research of the leaves, petioles, and stems of *Litsea quinqueflora* shows the presence of highly cuticularized epidermis in these three organs. Oil cells are scattered in the petioles and stem (Hari et al. 2021). Based on the results of anatomical research that has been carried out; proving that the anatomical characterization of *L. cubeba* has never been done.

The existence of *L. cubeba* is now becoming rare, so *L. cubeba* status has become Least Concern ver 3.1 IUCN (Kok 2021). The leading cause of this species' scarcity is the distilling of the bark and leaves as raw materials for perfume (Qiu et al. 2021). Based on these facts, it is necessary to breed *L. cubeba* so it does not become extinct. Currently, identification methods based on DNA barcodes have been widely used because they can provide species information quickly and accurately (Mathon et al. 2021).

One way to do this is by molecular characterization with the DNA barcode identification method. *L. cubeba* DNA barcodes have been identified based on *matK*, rbcL, and trnH-psbA gene markers. More than 30 *Litsea cubeba matK* gene sequences have been submitted on the National Center for Biotechnology Information (NCBI) website. Identification based on DNA barcodes has been carried out for *L. cubeba* samples growing in Cibodas Botanical Gardens (Fijridiyanto and Murakami 2016).

The potency of *L. cubeba* as a medicinal plant is important, but detailed character descriptions of this species have yet to be reported. Thus, the character marker to recognize these species is still in doubt. Therefore, it's important to research this species character traits. This research aims to characterize *L. cubeba* to provide accurate information about morphological, micromorphological, anatomical, and molecular markers.

MATERIALS AND METHODS

Plant materials

Litsea cubeba samples were collected from their growing locations in the Coban Watu Talang area of Mt. Anjasmoro, Tahura Raden Soerjo, Madiredo Village, Pujon Sub-district, Malang District, East Java, Indonesia (coordinate 7°47'21" S, 112°27'39" E) with a 100-m interval from 1,400 to 1,600 m asl. in elevation. Collections were in twigs, 30-40 cm long complete with leaves, flowers, and fruit; therefore, 27 specimens of *L. cubeba* were collected. The *L. cubeba* specimens in this study were named *L. cubeba* 1 (1,400 m asl.), *L. cubeba* 2 (1,500 m asl.), and *L. cubeba* 3 (1,600 m asl.) (Figure 1).

Procedures

Observation of morphological characters

Morphological characters and their attribute were observed based on Azah and Susiarti (2021). Morphological characters observed were 106 characters, including 89 qualitative characters and 17 quantitative characters.

Preparation and observation of micromorphological characters

Micromorphological characters were observed using two methods, namely SEM (scanning electrone microscope) and the leaf-clearing method. SEM sample preparation refers to the Prawasti et al. (2014) method. Samples for SEM observation are placed on stubs with self-adhesive double-sided carbon discs. Then they are coated with gold-palladium (AuPd) for 180 seconds. Observations and photography of SEM preparations are carried out using SEM FEI (INSPECT-S50). The second method uses the leaf-clearing method regarding Vasco et al. (2014), namely by cutting leaves measuring 1 × 1 cm (without main leaf veins) soaked in a 1:3 HNO_3 solution to separate the adaxial and abaxial epidermis of the leaves and then stained with 1% safranin. There were 25 observed micromorphological characters, including 16 laminae and 9 pollen micromorphological characters.

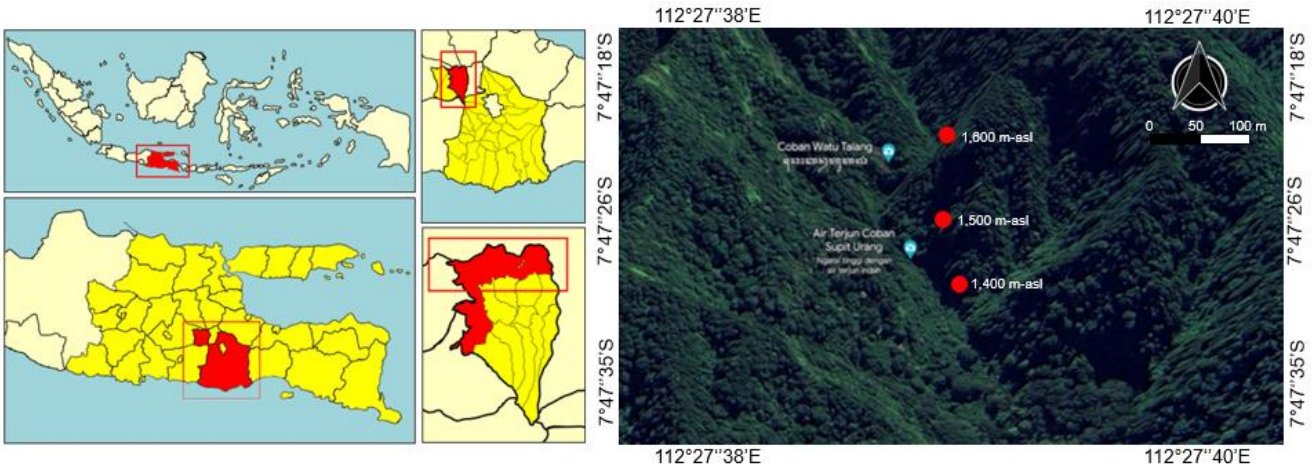


Figure 1. Sampling locations in the Coban Watu Talang area of Mt. Anjasmoro, Malang District, East Java, Indonesia

Preparation and observation of leaf anatomy

Observation of anatomical characters using cross-sectional slices of leaves was made using the paraffin embedding method (Retamales and Scharaschkin 2014). Samples were fixed using FAA solution (Formalin-acetic acid-alcohol). Samples were dehydrated using graded alcohol, followed by xylol-alcohol (1:3, 1:2, 1:1, and 1:0) for 30 minutes each. Furthermore, the infiltration and envelopment stages were carried out using pure paraffin. Samples were cut crosswise with a thickness of 6 μm using a rotary microtome (Shibuya manual), then stained with 1% safranin and covered with Canada balsam. There were 19 anatomical characters observed, including seven qualitative and 12 quantitative characters.

Molecular identification

Molecular identification was carried out in three stages of preparations: DNA isolation from fresh young leaves of *Litsea cubeba*, PCR amplification, and DNA sequencing. Total DNA was isolated from *L. cubeba* leaves using procedures according to the DNAsecure Plant kit (Tiangen). Amplification using PCR consisting of forward primer for the *matK* gene, which is *matK*472F (5'-CCC RTY CAT CTG GAA ATC TTG GTT C-3 '), and reverse primer for the gene *matK*1248R (5'-GCT RTR ATA ATG AGA AAG ATT TCT GC-3'). The PCR products were then sequenced using the service provider First Base Laboratories Sdn Bhd Malaysia.

Data analysis

Variations in morphological, micromorphological, and anatomical characters for each height were analyzed using the one-way ANOVA method SPSS statistics 23 software to determine the significance of *L. cubeba* characteristic variations at three locations with different heights. The DNA sequencing results were obtained as a chromatogram and edited using FinchTV 1.4.0 software. DNA sequence was translated into a protein using the Expasy website. Sequences are identified using the BOLD System. Alignment of DNA sequences was carried out using ClustalX 2.1 software (Ferrari and Patrizio 2021) to detect differences in nucleotides between each *L. cubeba* plant sample and its close relatives in GenBank (Chen et al. 2020; Zhang et al. 2021). Analysis of nucleotide base variation between each *L. cubeba* plant sample and its close relatives in GenBank using BioEdit software (Alzohairy 2011). The arrangement of phylogenetic trees was reconstructed using MEGA 6 software (Tamura et al. 2013) to obtain genetic distance and phylogenetic trees. The genetic distance was calculated with the Kimura-2 parameter model to obtain the matrix calculation. Phylogenetic tree reconstruction was analyzed using the Neighbor-Joining Tree method with a bootstrap value off 1,000 replications.

RESULTS AND DISCUSSION

Characterization of *Litsea cubeba* morphology

Morphological characterization data were obtained based on observations of the morphological characters of *L. cubeba* growing at three locations with different heights. *L. cubeba* has five qualitative characteristics: bark color, stem surface texture, branching growth direction, branching density, and leaf density (Table 1). These five characteristics are stable in all individuals in each study location.

Litsea cubeba bark shows color variations. The *L. cubeba* samples at location 1 are granite gray, while the *L. cubeba* samples at locations 2 and 3 are cinereous in color (Figure 2). Likewise, the surface texture of the *L. cubeba* stem also shows variations. Texture criteria are determined based on the number of lenticels (Pudjiono and Septina, 2008). *Litsea cubeba* in locations 1 and 2 had a very rough stem surface texture (lenticel count >20/cm^2), while location 3 had a reasonably rugged structure (lenticel count 10-20/cm^2) (Figure 3A).

Litsea cubeba flowers in three locations showed similarities in the type, namely unisexual flowers. Werff's (2019) and Chen's research (2013) shows that unisexual flowers in the Lauraceae family are only found in the *Litsea* genus in Asia. *Litsea cubeba* flowers include axillary inflorescences (Figure 3C-D). This character is similar to its close relatives, namely *Litsea glutinosa* and *Litsea deccanensis* (Kunuku et al. 2019; Kunuku and Aluri 2019). Another characteristic of *L. cubeba* is the fruit (Figure 3D). Mesocarp has a watery texture with a nutty taste and is edible. In addition, *L. cubeba* shows characteristics on *L. cubeba* leaves, and its bark has a pungent lemon scent. Li et al. (2016) showed that extracts of roots, leaves, flowers, and fruit contained essential oils with a lemon flavor.

Table 1. Variation of vegetative characters of *Litsea cubeba* that grows in the Mt. Anjasmoro, Malang District, East Java, Indonesia

Characters	Location		
	1	2	3
Bark color	Granit gray	Cinereous	Cinereous
Stem surface texture	Very rough	Very rough	Rough
Branching growth direction	Obliquely	Horizontal	Horizontal
Branch density	Currently	Currently	Tight
Leaf density (Σ node/m)	Tight	Tight	Very tight

Note: location 1, altitude 1,400 m asl.; location 2, altitude 1,500 m asl.; location 3, altitude 1,600 m asl.

A **B**

Figure 2. The color of the *Litsea cubeba* stem. A. Granite gray, B. Cinerous

The separate flat stamens on *L. cubeba* consist of nine stamens. The anther consists of fourth locules of the same size and shape. This is supported by research by Xu et al. (2017) which states that each anther has four pollen sacs, and the development of the anther wall follows the basic model. Stamens have glands at the stalk's base, as Yang et al. (2022) researched *Sassafras tzumu* (Lauraceae), a close relative of *L. cubeba.*

The quantitative characteristics of *L. cubeba* growing at different heights were analyzed using the one-way ANOVA (Table 2). Seven characters show significant differences ($\alpha = 0.05$). These characters include inflorescence stalk length, inflorescence bud length, inflorescence bud width, stamen stalk length, fruit stalk length, fruit diameter, and fruit weight. *Litsea cubeba* grown in location 3 showed significant differences in petiole length, lamina length, fruit stalk diameter, mesocarp thickness, and seed diameter.

Micromorphological characterization of the lamina and *Litsea cubeba* pollen

Based on SEM observations, *L. cubeba* stomata are anomocytic. This type can also be found in *Neolitsea javanica* and *Cinnamomum sintoc* (Lauraceae), which are close relatives of *L. cubeba* (Fadhila et al. 2023). The stomata length of *L. cubeba* that grew in location 3 had the smallest average value compared to the other two locations, at 1.32 $\pm$ 0.25 µm. Meanwhile, the stomata width of *L. cubeba* that grows in location 1 has the most significant size than the other two locations, namely 7.62 $\pm$ 1.48 µm (Table 3). Stomata width is affected by the time of sample collection. Most of the stomata observed were open with a larger porous width. Kingston et al. (2016) stated that stomata with these conditions were found on leaves collected in the morning before noon.

Stomata density is classified into three categories: low (<300/mm^2), medium (300-500/mm^2), and high (>500/mm^2) (Susilowati et al. 2022). Based on the research results, the stomatal density of the *L. cubeba* sample has a low density. Environmental factors like light intensity and high temperature affect stomata density (Kathryn et al. 2015). The higher the light intensity and temperature, the stomata density also increases (Idris et al. 2019). This is by the light intensity in the Mt. Anjasmoro, which is low because there is much shade, which is less than 850 lux, causing the density of *L. cubeba* stomata in the Mt. Anjasmoro to be low. The periclinal walls of the *L. cubeba* epidermis do not have reticulate ornamentation, and the anticlinal walls are straight. The periclinal walls of the abaxial epidermal cells of *L. cubeba* leaves look like papillose at SEM observation. This structure is similar to the abaxial epidermal cells of *Cryptocarya impressa* (Lauraceae) leaves observed by Nishida et al. (2016). In contrast, the adaxial epidermis of a leaf has a periclinal cell wall and a smooth anticlinal cell wall. This is by research conducted by Nishida et al. (2016) on 26 *Cryptocarya* species, all of which have adaxial epidermis of leaves with smooth periclinal cell walls, smooth anticlinal cell walls, and no stomata.

A **B** **C** **D** **E**

Figure 3. Organs of *Litsea cubeba.* A. Stem, B. Leaves, C-D. Inflorescence, E. Fruit. AB: abaxial, AD: adaxial, BS: stamens, L: lenticels

Table 2. Variation of quantitative characters of *Litsea cubeba* growing in the Mt. Anjasmoro, Malang District, East Java, Indonesia [(minimum-maximum) Mean ± Standard deviation, n = 27]

Characters	Location		
	1	2	3
Petiole length (cm)	(1.4-1.7) 1.54 ± 0.09^a	(1.5-1.7) 1.60 ± 0.09^a	(1.7-1.9) 1.82 ± 0.08^b
Lamina length (cm)	(8.5-9.6) 9.11 ± 0.39^a	(9.5-10.1) 9.78 ± 0.23^a	(10.3-11.5) 11.08 ± 0.35^b
Lamina width (cm)	(1.5-2) 1.77 ± 0.18^a	(2-2.4) 2.13 ± 0.14^b	(2.4-2.7) 2.52 ± 0.08^b
Inflorescence stalk length (mm)	(6-7) 6.56 ± 0.53^a	(7-9) 8.56 ± 0.73^b	(9-11) 9.89 ± 0.78^c
Inflorescence bud length (mm)	(5-6) 5.67 ± 0.50^a	(5-7) 6.11 ± 0.78^b	(8-9) 8.67 ± 0.50^c
Inflorescence bud width (mm)	(3-4) 3.33 ± 0.50^a	(4-5) 4.56 ± 0.53^b	(5-7) 5.89 ± 0.60^c
stamen stalk length (mm)	(0.6-0.7) 0.66 ± 0.05^a	(0.6-0.8) 0.73 ± 0.07^b	(0.9-1) 0.97 ± 0.05^c
Fruit stalk length (mm)	(5-7) 6.00 ± 0.87^a	(6-8) 7.00 ± 0.87^b	(9-10) 9.44 ± 0.53^c
Fruit stalk diameter (mm)	(0.8-1) 0.87 ± 0.07^a	(0.9-1) 0.99 ± 0.03^a	(1.1-1.2) 1.14 ± 0.05^b
Fruit diameter (mm)	(6-7) 6.44 ± 0.53^a	(7-8) 7.33 ± 0.50^b	(8-10) 9.00 ± 0.71^c
Mesocarp thickness (mm)	(1.01-1.07) 1.04 ± 0.02^b	(1.06-1.09) 1.07 ± 0.01^b	(1.14-1.16) 1.15 ± 0.01^a
Fruit weight/10 seeds (gr)	(1.50-1.59) 1.55 ± 0.05^a	(1.60-1.66) 1.63 ± 0.003^b	(1.77-1.89) 1.82 ± 0.06^c
Seeds diameter (mm)	(3-3.5) 3.22 ± 0.26^b	(3.5-4) 3.67 ± 0.25^b	(4-5) 4.50 ± 0.35^a

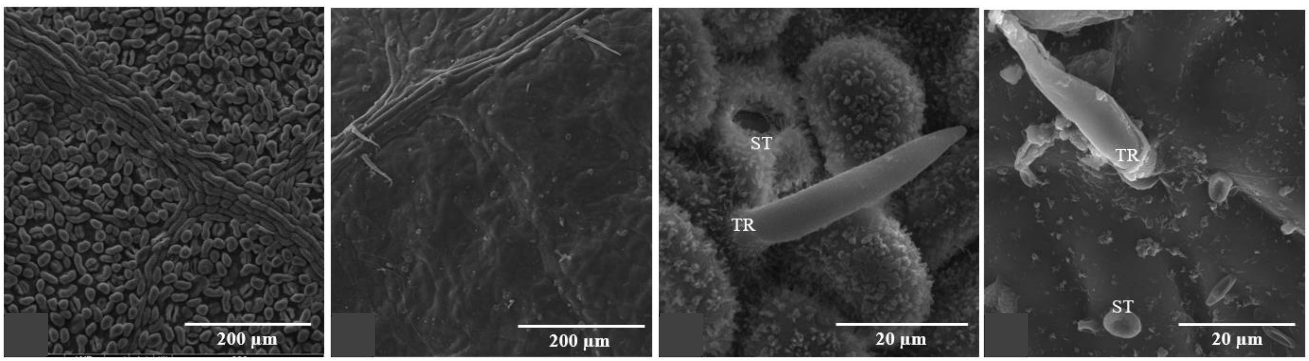
Note: location 1, altitude 1,400 m asl.; location 2, altitude 1,500 m asl.; location 3, altitude 1,600 m asl. The value on a similar line was followed by different superscripts, representing a significant difference, $\alpha = 0.05$

The upper and lower epidermis of *L. cubeba* leaves observed using SEM have several differences (Figure 4). Upper leaf vein epidermal cells are round and elongated (cylindrical). The lower epidermis has a very dense cell arrangement, while the upper epidermis has a smooth periclinal wall. Lower epidermal cells are round, oval, irregular, and cylindrical. Glands cover the entire surface of the lower epidermal cells. This is supported by the research results by Zhang et al. (2021) regarding the presence of glands in the leaves of *Cinnamomum chartophyllum*, which is a close relative of *L. cubeba*. The shape of the upper epidermal cells is rectangular, pentagonal to hexagonal.

The position of the abaxial stomata of lamina *L. cubeba* is half sunk and oval, whereas in adaxial, it is not. Other epidermal derivatives on the *L. cubeba* lamina are trichomes found on both abaxial and adaxial surfaces. Trichomes are unicellular non-glandular types, similar to *Litsea parvifolia*, a close relative of *L. cubeba* (Jiménez-Pérez and Lorea-Hernández 2009).

The quantitative character of the *L. cubeba* lamina micromorphology was analyzed using the one-way ANOVA method (Table 3). Successively the three locations show different averages. Although the average locations 1, 2, and 3 are not too sharp, the statistical analysis results show significant differences. Three characters showed significant differences: number of stomata, density of stomata, and average length of epidermal cells. The average stomata length at location 2 differed significantly from locations 1 and 3, while the average stomata width differed significantly from *L. cubeba* at location 1.

Based on pollen observations with SEM, *L. cubeba* pollen units are single pollen grains (monad) (Figure 5). Pollen grains are separated from one another and independent of each other. This was confirmed by Agashe and Caulton (2019), who stated that most Angiosperm pollen is solitary and single pollen (monad). The characteristics of *L. cubeba* pollen are circular and do not have an aperture (inaperturate). Inaperturate pollen types represent Lauraceae (Rohwer 2018; Xu et al. 2017). Rashid et al. (2021) said that the type of aperture is essential in classifying the genus level. The polarity of *L. cubeba* pollen shows the apolar type because the polar pole cannot be clearly distinguished. The research of Wang and Chen (2001) stated that *Litsea cubeba* has an apolar pollen polarity.



A **B** **C** **D**

Figure 4. Micrograph of the epidermis and *Litsea cubeba* epidermis derivatives. A SEM of the abaxial epidermis of a leaf; B. SEM of adaxial epidermis of the leaf; C. Abaxial epidermal trichome and stoma details; D. Details of adaxial epidermal trichomes and stomas. ST: stoma and TR: trichomes

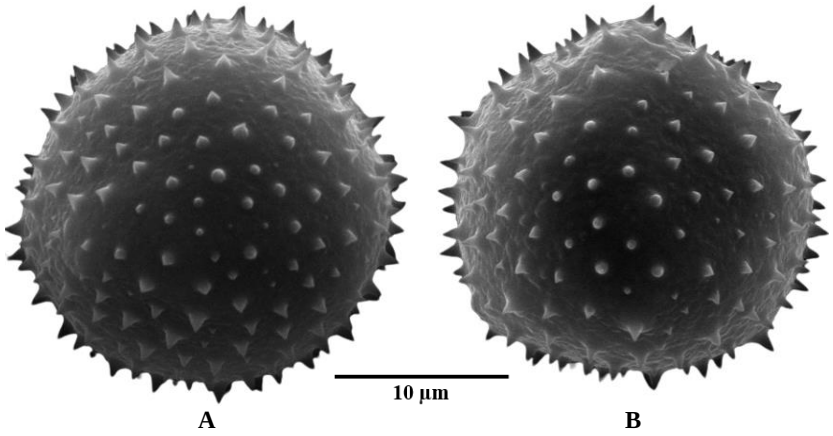


Figure 5. Micrograph of *Litsea cubeba* pollen. A. Appears polar; B. Equatorial view

Table 3. The quantitative character of abaxial micromorphology of *Litsea cubeba* leaf lamina using leaf clearing method [(minimum-maximum) Mean ± Standard deviation, n = 27]

Characters	Location		
	1	2	3
Number of stomata	(37-41) 38.67 ± 2.08[a]	(39-41) 40.0 ± 1.0[b]	(40-45) 42.67 ± 2.52[c]
Stomata average length (µm)	(17.11-17.89) 17.41 ± 0.42[a]	(17.54-17.98) 17.71 ± 0.23[b]	(17.13-17.61) 17.32 ± 0.25[a]
Stomatal width average (µm)	(6.29-9.21) 7.62 ± 1.48[b]	(5.19-8.17) 6.91 ± 1.54[a]	(5.68-8.35) 6.78 ± 1.39[a]
Stomatal density	(189-209 /mm^2) 195.6 ± 11.5[a]	(199-209 /mm^2) 202.3 ± 5.7[b]	(204-229 /mm^2) 212.3 ± 14.4[c]
Average length of epidermal cells (µm)	(21.67-27.58) 24.36 ± 2.99[a]	(25.71-29.2) 27.91 ± 1.91[b]	(23.49-28.56) 26.47 ± 2.65[c]
Average width of epidermal cells (µm)	(13.24-17.93) 15.34 ± 2.38[a]	(13.59-17.46) 15.16 ± 2.04[a]	(15.28-17.17) 16.20 ± 0.95[b]

Note: location 1, altitude 1,400 m asl.; location 2, altitude 1,500 m asl.; location 3, altitude 1,600 m asl. The value on a similar line was followed by different superscripts, representing a significant difference, $\alpha = 0.05$

Litsea cubeba has a radial pollen symmetry. This is supported by the statement of Agashe and Caulton (2019) that most of the dicotyledonous have radial symmetry pollen. The exine differentiates into a regularly distributed, cone-shaped spinulate sexin. Spinula height ± 1.22 µm, supported by the results of research by Wang and Chen (2001), which showed that spinula height was less than 1.8 µm. The characteristics of *L. cubeba* pollen grains have similarities with pollen in the species *Ocotea bullata*, *Ocotea Kenyensis*, *Beilschmiedia mannii*, *Lindera subumbelliflora*, *Hypodaphnis zenkeri* (Merwe et al. 1990), *Lindera thunbergii*, *Litsea elongata*, *Neolitsea acuminatissima* (Wang and Chen 2001) and 21 species *Globba* of the Lauraceae family have been studied by Kajornjit et al. (2018). It shows that some plants in different genera but still in the same family have several characteristics in common. Geng-guo and Chih-bei (1995) grouped the genera *Litsea*, *Lindera*, and *Neolitsea* in one group based on their exine structure, namely the spinulate sexes (thorns) which are clear, without mat-shaped thickening near the base of the spinules. Usually, the spinules are scattered at a significant distance. Regular (not too dense).

Anatomical characterization of *Litsea cubeba* leaves

The results of the *L. cubeba* research at three different locations did not show qualitative variations in anatomical characters. The anatomical characteristics of the *L. cubeba* lamina are thick adaxial cuticle, abaxial epidermal cells shaped like papillose, and the presence of secretory cells, namely lithochic cells (Figure 6). Lithochic cells are found beneath the adaxial epidermal tissue and contain calcium carbonate crystals called cystoliths. Choopan and Grote (2015) stated that cystoliths are more commonly found on plant leaves and are limited to several families, especially Cannabaceae, Moraceae, Urticaceae, and Acanthaceae. However, this study found cystolith in *L. cubeba* leaves belonging to Lauraceae. This research is supported by the results of Gonçalves' research et al. (2018) on the species *Endlicheria paniculata* and *Ocotea indecora* (Lauraceae), which are also close relatives of *L. cubeba*.

The anatomical characteristics of *L. cubeba* leaves growing at different heights, which are quantitative, are analyzed using the one-way ANOVA method. The results of the 12-character statistical analysis showed that the quantitative characters of the range anatomy varied between heights except for the length of the secretory cells ($\alpha = 0.05$) (Table 4). Lamina thickness, cuticle thickness, leaf mesophyll thickness, spongy tissue thickness, leaf vein thickness, and leaf vein width showed significantly different values between heights. The range in locations 1 and 2 have similarities in the adaxial epidermis thickness and the secretory cells' width. *Litsea cubeba* at locations 1 and 3 also have similarities in the palisade tissue's thickness, the abaxial epidermis's thickness, and the trichomes length.

Table 4. Variation of quantitative anatomical characters of *Litsea cubeba* growing in the Mt. Anjasmoro, Malang District, East Java, Indonesia [(minimum-maximum) Mean ± Standard deviation, n = 27]

Characters	Location		
	1	2	3
Lamina thickness (µm)	(164.2-170.7) 166.37 ± 3.75^{b}	(168.9-216.6) 185.17 ± 27.23^{c}	(137.8-154) 143.33 ± 9.24^{a}
Cuticle thickness(µm)	(3.5-4.8) 4.23 ± 0.67^{a}	(4.6-9.8) 6.93 ± 2.64^{c}	(3.5-6.8) 5.27 ± 1.66^{b}
Adaxial epidermis thickness (µm)	(12.2-17.2) 14.93 ± 2.53^{a}	(10.9-16.4) 14.50 ± 3.12^{a}	(12.7-15) 13.90 ± 1.15^{b}
Leaf mesophyll thickness (µm)	(121.3-128) 125.53 ± 3.68^{b}	(135.1-160.1) 143.73 ± 14.18^{c}	(100.6-118.4) 107.73 ± 9.41^{a}
Palisade tissue thickness (µm)	(52-67.6) 57.83 ± 8.51^{a}	(60.6-79.5) 72.67 ± 10.48^{b}	(47.1-65.2) 54.37 ± 9.56^{a}
Spongy tissue thickness (µm)	(67-77.7) 72.10 ± 5.37^{c}	(53.3-78.7) 68.33 ± 13.33^{b}	(49.9-58.6) 54.63 ± 4.40^{a}
Thickness of abaxial epidermis (µm)	(13.7-19.1) 15.87 ± 2.85^{a}	(10.5-17) 14.13 ± 3.32^{b}	(14.4-18,.4) 15.93 ± 2.16^{a}
Secretory cell length (µm)	(44-64.3) 56.86 ± 7.59^{a}	(48.2-63.8) 55.16 ± 5.40^{a}	(39.6-59.7) 53.08 ± 9.12^{a}
Secretory cell width (µm)	(33.4-53) 41.54 ± 7.86^{b}	(39-48.4) 44.67 ± 3.43^{b}	(25.1-44.6) 37.60 ± 8.59^{a}
Trichome length (µm)	(29.9-74.9) 53.78 ± 17.28^{a}	(29.2-96) 64.31 ± 26.18^{b}	(36.6-86.6) 56.58 ± 20.66^{a}
Leaf vein thickness (µm)	(827.6-900.4) 859.50 ± 37.23^{b}	(908.8-919.9) 914.87 ± 5.62^{c}	(581.2-711.3) 652.50 ± 65.94^{a}
Leaf vein width (µm)	(749.6-809.3) 804.37 ± 8.46^{b}	(925-948.6) 935.20 ± 12.12^{c}	(672.7-824.1) 769.60 ± 84.14^{a}

Note: location 1, altitude 1,400 m asl.; location 2, altitude 1,500 m asl.; location 3, altitude 1,600 m asl. The value on a similar line was followed by different superscripts, representing a significant difference, α = 0.05

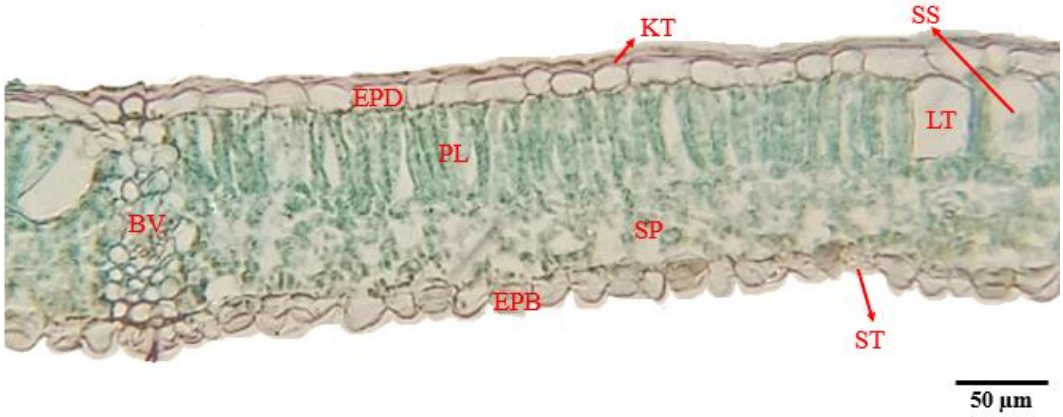


Figure 6. Anatomy of the *Litsea cubeba* lamina, BV: vascular bundles, EPB: abaxial epidermis, EPD: adaxial epidermis, KT: cuticle, LT: lithocis cells, PL: palisade tissue, SP: spongy tissue, SS: secretory cells, ST: stomata

Most of the *L. cubeba* anatomical attribute in location 2 is more valuable than other locations (Table 4). It is assumed that there is the influence of environmental factors. The *L. cubeba* population in locations 1 and 3 grew on flat to steep ground with more soil layers than in location 2. The *L. cubeba* population in location 2 grew on rocky cliffs with fewer soil layers.

Molecular-based identification of *Litsea cubeba* based on the *matK* gene

A research study was conducted by *L. cubeba* to collect samples from different altitudes in the Mt. Anjasmoro. The results showed that the DNA isolation process yielded a clear solution. The amplified DNA was then subjected to PCR using the *matK* gene before being electrophoresed using a 1.5% agarose gel. The resulting amplicons of *L. cubeba* samples 1, 2, and 3 were deemed suitable for DNA sequencing because they appeared clear and thick on the agarose gel medium (Figure 7).

Figure 7 shows the results of readings via Gel-Doc showing that the amplicon product has a fragment length of approximately 730 bp. The amplified product is continued to the sequencing stage because the DNA has been amplified properly. The resulting DNA sequences obtained had band lengths of 727 bp, 730 bp, and 731 bp. *Litsea cubeba* has also been identified on a molecular basis using the rbcL (ribulose-1.5-bisphosphate carboxylase Large) marker. There are 30 *L. cubeba* sequences with rbcl gene markers registered in NCBI. The shortest sequence, *L. cubeba* HQ415130.1, has a band length of 518 bp (Pei et al. 2011). In their research, Pei et al. (2011) said that *L. cubeba* sequences using recall markers showed compatibility and could differentiate between closely related species.

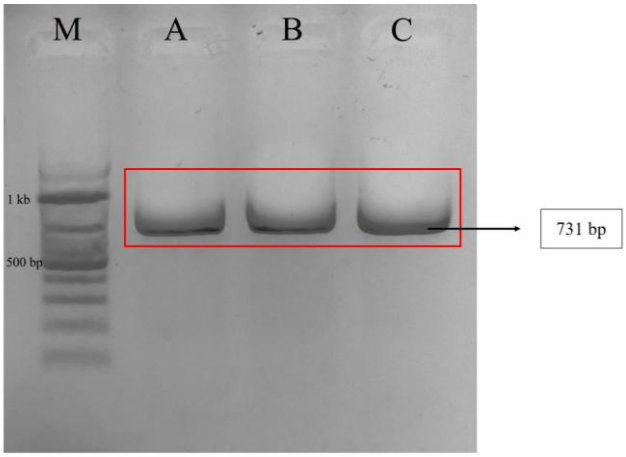
Three samples of *L. cubeba* from different heights were identified using the BOLD system resulting in 100% similarity (identical) with the *L. cubeba* species, which is in the first order. However, the 2nd to 71st ranks are owned by species with several genera of the Lauraceae family with the same high percentage of 99.73%. The entire sequence includes the genera *Actinodaphne*, *Sassafras*, *Lindera*, *Ocotea*, *Nectandra*, *Cinnamomum*, *Licaria*, *Machilus*, *Litsea*, *Rhodostemonodaphne*, *Endlicheria*, and *Aiouea*. In his research, Liu et al. (2017) wrote that there were 44

individuals in the Lauraceae family whom experts incorrectly identified, 34 individuals were incorrect at the genus level, and 10 individuals needed to be corrected at the species level. It causes the *matK* barcode to be unable to distinguish several closely related members of the Lauraceae family.

The search results through the BOLD system were also strengthened through the BLAST program. The cover query value of *L. cubeba* samples in the Mt. Anjasmoro was 100% for the *L. cubeba* species in NCBI, namely with the accession code AB259073.1. These results indicate that the *L. cubeba* samples in the Mt. Anjasmoro are identical to *L. cubeba* in NCBI. *L. cubeba* sequence AB259073.1 is taken from *L. cubeba* individuals in the Cibodas Botanical Garden (Fijridiyanto and Murakami 2016). The E-value of the sequence from the BLAST search results is 0.0, which means the sequence is identical to the sample sequence being compared.

The alignment results are constructed into a phylogenetic tree divided into two major clades (Figure 8). Clade I consists of ingroup species, while clade II consists of outgroup species. Clade I divides into two distinct clusters. This is due to DNA sequence mutations inherited from our ancestors. The first cluster consists of *Litsea japonica*, *L. acutivena*, *L. elongata*, *L. szemaois*, *L. cubeba* AB259073.1, and *L. cubeba* 1, 2, and 3. The second cluster comprises of *Litsea glutinosa*, *L. monopetala*, *L. chunni*, *L. garrettii*, and *L. dilleniifolia*. The outgroup species also form 2 different clusters because they come from different genera. This second clade consists of each outgroup species, namely *Persea americana* and *Liriodendron chinense*.

Based on pairwise distance analysis, the genetic distance of *L. cubeba* 1, 2, and 3 with the comparison species *L. cubeba* from NCBI was 0.000 and had a similarity value of 100% (Table 5). That shows *L. cubeba* 1, 2, and 3 have sequences that match (identical) with the *L. cubeba* sequence in NCBI. *L. cubeba* 1, 2, and 3 had the lowest genetic distance, namely between the *L. cubeba* samples and *L. elongata* and *L. japonica*, with a distance value of 0.010 and a similarity value of 99.04%, so it can be concluded that the three species are more closely related than the other species. The highest genetic distance was found between the *L. cubeba* and *Liriodendron chinense* samples with a distance value of 1.469 and a similarity value of 48.9%, so it can be concluded that the two species are quite distantly related. The higher the nucleotide base similarity, the greater the similarity value, which means it has a close kinship relationship.



Note :
M : Marker; A : Krangean 1; B : Krangean 2; C : Krangean 3

Figure 7. Visualization of DNA specimens of *Litsea cubeba* from Mt. Anjasmoro, Malang District, East Java Province, Indonesia, in a 1.5% agarose gel with a 100 bp DNA ladder

Table 5 The genetic distance of *Litsea cubeba* is based on *matK* gene sequences using Kimura-2 parameter model (percentage) calculation

Specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Litsea glutinosa*		0.010	0.011	0.011	0.003	1.008	1.008	1.008	1.008	1.027	1.020	1.020	1.005	1.568	1.620
2. *Litsea monopetala*	0.010		0.011	0.005	0.006	1.015	1.015	1.015	1.015	1.034	1.028	1.028	1.012	1.568	1.621
3. *Litsea dilleniifolia*	0.011	0.011		0.013	0.008	1.010	1.010	1.010	1.010	1.029	1.022	1.022	1.007	1.530	1.634
4. *Litsea garrettii*	0.011	0.005	0.013		0.008	1.009	1.009	1.009	1.009	1.028	1.021	1.021	1.006	1.555	1.649
5. *Litsea chunii*	0.003	0.006	0.008	0.008		0.997	0.997	0.997	0.997	1.015	1.009	1.009	0.994	1.555	1.634
6. *Litsea cubeba* AB259073.1	1.008	1.015	1.010	1.009	0.997		0.000	0.000	0.000	0.011	0.010	0.010	0.010	1.469	1.388
7. *Litsea cubeba* 1 (this study)	1.008	1.015	1.010	1.009	0.997	0.000		0.000	0.000	0.011	0.010	0.010	0.010	1.469	1.388
8. *Litsea cubeba* 3 (this study)	1.008	1.015	1.010	1.009	0.997	0.000	0.000		0.000	0.011	0.010	0.010	0.010	1.469	1.388
9. *Litsea cubeba* 2 (this study)	1.008	1.015	1.010	1.009	0.997	0.000	0.000	0.000		0.011	0.010	0.010	0.010	1.469	1.388
10. *Litsea elongata*	1.027	1.034	1.029	1.028	1.015	0.011	0.011	0.011	0.011		0.003	0.003	0.014	1.485	1.377
11. *Litsea japonica*	1.020	1.028	1.022	1.021	1.009	0.010	0.010	0.010	0.010	0.003		0.000	0.013	1.483	1.366
12. *Litsea acutivena*	1.020	1.028	1.022	1.021	1.009	0.010	0.010	0.010	0.010	0.003	0.000		0.013	1.483	1.366
13. *Litsea szemaois*	1.005	1.012	1.007	1.006	0.994	0.010	0.010	0.010	0.010	0.014	0.013	0.013		1.469	1.367
14. *Liriondendron chinense*	1.568	1.568	1.530	1.555	1.555	1.469	1.469	1.469	1.469	1.485	1.483	1.483	1.469		0.946
15. *Persea americana*	1.620	1.621	1.634	1.649	1.634	1.388	1.388	1.388	1.388	1.377	1.366	1.366	1.367	0.946	

Note : the highlight section shows the closest, farthest genetic distance
: Identic
: Closest genetic distance
: Farthest genetic distance

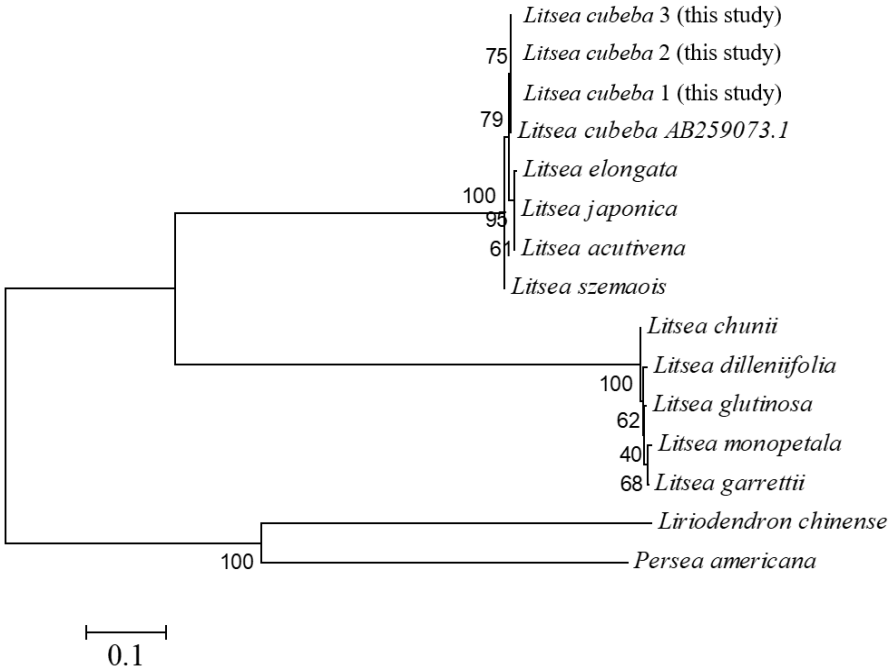


Figure 8. Phylogenetic tree of *Litsea cubeba* species with reference species constructed using the Neighbor-Joining MEGA 6 method. *L. cubeba* 1, 2, and 3 are collected from an altitude of 1,400, 1,500, and 1,600 m asl. respectively

The results of molecular analysis showed that 100% of the studied *L. cubeba* species were *Litsea cubeba*. This research resulted in findings in the form of 16 superior characteristics as markers of *L. cubeba* characters that grow in mountain forests, including seven morphological characters: inflorescence stalk length, inflorescence bud length, inflorescence bud width, stamen stalk length, fruit stalk length, fruit diameter, and fruit weight; three micromorphological characters: number of stomata, stomatal density, and average length of epidermal cells; six anatomical characters: lamina thickness, cuticle thickness, leaf mesophyll thickness, spongy tissue thickness, leaf vein thickness, and leaf vein width. In addition, this research can also be used as a means of identifying *L. cubeba* based on the *matK* gene marker. *L. cubeba* has potential as a drug, but *L. cubeba* has a low seedling survival rate, causing *L. cubeba* in Indonesia to have the status of least concern (IUCN red list). Thus, characterization and molecular-based identification of *L. cubeba* are appropriate for conservation efforts.

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