

Anatomical Characteristics and Genetic Relationship of *Curcuma gracillima* (Zingiberaceae)

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ABSTRACT. — *Curcuma gracillima* Gagnep. is a rare species and it has been found in Cambodia, Laos, Thailand, and some regions of southern Vietnam. This study provided the micro-morphological and molecular data of this species collected from Xuyen Moc Commune, Ho Chi Minh City, Vietnam for the first time. As a result, the Internal Transcribe Spacer (ITS) and *trnL* intron of *C. gracillima* were successfully amplified and sequenced. These sequences were also successfully deposited on the NCBI database with the accession numbers PQ813594 and PQ821005. The genetic relationship of *C. gracillima* in this study with other taxa of this species from different habitats was also reported. The pairwise alignment of *C. gracillima* from this study and its taxa from the prior reports showed the genetic variation in the ITS and *trnL* intron regions among *C. gracillima* collected from the different locations. In addition, the details of the anatomical characteristics of *C. gracillima* leaf, leaf sheath, petiole, root, and rhizome were first investigated.

KEYWORDS: *Curcuma gracillima*, anatomy, ITS, *trnL*

INTRODUCTION

Curcuma L. is one of the large genera of the Zingiberaceae family with about 120 species. Many geographical regions of the world like northern Australia, Southeast Asia, and some islands of the South Pacific have been reported as the major habitats of *Curcuma* species (Záveská et al., 2012; Sangvirodjanapat and Newman, 2023). Traditional medicine in Asian countries, especially Southeast Asia, have used members of the genus *Curcuma* to treat many diseases, including infectious wounds, abscesses, pneumonia, bronchial complaints, insect bites, diarrhea, and leucorrhea (Van and Le, 2023). Furthermore, the *Curcuma* plants have been used for many purposes such as flavoring (Ukil et al., 2003), possessing preservatives (Noorafshan et al., 2014), and medicinal properties (Rahaman et al., 2021).

Curcuma gracillima Gagnep. was first described by Gagnepain in 1903 (Gagnepain, 1903). This rare species has been found in Cambodia, Laos, Thailand, and some regions of southern Vietnam (Pham, 2000; Nguyen, 2017; Sangvirodjanapat and Newman, 2023; POWO, 2025). In 2024, we conducted field trips to Xuyen Moc Commune, Ho Chi Minh City, Vietnam and discovered some populations of *C. gracillima*. Because many species are found in the *Curcuma*

genus, they possesses genetic and morphological variation and extensive polyploidization and hybridization (Záveská et al., 2012). Additionally, the morphological traits of the vegetative and reproductive organs among *Curcuma* species are usually similar, and especially the annual flowering cycle of plants in this genus is short (Leong-Škorničková et al., 2008). This often leads to difficulty in determining the scientific names of studied species. Currently, some supporting assays have been used to solve the problem in taxonomy using vegetative and reproductive characteristics, of which molecular markers and anatomical traits have been shown to play important roles in classification as well as showing morphological and molecular diversity among species within a genus (Záveská et al., 2012; Khumkratok et al., 2015; Anu et al., 2020). Therefore, the micro-morphological characteristics and the genetic relationship of *C. gracillima* in the current study with other taxa of this species from different habitats were reported for the first time.

MATERIALS AND METHODS

Plant materials

Curcuma gracillima was collected from Xuyen Moc Commune, Ho Chi Minh City, Vietnam (Fig. 1). The

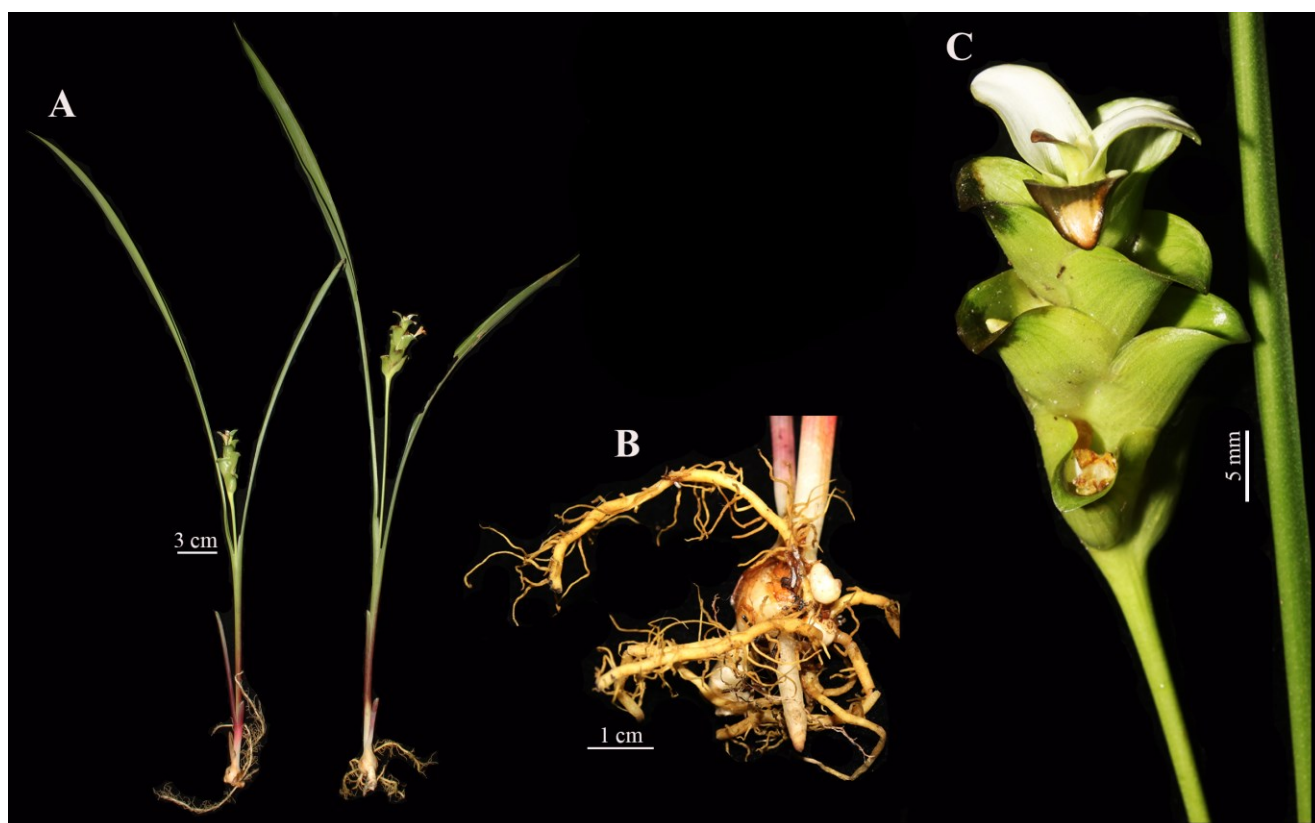


FIGURE 1. *Curcuma gracillima* Gagnep. A: whole plants. B: rhizome and root. C: inflorescence.

voucher specimen, HOI-TR7 was deposited at the Herbarium of Faculty of Biology and Biotechnology, University of Science, Vietnam National University, Ho Chi Minh City (PHH).

Macro-morphological comparison

Sample collection and processing were performed according to the guidelines of the Royal Botanic Gardens, Kew (Bridson and Forman, 1999). The scientific name of the studied species was determined by the comparative morphological method. The reproductive and vegetative traits of *C. gracillima* were compared with those of prior studies (Pham 2000; Závěská et al., 2012; Nguyen 2017; Huynh et al., 2024; Van et al., 2021).

Micro-morphological traits

The root, leaf, leaf sheath, petiole, and rhizome of *C. gracillima* were manually sectioned into thin freehand slices using a razor blade. The fresh sections were then bleached in Javel water. They were stained using the iodine green-carmin double staining method. These specimens were washed with water several times and finally, glycerol 10% was used to preserve them (Truong et al 2007). The Olympus BX53 Digital Upright Microscope was used to observe the plant cells and tissues.

DNA extraction, PCR reaction and sequencing data analysis

The CTAB 2X method (Aboul-Maaty and Oraby, 2019) was modified to extract the total DNA of *C. gracillima*. The internal transcribed spacer (ITS) and *trnL* intron regions were amplified using a Mastercycler PCR machine (Eppendorf, Germany) with the PCR components following: 12.5 μ L master mix (Phu Sa Company, Vietnam), 1.25 μ L of each forward and reverse primer (10 μ M); 9.5 μ L deionized water and 0.5 μ L DNA sample. The PCR cycle consists of 5 minutes at 95°C; 35 cycles including, denaturation (1 minute at 94°C), primer annealing (1 minute 30 seconds at 55°C) and a final extension (1 minute 30 seconds at 72°C). The PCR products were purified and sequenced using an ABI 3500: DNA analysis system 3500 Series Genetic Analyzer (Applied Biosystems™ 3500 XL Genetic Analyzer) at the LOCI Institute of Molecular Biology (Thu Duc City, Ho Chi Minh City, Vietnam). The ITS and *trnL* intron regions of *C. gracillima* were processed using FinchTV and Seaview softwares. The Bioedit and ClustalX2 software was used to align the studied sequences with those of other taxa of *C. gracillima* from the NCBI database.

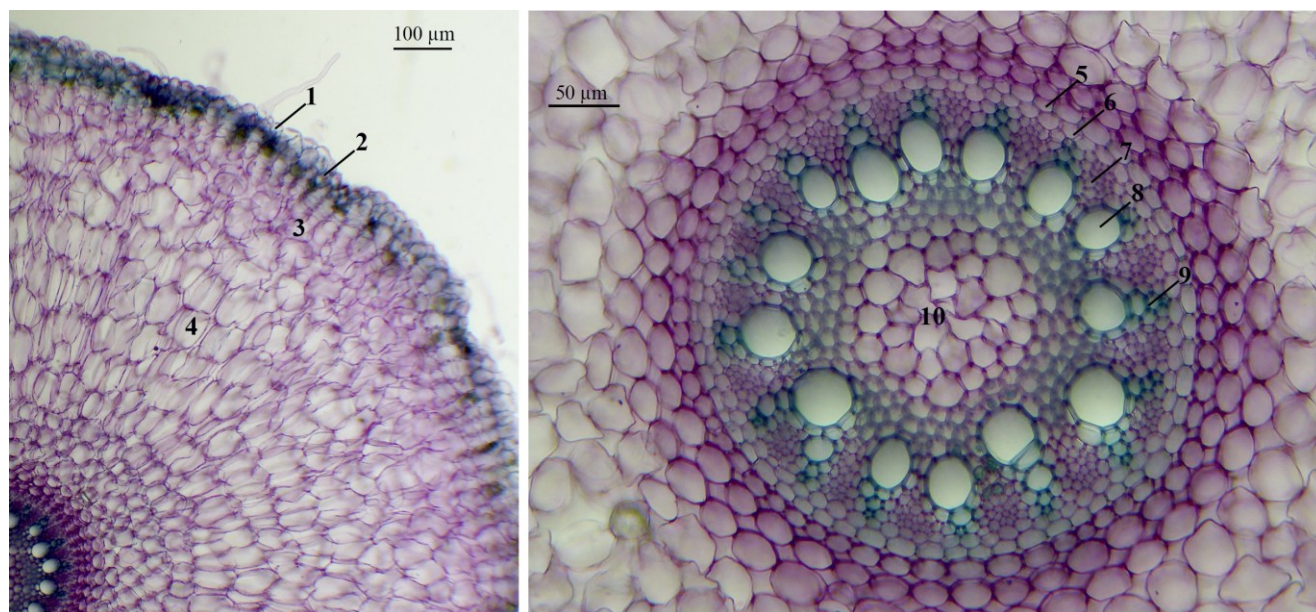


FIGURE 2. Cross-section of root; 1: piliferous layer, 2: exodermis, 3: outer cortical parenchyma, 4: inner cortical parenchyma, 5: endodermis with Casparian strip, 6: pericycle, 7: phloem, 8: metaxylem, 9: protoxylem, 10: pith.

RESULTS AND DISCUSSIONS

Micro-morphological characteristics

Root (Fig. 2)

The root's cross-section is nearly circular. Cortical region: The piliferous layer consists of a layer of polygonal cells, irregular in size, cellulose walls. The exodermis is radially arranged in 2–4 layers, polygonal, cork-impregnated walls. The cortical parenchyma is arranged with small polygonal or air cavity, consisting of 5–6 layers of outer cells, polygonal, wavy walls, arranged haphazardly, 6–8 layers of inner cells gradually smaller and arranged in radial rows and concentric rings and the endodermis with Casparian strip is presented. Stele: the pericycle consists of a layer of regular, polygonal cells with cellulose walls, arranged alternately with the endodermis. The vascular system consists of 16–18 primary phloem bundles alternating with 16–18 protoxylem bundles arranged in a ring, separated by medullary rays. Phloem bundles form clusters of irregular, polygonal cells. Protoxylem bundles consist of 2–4 vessels, polygonal, centripetally differentiated. There are 12–14 metaxylem vessels usually located immediately below the protoxylem. Medullary rays include 1–2 rows of narrow polygonal parenchyma cells horizontally, cellulose walls. The medullary parenchyma consists of polygonal cells, irregular size, closely packed, 3–5 outer cell layers with lignin walls, 3–4 inner layers with cellulose walls.

Leaves (Fig. 3)

The midrib is deeply concave on the upper surface and convex on the lower surface. The upper and lower epidermis consist of a single layer of rectangular cells, quite even, with many stomata in the lower epidermis. Vascular bundles are arranged in 1–2 rows above the lower epidermis. Each xylem bundle includes 6–10 small xylem vessels and 1–3 large metaxylem vessels; the phloem consists of polygonal cells, irregular in size, arranged haphazardly. Sclerenchyma is located at both ends of the vascular bundle, lignin-impregnated walls and irregular in size. The parenchyma surrounding the vascular bundles has many chloroplasts, in the middle of the vascular bundles, present of air cavity, and secretory cells. The upper and lower epidermis consist of a single layer of rectangular cells and cellulose wall, the upper epidermal cells are larger than the lower one. Hypodermis includes 1–3 layers of large cells and cellulose walls. Vascular bundles are arranged in a row, a structure similar to the vascular bundles in the midrib. The mesophyll contains most of chlorenchyma and has scattered scattered secretory cells.

Petiole (Fig. 4)

The petiole is concave above, convex below, and midrib-like structure. There are secretory cells scattered in the parenchyma.

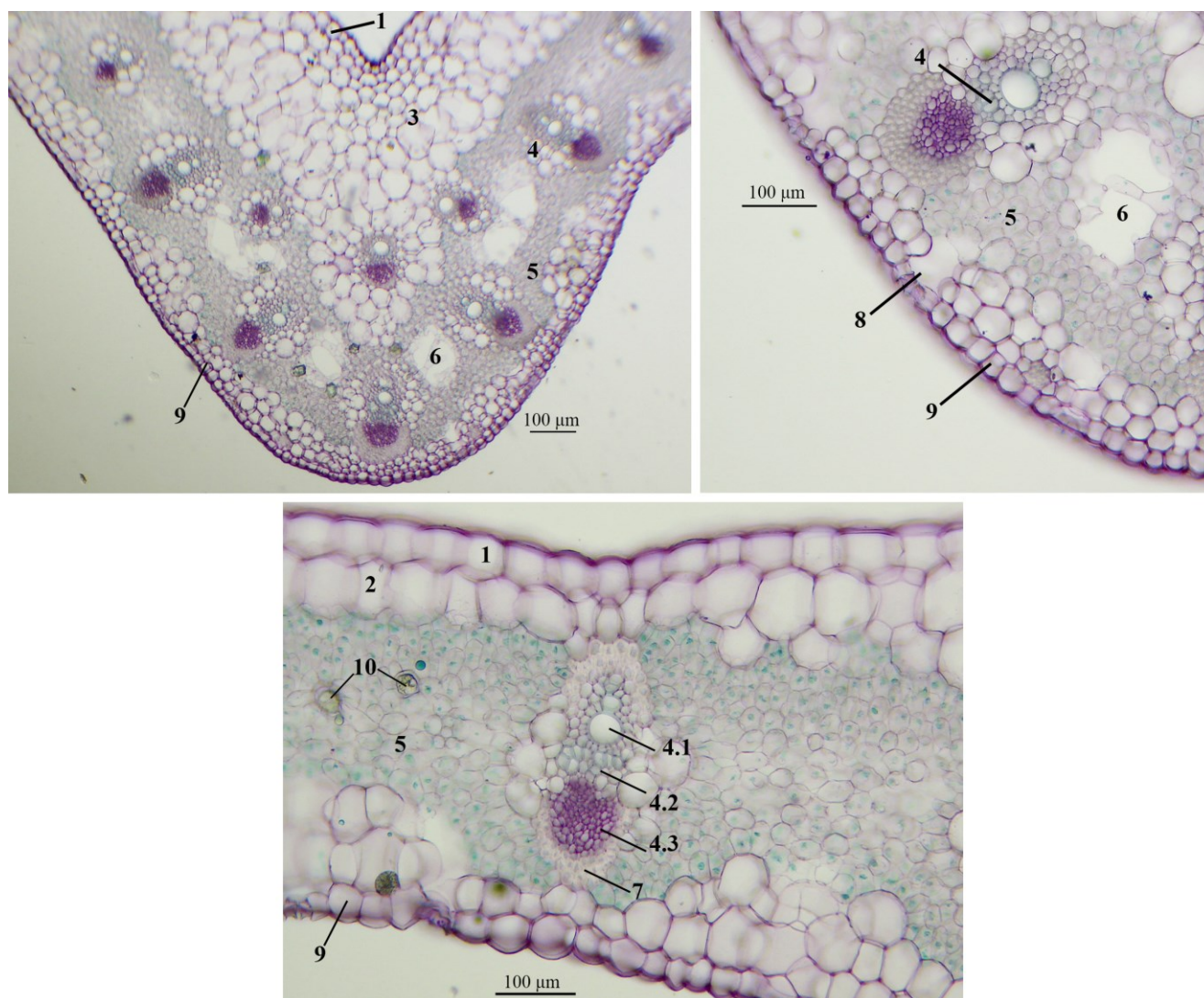


FIGURE 3. Cross-section of midrib (figure above) and lamina (figure below). 1: upper epidermis, 2: hypodermis, 3: parenchyma, 4: vascular bundle (4.1: metaxylem, 4.2: protoxylem 4.3: phloem), 5: chlorenchyma, 6: air cavity, 7: sclerenchyma, 8: guard cells, 9: lower epidermis, 10: secretory cell.

Leaf sheath (Fig. 5)

The leaf sheaths are rolled up and hug the petiole. The upper and lower epidermis consist of a layer of polygonal cells with cellulose walls. The parenchyma consists of irregular, polygonal cells, closely arranged. The vascular bundles are arranged in 1–2 rows in the parenchyma region, the regions are supported by sclerenchyma. There is a large air cavity between the vascular bundles.

Rhizome (Fig. 6)

The cross-section of the rhizome is a nearly circular shape. Cortex: the epidermis consists of a single layer of rectangular cells, and cellulose walls. The cortical parenchyma is usually solid or has small intercellular spaces. Some vascular bundles are scattered in the

parenchyma region and the endodermis with Casparian strip is presented. Stele: the pericycle consists of a single layer of rectangular cells, usually larger than the endodermis. The vascular bundles are scattered throughout the parenchyma. There are no sclerenchyma surrounding vascular bundles in the parenchyma (cortex and stele) but there are scattered secretory cells.

Anu et al. (2020) provided the micro-morphological characteristics of the petiole of 12 species belonging to the genus *Curcuma*, including *C. pseudomontana*, *C. amada*, *C. aeruginosa*, *C. zanthorrhiza*, *C. aromatica*, *C. vamana*, *C. zedoaria*, *C. aurantiaca*, *C. oligantha*, *C. longa*, *C. caesia*, and *C. haritha*. Five types of petiole shapes were presented. In this study, the outline

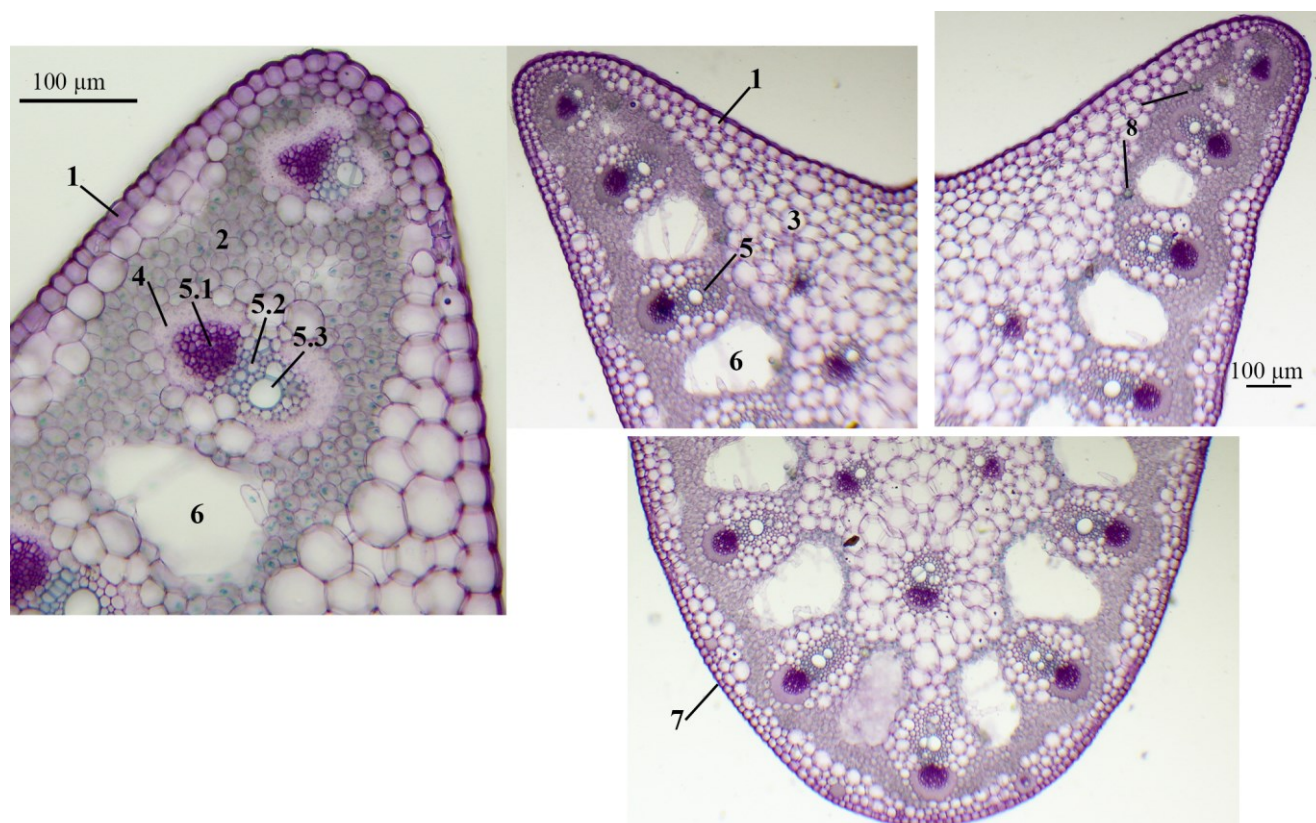


FIGURE 4. The cross-section of petiole. 1: upper epidermis, 2: clenchyma, 3: parenchyma, 4: sclerenchyma, 5: vascular bundle (5.1: phloem, 5.2: protoxylem, 5.3: metaxylem), 6: air cavity, 7: lower epidermis, 8: secretory cell.

of the petiole shape of *C. gracillima* is an “open V shape” and similar to those of *C. aromatica*, *C. vamana*, and *C. zedoaria*. Additionally, the epidermal cells of the *C. gracillima* petiole are rectangular or elliptical like those of *C. amada*, *C. aeruginosa*, and *C. aromatica*. The vascular bundles of *C. gracillima* are close collateral alternating with air cavities like Anu and colleagues’s work (Anu et al 2020).

Genetic relationship of *Curcuma gracillima*

The lengths of ITS and *trnL* intron regions of *C. gracillima* were 452 and 501 bps, respectively. These sequences were deposited in the NCBI database with the accession numbers PQ813594 and PQ821005. The nucleotide composition of ITS and *trnL* regions of *C. gracillima* from this study showed that the *trnL* regions possessed the A+T content of 69.26% and G+C content of 30.74%. In comparison, the A+T content of 46.68% and G+C content of 53.32% were found in the ITS region. Studies demonstrated that the nucleotide composition between the genomic and chloroplast DNA. Accordingly, the percentages of G+C were lower than A+T nucleotides for chloroplast DNA regions but the opposite occurred in ITS1-5.8SrRNA-ITS2 region (nuclear DNA) (Denk and Grimm, 2010; Yilmaz, 2021).

Studies showed that the different taxa of one species belonging to the genus *Curcuma* collected from various geographical locations exhibited the diversity in the genetic characteristics (Chen et al., 2015; Khumkratok et al., 2015; Záveská et al., 2012). For instance, Khumkratok et al. (2015) showed the genetic variation in the ITS sequence of 9 taxa of *C. gracillima* collected from the different locations in Thailand, including 2 taxa from Khong Chiam district (NCBI database’s accession numbers, KF709122.1 and KF709123.1), 2 taxa from Sai Thong National Park (KF709124.1 and KF709125.1), 2 taxa from Pa Hin Ngam National Park (KF709126.1 and KF709127.1) and 3 taxa grown in Mahasarakham University (KF709128.1, KF709129.1 and KF709130.1) (Fig. 7) (Khumkratok et al., 2015).

The pairwise alignment of ITS region among *C. gracillima* from this study (TR7) and its taxa from a prior report (Fig. 7) showed the genetic variation in the ITS sequence region among *C. gracillima* collected from the different locations. Accordingly, the studied species (TR7) from this study showed a closer relationship to the taxon JQ409851.1 which was also collected in Vietnam, than to the taxa distributed in Thailand (Khumkratok et al., 2015). Meanwhile, there

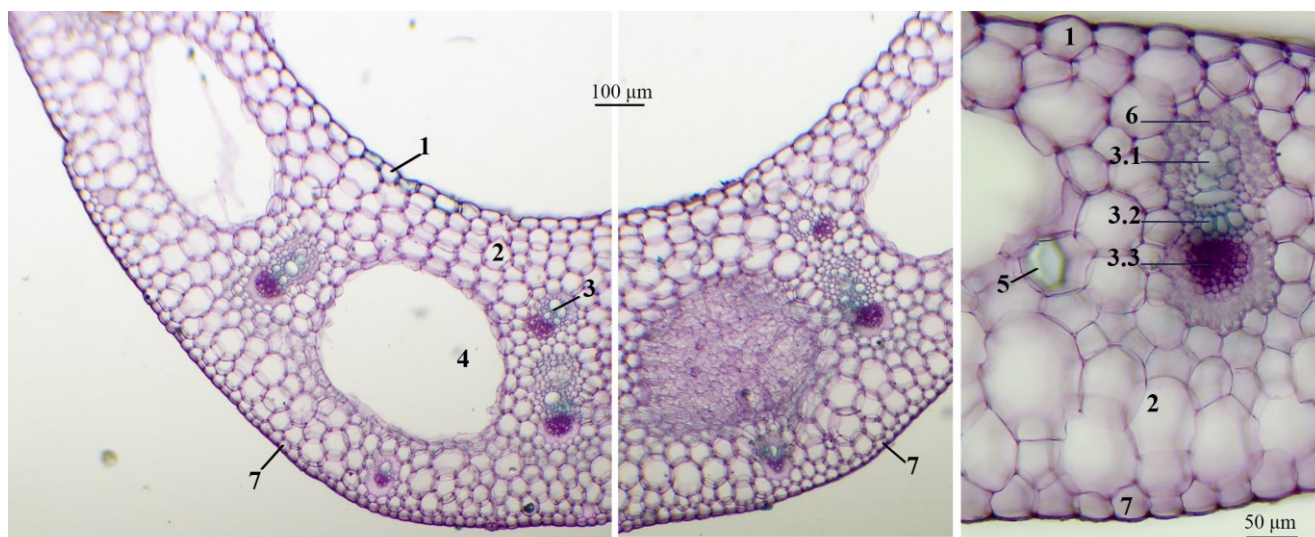


FIGURE 5. Cross-section of leaf sheath. 1: upper epidermis, 2: parenchyma, 3: vascular bundle (3.1: metaxylem, 3.2: protoxylem, 3.3: phloem), 4: air cavity, 5: secretory cell, 6: sclerenchyma, 7: lower epidermis.

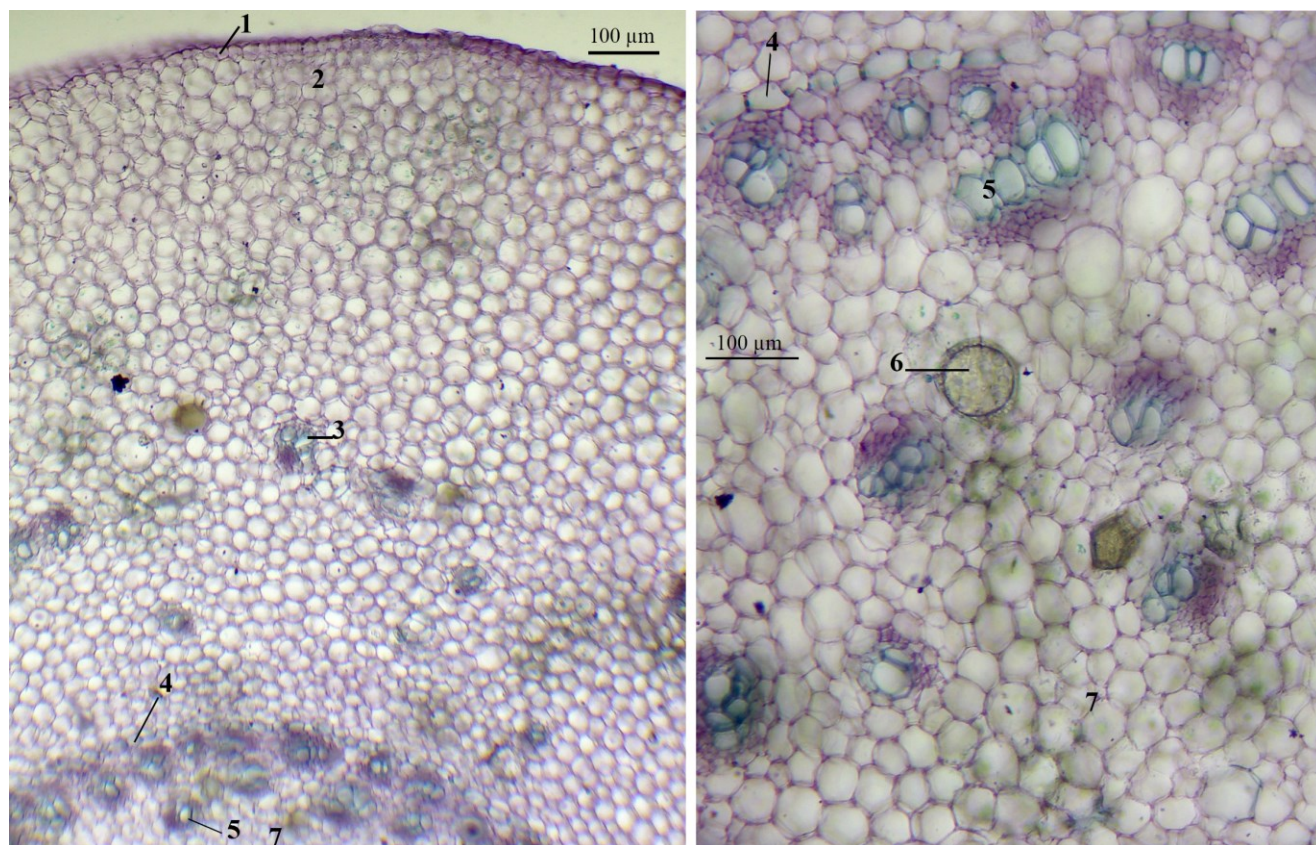


FIGURE 6. Cross-section of rhizome. 1: upper epidermis, 2: parenchyma, 3: vascular bundle in the cortex, 4: endodermis with Casparian strip, 5: vascular bundle in the stele, 6: secretory cell, 7: medullary parenchyma.

were from 3 to 6 non-homologous positions out of a total of 452 aligned sequences between the ITS regions of *C. gracillima* from this study (TR7) and 9 taxa from Thailand (Fig. 7).

Záveská et al. (2012) showed the ITS sequence obtained from the *C. gracillima* collected from Tan Phu district, Dong Nai province, Vietnam on the NCBI database with the accession numbers of JQ409851.1. The voucher specimen of this taxon, THD 48, was

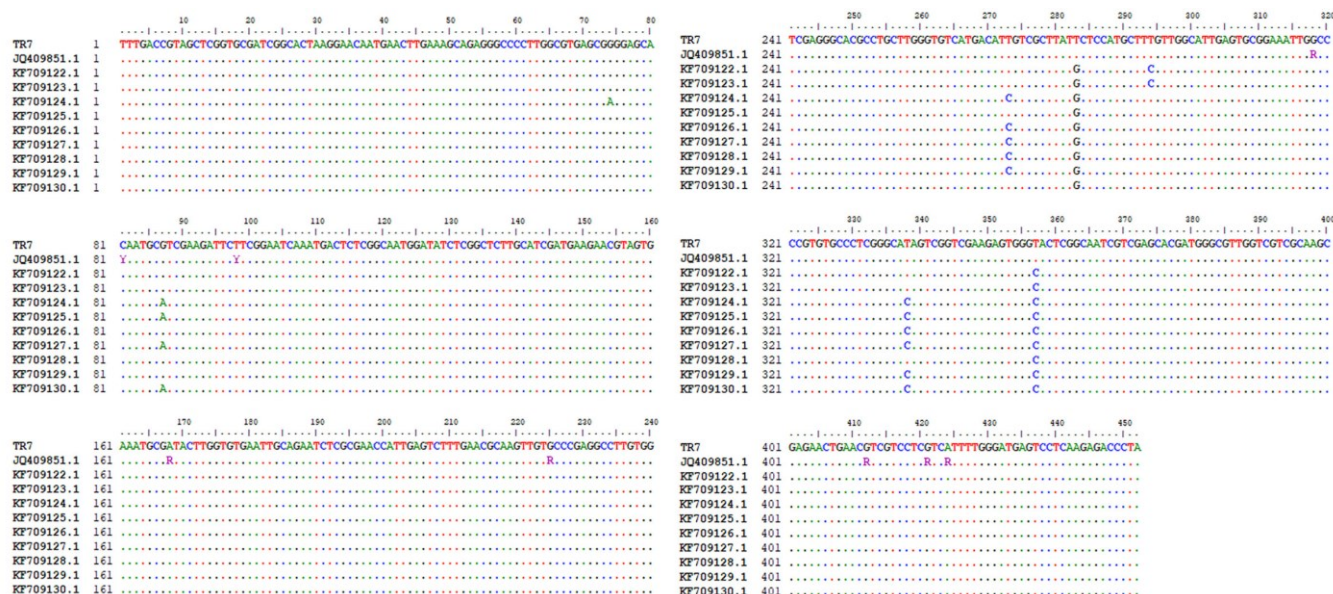


FIGURE 7. ITS pairwise sequence alignment of *C. gracillima* (TR7) from this study and its taxa from the prior report.

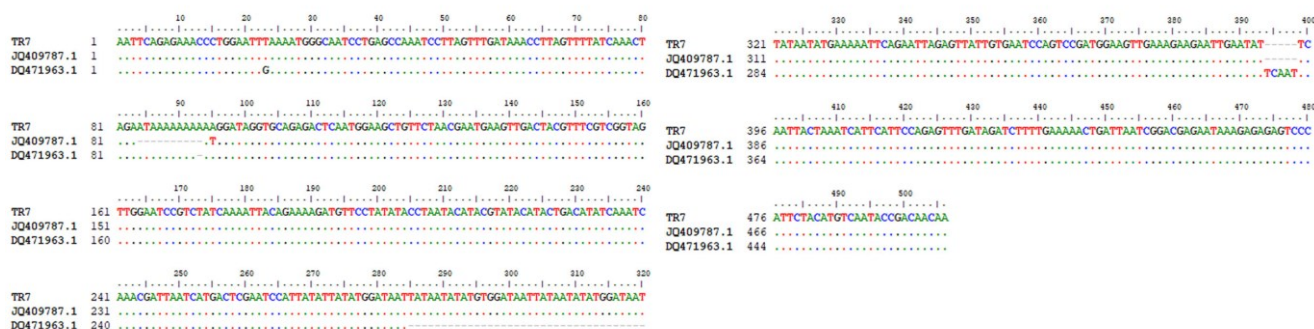


FIGURE 8. *trnL* intron pairwise sequence alignment of *C. gracillima* (TR7) from this study and its taxa from the prior report.

deposited at the Singapore Botanic Gardens Herbarium (Záveská et al., 2012). However, there were some ambiguous positions in the JQ409851.1 sequence and were denoted by letters that differed from those commonly used for purines and pyrimidines, which are A, T, G, and C (Bowden 1985). For example, the letter Y (Thymine or Cytosine) was used for nucleotide positions 81 and 98, and R (Adenine or Guanine) was used for nucleotide positions 168, 225, 318, 412, 421, and 424 (Fig. 7). The ITS region of the studied sample (TR7) from this study and JQ409851.1 sequence had 100% similarity, except 8 ambiguous positions in the JQ409851.1 sequence (C, T, A, G, G, G, A on TR7 sample vs. 8 ambiguous positions of JQ409851.1 sequence) (Fig. 7).

The pairwise sequence alignment in *trnL* intron of *C. gracillima* (TR7) from this study and 2 taxa collected from Tan Phu district, Dong Nai province,

Vietnam (NCBI database's accession number, JQ409787.1) (Záveská et al., 2012) and Thailand (DQ471963.1) showed that there were 1 mismatch and 11 gaps between the studied sequence (TR7) and JQ409787.1 whereas 1 mismatch and 42 gaps were found between sequence TR7 and the sample from Thailand (DQ471963.1) (Fig. 8).

Recently, DNA barcodes have become the solution for plant taxonomy and species determination as well as the identification of the phylogenetic relationship among plant species (Petersen et al., 2008; Erol et al., 2011; Harpke et al., 2013; Mosolygo et al., 2016; Yilmaz, 2021). Notably, the chloroplast regions, including *trnL-trnF*, *rbcL*, *matK*, *atpB-rbcL*, *trnK*, and *trnH-psbA* were used to identify the phylogenetic relationships whereas the ITS region, between rDNA genes in genomic DNA, was also chosen by botanist (Sramkó et al. 2014; Harpke et al 2013; Yilmaz 2021).

The DNA barcodes have been used to identify the scientific name or phylogenetic relationship of various plants belonging to the genus *Curcuma* by previous reports. Accordingly, Khumkratok et al. (2015) showed the genetic variation in the ITS sequence region of 9 taxa of *C. gracillima* collected from the different locations in Thailand, including 2 taxa from Kongchiam district (NCBI database's accession numbers, KF709122.1 and KF709123.1), 2 taxa from Sai Thong National Park (KF709124.1 and KF709125.1), 2 taxa from Pa Hin Ngam National Park (KF709126.1 and KF709127.1) and 3 taxa grown in Mahasarakham University (KF709128.1, KF709129.1 and KF709130.1) (Khumkratok et al., 2015). Závěská et al. (2012) established the phylogenetic tree of 80 *Curcuma* species using three plastid (*trnL-trnF*, *matK*, and *psbA-trnH*) and ITS regions to determine the delimitation and monophyly of the genus *Curcuma* (Závěská et al. 2012). Nguyen et al. (2023) described a new species belonging to the genus *Curcuma* based on morphological characteristics and ITS, *matK*, *trnL-F*, and *psbA-trnH* regions (Nguyen et al., 2023).

CONCLUSION

This study demonstrated the micro-morphological and molecular data of *C. gracillima* collected from Xuyen Moc Commune, Ho Chi Minh City, Vietnam. Based on the ITS and *trnL* intron regions, the results showed the genetic variation of *C. gracillima* from this study and its taxa collected from different locations. The anatomical characteristics of the leaf, leaf sheath, petiole, root, and rhizome of the studied plant were first investigated. The present study aims to provide the additional micromorphological and molecular data to establish a standardized approach for identifying *C. gracillima* and other medicinal plants in the future.

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