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MORPHO-MOLECULAR CHARACTERIZATION OF *LASIODIPLODIA THEOBROMAE* (PAT.) GRIFFON & MAUBL AND ITS FIRST REPORT ON THE ASSOCIATION WITH COCONUT KERNEL FROM BANGLADESH

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Abstract

This study marks the first report of *Lasiodiplodia theobromae* associated with coconut in Bangladesh, a pathogen known to cause wide range of diseases crippling coconut production worldwide. Two isolates, Lt_BD 1 and Lt_BD 4, were obtained from coconut samples and subjected to comprehensive morpho-molecular and phylogenetic analyses. Morphological observations, including colony characteristics (color, texture, and surface appearance), growth patterns and conidial dimensions and shapes, preliminarily identified the isolates as *Lasiodiplodia* species. Molecular analysis, through PCR amplification of the internal transcribed spacer (ITS) regions, confirmed the identity of the isolates as *L. theobromae*. A phylogenetic tree, constructed using sequences of the studied isolates alongside 48 reference *Lasiodiplodia* species (retrieved from NCBI) and one out-group species (*Pyricularia oryzae*), corroborated this identification. This study provides a foundation for further rigorous research on the diseases of coconut caused by *L. theobromae* in Bangladesh.

Introduction

The coconut (*Cocos nucifera* L.), a member of the Arecaceae family, is one of the most vital perennial crops in tropical regions. Often termed the "tree of life," coconut offers diverse applications, ranging from food, oil, and medicine to construction materials, fibers, and cosmetics. The white flesh of the coconut is nutrient-rich, containing high levels of fats, carbohydrates, iron, potassium, vitamin A, and vitamin B. Its endosperm is widely consumed raw, used in confections, and forms the basis for various dishes and desserts. Coconut oil, extracted and processed from dried coconut, is good for skin and hair care. Economically, coconut is significant for Bangladesh, which exports pure and natural coconut products globally.

Coconut production faces significant qualitative and quantitative challenges, with fungal diseases playing a critical role. Among these, phytopathogenic species from the genus *Lasiodiplodia* are responsible for approximately 500 plant diseases, including fruit rot, root rot, collar rot, stem-end rot, dieback, canker, and leaf necrosis (Huda-Shakirah *et al.*, 2022). As a globally distributed pathogen, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Botryosphaeriaceae, Botryosphaeriales, Dothideomycetes, Ascomycota) affects a wide range of hosts and can exist as a parasite, saprophyte or endophyte in nature (Alves *et al.*, 2008; Machado *et al.*, 2014; Rosado *et al.*, 2016). In coconut, *Lasiodiplodia theobromae* causes a wide range of diseases including nut fall (Venugopal and Mohanan, 2006; Sunpapao *et al.*, 2022), leaf blight (Santos, 2020; Ramjegathesh *et al.*, 2019; Ashokkumar *et al.*, 2018), nut rot disease (Taylor and Hyde, 2003; Dheepa *et al.*, 2018) and postharvest stem end rot (Rosado *et al.*, 2016; Zhang and Niu, 2019), all of which cause serious hindrance to coconut production. Based on morphological,

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phylogenetic and pathogenicity data, Santos *et al.* (2020) first addressed two species namely, *Botryosphaeria fabicerciana* and *Lasiodiplodia pseudotheobromae* in addition to *L. theobromae* as causal agents of leaf blight disease in coconut from Brazil. Previously, *L. theobromae* was the sole species linked to postharvest stem-end rot of coconut (Piepenbring, 2006; Taylor & Hyde, 2003). Rosado *et al.* (2016) expanded this understanding by reporting three additional species alongside *L. theobromae* namely, *L. brasiliense*, *L. egyptiacae*, and *L. pseudotheobromae* as causative agents of postharvest stem-end rot in Brazil. Their artificial inoculation experiments demonstrated that *L. theobromae* was the most prevalent and aggressive species causing the disease.

Lasiodiplodia spp. are capable of surviving endophytically, enabling them to evade detection during quarantine. These fungi can infiltrate the endosperm, rendering coconut water unsuitable for consumption. Additionally, research by Felix *et al.* (2018) underscores the health risks posed by toxic metabolites produced by *L. theobromae* strains. Therefore, identification and proper characterization of this fungus in coconut is desperately needed. Several studies on fungal association with coconut have been reported from Bangladesh (Bhuiyan *et al.*, 2021; Khan and Hossain, 2014). However, to the best of our knowledge, association of *Lasiodiplodia theobromae* with coconut has not yet been reported from Bangladesh. Therefore, this study aims to address this gap by characterizing *L. theobromae* isolates obtained from coconut kernels.

Materials and Methods

Sample collection and pathogen isolation

Coconut fruit with characteristic symptoms was collected for isolation. The rotted coconut kernel was associated with dark brown to blackish mycelial patches which is a typical feature of fungi belonging to the Botryosphaeriaceae. Fungus was isolated directly from symptomatic fruits using the method described by Hosen *et al.* (2023). Details of isolate ID, origin, and corresponding NCBI accession numbers are provided in Table 1. The fungal isolates were incubated at 25°C for five days to obtain pure cultures, which were subsequently used for morphological and molecular characterization. For long-term preservation and future molecular analyses, the isolates were grown on sterile 3 mm filter paper disks and stored in sterile Eppendorf tubes at −80°C.

Morphological identification

Preliminary identification of fungal isolates was performed based on morphological characteristics, encompassing both macroscopic and microscopic features including conidia, conidiogenous cells and mycelium. Spore images were measured at 40x magnification using a Nikon Optiphot-2 trinocular microscope (Japan) equipped with a digital camera and ImageFocus Alpha software. For each isolate, the length and width of ten spores were recorded, and the average size was calculated.

Molecular characterization and phylogenetic analysis

DNA extraction

Approximately 1 gram of mycelium from 7-day-old culture for each isolate was transferred into a 1.5 ml sterile Eppendorf tube. The mycelium was ground using a homogenizer in 400 µl of sterile extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Genomic DNA was extracted following the protocol described by Noman *et al.* (2021). The resulting DNA pellet was resuspended in 100 µL of 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and allowed to dissolve overnight at 4°C. The DNA samples were stored at −20°C for subsequent analyses.

DNA concentration was measured at 260 nm using a Nanodrop spectrophotometer, and quality was assessed by electrophoresis on a 1% agarose gel prior to PCR amplification.

Table 1. Details of the *Lasiodiplodia theobromae* isolates isolated from coconut kernel during the present study and the reference isolates retrieved from NCBI for phylogenetic analysis.

Sl. No. [a,b&c]	Name of the isolate	Isolate ID	Origin of the isolate		NCBI accession no.	References
			Location	Host		
1.	***Lasiodiplodia theobromae***	**Lt_BD 1**	**Bangladesh**	***Cocos nucifera***	**OQ438652**	**This study**
2.	***L. theobromae***	**Lt_BD 4**	**Bangladesh**	***C. nucifera***	**OQ438653**	**This study**
3.	*L. theobromae*	PCB	Malaysia	*Jatropha curcas*	GU228527	Sulaiman *et al*., 2012
4.	*L. theobromae*	RSGV/LK02	Malaysia	*J. curcas*	HM346873	Sulaiman *et al*., 2012
5.	*L. theobromae*	FH14K03	Mexico	Citrus tree	MK886711	Hernández *et al*., 2021
6.	*L. theobromae*	Lt1	India	Bottle Gourd	MN995068	Unpublished Jain S and Singh G
7.	*L. theobromae*	1_finish	China	Poplar stem	KF294005	Unpublished Sun XM and Yan DH
8.	*L. theobromae*	YLH2-2	China	Avocado	OM736159	Unpublished Yu HR and Wu JB
9.	*L. pyriformis*	CBS 121770	Namibia	*Acacia mellifera*	EU101307	Cruywagen *et al*., 2017
10.	*L. pyriformis*	CMW 25415	Namibia	*A. mellifera*	EU101308	Cruywagen *et al*., 2017
11.	*L. egyptiacae*	BOT-29	Egypt	*Mangifera indica*	JN814401	Ismail *et al*., 2012
12.	*L. egyptiacae*	BOT-10	Egypt	*M. indica*	JN814397	Ismail *et al*., 2012
13.	*L. subglobosa*	CMM 3872	Brazil	*Jatropha curcas*	KF234558	Gnanesh *et al*., 2022
14.	*L. subglobosa*	CMM 4046	Brazil	*J. curcas*	KF234560	Gnanesh *et al*., 2022
15.	*L. gilanensis*	IRAN1501C	Iran	*Unknown*	GU945352	Abdollahzadeh *et al*., 2010
16.	*L. gilanensis*	IRAN1523C	Iran	*Unknown*	GU945351	Abdollahzadeh *et al*., 2010
17.	*L. venezuelensis*	WAC12539	Venezuela	*Acacia mangium*	DQ103547	Burgess *et al*., 2006
18.	*L. venezuelensis*	WAC12540	Venezuela	*A. mangium*	DQ103548	Burgess *et al*., 2006
19.	*L. venezuelensis*	CMW 13513	Venezuela	*A. mangium*	DQ103549	Burgess *et al*., 2006
20.	*L. rubropurpurea*	WAC12535	Tully, Queensland	*Eucalyptus grandis*	DQ103553	Burgess *et al*., 2006
21.	*L. rubropurpurea*	WAC12536	Tully, Queensland	*E. grandis*	DQ103554	Burgess *et al*., 2006
22.	*L. rubropurpurea*	WAC12537	Tully, Queensland	*E. grandis*	DQ103555	Burgess *et al*., 2006
23.	*L. rubropurpurea*	WAC12538	Tully, Queensland	*E. grandis*	DQ103556	Burgess *et al*., 2006
24.	*L. citricola*	CBS124707a	Iran	*Citrus* sp.	GU945354	Abdollahzadeh *et al*., 2010
25.	*L. citricola*	CBS124706	Iran	*Citrus* sp.	GU945353	Abdollahzadeh *et al*., 2010
26.	*L. crassispora*	CBS125626	South Africa	*Vitis vinifera*	MT587424	Zhang *et al*., 2021
27.	*L. crassispora*	CMW33262	Unknown	*Adansonia* sp.	KU887068	Cruywagen *et al*., 2017
28.	*L. crassispora*	CMW 13488	Venezuela	*Eucalyptus europhylla*	DQ103552	Gnanesh *et al*., 2022

29.	*L. crassispora*	CBS 118741	Australia	*Santalum album*	NG_062741	Phillips *et al.*, 2005
30.	*L. euphorbicola*	CMM3651	Brazil	*Jatropha curcas*	KF234553	Machado *et al.*, 2014
31.	*L. euphorbicola*	CMW33268	Unknown	*Adansonia* sp.	KU887131	Cruywagen *et al.*, 2017
32.	*L. euphorbicola*	CMM3609	Brasil	*Jatropha curcas*	KF254926	Machado *et al.*, 2014
33.	*L. mahajangana*	CBS124925	Madagascar	*Terminalia catappa*	FJ900595	Begoude *et al.*, 2010
34.	*L. mahajangana*	CBS124926	Madagascar	*T. catappa*	FJ900596	Begoude *et al.*, 2010
35.	*L. hormozganensis*	CBS124709	Iran	*Olea* sp.	GU945355	Abdollahzadeh *et al.*, 2010
36.	*L. hormozganensis*	CBS124708	Iran	*Mangifera indica*	GU945356	Abdollahzadeh *et al.*, 2010
37.	*L. margaritacea*	CBS122519	Australia	*Adansonia gibbosa*	EU144050	Cruywagen *et al.*, 2017
38.	*L. margaritacea*	CBS122065	Australia	*A. gibbosa*	EU144051	Cruywagen *et al.*, 2017
39.	*L. margaritacea*	CBS138289	Namibia	*Combretum elaeagnoides*	KP872320	Zhang *et al.*, 2021
40.	*L. margaritacea*	CBS138290	Zambia	*Combretum collinum*	KP872321	Zhang *et al.*, 2021
41.	*L. parva*	CBS 356.59	Sri Lanka	*Theobromae cacao*	EF622082	Ismail *et al.*, 2012
42.	*L. parva*	CBS 494.78	Colombia	Cassava-field soil	EF622084	Ismail *et al.*, 2012
43.	*L. exigua*	BL 184	Tunisia	*Retama raetam*	KJ638318	Linaldeddu *et al.*, 2015
44.	*L. exigua*	BL 185	Tunisia	*R. raetam*	KJ638319	Linaldeddu *et al.*, 2015
45.	*L. exigua*	BL 187	Tunisia	*R. raetam*	KJ638321	Linaldeddu *et al.*, 2015
46.	*L. exigua*	CBS 137785	Tunisia	*R. raetam*	KJ638317	Linaldeddu *et al.*, 2015
47.	*L. brasiliense*	CBS123095	Cameroon	*Teobroma cacao*	MT587423	Zhang *et al.*, 2021
48.	*L. brasiliense*	CMM4015a	Brazil	*Mangifera indica*	JX464063	Marques *et al.*, 2013
49.	*L. brasiliense*	CSM11	Venezuela	*Teobroma cacao*	MF436018	Mohali-Castillo *et al.*, 2023
50.	*L. brasiliense*	CF/UENF436	Brazil	*Cocos nucifera*	KY655209	Santos *et al.*, 2020
51.	*Pyricularia oryzae*	BDC_10	Bangladesh	*Triticum aestivum*	MT358609	Noman *et al.*, 2021

[a] *Lasiodiplodia theobromae* isolates studied in the present investigation are shown in bold (1&2)
[b] Reference *Lasiodiplodia* isolates obtained from NCBI and used for phylogenetic analysis (03-50)
[c] Reference *Pyricularia oryzae* isolate obtained from NCBI and used as out-group for phylogenetic analysis (51)

PCR amplification and sequencing

The internal transcribed spacer (ITS) regions of the isolates were amplified using the forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Each 25 µl PCR reaction mixture contained 2.0 µl of template DNA, 12.5 µl of Master Mix (Clever Scientific Ltd., Warwickshire, UK), 1.0 µl of each primer, and 8.5 µl of nuclease-free water. The reaction mixture was thoroughly mixed before thermal cycling, which included an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step at 72°C for 5 minutes was included, ending with a hold at 4°C. Successful amplification of the ITS regions was verified by electrophoresis on a 1% agarose gel with compared to a 100 bp DNA ladder (Clever Scientific Ltd., Warwickshire, UK). Purified PCR products were sequenced using a SeqStudio Genetic

Analyzer (Thermo Fisher Scientific, USA) at the Centre for Advanced Research in Sciences (CARS), University of Dhaka, Bangladesh.

Sequence analysis and phylogenetic tree construction

The nucleotide homogeneity of the obtained consensus sequences was evaluated by comparing them with other sequences in the GenBank database using the BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST) and these sequences were subsequently deposited in the GenBank database. Sequence alignment was performed using the CLUSTAL W algorithm implemented in Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (Kumar *et al.*, 2016). A phylogenetic tree was constructed using the neighbor-joining method within the same software, and branch support was evaluated using 1000 bootstrap replicates.

Results and Discussion

Morphological characterization

Morphological characteristics such as colony color and texture, surface appearance, growth pattern, and conidial size and shape were examined. The isolates grown on culture media displayed typical *Lasiodiplodia* morphology. The mycelium grew vigorously in all directions, completely covering the surface of the Petri plates within 5 days (Fig. 1). The colony texture of the *Lasiodiplodia* isolates was fluffy, raised and irregular. Initially, the colonies were white, gradually changing to light gray within a week. After two weeks of incubation, the color turned dark gray or black when viewed from the top and dark olive green or black from the reverse side (Fig. 1). No variation in conidial shape was observed. The conidia were septate, oval in shape, dark brown in color with irregular longitudinal striations on the spores. Average conidial sizes of the isolates Lt_BD 1 and Lt_BD 4 were found 22.5 × 12.0 µm and 23.0 × 11.5 µm, respectively.

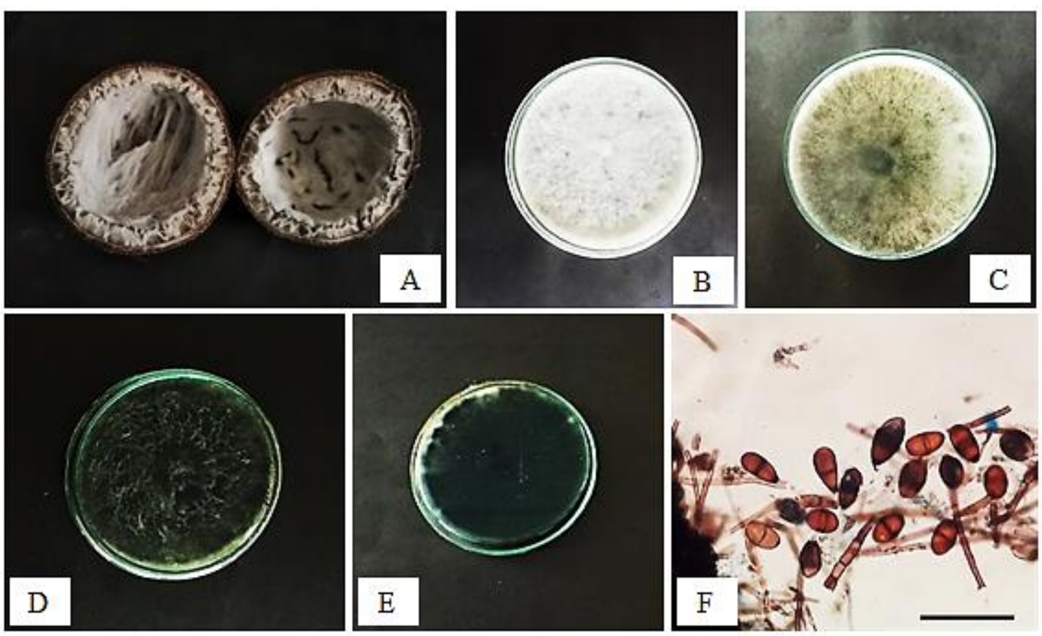

Fig. 1. Morphological characterization of *L. theobromae*. A. Infested coconut kurnel; B-C. 5 days old colony on PDA medium from upper (B) and reverse view (C); D-E. 14 days old mature colony on PDA medium from upper (D) and reverse view (E); F. Conidia under microscope (scale bar = 50 µm).

Molecular characterization and phylogenetic analysis

Molecular identification and phylogenetic analysis were conducted to accurately identify the isolates at the species level. PCR amplification of the internal transcribed spacer (ITS) regions produced an amplicon of approximately 550 bp for each isolate (Fig. 2). The amplicons were purified, sequenced, and analyzed using the NCBI BLAST search tool. The ITS sequences of the isolates were found to be identical and confirmed as *L. theobromae*. The newly generated sequences from this study were submitted to NCBI, and the corresponding GenBank accession numbers are listed in Table 1.

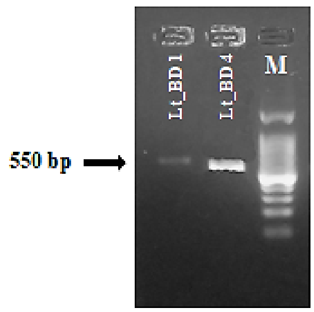

Fig. 2. Gel electrophoresis of amplified ITS region of the *L. theobromae* isolates using 1% agarose gel (M indicates 100 bp DNA ladder).

To analyze the phylogenetic position of the studied *L. theobromae* isolates, a neighbor-joining tree was also constructed based on ITS sequences. The ITS sequences of the isolates from the present study were aligned with 48 reference isolates of *Lasiodiplodia* species of different countries and plant hosts (retrieved from NCBI) and one outgroup taxon (*Pyricularia oryzae*) (Fig. 3). From the phylogenetic tree it was observed that out-group taxon, *P. oryzae,* clustered completely separately and remaining all the *Lasiodiplodia* species formed a major cluster among them. Species wise clustering was demonstrated in the dendrogram. Isolates of this study namely, Lt_BD 1 and Lt_BD 4 showed strong relationship with reference *L. theobromae* isolates and formed a different cluster. As a result, isolates of this study were verified as *L. theobromae* by virtue of molecular identification and phylogenetic analysis.

Lasiodiplodia theobromae has previously been identified as a pathogen of dragon fruit in Bangladesh (Briste *et al.*, 2021). However, despite being serious pathogen of coconut, there is no available report on the association of this fungus with coconut from Bangladesh till date. This study marks the association of *L. theobromae* with coconut from Bangladesh and demonstrated detailed morpho-molecular characterization with phylogenetic relationship. The isolates studied here are preliminarily identified as *Lasiodiplodia* species based on their morphological features, consistent with descriptions provided by other researchers studying *Lasiodiplodia* (Alves *et al.*, 2008; Marques *et al.*, 2013; Machado *et al.*, 2014; Linaldeddu *et al.*, 2015; Rosado *et al.*, 2016; Huda-Shakirah *et al.*, 2022).

Morphological methods have traditionally been central to fungal taxonomy. However, morphology-based identification within the Botryosphaeriaceae family is limited to the genus level, as many *Lasiodiplodia* species share overlapping morphological traits. This limitation highlights the importance of molecular techniques. As a result, molecular and phylogenetic investigations incorporating ITS DNA sequences are critical for avoiding ambiguous and misleading results and resolving species-level identification issues. ITS region is recognized as a universal fungal barcode and an effective molecular tool for identifying fungal species and analyzing the phylogenetic relationships of various species and geographic isolates (Rosado *et al.*, 2016; Noman *et al.*, 2021). In this study, molecular characterization of the isolates was carried out using rDNA sequences of the ITS region. Neighbor-joining tree inferred from *L. theobromae* isolates of this study together with 48 reference *Lasiodiplodia* species and one outgroup taxon (*Pyricularia oryzae*) revealed that studied Lt_BD 1 and Lt_BD 4 isolates and other reference *L. theobromae* isolates showed strong relationship and formed a completely separate cluster, confirming that studied fungal isolates were *L. theobromae.*

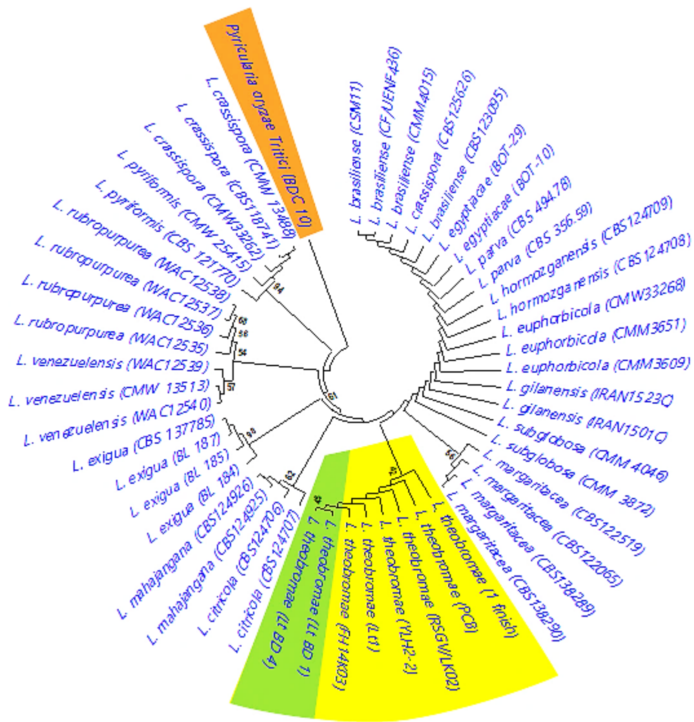

Fig. 3. Phylogenetic relationship of *Lasiodiplodia theobromae* isolates with other reference *Lasiodiplodia* isolates based on ITS sequence similarity using neighbour- joining method. *L. theobromae* isolates of this study, reference *L. theobromae* isolates and reference *Pyricularia oryzae* isolate (out-group) were marked in green, yellow and light orange zones, respectively. Numbers besides each branch represent bootstrap values obtained after a bootstrap test with 1000 replications. Branch support less than 40 was not shown in the dendrogram.

As a globally significant pathogen, *L. theobromae* is responsible for severe diseases in coconut, impacting its production and economy. This study provides the first comprehensive evidence of the association of *L. theobromae* with coconut in Bangladesh through morpho-molecular approaches. The findings of this study enhance the deeper understanding of *L. theobromae* in coconuts and this research will lay the groundwork for more in-depth studies on coconut diseases caused by this fungus.

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