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MOLECULAR IDENTIFICATION AND MORPHOLOGICAL CHARACTERIZATION OF *Ficus* sp. (MORACEAE) IN BOGOR BOTANIC GARDENS

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Abstract. *Ficus spp. belongs to the tribe Ficeae in the Moracea family. Many members of this genus have been collected and grown in Bogor Botanic Gardens. There are 519 living collections of Ficus conserved since 1817, and 13 of them have not been identified until the species level. This research aimed to identify the Ficus sp. originated from Kaur Selatan (Bengkulu) using morphological and molecular approaches. Morphological characterization and herbarium specimen observation have been carried out to identify the Ficus sp. The molecular approach was conducted through DNA barcoding using ITS primer. The molecular identification using ITS sequence showed that Ficus sp. is Ficus crassiramea with 99.87% similarity to the sequence in NCBI. Morphological observation through herbarium specimen showed that there are 9 vegetative characters specific to Ficus crassiramea.*

Keywords: *Bogor Botanic Gardens, DNA barcoding, living collection, molecular identification, Sumatra.*

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INTRODUCTION

Bogor Botanic Gardens (BBG) is the oldest ex-situ conservation institution in Southeast Asia (Ariati & Widyatmoko, 2019). The garden has more than 15,000 living collections and one of them is *Ficus* L. (Moraceae) (Hotimah et al., 2015). The genus is mainly distributed in the tropics and 367 species occurring in the Malesia region (Berg & Corner, 2005). Since 1914 – 2019, BBG has collected 519 living collections of *Ficus*,

which divided into 87 identified species and 13 remains unidentified (Peniwidiyanti & Hariri, 2019). *Ficus* spp. at the BBG enriches germplasm and plays a role in various research and world references for living plant collections. The previous researches compared the species of pollinators in *Ficus elastica* in the Bogor Botanic Gardens with those found in India, Thailand, and Singapore (Harrison et al., 2014; Chantarasuwan et al., 2016; Harrison et al., 2017). The studies showed that the accuracy of species identification in botanical gardens is very important. However, there are 13 living collections of *Ficus* that still unidentified.

The identification process through direct observation of *Ficus* morphological characters, especially in generative organs is an important part. The generative organs in *Ficus* are complex because they have a high variation of morphological features. For example, the characteristics of fig without interflora bract, staminate flower ostiolar, and subtended or enveloped by bracteoles, are the key features that separate the subgenus *Sycomorus* from the other subgenus (Berg & Corner, 2005). However, some of the living collections at BBG never produce reproductive organs that make morphological identification challenging. Therefore, identification through molecular approaches is needed.

The molecular approach is commonly used to identify species' levels through DNA barcoding sequences. Several sequences are designated as universal DNA barcoding to identify plant species derived from the plastid or nucleus genome. Those sequences are *rbc*L, *mat*K, *trn*H-*psb*A intergenic spacer region, and internal transcribed spacer (ITS) (CBOL Plant Working Group, 2009; Li et al., 2012; Samsuddin et al., 2012; Olivar et al., 2014; Balkanska et al., 2020). Besides, two sequences have been evaluated and applied in *Ficus*, namely *psb*K-*psb*I, *atp*F-*atp*H (Li et al., 2012). ITS has been evaluated as DNA barcoding and provides higher variable and parsimony-informative characters with greater intra- and interspecific divergences. The ITS sequence also showed the highest species discrimination rate and primer universality, making it reliable to be used as a single DNA barcode in *Ficus* (Rønsted et al., 2008; Li et al., 2012). ITS can help resolve the disclosure of the *Ficus* identity, which is difficult to identify morphologically. Thus, this study aimed to reveal the identity of *Ficus* in BBG that never produces reproductive organs using ITS sequences.

MATERIALS AND METHODS

DNA Extraction, Amplification, and Sequencing

According to the manufacturer's protocol, total DNA extraction was obtained from young leaf tissue using Tiangen Plant Genomic DNA Kit (Tiangen Biotech Co., Ltd.). The PCR reactions were performed in a final volume of 50 µL, using MyTaq™ Master Mix 2X (Bioline), 1 µM for each forward and reverse primers, and 10 ng genomic DNA. The amplification process was achieved using the ITS primers previously used by Sun et al. (1994). The amplification process was carried out following the thermal profile: [95°C 3 min, (95°C for 30 s, 58°C for 45 s, 72°C for 45 s) × 35 cycles), 72°C for 5 min. The PCR products were observed using GelDoc (BioRad) through 1% GelRed-stained agarose. The sequencing process was conducted at 1st Base, Singapore, through PT. Genetika Science Indonesia service.

Sequence Editing, Alignment, and Phylogenetic Tree Reconstruction

The ITS sequences were processed using the BioEdit program and aligned to their homologous sequences using the Codoncode Aligner tool. The molecular identification was carried out using BLAST through National Center for Biotechnology Information (NCBI) website. The contig sequence was analyzed using the MEGA X software using the Kimura 2-parameter model (Kimura, 1980; Kumar et al., 2018). The reconstruction of a phylogenetic tree was achieved through the Neighbor-Joining (NJ) method with 1000 replicate bootstraps (Felsenstein, 1985; Saitou & Nei, 1987). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair (pairwise deletion option). Bootstrap support values were categorized as strong, moderate, weak, or poor following Kress et al. (2002).

Morphological Characterization and Herbarium Specimen Observation

The quantitative and qualitative morphological characters were observed directly through living specimens and herbarium collections. *Ficus* sp., which was identified through molecular approach, is one of the Bogor Botanic Gardens' living collections located in VII.F.58 and originating from South Kaur, Sumatra. Among the unidentified *Ficus* collections in BBG, this specimen never produced reproductive organs that make the morphological identification become very challenging because the important characters lie in the syconium. *Ficus callosa* Willd. grown in VIII.B.123 was used as a comparison and outgroup due to the similarities in *Ficus callosa* and *Ficus* sp. which was identified in this study.

The plant materials collected in this study were only vegetative organs. The observed data were recorded from *Ficus* sp. and *Ficus callosa* specimens. The materials were preserved and identified in Herbarium Kebun Raya Bogor (KRB) and Herbarium Bandungense (FIPIA), School of Life Sciences and Technology (SITH), Institut Teknologi Bandung (ITB). The preservation method following Djarwaningsih et al. (2002).

RESULTS AND DISCUSSION

The living collection of *Ficus* sp. in VII.F.58 has been planted since the year 2000 and has never been a generative organ appear. Morphological identification based on vegetative organs can only show the species belong to *Ficus* subg. *Urostigma*. The living collection characteristics belonging to the *Ficus* subg. *Urostigma* are spiral leaf arrangement and one wax gland at the midrib base. The observed vegetative traits cannot be used for species identification because of the special character on the *Ficus* subg. *Urostigma* is in its generative organs, both in the bractea and receptacle (Berg & Corner, 2005). Also, the morphological features used in the identification of the *Ficus* subg. *Urostigma* overlap among species.

The DNA samples of *Ficus* sp. and *Ficus callosa* were successfully extracted and amplified using ITS primer. The sequencing results show that the ITS sequence from *Ficus* sp. and *Ficus callosa* were amplified successfully along 849 and 896 base pairs, respectively. The amplified sequence consisted of the partial sequence of the 18S ribosomal RNA gene, the complete sequence of internal transcribed spacer 1, the complete sequence of 5.8S ribosomal RNA gene, the complete sequence of internal transcribed spacer 2, and the partial sequence of 28S ribosomal RNA gene (Figure 1). After the BLAST

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was carried out through the NCBI website, the ITS sequence for *Ficus* sp. has a 99.97% similarity to the *F. crassiramea* sequence with accession number EU091585.1 (Table 1). The high similarity value indicates that the living collection of *Ficus* sp. in BBG is *F. crassiramea*. The result of the sequence's alignment between *Ficus* sp. with *F. crassiramea* EU091585.1 showed a difference of one nucleotide base at the 57th position, namely the nucleotide G in *Ficus* sp. and N nucleotides in *F. crassiramea* EU091585.1 (Figure 2).

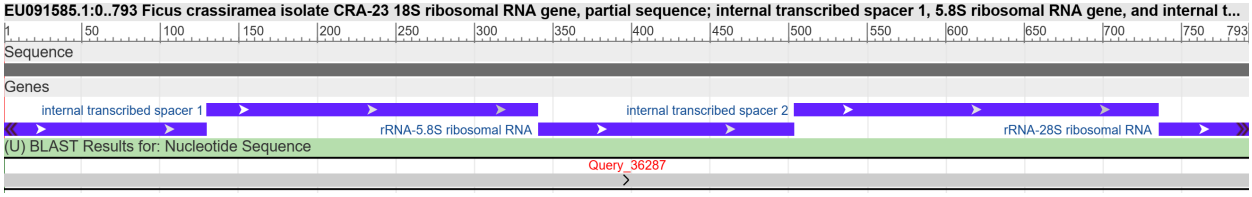

Figure 1. The composition of amplified ITS sequence from *Ficus* sp.

Table 1. Statistical simulation of BLAST sequence of observed specimens with ITS region

Specimen	ID Species	BLAST Similarity (%)	Query Coverage (%)	E-value
Ficus sp.	*Ficus crassiramea*	99.87	88	0

Score	Expect	Identities	Gaps	Strand	Frame
1461 bits(791)	0.0()	792/793(99%)	0/793(0%)	Plus/Plus	

```
Query  46   GCCCGTGACGTCGCGAGAAGTCCACTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAA  105
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GCCCGTGACGTCGCGAGAAGTCCACTGAACCTTATCATTTAGAGGAAGGAGAAGTCNTAA  60

Query  106  CAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCAGCAGAAGGA  165
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCAGCAGAAGGA  120

Query  166  CCGGCGAACACGTTACAACACTCGAgggggggCAAGGGGCGCGAACACGCCCCGGACCCT  225
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CCGGCGAACACGTTACAACACTCGAGGGGGGGGCAAGGGGCGCGAACACGCCCCGGACCCT  180

Query  226  CCTCGCCGGGTGCGTGTGGCCCCGCCCCTCGCCCCCGGCACCAAACGAACCCGGGCGCGG  285
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CCTCGCCGGGTGCGTGTGGCCCCGCCCCTCGCCCCCGGCACCAAACGAACCCGGGCGCGG  240

Query  286  AATGCGTCAAGGAAAGACAACGAGACGATCCCTGCCGTCGGGGCCCCGGAAACGGTGACT  345
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AATGCGTCAAGGAAAGACAACGAGACGATCCCTGCCGTCGGGGCCCCGGAAACGGTGACT  300

Query  346  CTGCCTCGGTGGTTGCTTCGGGATCGGTTTGAGTACGAAGAACGACTCTCGGCAACGGAT  405
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CTGCCTCGGTGGTTGCTTCGGGATCGGTTTGAGTACGAAGAACGACTCTCGGCAACGGAT  360

Query  406  ATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAG  465
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  ATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAG  420

Query  466  AATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAGGCCATCAGGTCGAGGG  525
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAGGCCATCAGGTCGAGGG  480

Query  526  CACGTCTGCCTGGGCGTCACACGCCGTTGccccccccAAACCCCCTTTCCCGCTCCTAGCC  585
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CACGTCTGCCTGGGCGTCACACGCCGTTGCCCCCCCCCAAACCCCCTTTCCCGCTCCTAGCC  540

Query  586  GGGGCGAGCGGGGACCGCGGGGGGCGGACGATGACCTCCCGTGCGCGCGCTTCGACCCGC  645
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GGGGCGAGCGGGGACCGCGGGGGGCGGACGATGACCTCCCGTGCGCGCGCTTCGACCCGC  600

Query  646  GGTTGGTCCAAAAATCGAGTCCCCTGTCACGTCGTCTTGGCAACAGGTAGTCGATCATTC  705
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  GGTTGGTCCAAAAATCGAGTCCCCTGTCACGTCGTCTTGGCAACAGGTAGTCGATCATTC  660

Query  706  GGTGCCACGGCCGTGCGCGTCGTGCACGTATCGGGACTCCGACAGACCCCAGTGCGCCCG  765
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  GGTGCCACGGCCGTGCGCGTCGTGCACGTATCGGGACTCCGACAGACCCCAGTGCGCCCG  720

Query  766  TCACGGGTGCCTCCAACGCGACCCCAGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATA  825
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  TCACGGGTGCCTCCAACGCGACCCCAGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATA  780

Query  826  TCAATAAGCGGAG  838
            |||||||||||||
Sbjct  781  TCAATAAGCGGAG  793
```

Figure 2. Alignment result of *Ficus* sp. sequence and *Ficus crassiramea* EU091585.1 with nucleotide difference marked by the red line.

The ITS sequence was able to determine *Ficus* species clearly (Li et al., 2012). In this study, phylogenetic tree construction was carried out to evaluate the species determination accuracy by comparing ITS sequences from the NCBI database. A total of 19 sequences were used to construct phylogenetic trees consisting of 2 living collection samples from BBG, namely *Ficus* sp. and *Ficus callosa* (*Ficus* subg. *Pharmacosycea*). The other 15 species derived from the NCBI database consisted of 13 members of the *Ficus* subg. *Urostigma* as ingroup and 2 other species as outgroups, namely *Ficus erecta* (*Ficus* subg. *Ficus*) and *Ficus nota* (*Ficus* subg. *Sycomorus*) (Table 2). Phylogenetic trees constructed using the NJ method with 1000 bootstraps showed that the entire *Ficus* subg. *Urostigma* was clustered simultaneously and separated from the other subgenus (Figure 3). On this tree, *Ficus* sp. remains clustered to *Ficus crassiramea*.

Morphological observations of herbarium specimens of *Ficus* sp., *Ficus crassiramea*, and *Ficus callosa* were carried out again to look for the characteristics of vegetative organs that can be used as specific characters (Figure 4). Observations were made by comparing sample herbarium specimens from the BBG with herbarium specimens stored in Herbarium Bogoriense (BO). Based on the observations, 15 morphological features on the leaves, stipules, and twigs could be compared between species and 9 of them are specific to *Ficus crassiramea* (Table 3).

Table 2. Sequence length variation of ITS region within observed samples and NCBI database

No		Species	No Accession	Sequence Length (bp)
	Observed samples			
Ingroup	1	*Ficus callosa*	-	849
Outgroup	2	*Ficus* sp.	-	896
	NCBI database			
	3	*Ficus callophylla*	EU091582.1	713
	4	*Ficus consociata*	AY063558.1	693
	5	*Ficus cordatula*	EU091584.1	720
	6	*Ficus crassiramea*	EU091585.1	793
	7	*Ficus elastica*	HM368192.1	691
	8	*Ficus forstenii*	EU091587.1	738
Ingroup	9	*Ficus kochummeniana*	EU091590.1	789
	10	*Ficus microcarpa*	JN407488.1	710
	11	*Ficus paracamptophylla*	EU091584.1	720
	12	*Ficus pellucidopunctata*	AF165399.1	707
	13	*Ficus spathulifolia*	EU091594.1	722
	14	*Ficus sumatrana*	EU091597.1	695
	15	*Ficus sundaica*	AY730068.1	785
Outgroup	16	*Ficus erecta*	KX572967.1	728
	17	*Ficus nota*	EU091626.1	598

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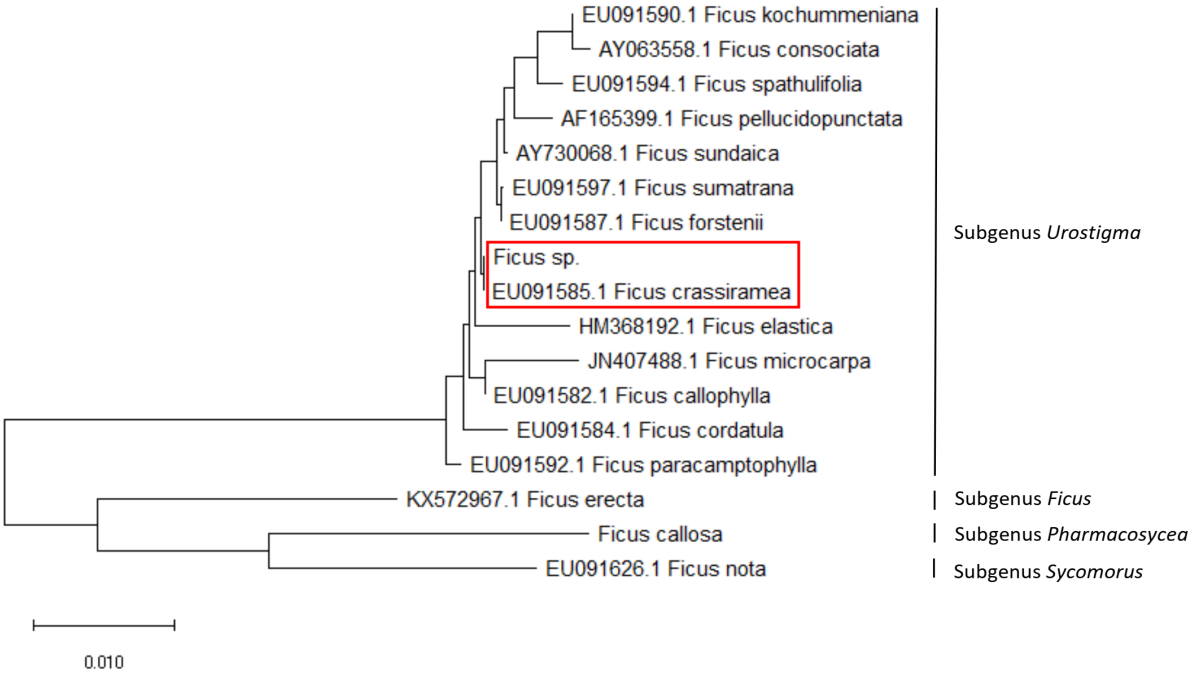

Figure 3. Phylogenetic tree of *Ficus* sp. generated through Neighbor-Joining method and 1000 replicate of boot-straps.

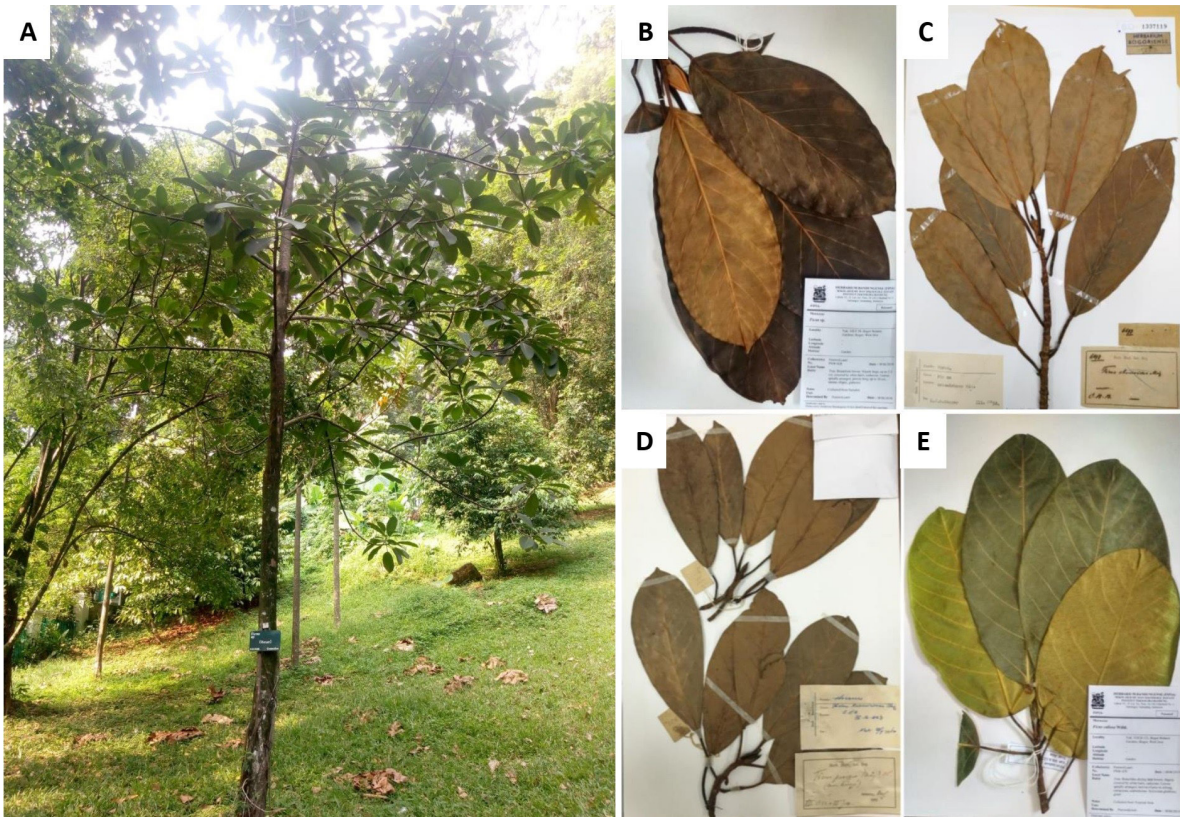

Figure 4. Habits of *Ficus* sp. in Bogor Botanic Gardens (A) and specimen herbarium of *Ficus* sp. (B), *Ficus crassiramea* (BO) (C), *Ficus crassiramea* (KRB) (D), and *Ficus callosa* (E).

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Ficus crassiramea is distributed from Myanmar, Thailand, to throughout Malesia, including Solomon islands (Berg & Corner, 2005; Ng et al., 2014). This species is found growing from coastal forests, riverbank areas to tropical rainforests at an altitude of 1500 m asl (Ng et al., 2014). Taxonomically, the species are grouped into 2 subspecies, namely *F. crassiramea* subsp. *crassiramea* and *F. crassiramea* subsp. *stupenda* (Miq.) C.C.Berg (Berg & Corner, 2005). In Indonesia, *F. crassiramea* subsp. *crassiramea* is found in Sumatra, Java, Kalimantan, Sulawesi, Maluku, and Papua. Meanwhile, the latter subspecies only occurred in Western Java and Borneo (Berg & Corner, 2005). Thus, based on their distribution, the living collection in BBG could be identified as *F. crassiramea* subsp. *crassiramea*. Morphologically, both subspecies can be distinguished based on several morphological characteristics (Table 4).

Table 3. Morphological comparison of *F. callosa*, *F. crassiramea* (BO), and *Ficus* sp.

Morphological Characters	*Ficus callosa*	*Ficus crassiramea*	*Ficus* sp.
Branchlets	Drying brown	Blackish-brown	Drying yellowish
Stipule length	1.5 cm	1.3-2.8 cm	4 cm
Indument on stipule*	Sericeous	Puberulous	Puberulous
Dried petiole colour*	Brown	Blackish-brown	Blackish brown
Petiole length	3-8 cm	3.4-4 cm	6.5-10 cm
Petiole width	5-6 mm	3-4 mm	2-4 mm
Lamina shape*	Elliptic to oblong	Elliptic to elliptic-oblong	Elliptic to elliptic-oblong
Leaf base*	Subcordate to rounded	Cuneate	Cuneate
The margin of juvenile leaves*	Lobed	Entire	Entire
Leaf apex*	Rounded to shortly acuminate	Short acuminate to obtuse	Short acuminate to obtuse
Lateral veins	8-12 pairs	7-8 pairs	9-10 pairs
Tertiary venation*	Scalariform	Reticulate	Reticulate
Adaxial surface	Glabrous	Glabrous	Glabrous
Abaxial surface*	Scabridulous	Glabrous	Glabrous
Waxy gland*	Absent	At the base of midrib	At the base of midrib

Note: The morphological features marked with an asterisk indicate a notable characteristic for the *Ficus crassiramea* species

Table 4. Morphological comparison of *F. crassiramea* subsp. *crassiramea* and *F. crassiramea* subsp. *stupenda* from Berg & Corner (2005)

Morphological Characters	*Ficus crassiramea* (BBG)	*F. crassiramea* subsp. *crassiramea*	*F. crassiramea* subsp. *stupenda*
Branchlets	Glabrous	Glabrous or sparsely puberulous	Densely to sparsely minutely white puberulous
Leaf base	Cuneate	Cuneate to rounded	Obtuse to subcordate or cordate
Tertiary venation	Reticulate	Reticulate	Subscalariform
Lateral veins	8 pairs	6-8 pairs	9-12 pairs

Based on molecular identification using ITS sequence and morphological characters, the living specimen of *Ficus* sp. in BBG is identified as *Ficus crassiramea* subsp. *crassiramea* with a sequence similarity level of 99.87%. Observations of the specimen's twigs and leaves' morphological characteristics show that nine vegetative characters can be used as *Ficus crassiramea* specific markers.

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