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Research Article

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Anatomical and molecular characteristics of *Limnophila rugosa* (Roth) Merr.

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ABSTRACT

Limnophila rugosa is a perennial plant and has been used as a medicinal and agricultural plant in some Asian countries. In this study, we provided the ITS and *trn*L-F regions and the anatomical characteristics of *L. rugosa* for the first time. Based on these molecular regions, phylogenetic trees showing relationships between *L. rugosa* and other species belonging to the Scrophulariaceae and Plantaginaceae families were performed and the result showed that *L. rugosa* was nested within the family Plantaginaceae instead of Scrophulariaceae. Additionally, the details of the micro-morphological characteristics of *L. rugosa* were investigated for the first time.

KEYWORDS: *Limnophila rugosa*, ITS, *trn*L-F, Plantaginaceae, Scrophulariaceae

INTRODUCTION

Limnophila is a small genus, comprises about 40 aquatic or marshy species widely found in subtropical and tropical areas of the Old World, especially in tropical Asia (Poudel *et al.*, 2024). The genus *Limnophila* was formerly belonged to the family Scrophulariaceae in 1970 (Philcox, 1970) but all members of the genus have been transferred to the tribe Gratioleae of the Plantaginaceae family by Albach *et al.* (2005) and Rahmanzadeh *et al.* (2005). *Limnophila rugosa*, one of the species belonging to the genus *Limnophila*, is a perennial plant and has been used as a medicinal and agricultural plant in some Asian countries (Hota *et al.*, 2022). For instance, this species was used as the agent for treatment of stomachic, diuretic, digestive tonic, diarrhoea, dyspepsia, elephantiasis, urinary burning, and dysentery (Hota *et al.*, 2022). So many biological activities of *L. rugosa* have been reported by previous studies, including antimicrobial, anti-inflammatory, hypotensive, diuretic, antibacterial and antifungal activities (Ahmad *et al.*, 1998; Madhumitha *et al.*, 2009; Acharya *et al.*, 2014; Hota *et al.*, 2022).

So far, many publications have been placed *L. rugosa* in the Scrophulariaceae family (Linh & Thach, 2011; Acharya *et al.*, 2014; Patel *et al.*, 2014; Hota *et al.*, 2022, 2023) although this species was transferred to the Plantaginaceae family (Albach *et al.*, 2005; Rahmanzadeh *et al.*, 2005). In this study, thus, the ITS and *trn*L-F regions of *L. rugosa* were firstly provided to determine the position classification of the studied species. In additison, the details of micro-morphological characteristics of *L. rugosa* were investigated for the first time.

MATERIALS AND METHODS

Materials

The samples of *Limnophila rugosa* were collected from a local vegetable farm in Cu Chi district, Ho Chi Minh City, Vietnam (Figure 1). The voucher specimen, Le H.T 15, was deposited at the Institute of Environmental Science, Engineering and Management, Industrial University of Ho Chi Minh City.

Anatomical Characteristics

The thin slices of the root, leaf, petiole, and stem of *L. rugosa* were soaked in Javel solution. These thin slices were stained using the iodine green-carmine double staining method. The

distilled water was used to wash and glycerol (10%) was used to preserve these samples (Truong *et al.*, 2007). The Olympus BX53 Digital Upright Microscope was used to observed and take the picture.

DNA Extraction, PCR Reaction and Sequencing Data Analysis

The total DNA of *L. rugosa* was isolated from young leaves of *L. rugosa* using the DNA Gene Jet Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA). The ITS and *trn*L-F regions were amplificated using a Mastercycler PCR machine (Eppendorf, Germany) with the constituents as follows: master mix (12.5 µL); primers 10 µM (forward-1.25 µL and reverse-1.25 µL); deionized water (9.0 µL); DNA sample (1.0 µL). PCR cycling process: 95 °C (5 minutes); 35 cycles (1 minute at 94 °C, 1 minute at 56 °C, 1 minute 30 seconds at 72 °C); 72 °C for 10 minutes. PCR products were purified and sequenced at the Nam Khoa BioTek Company (Ho Chi Minh City, Vietnam). The FinchTV and Seaview softwares were used to process the ITS and *trn*L-F regions of *L. rugosa*. The phylogenetic trees of the studied species and other related species were conducted using PAUP* software.

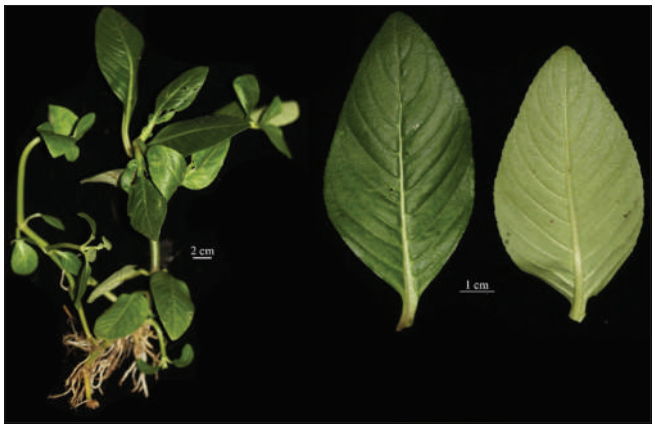

Figure 1: *Limnophila rugosa* from a local vegetable farm in Cu Chi district, Ho Chi Minh City, Vietnam

RESULTS AND DISCUSSION

Phylogenetic Tree

The final lengths of the ITS and *trn*L-F sequences of the studied sample were 631 and 798 bps, respectively. These sequences were deposited on GenBank database with the accession numbers of PQ787496 and PQ790045, respectively.

The genus *Limnophila* formerly belonged to the family Scrophulariaceae (Philcox, 1970) but all members of the genus have been transferred to the tribe Gratioleae of the Plantaginaceae family by (Albach *et al.*, 2005; Rahmanzadeh *et al.*, 2005). Unfortunately, so many publications have placed *L. rugosa* in the Scrophulariaceae family so far (Linh & Thach, 2011; Acharya *et al.*, 2014; Patel *et al.*, 2014; Hota *et al.*, 2022, 2023). In this study, thus, the position classification of *L. rugosa* was determined using molecular markers. The result showed that *L. rugosa* was nested within the family Plantaginaceae instead of Scrophulariaceae on both the phylogenetic trees conducted on ITS (Figure 2) and *trn*L-F (Figure 3) regions.

In this study, the DNA barcode of *L. rugosa* was provided for the first time. However, the DNA markers of other *Limnophila* species have been reported by prior studies. For instance, Padiya *et al.* (2013) showed the genetic diversity of *L. heterophylla* collected from different regions of India using Random Amplified Polymorphic DNA (Padiya *et al.*, 2013). Lonthor *et al.* (2023) also demonstrated the genetic diversity between some *Limnophila* species and other aquatic plants in the Rote Dead Sea area, Indonesia using rbcL marker (Lonthor *et al.*, 2023). Recently,

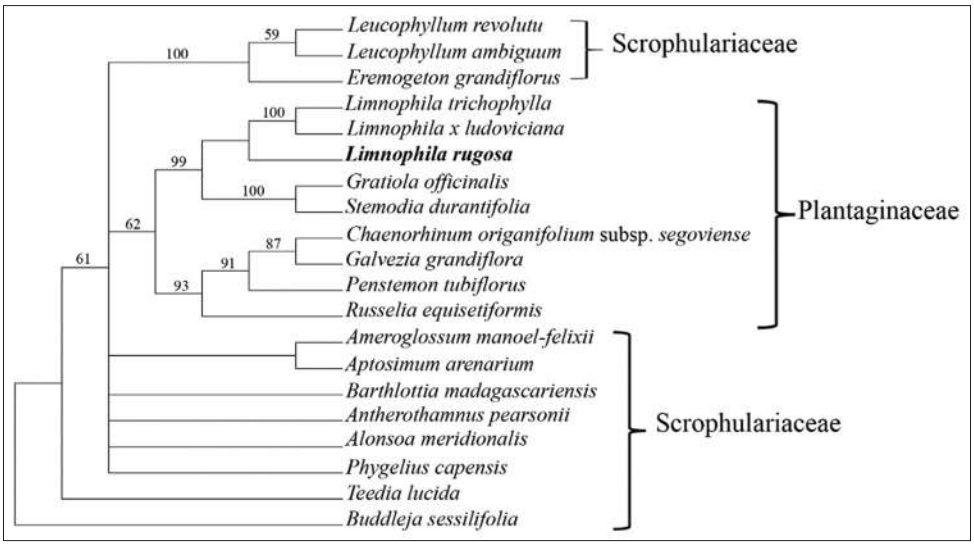

Figure 2: Phylogenetic tree based on the ITS sequence region showing the relationship between Limnophila rugosa and other species of Scrophulariaceae and Plantaginaceae, using data obtained from the NCBI database

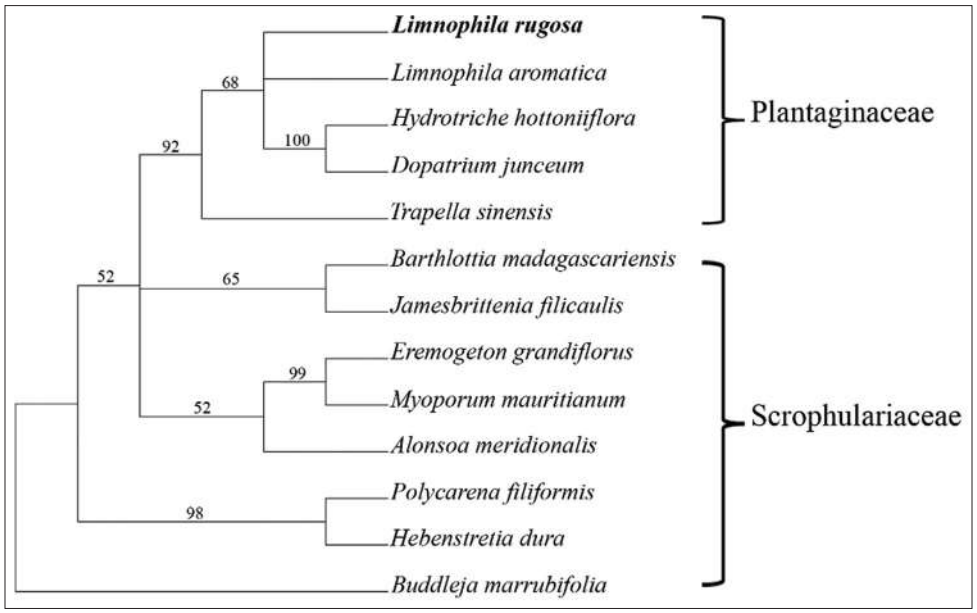

Figure 3: Phylogenetic tree based on the *trn*L-F sequence region showing the relationship between Limnophila rugosa and other species of Scrophulariaceae and Plantaginaceae, using data obtained from the NCBI database

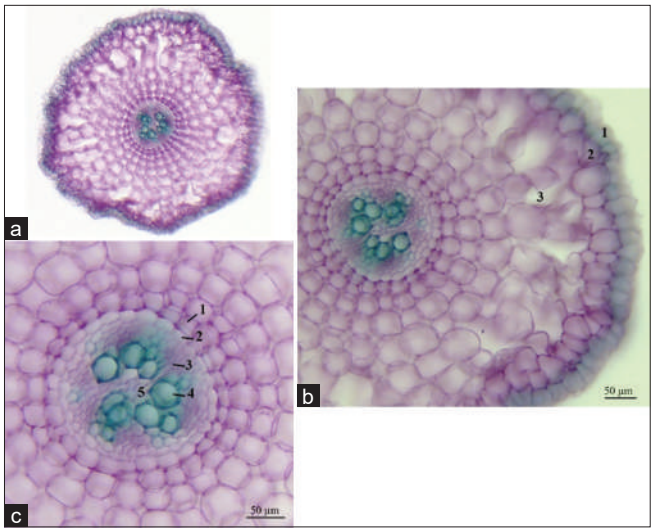

Figure 4: The cross-section of the root. a) whole cross-section, b) cortex (1: piliferous layer, 2: exodermis, 3: spongy parenchyma) and c) stele (1: endodermis with casparian strip, 2: pericycle, 3: phloem, 4: xylem, 5: medullary parenchyma)

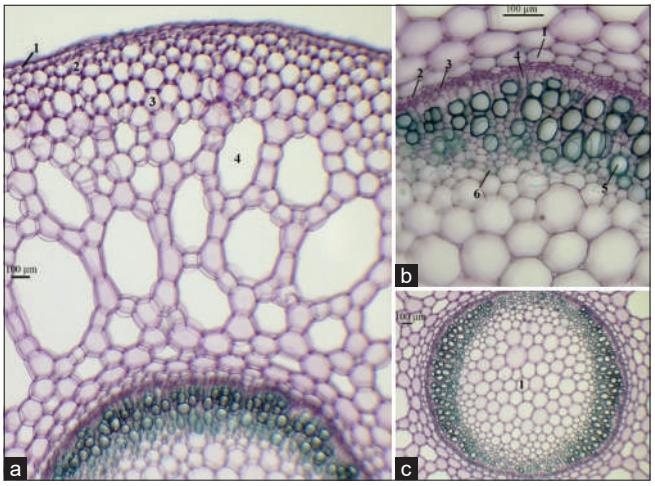

Figure 5: The cross-section of stem. A) cortex (1: epidermis, 2: collenchyma, 3: parenchyma, 4: spongy parenchyma), b) stele (1: endodermis, 2: pericycle, 3: primary phloem, 4: medullary ray, 5: primary xylem, 6: secretory cell) and c) medullary parenchyma (1).

Dong *et al.* (2023) provided the complete chloroplast genome sequence of *L. sessiliflora* (Dong *et al.*, 2023).

Anatomical characteristics of *Limnophila rugosa*

Roots

The cross-section of the root is axially symmetrical, nearly circular, consisting of two regions: the cortex occupies 2/3 of the radius, the stele occupies 1/3 of the radius. Cortical region: The piliferous layer consists of 1 layer of polygonal cells, irregular in size, the wall is impregnated with cork and there are root hairs on the surface. The exodermis layer has 1 layer of cells, the wall still has cellulose. The cortical parenchyma is divided into 2 regions: the outer cortical parenchyma is the spongy parenchyma, composed of cells of irregular shape and size, arranged to open the intercellular spaces containing air (intercellular spaces); the inner cortical parenchyma consists of 4 layers of nearly round polygonal cells arranged in radial rows. The endodermis has a clear casparian strip, rectangular cells, the cell walls on both sides are thick. Stele: the pericycle consists of 1-2 layers of polygonal cells with cellulose walls, irregular in size, arranged alternately with the endodermis. The vascular system consists of 6 phloem bundles alternating with 5 xylem bundles arranged in a ring. Phloem bundles are cells with cellulose walls, small in size, polygonal in shape, irregular and arranged haphazardly (Figure 4).

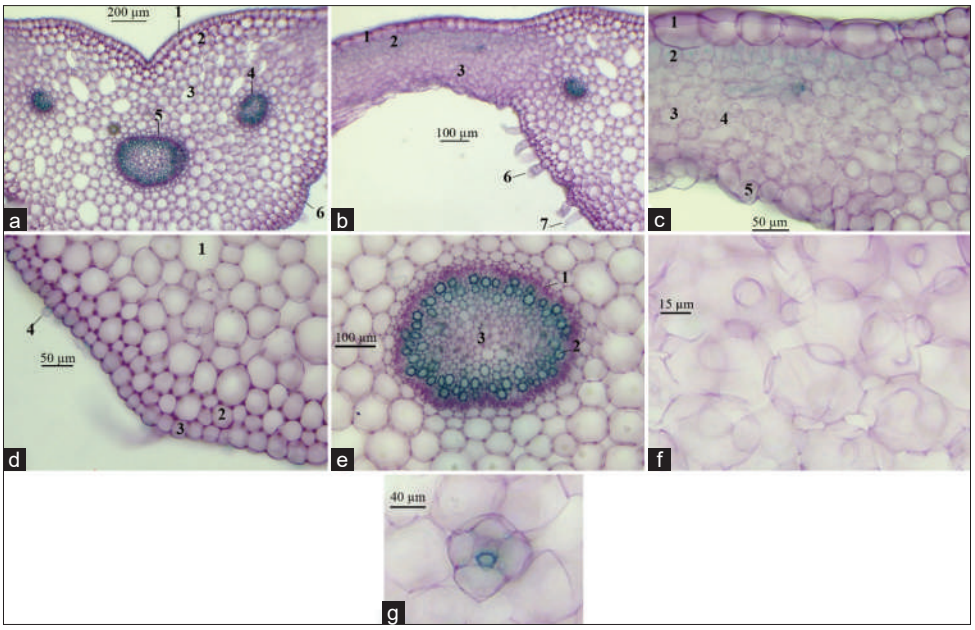

Figure 6: The cross-section of leaf. a) midrib (1: upper epidermis, 2: collenchyma, 3: spongy parenchyma, 4: secondary vascular bundle, 5: major vascular bundle), b & c) main lamina (1: upper epidermis, 2: chlorenchyma, 3: spongy parenchyma, 4: starch, 5: lower epidermis), d) lower of midrib (1: schizogenous cavity, 2: angular collenchyma, 3: lower epidermis, 4: secretory trichomes), e) vacular bundle (1: phloem, 2: xylem, 3: medullary parenchyma), and f) plastids with starch storage; g: calcium oxalate crystals.

Stem

The cross-section of the stem is divided into 2 regions, the cortex is smaller than the stele. Cortical region: epidermis has a layer of polygonal cells, irregular in size, cork-impregnated walls, and hairs covering the surface. The cortical parenchyma is divided into two regions: the outer layer is the parenchyma, composed of polygonal cells of irregular shape and size; the inner parenchyma consists of polygonal or round cells, arranged to leave open intercellular spaces containing air. The endodermis is a row of rectangular cells with thick cell walls. Stele: the outermost is the pericycle composed of 1 cell, arranged alternately with endodermal cells, cellulose wall. Vascular bundle: located below the pericycle, the phloem bundle overlaps the xylem bundle; arranged in a circle, the xylem differentiates centrifugally. Between the vascular bundles, there are straight rows of parenchyma called medullary rays. Inside the vascular bundles is a mass of parenchyma called the medullary, there is secretory tissue in the medullary parenchyma (Figure 5).

Leaf

The cross-section of a leaf consists of two parts: the enlarged part in the middle is the midrib and the main lamina on both sides is the leaf flesh.

Midrib: upper surface is concave in the middle, bulging and rounded on the lower surface. Upper and lower epidermis consists of 1 layer of irregular polygonal cells, a thin cutinized outer surface, scattered with air holes. Angular collenchyma consists of 2-3 layers below the epidermis, polygonal cells. Parenchyma includes polygonal cells, some areas have distorted cell walls, irregular size, intercellular spaces and secretory cells.

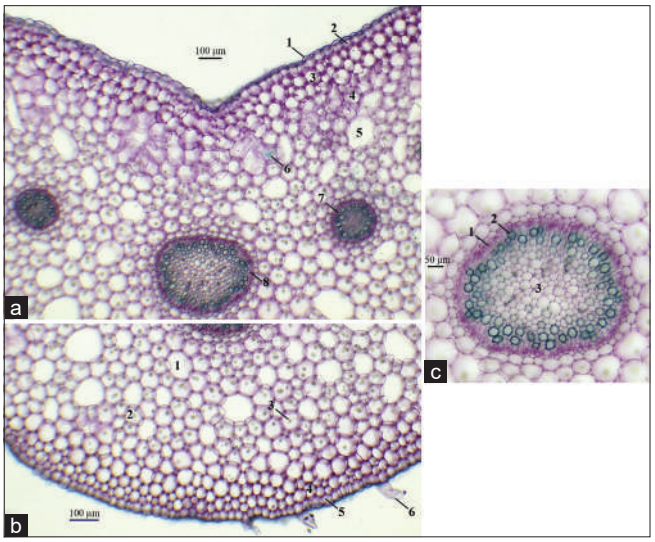

Figure 7: The cross-section of petiole. a) upper of petiole (1: cuticle layer, 2: upper epidermis, 3: angular collenchyma, 4: spongy parenchyma, 5: schizogenous cavity, 6: calcium oxalate crystals, 7: secondary vascular bundle, 8: major vascular bundle), b) upper of petiole (1: schizogenous cavity, 2: spongy parenchyma, 3: chloroplast, 4: angular collenchyma, 5: lower epidermis, 6: protective hairs) and c) vascular bundle (1: phloem, 2: xylem, 3: medullary parenchyma).

The vascular tissue system includes accessory tendon bundles located in the same parenchyma, main vascular bundle arranged in a circle: phloem covers the xylem inside (xylem above phloem below), there are parenchyma and spherical calcium oxalate crystals in the medullary area.

Lamina: upper and lower epidermis consists of a layer of polygonal cells, cellulose walls and the upper epidermal cells are

larger than the lower epidermis. Under the upper epidermis is a layer of chlorenchyma cells, rectangular cells arranged closely together and perpendicular to the upper epidermis, containing many chloroplasts, irregular, arranged haphazardly. There are layers of spongy parenchyma above the lower epidermis. Both the upper and lower epidermis of the mesophyll region have many stomata, more of which are present on the lower surface. In addition, there are secretory vesicles, starch-containing plastids, calcium oxalate crystals, and sclerenchyma fibers in the spongy parenchyma (Figure 6).

Petiole

The main petiole and petiole have the same anatomical traits to the leaf blade (Figure 7).

CONCLUSION

The current study provided the ITS and *trn*L-F regions and the micro-morphological traits of *L. rugosa*. The results from this study are expected to provide additional important data for *L. rugosa*, an important medicinal and crop plant.

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