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# Research Article
# Morphological and Molecular Characteristics of *Parashorea stellata* Kurz. in Central Vietnam

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## Abstract

**Background and Objective:** *Parashorea stellata* Kurz. is a large tree species with many values in Vietnam. Currently, the identification of *P. stellata* is mainly based on basic morphological indicators, which are difficult, inaccurate and easily mistaken. Therefore, as a basis for effective species identification, this study aims to analyze morphological, microscopic and molecular characteristics. **Materials and Methods:** The specimens were collected from 14 representative *P. stellata* individuals from five sites in Central Vietnam. Methods of micromorphological characteristics were observed and compared with previous research. The stems, leaves and roots were then analyzed histologically using the green carmine stain. Powder characteristics were also examined microscopically and finally, two DNA barcodes *rbc*L and *trn*H-*psb*A belonging to the chloroplast gene region were used to analyze the molecular characteristics. Microscopes Olympus LC30, ImageJ 1.5 bp and MEGA X were used for equipment and software data analysis. **Results:** Leaf stomatal structure is oval and elongated and leaves, stem and roots have calcium oxalate. Two barcode gene segments were cloned and sequenced with a total length of 731 bp for *rbc*L and 236 bp for the *trn*H-*psb*A region. The coverage was high and the similarity reached 100% with an alignment E-value of 0.000. Such anatomical and molecular characteristics could be able to be used to distinguish this species from other species with similar morphological characteristics. **Conclusion:** This study is the first to describe in detail the morphological characteristics, microanatomy and DNA barcode analysis of *P. stellata* in Central Vietnam. It is an important contribution to the classification of plant species with similar morphological characteristics or closely related.

Key words: Botanical characteristics, Central Vietnam, DNA barcode, molecular features, *Parashorea stellata*, powdery characteristics

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The species of the Dipterocarpaceae have created the most unique plant family of the tropics. They play an important ecological and economic role in producing valuable non-timber forest products to serve the lives of people in Asian countries, especially Southeast Asian countries[1]. Statistics indicate 44 species belonging to 6 genera in Vietnam, with 11 species listed as rare and endangered plant species[2]. Among the Dipterocarpaceae family, the *Parashorea* genus, first described by Kurz in 1870, belongs entirely to Asia distributed in tropical forests from South China to most Southeast Asian countries with 24 species[3]. The *Parashorea* genus has been recorded and described in 2 species, including *P. chinensis* Wang Hasie. and *P. stellata* Kurz.[3]. In Central Vietnam, *P. stellata* is a woody plant with significant value for timber and non-timber forest products. Additionally, it plays a crucial role in shaping the structure of the evergreen forest complex in tropical regions, with a minimum vitality index (IVI) of 18.9%[4] and it was categorized as Critically Endangered (CR) in IUCN Red List (2018) and as Vulnerable (VU) in Red Data Book of Vietnam (2007)[3]. *Parashorea stellata* is typically distributed in the mountainous areas of Phu Loc, Nam Dong and A Luoi in Thua Thien Hue Province, with soil characteristics of red-yellow feralit soil, growing on magma rocks with many exposed rocks and thin soil layers. *Parashorea stellata* has been identified as a native tree species belonging to the group of trees planted for forest restoration in Thua Thien Hue Province. However, biological information about the species is mainly a preliminary description of the morphology, distribution, ecology, use value and conservation status[4]. Comprehensive information on biological characteristics of the species, including their anatomical and molecular morphology which are closely related to their taxonomic position[5,6]. Therefore, in-depth studies on the morphological, anatomical and silvicultural characteristics of *P. stellata* will be important data in improving understanding and developing conservation strategies for the species.

For plants in general, morphological description is the classic method commonly used to classify species[7,8]. Based on morphological characteristics, two species *P. chinensis* and *P. stellata* in the *Parashorea* genus (Dipterocarpaceae) distributed in Vietnam were previously classified[3]. Still, many *Parashorea* and *Shorea* genus species have so many morphological characteristics that it is easy to make mistakes in species identification[8]. Furthermore, to gather key morphological indices used in plant classification, such as flowers, fruits, etc.[9], researchers must collect data seasonally; this seasonal collection requirement poses challenges and limitations to research progress and classification efficiency. Therefore, many researchers are adopting the combined use of molecular and morphological markers in plant classification as a highly accurate method in phylogenetic research. It can be performed quickly at any time independent of genetic or seasonal factors[10-12].

Recently, the use of DNA barcodes is proving valuable in identifying species, understanding species boundaries, community ecology, functional trait evolution, trophic interaction and the conservation of biodiversity[13,14]. Using the DNA indicator was more accurate without depending on any objective factors. For plant taxonomy, the Ribulose-1,5-Bisphosphate Carboxylase (*rbc*L) gene and the *trn*H-*psb*A gene region are two of the plastid genome markers commonly recommended[14-18]. The *rbc*L and *trn*L-*trn*F barcode gene segments have also been successfully used in the taxonomy of tropical plants including Dipterocarpaceae[17,18]. The *rbc*L is proposed as the standard barcode locus while *trn*H-*psb*A is considered an additional locus commonly used in plant identification[19]. Therefore, this study aims to provide detailed information on the biological characteristics of *P. stellata* species in Central Vietnam, especially morphological, microscopic and molecular features to identify and distinguish this species from plant species with similar morphological characteristics or closely related.

## MATERIALS AND METHODS

**Materials:** About 14 natural specimens of *P. stellata* were collected from 5 localities in Central Vietnam (12 in Thua Thien Hue, 01 in Da Nang and 01 in Quang Nam) from September, 2023 to May, 2024 for morphological and molecular studies (Table 1). The tissues from the adductor muscle were dissected from fresh leaves and frozen at -80°C until DNA extraction.

**Morphological study:** The macromorphological features of *P. stellata* were conducted at the Silviculture Laboratory, Faculty of Forestry, University of Agriculture and Forestry, Hue University. Morphological characteristics were described, measured and counted based on the methods of James and Bell[20]. The macromorphological features were compared with the taxonomy key, pictures and descriptions in the references of Van Sam and Nanhe[3].

Epidermal characteristics of leaves were determined according to the method described by Van *et al.*[21] with modifieds at the Laboratory of the Faculty of Agronomy, University of Agriculture and Forestry, Hue University. Twelve

Table 1: Biology database and analytical indicators for samples collected

| Sample | Location | Date collection | Sample characteristics | Analysis index |
|---|---|---|---|---|
| ND.01 | Nam Dong, TT.Hue | 07/09/2023 | Adult with fruit, 15-25 m (tree height) | Morphological (stem, leaf, fruit) and molecular |
| ND.02 | Nam Dong, TT.Hue | 07/09/2023 | Adult with fruit, 25-30 m (tree height) | Morphological (stem, leaf, fruit) |
| ND.03 | Nam Dong, TT.Hue | 28/04/2024 | Adult with flower, 15-20 m (tree height) | Morphological (stem, leaf, flower), epidermal, micromorphological and powder features (leaf) |
| ND.04 | Nam Dong, TT.Hue | 28/04/2024 | Sapling under forest canopy, 1-2 years old, 35 cm (height) | Morphological, micromorphological, powder features (leaf, stem, root) and epidermal (leaf) |
| DNang.01 | Da Nang | 15/12/2023 | Mature tree, no flowers and no fruits, 12-15 m (tree height) | Molecular |
| AL.01 | A Luoi, TT.Hue | 02/09/2023 | Adult with fruit, 15-20 m (tree height) | Morphological (stem, leaf, fruit) and molecular |
| AL.02 | A Luoi, TT.Hue | 02/09/2023 | Adult with fruit, 20-25 m (tree height) | Morphological (stem, leaf, fruit) and molecular |
| AL.03 | A Luoi, TT.Hue | 25/04/2024 | Adult with flower, 25-30 m (tree height) | Morphological (stem, leaf, flower), epidermal, micromorphological and powder features (leaf) |
| AL.04 | A Luoi, TT.Hue | 25/04/2024 | Sapling under the forest canopy, 1-2 years old, 30-35 cm (tree height) | Morphological, micromorphological, powder features (leaf, stem, root) and epidermal (leaf) |
| PL.01 | Phu Loc, TT.Hue | 05/09/2023 | Adult with fruit, 20-25 m (tree height) | Morphological (stem, leaf, fruit) and molecular |
| PL.02 | Phu Loc, TT.Hue | 05/09/2023 | Adult with fruit, 15-20 m (tree height) | Morphological (stem, leaf, fruit) and molecular |
| PL.03 | Phu Loc, TT.Hue | 02/05/2024 | Adult with flower, >20 m (tree height) | Morphological (stem, leaf, flower), epidermal, micromorphological and powder features (leaf) |
| PL.04 | Phu Loc, TT.Hue | 02/05/2024 | Sapling under the forest canopy, 1-2 years old, 30-35 cm (tree height) | Morphological, micromorphological, powder features (leaf, stem, root) and epidermal (leaf) |
| QNam.01 | Quang Nam | 02/12/2023 | Mature tree, no flowers, no fruits, 10-15 m (tree height) | Molecular |

leaf samples with full development were collected from four mature trees from Phu Loc site to estimate stomatal density. Surface impressions of the leaf were estimated by preparing slides from the clear nail polish impression at two opposite middle positions of the lower surfaces of each leaf. Ten images were obtained from each site with a total area of 0.2378 $mm^2$ per picture (at 100 µm scale) using the Digital camera LC30-Olympus Microscope, Japan. Leaf area and stomatal counts were performed with ImageJ 1.5 p under a freeware license (Wayne Rasband, National Institutes of Health, USA).

The micromorphological features of stems, roots and leaves were determined using the iodine green-carmine double staining method by Van Chen *et al.*[22] at the Laboratory of the Faculty of Pharmacy, University of Medicine and Pharmacy, Hue University. The root, stem and leaf samples from 3 collected saplings were cut crosswise into thin slices using a razor blade and around 20-30 representative samples were selected to be stained. The stem, leaf and root samples of *P. stellata* were soaked in java solution for 15-30 min and then washed under water. Next, the specimens were soaked in 5% acetic acid for 3-5 min, then washed with water. The specimens were microscopically stained with methylene blue and carmine red, respectively. After each staining, the specimens were washed several times with water. Finally, the stained specimens were placed in a drop of 10% glycerine on a glass slide, covered with a coverslip, observed under a microscope, photographed and described.

The powder features of the stems, roots and leaves of *P. stellata* were determined by using the protocol described by Van Chen *et al.*[22]. The samples after harvest were washed, exposed to sunlight dried completely at 60-70°C and ground into a coarse powder. The powders of stems, roots and leaves were passed through a sieve (mesh size: 150 µm) to remove coarse particles. The fine powder is then soaked in water to remove air bubbles. An appropriate amount of hydrated powder is placed on a slide containing a drop of 10% glycerine, covered with a cover slip and observed under a microscope (LC30-Olympus, Japan), looking for features and photographs.

Cell size and cell composition are measured using an eyepiece micrometer (LC30-Olympus Microscope, Japan) at magnifications of 4, 10, 20 and 40X. All values measured in the morphological and anatomical analyses were shown as Mean±SD (Standard Deviation), maximum and minimum values. Calculations were performed using Microsoft Excel 2016 software.

**Molecular analysis:** The molecular analysis were performed at the Molecular Biology Laboratory, University of Agriculture and Forestry, Hue University. Total DNA from *P. stellata* leaf samples from trees of ND.01, DNang.01, AL.01, PL.01, PL.02, QNam.01 in Table 1 was extracted using the method described by FastPure Plant DNA Isolation Mini Kit (Vazyme Biotech Co., Ltd., China) according to the manufacturer's protocol. They were purified using the phenol: Chloroform method. The DNA quality was then checked by electrophoresis on a 1.0% agarose gel in 1X TAE Tris-Acetate-EDTA buffer and stained with Gelred dye (Biotium, USA). Two

Table 2: Reference sequences from GenBank database

| Species name | Accession numbers | | |
|---|---|---|---|
| | *rbc*L | | *trn*H-*psb*A |
| *Parashorea stellata* | OR922785-OR922790* | | OR913554-OR913559* |
| *Parashorea stellata* | | MZ901836.1** | |
| *Parashorea chinensis* | NC_046579.1** | | NC_046579.1 ** |
| | | | MW074187.1* |
| *Shorea zeylanica* | NC_040965.1** | | - |
| *Parashorea macrophylla* | | MH791330.1** | |
| *Shorea pachyphylla* | | NC_040966.1** | |
| *Shorea macrophylla* | | NC_064148.1** | |

*Partial sequence and **Complete genome

gene regions in the chloroplast genome (*rbc*L and *trn*H-*psb*A) of *P. stellata* were selected for PCR amplification with the corresponding specific primer pairs by 1F-5'ATGTCACC ACAAACAGAAAC3' and 724R-5' TCGCATGTACCTGCAGTAGC3' for *rbc*L gene; *trn*H-5'ACTGCCTTGATCCACTTGGC3' and *psb*A-5' CGAAGCTCCATCTACAAATGG3' for *trn*H-*psb*A gene region[23].

The PCR reaction components: 25 µL GoTaq® Green Master Mix, 2X (Promega), 1 µL forward primer (10 pmol/µL, IDT, USA), 1 µL reverse primer (10 pmol/µL, IDT, USA), 100 ng template DNA and additional water store aseptically to a total volume of 50 µL. Thermal cycle for gene fragment amplification reaction: 95°C/5 min; then 30 cycles with 95°C/45 sec, 55°C/45 sec, 72°C/60 sec and finally 72°C/10 min to complete the reaction. The reactions were performed in a thermal cycler SimpliAmp™ Thermal Cycler (Thermo Fisher, USA). The PCR products were checked by electrophoresis on 1% agarose gel in 1X TAE buffer at 100 V for 30 min. Based on the DNA 1 kb standard ladder (Bioline, UK) to determine the size of the amplified product. The PCR products are purified to remove the remaining components after the PCR process using the ISOLATE II PCR kit and Gel kit (Bioline, UK). The purified PCR products will be used for direct gene sequencing reactions from specific primer pairs using the Sanger method on the ABI 3100 analysis system (Applied Biosystems, USA) at 1st Base-Malaysia company. The DNA marker gene regions' nucleotide sequences were edited using BioEdit 7.0.5 software by Sofi *et al.*[24]. After being aligned and compared using the Clustals program[25,26], the nucleotide sequences were registered with the corresponding code number in the GenBank database (Table 2).

The BLAST tool on the GenBank database (NCBI)[27] was used to identify the species name. The nucleotide sequences of target genes of *P. stellata* and other species of the *Parashorea* and *Shorea* genus of the Dipterocarpaceae family published in GenBank were used as outgroups in determining species composition and constructing phylogenetic trees. The kimura 2-parameter (K2P) distance model was used to determine the level of genetic differentiation and construct phylogenetic trees using MEGA 10.0 (The Molecular Evolution Genetics Analysis) software, based on the Maximum Like hood (ML) method[28] with a bootstrap value of 2,000 replicates[29].

## RESULTS

**Morphological characteristics:** *Parashorea stellata* is a large, straight, evergreen tree, with a cylindrical trunk occasionally featuring buttresses and a spherical canopy shape. The tree can achieve a height of 30-40 m, with trunk diameters spanning 80-120 cm and clear height beneath the branches reaching 20-30 m (Fig. 1a-b). The bark exhibits a brownish-grey hue with vertical fissures and emits a slightly aromatic resin (Fig. 1c). Branches are typically large, curved and twisted and young twigs tend to be glabrous. Leaves are characterized by their simple, alternate arrangement, with an entire leaf margin and an elliptical or spear shape. The upper leaf surface is glabrous and smooth, while the lower surface may bear sparse, whitish short hairs. Young leaves have pinkish, mature leaves that have a glossy green coloration on both sides, featuring a curved acuminate apex (Fig. 1d). The leaf area of *P. stellata* has a total area of 57.18±28.24 $cm^2$ with a total length of 126.51±32.438 mm; width of 55.49±20.716 mm; length/width ratio of 2.40±0.462. The leaves have a fresh and dry weight ranging from 0.08-2.1 and 0.06-0.86 g, respectively (Table 3). The petiole of the leaf appeared light brown, smooth and slightly bulging both petioles with length and diameter measuring 19.37±7.439 mm and 1.67±0.534 mm, respectively. Leaf veins are prominently visible on the lower surface, characterized by a smooth, straight midrib, 9 to 13 pairs of lateral veins that are parallel but curved near the margin of leaves and sublateral veins that are scarcely discernible (Fig. 1d and Table 3). Stipules are pale yellow, 4-6 cm in length and 2-4 mm in width, with three veins at the base, covered in star-shaped hairs and soon shed, leaving behind bundle scars (Fig. 1d).

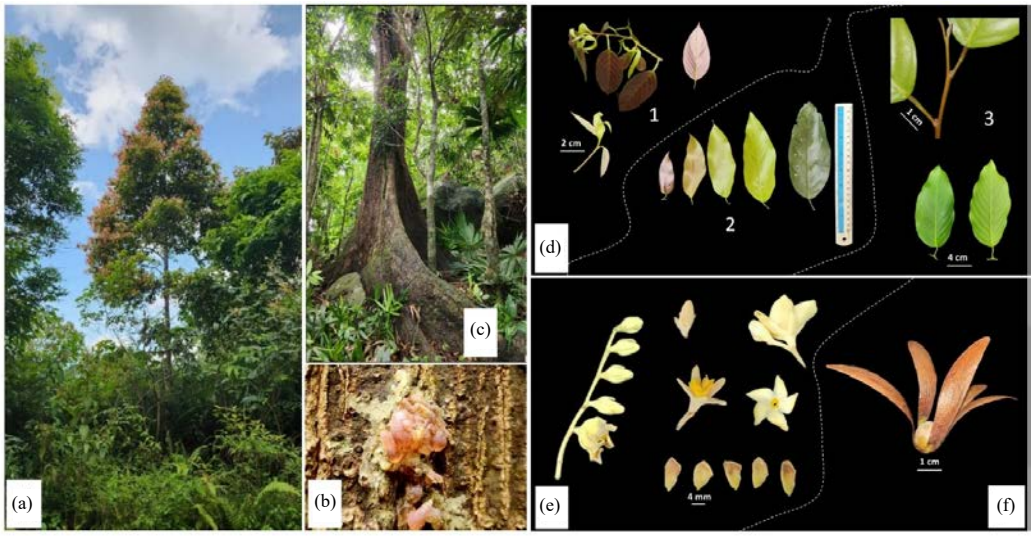


Fig. 1(a-f): Morphological characteristics of *P. stellata* collected in Central Vietnam, (a) Adult tree in the forest, (b) Bark of the stem (Resin and Bark brownish grey with slight fissures), (c) Trunk, (d) Leaf, (e) Flower and (f) Fruit with winds and seed
1: Young leaf branch with pinkish, 2: All leaf types and 3: Adult leaf with branch and petioles

Table 3: Morphological and stomata characteristics of *P. stellata* in Central Vietnam

| Indicators | Range (min-max) | Average±SD |
|---|---|---|
| **Leaf morphological indicators** | | |
| Leaf total length (mm) | 55.0-200.0 | 126.51±32.438 |
| Maximum width (mm) | 20.0-101.0 | 55.49±20.716 |
| Length/width | 1.56-4.0 | 2.40±0.462 |
| Leaf area ($cm^2$) | 34.70-115.75 | 57.18±28.24 |
| Fresh mass (g) | 0.08-2.1 | 0.73±0.444 |
| Dry mass (g) | 0.06-0.86 | 0.37±0.182 |
| Water content (%) | 20.0-59.05 | 45.58±11.05 |
| Number of secondary leaf veins (pairs) | 9-13 | - |
| Petiole length (mm) | 7.0-46.0 | 19.37±7.439 |
| Petiole diameter (mm) | 0.67-2.68 | 1.67±0.534 |
| Petiole length/leaf total length | 0.075-0.247 | 0.153±0.037 |
| **Leaf anatomy** | | |
| Stomatal density ($mm^{-2}$) | 391.8-976.6 | 691.6±117.2 |
| Stomatal length (μm) | 25.31-64.46 | 61.4±6.92 |
| Stomatal width (μm) | 32.74-83.66 | 42.7±8.32 |
| **Flower morphological indicators** | | |
| Number of petals | 5 | 5 |
| Petals length (mm) | 6.95-9.72 | 8.20±0.813 |
| Petals width (mm) | 4.09-6.13 | 4.88±0.493 |
| Length/width | 1.4-2.02 | 1.69±0.176 |
| Number of stamens | 15 | 15 |
| **Fruit morphological indicators** | | |
| Number of fruit wing | 5 | 5 |
| Length of fruit wing (mm) | 41.0-105.0 | 71.0±12.48 |
| Width of fruit wing (mm) | 8.0-29.0 | 16.2±3.71 |
| Length/width of wings | 1.86-9.0 | 4.62±1.333 |
| Length of seed (mm) | 13.36-18.71 | 16.70±1.243 |
| Width of seed (mm) | 13.01-17.23 | 15.03±0.902 |
| Length/width of seed | 0.95-1.22 | 1.11±0.056 |
| Fresh mass of seed (g) | 1.97-2.12 | 2.06±0.081 |
| Dry mass of seed (g) | 1.24-1.26 | 1.25±0.007 |
| Water content of seed (%) | 36.7-40.8 | 39.1±2.10 |

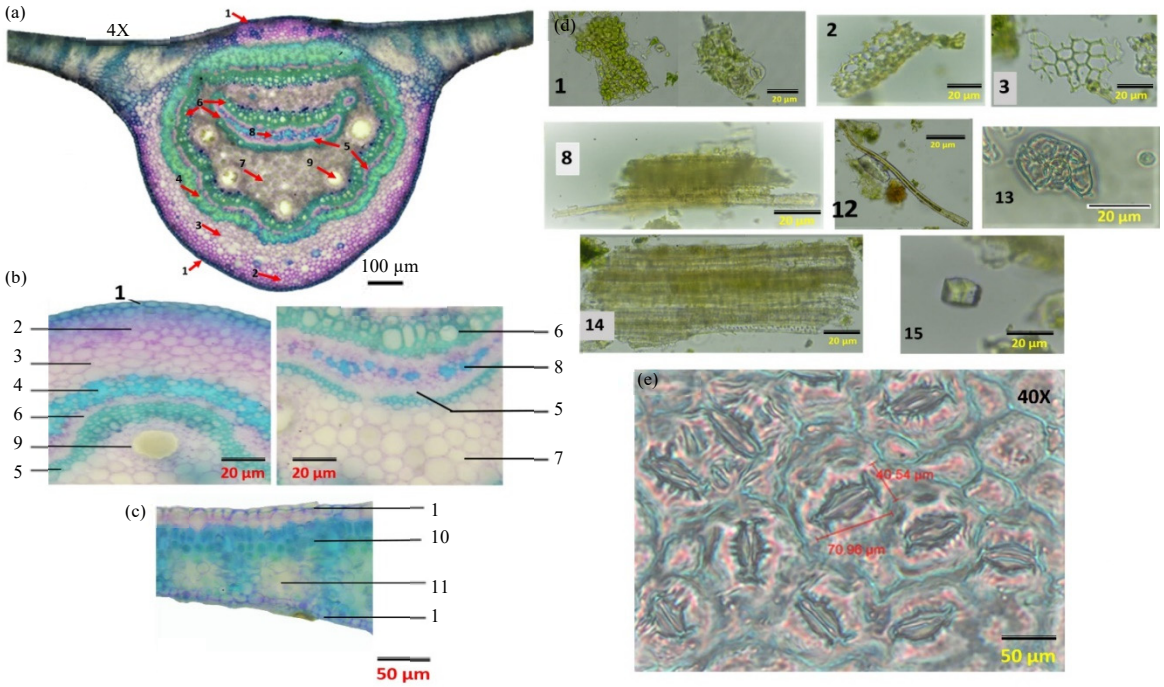


Fig. 2(a-e): Anatomical characteristics of the leaves of *P. stellata* in Thua Thien Hue, (a) Leaf microscopy under 4X objective, (b) Leaf vein microscopy under 40X objective, (c) Leaf blade microscopy under 40X objective, (d) Leaf powder microscopy under 40X objective and (e) Image of leaf stomata under 40X objective

1: Epidermises/pieces of epidermis bearing stomata in power (a, b, c, d), 2: Hypodermis cells (a, b, d), 3: Parenchymatous cells (a, b, d), 4: Sclerenchymatous bundle sheath (a, b), 5: Phloem (a, b), 6: Xylem (a, b), 7: Parenchymatous cells of the intestine (a, b), 8: Vascular bundles (a, b, d), 9: Secretory tissue (a, b), 10: Palisade parenchyma (c), 11: Spongy parenchyma (c), 12: Trichomes (d), 13: Sclereid fibers (d), 14: Point circuit fragments (d) and 15: Calcium oxalate crystals (d)

Inflorescences range from 6-14 cm in length and can be terminal or axillary. Each flower has 5 petals, while petals are ovate and whitish and have a distinctive aroma. Sepals are imbricate and oblong, measuring range in length from 6.95-9.72 (8.20±0.813) mm, petal width is about 4.09-6.13 (4.88±0.493) mm; petals are even with a length/width ratio of 1.69±0.176 mm. Stamens, numbering 15, are arranged in two rows. The ovary is ovoid, divided into three compartments, each containing two ovules, with slightly greyish hairs measuring 1 mm in length. Flowers of *P. stellata* usually bloom around the end of April to the first of May in Thua Thien Hue Province, Vietnam (Fig. 1e, Table 3).

The fruits of *P. stellata* develop from April to about September each year in Thua Thien Hue, Vietnam. The fruit bears five nearly equal wings with the length of the fruit wing ranging from 41.0-105.0 (71.0±12.48) mm, the width of the fruit wing is about 8.0-29.0 (16.2±3.71) mm; the length/width ratio of the fruit wing ranges from 1.86 to 9.0 (4.62±1.333) mm. The seeds are distributed mainly between the wings which are linked together. The seeds are globular, featuring grey stomata and a star-shaped hair pattern the length of the fruit ranges from 13.36-18.71 (16.70±1.243) mm and the width of the fruit ranges from 13.01 to 17.23 (15.03±0.902) mm. The length/width ratio of the seed was 1.11±0.056 mm. The fresh weight and dry weight of the seed ranged from 1.97 to 2.12 (2.06±0.081) g and 1.24 to 1.26 (1.25±0.007) g, respectively and the water content of the seed was 39.1±2.10% (Fig. 1f, Table 3).

## Micromorphological features

**Leaf:** The structure of the leaf included the following parts: Midrib, vascular bundles, leaf blade, stomata and powder.

**Midrib and vascular bundles:** Microscopic analysis of *P. stellata* leaf veins shows that the upper surface is slightly convex and round, while the lower surface is convex and has many arc-shaped veins (Fig. 2a). The upper and lower epidermises (1) consist of a layer of polygonal, slightly round and small cells, an average size of about 4.39 µm, a cellulose wall and a cuticle covering the outer cell wall. Hypodermis cells (2) consist of polygonal, slightly round cells, in which the upper hypodermis tissue consists of 5-7 cell layers, the lower

Table 4: *Parashorea stellata* identification by using BLASTn of *rbc*L and *trn*H-*psb*A sequences with MZ901836.1 on NCBI database

| | | Quantity (values in parentheses are calculated in percentage) | | | | | Specie identification using BLASTn on NCBI | | |
|---|---|---|---|---|---|---|---|---|---|
| Gene | Accession GenBank | T | C | A | G | Total | Cover (%) | Max identity (%) | E-value |
| *rbc*L | OR922785-OR922790 | 204 (27.91) | 153 (20.93) | 214 (29.26) | 160 (21.89) | 731 (100) | 100 | 100 | 0.000 |
| *trn*H-*psb*A | OR913554-OR913559 | 80 (33.90) | 27 (11.44) | 56 (36.86) | 42 (17.80) | 236 (100) | 100 | 100 | 0.000 |

hypodermis tissue consists of 3-5 cell layers, cell size is about 11.17 μm. The parenchymatous cells (3) behind the hypodermis cells layer are round and polygonal cells, of irregular size, averaging about 21.57 μm. The layers of sclerenchyma cells (sclerenchymatous bundle sheath) (4) an arc around the central conductive bundle, are about 82.59 μm thick and consist of round or oval thick-walled cells, with an average size of about 13.04 μm.

The vascular bundle system consists of two parts, the outer vascular bundle and the inner vascular bundle. The outer vascular bundle consists of a secondary phloem ring (5) surrounding the secondary xylem ring (6) on the inside; the cells of the phloem layer are about 6.72 μm in size and about 11.35 μm for the xylem cells. Surrounding the inner vascular bundle is the parenchymatous tissue (7), consisting of round or polygonal cells of uneven size, scattered in the parenchyma are calcium oxalate crystals and secretory tissue (9). The inner vascular bundle has the opposite arrangement to the outer vascular bundle, the primary xylem ring (6) surrounds the primary phloem layer (5) on the inside, in the phloem there are many scattered vascular bundles (8) (Fig. 2a-b).

**Leaf blade:** In the transverse section of the leaf blade, the upper and lower epidermises are composed of polygonal cells with a size of about 14.98 μm. The cells in the upper epidermis are larger than in the lower epidermis. Located between the two epidermal cell layers of the blade leaves is mesophyll composed of two kinds of tissues: The palisade parenchyma (10) is a layer of elongated chlorenchyma cells rectangular or trapezoidal, elongated, closely arranged and perpendicular to the upper epidermis, containing large amounts of chloroplasts; the spongy parenchyma (11), a lower layer of irregular cells with few chloroplasts and very prominent intercellular air spaces. The middle mesophyll cells contain scattered calcium oxalate crystals (Fig. 2c).

**Leaf powder:** The leaf powder of *P. stellata* is light green, has a light aroma and an astringent taste. Observing the leaf powder under the microscope presented different components, such as the polygonal fragments of epidermises with stomata (1), hypodermis (2), parenchymatous (3), vascular bundles with long, thin-walled fibers clustered in bundles (8), trichomes which intact or broken (12), starch granules which 4-petaled flower-shaped (13), point circuit fragments (14) and calcium oxalate crystals which cubic-shaped (15) (Fig. 2d).

**Stomata:** The stomatal structure of *P. stellata* is oval and elongated (Fig. 2e), found on the lower side of the leaves. The average length and width of stomata are 61.4±6.92 μm and 42.7±8.32 μm, respectively and the stomatal density is 691.6±117.2 $mm^{-2}$ (Table 4).

**Stem:** The morphological anatomy of *P. stellata* stem shows that the cross-section of the stem is circular and slightly distorted (Fig. 3a). The structure from the outside includes the outermost cuticle layer (1), which consists of a layer of rectangular dead cells arranged regularly with an average size of about 9.52 μm. The hypodermis (2), located close to the cork layer, consists of 4-6 rows of cells with a size of about 10.50 μm arranged in a continuous circle. The parenchymatous of bark (3) are polygonal, thin-walled cells with different sizes from 9.77-24.15 μm, averaging about 14.14 μm. The sclerenchymatous bundle sheath, also known as sclerenchyma (4), is polygonal with thick walls, arranged closely in clumps at the boundary between the soft bark tissue and phloem, with a size of about 9.31 μm. Xylem (5) is arranged in a continuous ring, consisting of many cells with a size of about 7.66 μm. Phloem (6) is arranged in a polygonal ring, the wood tissue cells have thick walls with an average size of about 11.77 μm. The secretory tissue (7) has an average size of about 96.98×46.53 μm and is where essential oils are stored, etc. In the innermost layer is the parenchymatous of the pith (8), consisting of many polygonal cells and thin walls, with an average uniform size of about 25.58 μm. Interspersed between the hypodermis (2) and the soft parenchymatous tissue layer (3) are vascular bundles (9) with irregular sizes and sparse distribution. Calcium oxalate crystals, cubes or spheres, are scattered throughout the tissue layers (Fig. 3a-b).

In the powder of *P. stellata* stems have earthy yellow, besides, fragments of cork (1), hypodermis (2), fragments of parenchymas (3), xylem vessels with spiral vessels (6), the fragments of forming spiral vessels, bunched (9), crystal fibers, which are intact or broken (12), point circuit fragments, fragments of spiral vessels (5), calcium oxalate crystals have shape cubic or spherical (6), starch granules have single or double, triple structure (7) and fragments of tissue with starch granules (8) were observed in the powder sample (Fig. 3c).

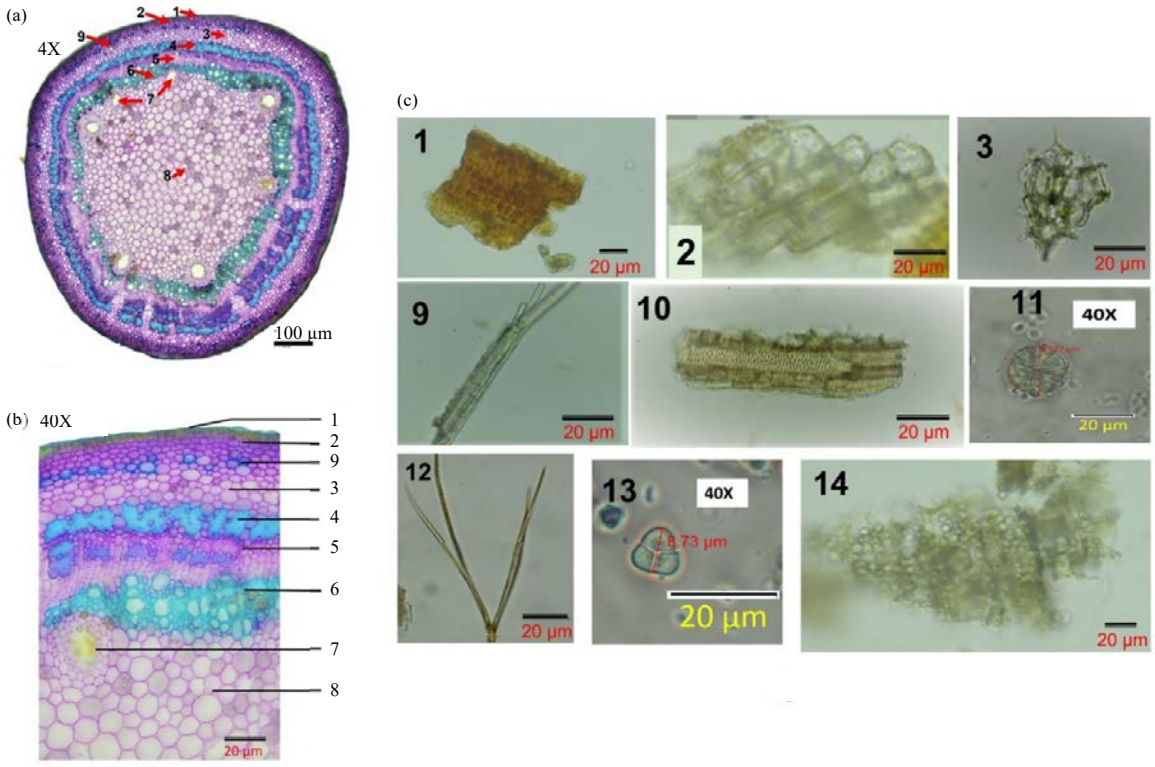


Fig. 3(a-c): Anatomical characteristics of the *P. stellata* stem in Thua Thien Hue, (a) Stem microtomy at 4X objective: 1-9, (b) Stem microtomy at 40X objective: 1-9 and (c) Stem powder microscopy image: 1-3, 9-14

1: Fragment of cork (a, b, c), 2: Hypodermis (a, b, c), 3: Fragment of parenchyma cell (a, b, c), 4: Sclerenchymatous bundle sheath (a, b), 5: Xylem (a, b), 6: Phloem (a, b), 7: Secretory tissue (a, b), 8: Parenchymatous cells/tissue of the pith (a, b), 9: Bundle of fibers (a, b, c), 10: Fragment of vasculars (c), 11: Calcium oxalate crystals (c), 12: Crystal fibers (c), 13: Starch granules (c) and 14: Starch-bearing tissue/fragments of tissue with starch granules (c)

**Root:** The roots of *P. stellata* have a strongly developed taproot structure in the middle, surrounded by a well-developed fibrous root system (Fig. 4a). The microscopic characteristics of the black poplar tree roots show that the root cross-section is circular (Fig. 4b). The structure from the outside to the inside includes: The cork (1) consists of 3-5 rows of rectangular cells, arranged in concentric rings, with many cracks and tears, with an average size of about 5.11 µm. The parenchymatous (2) consists of 4-7 rows made of thin-walled cells, the outside is flattened and the inside is ovoid, the size of the soft cortex tissue cells is about 11.47 µm. The phloem (3) consists of polygonal cells about 10.08 µm in size forming phloem bundles; the bundles are arranged in discontinuous rings interspersed with fiber clusters. The fiber bundles (4) have 7-9 rows of discontinuous cells forming clusters, the polygonal fiber cells are about 10.21 µm in size. The phloem (5) consists of polygonal cells arranged in a ring around the soft intestinal tissue, the wood cell size is about 18.11 µm. The parenchymatous intestinal tissue (6) is the innermost layer, consisting of many polygonal cells about 25.06 µm in size (Fig. 4b).

The root powder of *P. stellata* is greenish-yellow, astringent and odorless. Microscopic observation of the structure of the fragment of cork (1), fiber bundles (3), tissue fragments bearing calcium oxalate crystals (1), chromophore fragments (7), fragments of spiral vessels (8) and cubic or spherical calcium oxalate crystals (9) shows that they are separated and have a stable structure (Fig. 4c).

## Molecular characteristics

**Molecular classification:** All PCR products from the studied *P. stellata* samples exhibited a single band with a consistent 100% amplification ratio, high DNA concentration and differences between the targeted genomic regions. The size of the isolated genomic regions, approximately 731 bp (*rbc*L) and 236 bp (*trn*H-*psb*A), aligns with the originally expected sizes (Fig. 5).

All samples were successfully sequenced and deposited in the US gene bank (Genbank) with the corresponding assigned codes: *rbc*L (OR922785-OR922790) and *trn*H-*psb*A (OR913554-OR913559). The BLAST analysis conducted on NCBI verified and compared these sequences with those of the

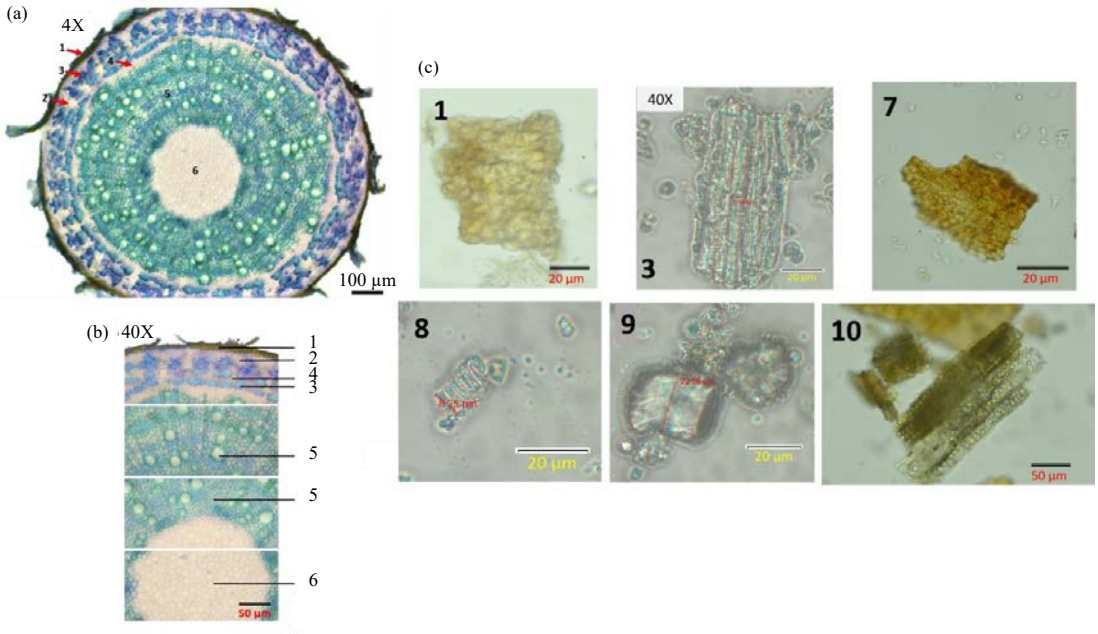


Fig. 4(a-c): Microscopic characteristics of roots of *P. stellata* in Thua Thien Hue, (a) Root microsurgery at 4X objective, (b) Root microsurgery at 40X objective and (c) Root powder examination at 40X objective

1: Fragment of cork (a, b, c), 2: Parenchymatous of bark (a, b), 3: Xylem (a, b, c), 4: Fiber bundles (a, b), 5: Phloem (a, b), 6: Parenchymatous (a, b), 7: Chromophore fragments (c), 8: Fragments of spiral vessels (c), 9: Calcium oxalate crystals (c) and 10: Circuit fragments bearing calcium oxalate crystals (c)

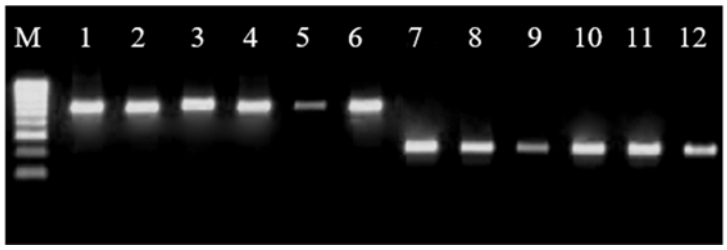


Fig. 5: Electrophoresis of PCR amplified products

M: DNA Standard Marker (100-1000 bp, Biobase), Wells 1-6: PCR products of the *rbc*L gene and Wells 7-12: PCR products of the *trn*H-*psb*A gene

species *P. stellata* (code: MZ901836.1) showing high coverage and similarity reaching 100% with an alignment E-value of 0.000 (Table 4).

**Phylogenetic tree:** Based on the sequences of the *rbc*L and *trn*H-*psb*A gene segments of *P. stellata* in Central Vietnam and reference sequences taken from GenBank, a phylogenetic tree was constructed using Mega X software (Fig. 6).

All *P. stellata* samples collected in the center of Vietnam clustered together and with the carpet sample MZ901836.1 (*P. stellata*) into a group located on the same branch on the phylogenetic tree with K2P distances created from *rbc*L and *trn*H-*psb*A both equal to 0.000 and far different from some other species of *Parashorea* and *Shorea* genus with K2P distances of 0.0041-0.0152 and 0.078-0.088, respectively (Fig. 6).

## DISCUSSION

In this study, detailed information on the basic morphological characteristics of the stem, leaves, roots, flowers and fruits of *P. stellata* (Fig. 1, Table 3) was described: Cylindrical stem, brown bark, straight stem, few branches; oval-shaped leaves-oblong, young leaves are light pink, when old the leaves are smaller green; leaves have 9-13 pairs of veins, prominent on the underside. Stipules are curved and fall early; flowers are milky white, have a light fragrance, grow in clusters and bloom at the end of April-the first of May,

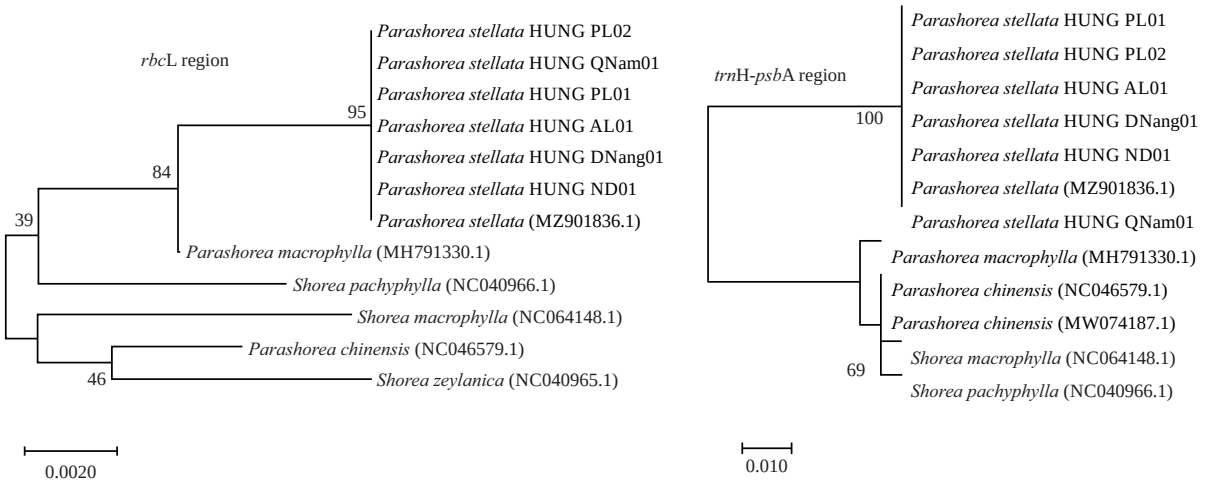


Fig. 6: Phylogenetic trees based on *rbc*L and *trn*H-*psb*A gene region sequence

stamens have two rows, each row has 15; fruit develops from April-August and falls in September, fruit has five nearly equal petals, oval seeds. These characteristics were consistent with previous descriptions by van Sam and Nanhe[3].

The morphological and anatomical characteristics of *P. stellata* presented in this study (Fig. 1-4, Table 3) show the typical adaptive characteristics of a light-loving, high-altitude tree species distributed in mountainous areas at an altitude of 300-800 m above sea level with soil characteristics on red-yellow fertility soil, growing on magma rocks with many exposed rocks, thin soil layers and complex terrain. *Parashorea stellata* has a straight, woody stem with few branches, the root system is a taproot system with strongly developed secondary roots consistent with the characteristics of the dicotyledonous woody plant group (Fig. 1). The histological structure of the stem and root shows the arrangement and proportion between the tissue layers: Epidermises (1), hypodermis cells/tissue (2), parenchymatous cells/tissue of bark (3), sclerenchymatous bundle sheath (4), xylem (5), phloem (6) combined with the system of secretory tubes, vascular bundles, calcium oxalate crystals (Fig. 3-4) along with structures such as crystal fibers (stem) (Fig. 3c) along with structures such as crystal fibers (stem) (Fig. 3c) and spiral vessels (roots) (Fig. 4c). The secondary phloem layers in the stem and the roots develop; xylem fibers and phloem fibers are distributed widely in the stem and roots, supporting strong growth and development, forming a strong, flexible stem structure, suitable for harsh living conditions with strong winds and drought.

The leaves of *P. stellata* are not large, the more mature the tree is, the smaller the leaves are; the leaf surface has a thick cuticle layer, developed stomata, leaf vein system developed (9-13 pairs) and the palisade tissue develops with a strong vascular system, the intercellular space in the hexagram is relatively large, the sclerenchyma tissue rings surrounding the vascular bundles (Fig. 2, Table 3) help the leaves to be strong against the mechanical impacts of the external environment. The leaves of *P. stellata* have a stomatal system with a smaller area and higher density (Fig. 2e, Table 3) compared to the plants living in the lower layer such as *Distichochlamys citrea*[22], *Eurycoma longifolia*, the middle layer such as: *Vinca major*, *V. major* var. *variegata*, *V. herbac*[30] and the upper layer of forest vegetation such as *Eucalyptus globulus* ssp. *Globulus*[20]. In addition, the flower, fruit and seed characteristics of *P. stellata* described (Table 3, Fig. 1e-f) show conformity with the descriptions of Dipterocarpaceae family and natural distribution conditions in high mountainous areas, strong winds, heavy rain, hot and humid weather. The anatomical morphological characteristics of *P. stellata* are consistent with the observations on the adaptation of photophilous, highland and coastal mountainous plants by previous authors such as Heckenhauer *et al.*[6], Thach and Dieu[31] and Ha *et al.*[32].

In this study, the powder microscopy method was applied for the first time to *P. stellata*. Powder microscopy analysis of stems, leaves and roots of *P. stellata* generally observed most of the components present in fresh samples. Some components, such as upper epidermal cells, lower epidermal cells and hypodermal cells, were difficult to distinguish in powder microscopy samples because the structures were deformed after drying, they became similar in plant species and their locations could not be determined[22]. However, powder microscopy analysis showed intact structures or parts not observed in fresh samples such as vascular fragments, crystal fibers and calcium oxalate crystals (Fig. 2d, 3c, 4c).

Therefore, powder analysis combined with histological anatomy will help identify unique micromorphological characteristics of species with higher accuracy[33] in plant research. In addition, in the present study, a unique feature was added to *P. stellata*, which is the presence of calcium oxalate in the leaves, stem and roots (Fig. 2d, 3c and 4c). Calcium oxalate is a common substance identified in more than 200 plant families[34] with the shape, size, distribution and number of calcium oxalate crystals varying among species[35,36]. Several functions of calcium oxalate have been demonstrated, such as calcium regulation, plant defense and heavy metal detoxification[37]. Therefore, the identification of calcium oxalate crystals in *P. stellata* can be used to identify the potential of this species and further investigations on the anatomy of Dipterocarpaceae family.

In Vietnam, molecular marker classification studies have proven effective across various plant species. For instance, in the case of the *Eucalyptus hybrid* UG24 (*E. urophylla*× *E. grandis*), the *mat*K, *rbc*L, *trn*H-*psb*A, *ITS1* and *ITS2* gene segments were cloned via PCR, yielding to facilitating the classification within the species[37]. The *trn*H-*psb*A fragment has served as a vital indicator to identify certain plant species such as *Paphiopedilum orchids*[16]. In the Dipterocarpaceae family, DNA barcoding has been used to classify and build genetic phylogeny between genera and species based on the sequence analysis of barcode gene segments, including *rbc*L, *mat*K, *trn*L-*trn*F, *ITS1* and *ITS2*[17,18]. In this study, a pioneering approach was adopted, marking the first utilization of DNA barcoder molecular markers to classify and build a phylogenetic model for *P. stellata* in Central Vietnam based on the sequences of two gene fragments, *rbc*L and *trn*H-*psb*A. Based on the sequences of the two gene fragments, *rbc*L *and trn*H-*psb*A were successfully cloned with a length of 731 bp and 236 studies were identified as *P. stellata*, which belongs to the genus *Parashorea*, in the Dipterocarpaceae family by molecular markers (Table 4). Phylogenetic analysis based on the *rbc*L and *trn*H-*psb*A sequences showed a clear genetic relationship between *P. stellata* and other species in the Dipterocarpaceae family (Fig. 6). Notably, the subspecies and species examined in this study were correctly grouped into genera and sections, compared to previous taxonomic methods reported by van Sam and Nanhe[3], Hu *et al.*[17], Osathanunkul and Madesis[18] and Newman *et al.*[38]. These results underscore the suitability of the *rbc*L and *trn*H-*psb*A barcode gene segments as effective markers in the classification of *P. stellata* species. Such insights are pivotal for ongoing research endeavors aimed at devising strategies for the conservation and sustainable management of *P. stellata* populations in Vietnam and beyond.

## CONCLUSION

The study has built a data set on growth characteristics and morphology of leaves, stems, roots, flowers and fruits combined with molecular biology to determine the scientific name of *P. stellata* in Thua Thien Hue Province. The study has provided information on the morphological and anatomical characteristics of roots, stems and leaves of *P. stellata* species collected in the natural environment in Central Vietnam. For the first time, it was successfully isolated and used molecular markers based on *rbc*L and *trn*H-*psb*A gene sequences by DNA barcoding to determine the composition of species and genetic diversity of *P. stellata* in Central Vietnam. The research results provide and supplement the biological database and premise to expand other studies on silvicultural characteristics, conservation, propagation and development of *P. stellata* species in Vietnam and worldwide.

## SIGNIFICANCE STATEMENT

In Vietnam, *Parashorea stellata* belongs to the family of Dipterocarpaceae and is considered to be a large tree species of high economic, ecological and conservation value. Specific ecological factors limit the species' natural distribution. In particular, the Province of Thua Thien Hue is known as the first distribution area in the North-South direction of Vietnam. In addition to preliminary descriptions of macromorphological characteristics and distribution area, morphological, anatomical, molecular biological and distributional adaptation characteristics are still minimal. Biological data are important for plant classification, conservation, propagation and species development programs. Therefore, this study will provide comprehensive information on biological characteristics related to macromorphological, micromorphological and molecular features for identifying the *P. stellata* species in Central Vietnam.

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