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New report of isolates of *Lasiodiplodia* spp. from the coastal region of Odisha (India), causing crown rot disease in Banana

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Abstract

Crown rot disease in banana caused by the fungus *Lasiodiplodia brasiliensis* and *Lasiodiplodia theobromae* is one of the major post-harvest diseases causing a massive loss in the economic yield of banana. Isolates of *Lasiodiplodia brasiliensis* and *Lasiodiplodia theobromae* were isolated from infected fruits and were characterized based on their morphological and molecular characteristics. The isolates produced mustard seed-like pycnidia with conidia after 15 days after inoculation (DAI) and chlamydospore was observed after 16 DAI. The immature conidia were hyaline, aseptate, thin-walled and cylindrical to sub-ovoid in shape which became brown with central transverse septum at maturity. The isolates were sequenced and submitted in NCBI gene bank and were assigned with accession no. OQ921841 and OR764951. Further, the evolutionary relationships of the pathogen isolates were studied concerning different related genera and species of *Lasiodiplodia*.

Key words: *Lasiodiplodia brasiliensis, Lasiodiplodia theobromae*, banana, crown rot, morphology, pathogen

Introduction

Banana (*Musa paradisica*) is one of the oldest cultivated plants and it belongs to the family Musaceae (Jones and Daniells, 2019). Being a highly perishable fruit, banana endures serious post-harvest losses both in terms of quality and quantity. Post-harvest diseases of fruits mainly occur due to some phytopathogenic fungi and bacteria which lead to significant losses throughout the storage and transportation period (Ruffo *et al.*, 2019). Major post-harvest diseases of banana which affect the quality of the fruits across the banana growing countries are Anthracnose and Crown rot which is caused by the fungus *Colletotrichum musae* and *Lasidiplodia theobormae*, respectively (Kulkarni *et al.*, 2022). Crown rot is an important postharvest disease which is caused by several fungi involving fungal complex of *Lasiodiplodia theobromae*, *Colletotrichum musae* and *Fusarium* spp. (Sangeetha *et al.*, 2012). Typical symptoms of Crown rot appear as blackening of the fruit tissues and mold in the crown area (Kamel *et al.*, 2016). The rotting starts with mycelial development on the surface of the crown followed by the peduncles and fruits. In case of severe infection, the banana fruits may detach from the peduncle. Rotting of crown leads to softening and blackening of the tissue due to development of necrosis on the fruit which affects the fruit quality (Lassois *et al.*, 2010). According to Thangavelu *et al.* (2007) the major pathogen responsible for crown rot disease in India is *Lasiodiplodia theobromae*. Santos *et al.* (2023) first reported that *Lasiodiplodia brasiliensis* to be responsible for causing crown rot on banana in Brazil. In best of our knowledge up to now there is currently no report of *Lasiodiplodia brasiliensis* and *Lasiodiplodia theobromae* causing crown rot disease in banana from Odisha, India. Thus, aim of the study was to identify and characterize the two species *Lasiodiplodia brasiliensis* and *Lasiodiplodia theobromae* collected from coastal region of Odisha, India.

Materials and methods

Collection of disease sample and isolation of fungal isolates: During the year 2022-23, survey was undertaken and disease samples with crown rot symptoms like the blackening and mold in the crown area of the fruits were taken from banana growing regions of Bhubaneswar (20.2961°N, 85.8245°E), Odisha. The contaminated fruits were treated with 1% sodium hypochlorite solutions for 2 minutes. A lesion with apparent symptoms was examined under a microscope to confirm the presence of fungal spore. Both diseased and healthy portion of infected fruit samples were cut into (2-3mm) small bits and surface sterilized for two minutes with 1% sodium hypochlorite solution followed by sterilised distilled water washes. Samples were then transferred into PDA plates and the plates were incubated at 28 °C. The pure culture of isolated fungus was maintained on PDA at 28 °C and sub cultured as needed. At different periods of time, cultural characteristics like colour, texture, margin, conidial pattern and diameter of the fungal colonies were observed.

Pathogenicity test: Banana (var. Robusta) in green mature stage, devoid of stains and imperfections was collected for the pathogenicity test. They were cleaned with tap water followed by distilled water and then disinfected for 5 minutes with 1% sodium hypochlorite solution before being air dried. Wounding, pin pricking and spraying methods were carried out to study the pathogenicity of the isolated fungus. For conducting wounding method, with the help of cork borer (5mm) the fruits were wounded and 5mm diameter mycelia plug was removed from 7

days old culture and deposited on the wounded location. Fruits were injured with a sterile pin and spore suspension of (1×10^6 spores per mL) was injected in the pin prick method. Spore suspension of (1×10^6 spores per mL) was sprayed all over the fruit surface in spraying method. For three days the inoculated fruits were stored in a wet chamber. After the symptoms appeared, the fungus was re-isolated to prove the Koch- postulates (Renganathan *et al.*, 2020).

DNA Isolation: The pure fungal culture was sub-cultured in potato dextrose broth (PDB) media and incubated for 8-10 days at 28±1 °C. The mycelia mats were harvested from the broth using what man No.1 filter paper. The fungal mats were properly dried using and sterile blotting paper. Newly obtained mycelium (1g) was crushed to a very fine powder using liquid nitrogen in a pre-chilled mortar and pestle. Fungal DNA was isolated using CTAB (Cetrimide tetradecyl trimethyl ammonium bromide) technique of DNA extraction and purification (Murray and Thompson, 1980).

Polymerase Chain Reaction (PCR) amplification: The isolated DNA was amplified using ITS1 - (TCCGTAGGTGAACCTGCGG) and ITS4 - (TCCTCCGCTTATTGATATGC) primers through PCR. It was carried out in a 25 µL volume containing 10 pmol of forward and reverse primers, 2.5mM of $MgCl_2$, 200 µM of the four dNTPs (deoxyribonucleotide triphosphates), and 0.5 U of Taq DNA polymerase, 2.5 µL PCR buffer and 50 to 100 ng of isolated bacterial genomic DNA. The template was initially denatured by heating at 95 °C, for 5 minutes followed by primer annealing at 60 °C for 40 seconds, elongation at 72 °C for 1 minute, and a final extension at 72 °C for 7 minutes. The amplicon was resolved in 1.5 % agarose gels with 0.5 X Tris-acetate - EDTA (TAE) buffer. Gel electrophoresis (0.8% agarose gel run in 1×TAE buffer at 50V for 45 minutes) was used to evaluate and quantify the PCR amplification products until the DNA fragments moved well and were viewed under UV light using Ethidium bromide as a staining agent. A 100 bp DNA ladder was used as a reference standard molecular marker.

Elution of DNA fragment and sequencing: The amplified DNA was purified using the PROMEGA GEL kit and the PCR purification kit. After being seen under UV light, the appropriate DNA pieces were cut from the gel with a sterile knife. The DNA-containing agarose slice was placed in a micro centrifuge after adding membrane binding solution, and the gel was melted by running the device at 70 °C. Using Mini column assembly, the DNA was purified after being removed from the gel slice. A commercial service provider used the ABI 3730XL to sequence the purified DNA fragment after it had been kept at -20°C for storage. The sequence of fungal isolate was submitted in NCBI gene bank.

Phylogenenetic analysis: Phylogeny was established by utilizing the neighbour joining approach in MEGA 7.0 to establish the link between isolated fungal strains from the genus *Lasiodiplodia* and *Lasiodiplodia* analogous species (Atteson,1999).

Results and discussion

Morphological characterization: The isolates of the fungus *Lasiodiplodia brasiliensis* and *L. theobromae* were cultured on the Petri plate in potato dextrose media (PDA) for 30 days to observe the morphological characteristics and growth rate. The mycelium of both the species were found to be fast spreading. It covered the 90 mm Petri dish within three days of inoculation. The radial growth of the fungus *L. brasiliensis* was recorded as 6.6 cm two days after incubation. The optimum growth rate of the fungus was observed at temperature 28 to 30 °C. It was observed that the texture of the mycelium was feathery, smooth margin and the colour of the colonies is olivaceous - grey to dark grey. The fungal mycelium was found to be whitish grey in colour after 3 days of inoculation. After 7 days of inoculation, the mycelia were found to be dark greyish which was feathery in nature. The fungal sporulation was observed after 14 days after inoculation. After 15 days of incubation, the fungus produced mustard seed like pycnidia with conidia at periphery region of the petri plates at 28 °C. The reverse side of the plate was marked with black pigment at 28 days after inoculation which subsequently led to complete black colouration of the plate due to enormous spore production. The mycelium of fungus *L. theobromae* was also found to be fast spreading from 3 days after incubation similar to *L. brasiliensis*. The radial growth of the fungus *L. theobromae* was recorded as 6.4 cm three days after incubation (Table 1). The greyish-white mycelia of the fungus covered the 90 mm petri dish with in three days of incubation. Initially the colour of the colony was whitish and with ageing it turned greyish to dark grey. Sunken lesions were seen in the petri plate at 7 days after incubation which differentiated the species from each other. Sporulation started at 14 days after incubation and enormous spores were seen 21 days after incubation. After 28 days of incubation at 28 °C the fungus produced pycnidia with conidia at centre region of the petri plate (Santos *et al.*, 2023; Sangeetha *et al.*, 2012; Renganathan *et al.*, 2020).

Microscopic characterization: The isolated fungus was observed under microscope at 14 days after incubation to study the conidial, conidiophore and thallus structures. Immature conidia (14 days after incubation) were found to be hyaline, aseptate, thin walled and cylindrical to sub-ovoid in shape and they became brown with central transverse septum when mature (40 days after incubation). Mature conidia were ovoid with a broad, rounded apex and tapered at the base. The single conidium measured about 18-26 µm in length and 9-13 µm in width. Brownish to black in colour with branched mycelium was observed on compound microscope. After 16 days of incubation the fungus produced chlamydospore (Santos *et al.*, 2023).

Pathogenicity test: Pathogenicity test was carried out in banana fruit (var. Robusta) in 3 different methods like wounding, pin pricking and spraying method and one control was taken. The non-inoculated fruits were taken as control. The fungal isolate was mass cultured on sorghum grain was used for artificially inoculating the fruits. The symptoms initiation was observed at 3 days of inoculation on the inoculated fruits whereas the non-inoculated fruits did not show any symptoms. It has been observed that after 3 days of inoculation black colour lesion was seen on the fruit surface. Then after 7 days of inoculation the total surface of the fruits were covered by greyish mycelium. It was also noted that after 10 days of inoculation immature conidia were seen under compound microscope. Re isolation of the tested fungus was done from the 10 days of inoculated fruits to prove the Koch postulates (Santos *et al.*, 2023; Waliullah *et al.*, 2022).

Molecular characterization of the isolated fungus: The ITS 1- TCC GTA GGT GAA CCT GC GG and ITS 4- TCC TCC GCT TAT TGA TAT GC primer combination amplified the extracted DNA as mentioned in materials methods. The sequences of the fungal isolates assigned with the accession no. OQ921841 and OR764951 were analysed for similarity with already deposited

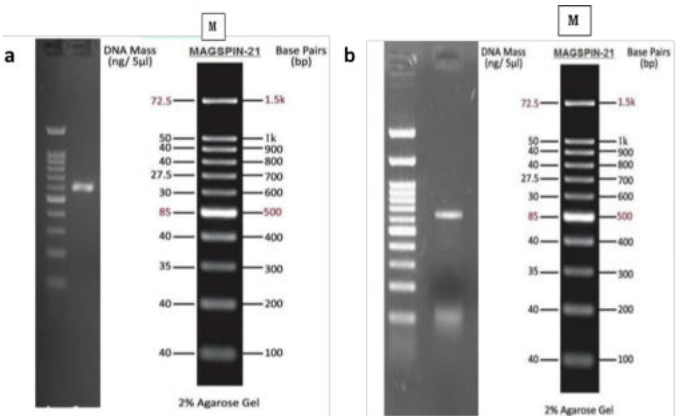

Fig. 1. PCR amplified Internal Transcribed Spacer (ITS) region of (a) *L. brasiliensis*. (b) *L. theobromae* M represents the standard molecular marker 100 bp ladder

sequences in NCBI. The pathogen was identified as *L. brasiliensis* and *L. theobromae,* respectively which produced amplicons on 600 bp separated through 1.8% agarose gel electrophoresis. Utilizing ClustalX, which was incorporated into the Mega 7 program, sequence alignments and comparisons served as the foundation for the phylogenetic analyses. The tree is scaled, with branch lengths measured in the same units as the evolutionary distances used to estimate the phylogenetic tree (Videira *et al.*, 2017). Based on the phylogenetic tree depicting the links between distinct *Lasiodiplodia* species and other closely linked genera, it was found that SOA 1 (*L. brasiliensis*) and SOA 4 (*L. theobromae)* shared a very close common ancestor but they are related to other *Lasiodiplodia* species. It was noticed that they shared ancestors with other genera like *Sphaeropsis*, *Neodeightonia, Tiarosporella*. The genera *Lasiodiplodia* and *Sphaeropsis* were found to have more immediate ancestor than between *Lasiodiplodia* and *Tiarosporella* (Phillps *et al.*, 2013).

From the analysis of phylogenetic tree, it is implied that the species of SOA 4 (*L. theobromae)* is more similar to *L. curvuta*, *L. citricola, Lasiodiplodia brasiliensis* and the species of SOA 1 (*L. brasiliensis*) is more similar to *L. citricola* and *L.macroconidia*.

The results of the current phylogenetic analysis indicate a closer relationship between *Lasiodiplodia* and the clade *Sphaeropsis,* which appears as a sister group. *Lasiodiplodia* and *Sphaeropsis* possess comparable morphological characteristics in the pattern of present of pycnidial paraphyses (Phillips *et al.*, 2013). The clade *Lasiodiplodia* and *Neodeightonia* also found to share common ancestor but they differ from each other in respect to present of pycnidial paraphyses (Phillips *et al.*, 2013). The phylogenetic tree revealed that the species SOA 4 *(L. theobromae*) share similar microscopic characteristics with the species *L.curvuta* in respect to conidiospore that the immature conidiospore were hyaline and aseptate whereas the mature conidiospore were brownish with prominent septation but *L. curvuta* had curved conidiospore which differentiate the species from *L. theobromae* (Munirah *et al.*, 2017; Wang *et al.*, 2019). From the phylogenetic tree it is notified that the species SOA 1 (*L. brasiliensis*) share similar morphological and microscopic characteristics like both the species produced initially greyish mycelium which became dark grey to black with age and also the immature conidiospore were hyaline and aseptate whereas the mature conidiospore were brownish with prominent septations (Santos *et al.*, 2023; Wang *et al.*, 2019).

In conclusion, from our study, the evolutionary relationship of two species of *Lasiodiplodia i.e. L. brasiliensis* and *L. theobromae* could be be established with different other species of *Lasiodiplodia* and related genera of the genus in order to study the diversity of the pathogen on the basis of morphological and molecular characteristics.

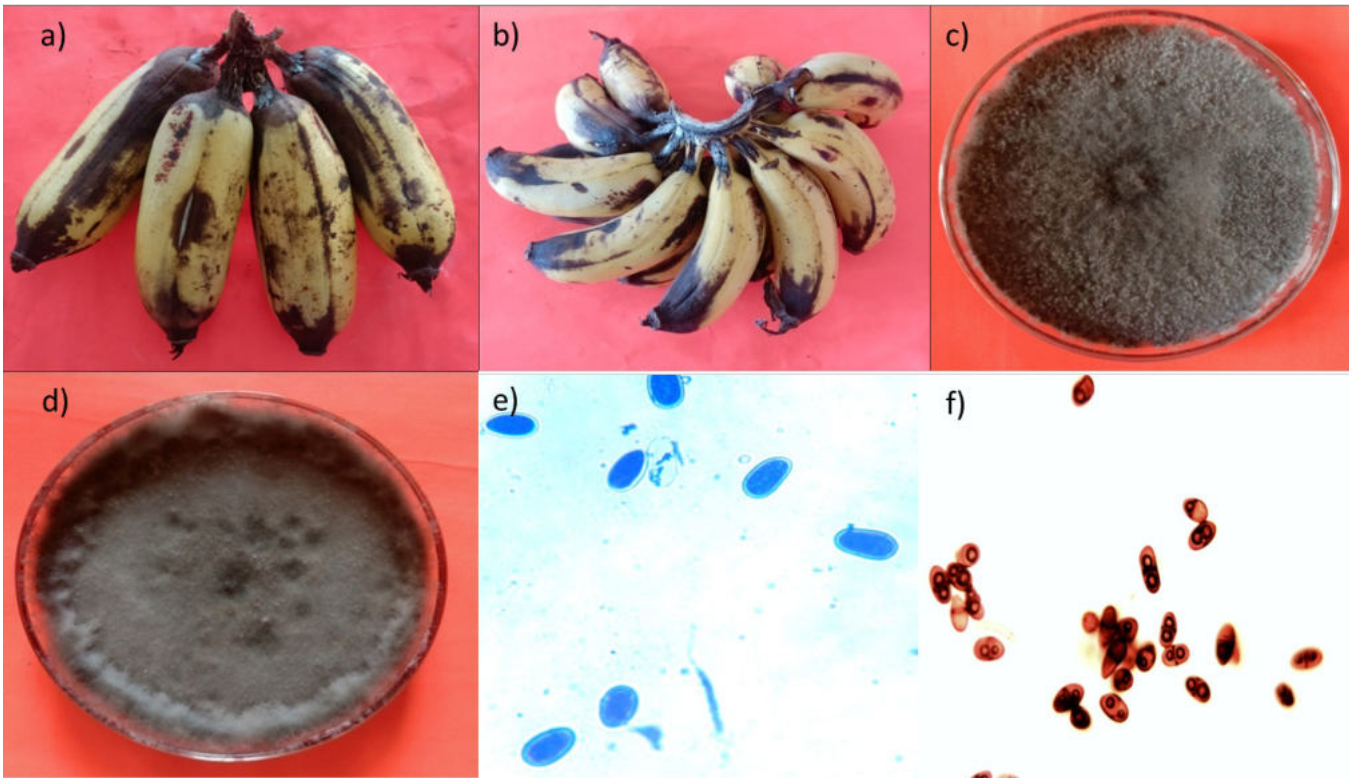

Fig. 2. (a, b) Infected banana fruits depicting crown rot sysmptouis; morphological characteristics of (c) *L. brasiliensis* (d) *L. theobromae* at 14 dai; microscopic characteristics of *Lasiodiplodia*. spp. (e) Immature conidia (f) Mature conidia

Fig. 3. Neighbour-Joining tree representing the phylogenetic relationship of *Lasiodiplodia* genus and its closely related genera.

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Conflict of Interest: The authors certify that they do not have any known competing financial interests or personal relationships which could have appeared to influence the research work reported in the article.

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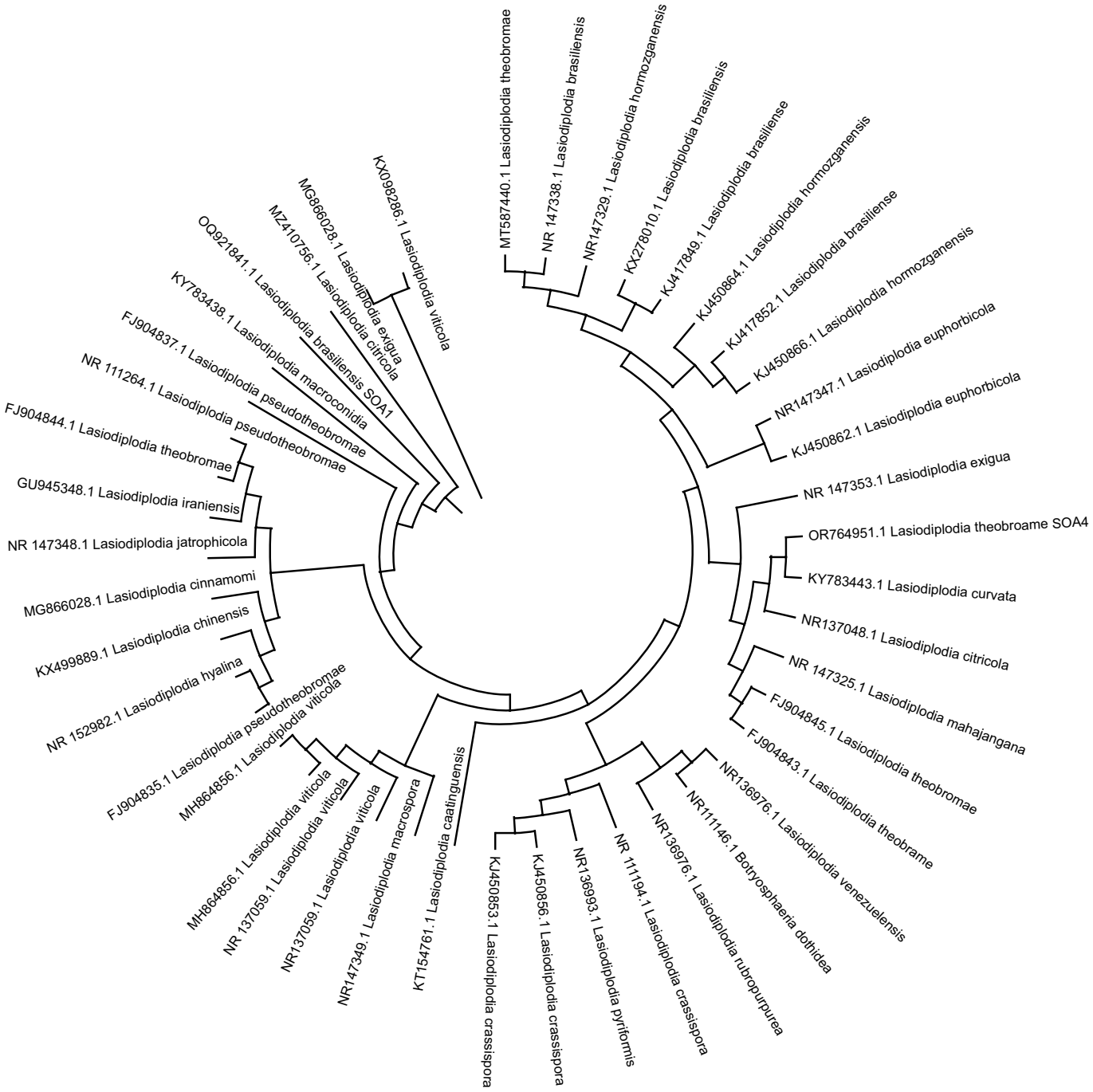

Fig. 4. Phylogenetic tree of genus *Lasiodiplodia* based on ITS nucleotide sequences constructed using neighbor joining method in MEGA 7.0.

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