

## Morphological Characterization and Molecular Identification of Stem-end Rot Associated Fungal Species Isolated from ‘Karutha Colomban’ Mango Fruits in Sri Lanka

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### ABSTRACT

**Purpose:** Stem-End Rot (SER) is one of the most frequently found mango postharvest diseases in many countries including Sri Lanka. Availability of a significantly detailed study on morphological and molecular characterization of the SER associated fungal species is comparatively low. The present study has been carried out considering the above fact.

**Research Method:** SER disease associated fungi were isolated and identification was carried out using morphological characteristics. Identification was confirmed by phylogenetic analysis of newly generated ITS sequencing data using Maximum Likelihood (ML) with RAxML software (Ver.8.2.10).

**Findings:** From the morphological studies it was observed that the fungal isolates SER1, SER2, SER3 and SER6 produced conidia on Potato Dextrose Agar. Both morphological and molecular results revealed that the isolates of SER1, SER4, SER5, and SER6 which were isolated from diseased Karutha Colomban mango fruits with SER (in Sri Lanka) belong to *Lasiodiplodia* sp., *Nodulisporium* sp., *Xylaria feejeensis* and *Pestalotiopsis* sp. respectively. Both SER2 and SER3 were confirmed as *Phomopsis* sp. During the present study two new fungal species (*Xylaria* spp. and *Nodulisporium* spp.) were also identified for the first time from SER of Karutha Colomban mango fruits.

**Research Limitations:** The pathogenicity of these isolates will be tested in near future, since the study was mainly focused on the isolation and identification of the SER associated fungal species.

**Originality/ Value:** Results of the present study will be very valuable for stakeholders in Agricultural sector, before designing control strategies at post-harvest level in order to minimize the loss and extend the storage life of mango.

**Keywords:** *Mangifera indica* L., stem-end rot pathogens, *Lasiodiplodia theobromae*

### INTRODUCTION

Mango (*Mangifera indica* L.) which belongs to family Anacardiaceae is one of the most important and widely cultivated fruit species of the tropical world (Bandyopadhyay *et al.*, 2014). Asia is the largest mango producer, representing more than 75% of global production. This crop is adaptable to a wide range of climate, from wet tropical to dry subtropical (Abd-Alla and Haggag, 2013). Due to the delicious taste and high calorific value, it has become one of the most desirable fruits in the international market

(Diedhiou *et al.*, 2007). Present extent under mango cultivation in Sri Lanka is about 27,500 acres. It is Predominantly, grown in *Kurunegala*, *Anuradhapura*, *Hambanthota*, *Puttalam*, *Moneragala*, Jaffna Districts and in *Mahaweli* Systems H and C. As a fresh fruit, mango has a high demand in local market. There is potential to earn, a considerable amount of foreign

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exchange by exporting both fresh and processed mango products. At present Sri Lanka exports about 80,000 Mt (FCRDC, 2015) to countries including, Middle East, Europe and Singapore by air freight. ‘*Karutha Colomban*’ is one of the most popular cultivars of mango in Sri Lanka due its delicious taste and orange colour flesh. In addition, cultivars like *Vellai Columban*, Parrot, *Peterpassand*, *Malwana*, *Willard*, TOM EJC, etc., are also being cultivated with the same preference in certain districts of Sri Lanka (Kotalawala, 1972).

However, the availability of fresh mango for the local and international consumers has been limited by its highly perishable nature and its susceptibility to postharvest diseases. Postharvest diseases can reduce mango fruit quality and cause severe losses (Narayanasamy, 2006). The postharvest loss of mango in Sri Lanka is estimated to be 17-36% (Haggag, 2010). Stem-end rot and anthracnose are the two major postharvest diseases of mango, which reduce the fruit quality, shelf life and marketability (Krishnapillai and Wijeratnam, 2013).

In Sri Lanka, Stem-end rot (SER) disease in mango is caused by a complex of fungi, namely, *Lasiodiplodia theobromae* (also known as *Botryodiplodia theobromae*), *Dothiorella* spp. *Colletotrichum gloeosporioides*, *Phomopsis mangiferae* and *Pestalotiopsis mangiferae* (Abeywickrama, 2006; Adikaram *et al.*, 2010; Syed *et al.*, 2014). This disease results in darkening of the pericarp near the base of the pedicel, which enlarges within two or three days, limiting to very short storage life of the fruit commodity and therefore restricting export over long distances.

As of today, the availability of significant detailed research studies on the characterization of the SER causing organisms from mango fruits (cv. “*Karutha Colomban*”) in Sri Lanka, under both Molecular and Morphological aspects are comparatively few. Thus, the main objective of the current research was to isolate and identify the stem-end rot associated fungi (potential

pathogens) in *Karutha Colomban* mango fruits and characterize them using morphological and molecular parameters.

## MATERIALS AND METHODS

### *Isolation and Identification of Stem End Rot associated fungi*

*Sample collection:* Mango fruits (cv. “*Karutha Colomban*”) with visible symptoms of Stem-End Rot (SER) were collected from the local markets in *Kiribathgoda*, *Kelaniya*, *Kadawatha*, *Gampaha*, *Kurunegala*, *Kandy*, *Polonnaruwa*, *Vavuniya*, *Galle*, *Dambulla*, *Batticaloa* and *Trincomalee* in Sri Lanka, for the isolation of SER pathogens.

### *Isolation of fungi and identification using molecular characterization*

Diseased mango fruit tissues (4 mm<sup>2</sup>) were cut from margins of SER symptom areas under aseptic conditions. The tissues were surface sterilized by soaking the sections in freshly prepared NaOCl (3% w/v) for 3 minutes. After 3 serial washings in sterile distilled water, mango tissues (4 pieces per plate) were placed on sterile Potato Dextrose Agar (PDA), and incubated at room temperature (28 ± 2 °C). Resulted fungal cultures were transferred to fresh, sterile PDA plates in order to obtain pure cultures. The isolated fungi were identified using their morphological characteristics under the Phase Contrast Microscopy (×400 magnification) (Olympus CX41 model, Tokyo, Japan) using slide cultures, by comparing with an Atlas of pathogens and previous literature (Ploetz 2003; Abeywickrama, 2006). Identification of the isolated fungi was confirmed by molecular techniques, carried out at Genetech Institute, Sri Lanka and Macrogen Inc. Seoul, Republic of Korea.

### *Molecular Characteristics of SER*

The study was conducted for six isolates labelled; SER 01, SER 02, SER 03, SER 04, SER 05 and SER 06. The total genomic DNA

was extracted from fungal cultures using Wizard® Genomic DNA Purification Kit (Cat NO. A1120) following the given protocol. Extracted DNA was dissolved in 100 µl of Tris EDTA pH 8.0 buffer and stored at -20 °C for further analyses. Primer pairs *ITS* 1 and *ITS* 4 (White *et al.*, 1990) were used to PCR amplify internal transcribed spacer region (*ITS*) following the protocols outlined by Green *et al.*, (2004).

Amplified PCR products were visualized on agarose gel electrophoresis stained with ethidium bromide in 0.5 x TBE, using UV illuminator (E-gel imager, Life technologies Israel). PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Cat NO. A9281) according to the manufacturer's instructions.

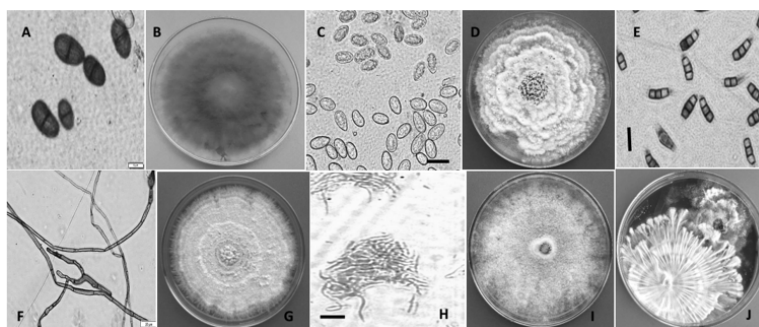
Purified PCR products were sequenced with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA, Cat no 4337455) on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA) using the same amplification primers for the gene regions (Macrogen Inc. Korea). The Sequences obtained were manually edited where necessary using BioEdit sequence Alignment Editor (Version 7.2.5). Sequences generated from this study were analyzed comparatively with other homologous sequences retrieved from GenBank. Sequences derived from this study were deposited in the GenBank (Table 01).

### Sequence alignment and phylogenetic analysis

Sequence homologies for the assembled consensus sequences were analyzed using the BLAST search engine available at the National Center for Biotechnology Information (NCBI) for the initial identification of fresh isolates, used in the analyses (Figure 01). Newly generated sequences were aligned using MUSCLE (Multiple Sequence Alignments) Alignment tool of MEGA sequence Alignment Editor (Version 7.0) along with 31 other reference taxa of *Lasiodiplodia*, *Phomopsis*, *Nodulisporium*, *Xylaria* and *Pestalotiopsis* species, obtained from the GenBank (Table 02). The alignments were visually checked and manually improved where necessary. Gaps were treated as missing data. Phylogenetic analysis using Maximum Likelihood (ML) trees were constructed using the MEGA software (Version 7.0). Kimura 2-parameter model with bootstrap values obtained for 1000 bootstrap replicates were applied following Kimura (1980). The best scoring tree was selected. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Trees were constructed in MEGA (Version 7.0) software.

**Table 01: Details of the locally collected stem-end rot disease associated fungal isolates and comparison with previously reported isolates.**

Sample code	Accession number (GENBANK)	Highest match with NCBI database	Location of previous isolate	Percentage similarity	Reference
Ser 1	MH005085.1	<i>Lasiodiplodia theobromae</i> (KY473044.1)	South Africa	100 %	Mehl <i>et al.</i> , 2017
Ser 2	MH005086.1	<i>Phomopsis sp.</i> (GQ352478.1)	Malaysia	99%	Sim <i>et al.</i> , 2010
Ser 3	MH005087.1	<i>Phomopsis sp.</i> (GQ352478.1)	Malaysia	99%	Sim <i>et al.</i> , 2010
Ser 4	MH005088.1	<i>Nodulisporium sp.</i> (AF201751.1)	USA	99%	Polishook <i>et al.</i> , 2001
Ser 5	MH005089.1	<i>Xylaria feejeensis</i> (KF619557.1)	Kenya	100%	Babajide <i>et al.</i> , 2015
Ser 6	MH005090.1	<i>Pestalotiopsis sp.</i> (KF746139.1)	USA	100%	Higginbotham <i>et al.</i> , 2014



**Figure 01:** Morphological variation of the isolates (B, D, G, I and J) and Phase contrast microscopic view of *Lasiodiplodia theobromae* conidia (A and C) and mycelia (F), conidia of *Pestalotiopsis* sp. (E) and conidia of *Phomopsis*. A scale of 20 µm is indicated by each bar.

**Table 02:** Details of reference taxa used for the phylogenetic analysis

Organism	GenBank Accession number (ITS)	Query cover
	MF480343.1	100%
	KU377460.1	100%
	KY473044.1	100%
<i>Lasiodiplodia iranensis</i>	KU997453.1	100%
	KP998515.1	100%
	KY583266.1	100%
	KU997383.1	100%
	KY284596.1	100%
	KU507476.1	100%
	KT164784.1	100%
<i>Phomopsis</i> sp.	KT953322.1	100%
<i>Phomopsis</i> sp.	GQ352478.1	100%
<i>Phomopsis</i> sp.	AF103000.1	100%
<i>Phomopsis</i> sp.	AB255260.1	100%
<i>Diaporthe</i> sp.	KY011895.1	100%
<i>Diaporthe</i> sp.	KY011896.1	100%
<i>Phomopsis liquidambari</i>	AY601919.1	100%
<i>Xylaria feejeensis</i>	KF619557.1	100%
<i>Xylaria feejeensis</i>	GU322453.1	100%
<i>Xylaria feejeensis</i>	GU322452.1	100%
<i>Xylaria</i> sp.	HQ435672.1	100%
<i>Nodulisporium</i> sp.	AF201749.1	99%
<i>Nodulisporium</i> sp.	AF201750.1	99%
Fungal endophyte	KR016438.1	100%
<i>Nodulisporium</i> sp.	AF201751.1	99%
<i>Nodulisporium</i> sp.	GQ334429.1	95%
<i>Pestalotiopsis theae</i>	JN943624.1	100%
<i>Pestalotiopsis theae</i>	JN651171.1	100%
<i>Pseudopestalotiopsis</i> sp.	KU252266.1	100%
<i>Pestalotiopsis</i> sp.	JF304634.1	100%
<i>Pestalotiopsis</i> sp.	KF746139.1	100%

From the BLASTn results, the organisms with  $\geq 95\%$  query cover percentages were selected as reference species in constructing the phylogenetic tree with the newly isolated organisms.

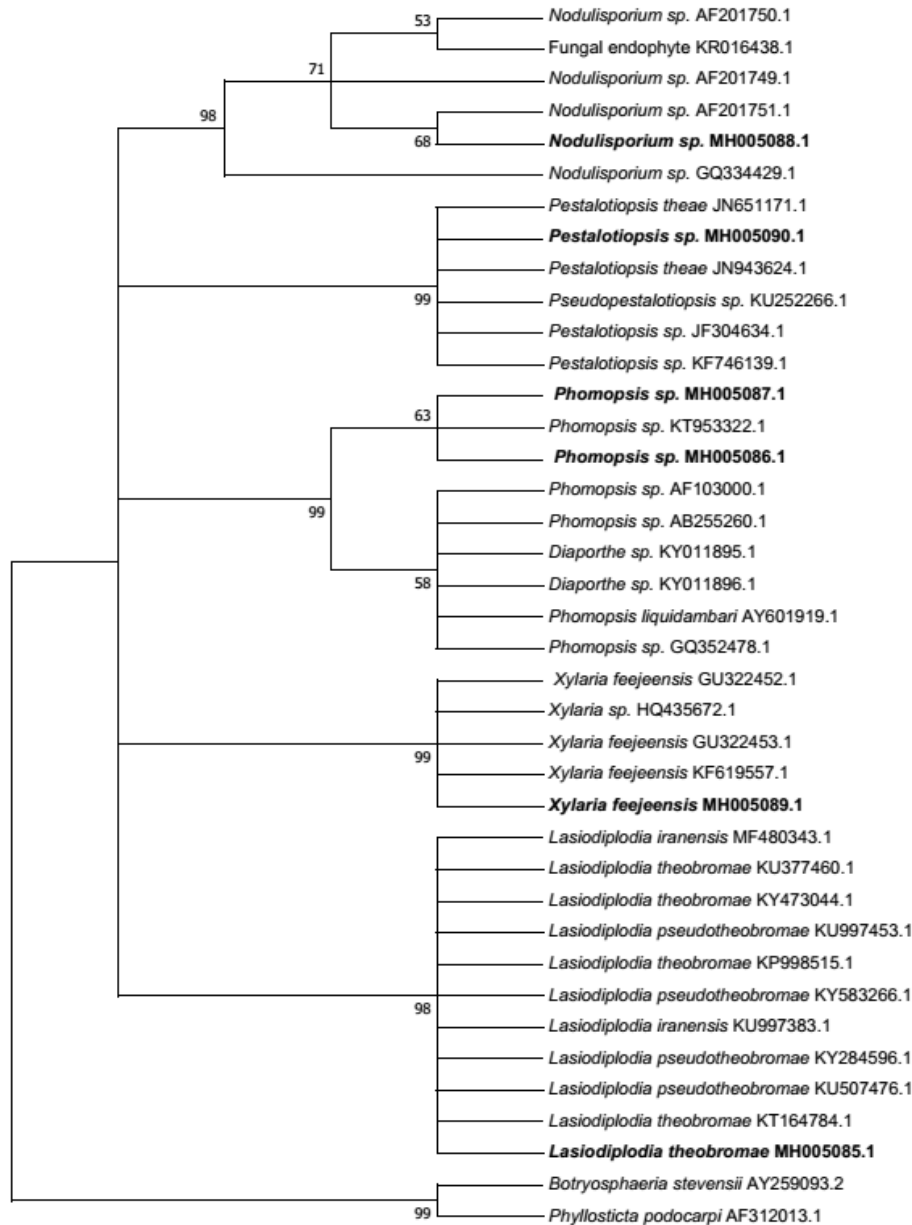


Figure 02: The phylogenetic tree inferred from likelihood analysis using *ITS* sequences. Newly derived *ITS* sequences of isolates MH005085.1, MH005086.1, MH005087.1, MH005088.1, MH005089.1 and MH005090.1 are in bold. Bootstrap values after 1000 replicates were expressed as percentages. *Botryosphaeria stevensii* and *Phyllosticta podocarpi* were used as outgroup taxa.

## RESULTS AND DISCUSSION

### *Isolation and Purification of the isolated fungi*

Six endophytic fungal strains were isolated and purified from diseased mango fruits, namely SER 01, SER 02, SER 03, SER 04, SER 05 and SER 06. Identification of the isolated fungal strains was performed using morphological characteristics and *ITS* sequencing.

### *Morphological characterization and identification of SER associated fungi*

SER 1 grew rapidly on PDA, producing light grey, cottony, mycelia, which turned dark grey within 2-3 days (Figure 02B). The hyphae were branched, septate and dark brown in color (Figure 02F). After about 20 days of maturity, black, flask shaped, pycnidia were produced on culture. Pycnidia contained simple, short conidiophores inside. Immature conidia borne

inside pycnidia were hyaline, but turned dark brown at maturity. Conidia are ovoid, two-celled and uni-septate. Conidia attained a size of  $22 \times 12 \mu\text{m}$  on maturity (Figures 02A and 02C).

SER 2 and SER 3 were similar in appearance and grew rapidly on PDA medium. Initially mycelia were white in color and turned greyish brown on maturity. Concentric rings were visible on the surface of mature culture (Figure 02G). Culture produced dark ostiolate pycnidia, which were immersed everywhere within the mycelium. Conidiophores were simple and hyaline. Two types of one-celled conidia were produced inside pycnidia; namely alpha conidia: relatively small in size, ovoid to fusoid in shape and beta conidia: curved, filiform, comparatively longer, which attained a size of  $12 \times 2 \mu\text{m}$  on maturity (Figure 02H).

SER 4 isolate was white in color when young, turned light gray with maturity on PDA. The reverse of the colony was yellowish brown. Center of the colony attained a dark brown color with time. The hyphae were, hyaline, branched and septate. No spore bearing structures or spores were observed under laboratory conditions, during the study period (Figure 02I). Whitish colonies with feather like appearance and with concentric rings on the PDA was observed in the SER 5 isolate. The reverse of the colony was whitish creamy color. Hyaline, branched and septate hyphae had no visible sporulation under laboratory conditions, during the period of study (Figure 02J). The isolated colonies of SER 6 strain contained creamy-white mycelia, which formed concentric rings gradually on the PDA on maturity. The center rings attained a yellow color with time. The reverse of the colony was creamy white in color (Figure 02D). They produced dark (black) acervuli. Conidiophores were simple and short and contained three to five dark conidia (2-3 septate), which were ellipsoid with two or more hyaline, apical appendages at maturity. These conidia contained hyaline pointed end cells and dark brown middle cells. Conidia attained a size of  $19 \times 6 \mu\text{m}$  on maturity (Figure 02E).

According to the comparison of colony morphology, sporulation, and phase contrast microscopic measurements and micrographs SER 1, SER 4, SER 5 and SER 6 were identified as *Lasiodiplodia theobromae*, *Nodulisporium sp.*, *Xylaria sp.* and *Pestalotiopsis sp.*, respectively. Further, both SER 2 and SER 3 were identified as *Phomopsis sp.*

### Phylogenetic analysis

Sequence analysis of the *ITS* regions showed a significant sequence similarity (100%) in SER 1 (492 bp), SER 5 (529 bp) and SER 6 (499 bp) with previously reported *Lasiodiplodia theobromae* (KY473044.1), *Xylaria feejeensis* (KF619557.1) and *Pestalotiopsis sp.* (KF746139.1), respectively. *ITS* sequences of SER 2 (521 bp), SER 3 (521 bp) and SER 4 (692 bp) showed a 99% sequence similarity with previously reported isolates of *Phomopsis sp.* (GQ352478.1) and *Nodulisporium sp.* (AF201751.1) respectively (Table 01.).

According to phylogenetic results, the isolate MH005085.1, which clustered with *Lasiodiplodia sp.* with a high bootstrap support of 98%, was identified as *Lasiodiplodia theobromae* (Figure 02). Isolates, MH005086.1 and MH005087.1 were identified as *Phomopsis sp.* HBS10, which clustered with other *Phomopsis sp.* with a bootstrap support of 99%.

MH005089.1, MH005088.1 and MH005090.1 isolates were clustered with known isolates of *Xylaria sp.*, *Nodulisporium sp.* and *Pestalotiopsis sp.*, respectively with bootstrap values ranging from 98% - 99%. It was identified that MH005085.1 is similar to the *Lasiodiplodia theobromae* (KT164784.1) from China (Figure 02), whereas, isolates MH005086.1 and MH005087.1 were similar to each other (Bootstrap support 63%) and similar to *Phomopsis sp.* (KT953322.1) from Malaysia with a 63% of Bootstrap support. Similarly, isolates of MH005089.1 and MH005088.1 clustered with *Xylaria feejeensis* (KF619557.1) from Kenya and *Nodulisporium sp.* (AF201751.1) from USA, respectively. Although BLASTn results revealed that the

isolate SER 6 (MH005090.1) is 100% identical with *Pestalotiopsis* sp. (KF746139.1) from USA (Table 01), with a bootstrap support of 99% the phylogenetic tree revealed that the isolate MH005090.1 was homologous to *Pestalotiopsis thea* (JN651171.1) from Italy, which is also a stem-end rot causative pathogen of mango fruits (Sandoval-sánchez *et al.*, 2013). Finally, considering all the morphological and molecular data, isolate MH005090.1 was confirmed as *Pestalotiopsis* sp.

*Lasiodiplodia theobromae*, *Phomopsis* sp. and *Pestalotiopsis* sp. have been frequently reported as stem-end rot pathogens of mango fruits in several previous studies (Johnson *et al.*, 1991; Adikaram *et al.*, 2010; Syed *et al.*, 2014). These fungal species are found to occur endophytically in the stem tissues (branches) of mango trees prior to inflorescence emergence. These fungi move in to flowers and subsequently to stem ends of fruits. They move in to healthy fruits after harvest and become active during fruit ripening to become pathogens. The symptoms therefore, appear during fruit ripening (Johnson *et al.*, 1991). Although the isolate of *Xylaria* species has not been recorded related to stem-end rot disease of mango, there is some evidence of *Xylaria feejeensis* strain being associated with dry rot of *Raphia hookeri* fruits (Esiegbuya, 2013). Similarly, *Nodulisporium* sp. has not been reported with any pathogenic activities relevant to postharvest fresh commodities. However, it has been reported that, this fungal strain identified as *Nodulisporium* spp. CMU-UPE34, has the ability to produce a volatile organic compound, which has the potential to act as a bio-fumigant in controlling postharvest diseases such as, green mold decay on *Citrus limon* caused by *Penicillium digitatum*, and blue mould decay of *Citrus aurantifolia* and *Citrus reticulata* caused by *Penicillium expansum* (Suwannarach *et al.*, 2013). A series of tests (*In-vivo*) are currently being carried out to investigate the pathogenicity of the isolated

fungal strains from the stem-end rot diseased “*Karutha Colomban*” mango fruits.

## CONCLUSION

Based on the morphological as well as molecular (*ITS1-5.8S-ITS4* sequence) characters, the associated fungal strains, MH005085.1, MH005086.1, MH005087.1 and MH005090.1 which were isolated from diseased *Karutha Colomban* mango fruits with SER (in Sri Lanka) were identified as *Lasiodiplodia theobromae*, *Phomopsis* sp., *Phomopsis* sp., and *Pestalotiopsis* sp. respectively. Two new fungal species (*Xylaria* spp. and *Nodulisporium* spp.) were also identified for the first time from rotting *Karutha Colomban* mango fruits. The pathogenicity of all SER associated fungi are being tested and will be published in the near future. Above research findings are invaluable to identify the SER disease associated fungal strains using their morphological and molecular features, before designing control strategies at post-harvest level in order to minimize the loss and extend the storage life of mango.

## Data Set policy

The datasets generated during and/or analyzed during the current study are available in the GenBank, and <https://www.ncbi.nlm.nih.gov/genbank/>

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