A Preliminary Checklist of Endomycorrhizal Fungi Associated with *Mauritia flexuosa* L.f. (Arecaceae-Calamoideae) in Trinidad, W.I.

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ABSTRACT

Endomycorrhizal fungi associated with the palm *Mauritia flexuosa* L. f. (Arecaceae-Calamoideae) were observed *in situ* and *ex situ* on the island of Trinidad, West Indies. Root endophytes varied between stage classes (seedling–adult) and amongst palms from different locations and individual species of endophyte were associated with more than one palm species. This suggests that palms with associated endophyte communities may have a greater chance of survival in stressful environments. Further studies on the relative abundance of endophytes and host specificity are warranted as different levels of endomycorrhizal associations and adaptations are expected.

Key words: Arborescent palms, Aripo Savanna Environmentally Sensitive Area, Endophytes, Fungus, Symbiosis.

INTRODUCTION

Mauritia flexuosa is one of the most common and widespread palms in the neotropics and plays a significant role in the economies of neotropical countries of South America (Virapongse *et al.* 2017). Trinidad represents the northern limit of the range of distribution for *M. flexuosa* (Comeau *et al.* 2003). Although these palms have been thoroughly studied (socioeconomic importance, livelihood, ecology, conservation, management, and sustainability (Virapongse *et al.* 2017), few records on root-endophyte associations with *M. flexuosa* can be found (Koolen *et al.* 2012, Koolen *et al.* 2013).

Endophytes or endophytic fungi are known to exist in every plant species found thus far on the planet (Arnold *et al.* 2001), however, there is not much information on their associations with individual plant species. Furthermore, little is known of endophyte associations in tropical trees (Asita *et al.* 2018, Schroeder *et al.* 2018), especially palms (Froehlich and Petrini 2000). At present, there is no checklist of endomycorrhizal fungi associated with *M. flexuosa*; leaf-endophytic fungi associations have been documented in palms (Rodrigues 1994, Froehlich and Petrini 2000, Arnold *et al.* 2001), but vaguely described for *M. flexuosa* (Delgado *et al.* 2007, Vasquez *et al.* 2008, Alvarez-Loayza *et al.* 2011).

Root endophytes represent a group of understudied microorganisms (Faeth and Fagan 2002) and can be defined as inconspicuous microbial organisms or endosymbionts adapted to mutualistic associations inside root tissues (Schulz and Boyle 2006, Kurissery *et al.* 2019). In many plant species, root endophytes are known to increase water and nutrient absorption (Kurissery *et al.* 2019), protect against plant pathogens (Faeth and Fagan 2002), and stimulate growth (Schulz and Boyle 2006). Results from this study will provide baseline information relevant to palm conservation and persistence in Trinidad, and by extension, its South American ecological range. This study records a preliminary checklist of endomycorrhizal fungi associated with *M. flexuosa* on the island of Trinidad.

METHODOLOGY

Study site and field collection

This study was conducted primarily in the savanna-forest ecotone landscapes of the Aripo Savanna Environmentally Sensitive Area (ASESA), located in Trinidad, (10°35′30′′ N, 61°12′0′′ W) altitude 45m (Fig.1). The ASESA ecosystem is a series of open treeless areas of grass and sedge marshland within an extensive area of forest. *M. flexuosa* palms are concentrated along the periphery of the savannas and are most abundant along the fire-impacted margins.

All palms sampled were placed in stage classes following Arneaud *et al.* (2017); new seedling (<0.25m), established seedling (0.25–0.5m), older seedling (0.5-1.0m, young juvenile (1–3m), juvenile-1 (3–8m), young adult (8–12m), adult-1 (12–22m), and adult-2 (>22m).

In October 2012, approximately 15 'new seedlings' were taken from the ASESA (Savannas 1 and 3) and transplanted at each of Talparo (along a gully), Tunapuna (in pots), and in a greenhouse in the Department of Life Sciences at the University of the West Indies in St. Augustine (also in pots). From these, only two palms survived in Talparo; the others died either within 15 months or upon reaching 1 metre.

In November 2014, a total of 30 established seedlings were collected from the ASESA (Savannas 1 and 3). From these, 15 were planted adjacent to the new wing of the Life Sciences Building, UWI (impeded soil), and 15 were planted in Tunapuna (well-drained soil). On this occasion, only one seedling survived at the UWI; in Tunapuna all died. In January 2018, several fallen fruit and seeds taken from the ASESA were planted (in a cluster) at the study

site in Tunapuna.

Between September 2017 and April 2018, root cross-sections (taken from aerial and underground roots) from the palms at each location; ranging from seedlings to adults (Table 1, Fig. 1) were observed under a microscope for fungal endomycorrhizal associations. In August 2018, more roots were collected from these palms and further investigated for endomycorrhizal associations. On this occasion, in addition to root samples, fruit samples were collected and cross-sections made, to investigate differences amongst fungal endomycorrhizal species.

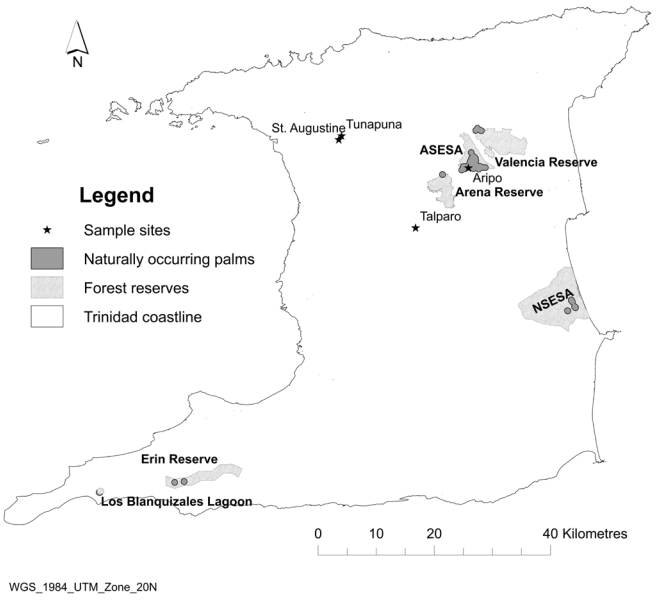

Fig. 1. Locations of naturally and non-naturally occurring *M. flexuosa* palms in Trinidad. ASESA, Aripo Savanna Environmentally Sensitive Area; NSESA, Nariva Swamp Environmentally Sensitive Area.
*Note. *M. flexuosa* palms do not occur in Tobago.

Extraction process

Collected fruit and root samples from the field were placed in sealed bags containing sterile water and immediately transported to the Biotechnology Laboratory (Department of Life Sciences at the UWI, St. Augustine). In the laboratory, samples were surface-sterilised by washing with detergent for one minute, followed by a 30-second rinse using tap water. Samples were then soaked in 95% alcohol for one minute and rinsed with distilled water. Immediately after, samples were placed on sterile potato dextrose agar plates containing rose bengal (50 µg/ml) and streptomycin (100 µg/ml; a broad spectrum aminoglycoside antibiotic) according to the Varma (2012) mycorrhizosphere microorganism screening methodology. Plates were then sealed and incubated at 30°C for 24 hours. Subsequently, samples were wrapped in Ziploc bags, and shipped to Plantwise Diagnostic and Advisory Service, Centre for Agriculture and Bioscience International (CABI) UK for identification. There, culture purity checks were performed, and mixed cultures were subcultured on diagnostic media prior to morphological and molecular analysis.

Morphological and molecular analysis of fungal isolates

For morphological analysis, the colony morphology of the cultures was examined by observing sporulating structures at x400 magnification. Molecular analyses were performed on each sample using nucleic acid as a template. A proprietary formulation (microLYSIS®-PLUS [MLP], Microzone, UK) was subjected to rapid heating and cooling of a thermal cycler to lyse cells and release DNA. Following DNA extraction, polymerase chain reaction (PCR) was employed to amplify copies of the rDNA in vitro. The quality of the PCR product was assessed by gel electrophoresis. The PCR purification step removed unutilised deoxyribonucleotide triphosphated

Table 1. Collection of *M. flexuosa* fruit and root samples in Trinidad.

Location	Stage class	N	Samples collected[1]	Yr. palms transplanted[2]	No. of palms transplanted	No. of palms surviving
ASESA	Seed	3	$1F_y$, $1F_m$, $1F_d$	—	—	—
ASESA	Juvenile 1	2	$2R_a$, $1R_u$	—	—	—
ASESA	Young adult	1	$1R_a$, $2R_u$	—	—	—
ASESA	Adult 2	2	$2R_a$, $2R_u$	—	—	—
St. Augustine	Older seedling	1	$2R_u$	2014	15	1
Talparo	Young juvenile	1	$2R_a$, $1R_u$	2012	10	2
Tunapuna	New seedling	3	$3R_u$	2018	15	3

1. F_y, Fruit (*young*); F_m, Fruit (*mature*); F_d, Fruit (*degraded*); R_a, Root (*aerial*); R_u, Root (*underground*).
2. transplanted from the Aripo Savanna Environmentally Sensitive Area (ASESA).
Note: R_a samples were collected at -5 to 5cm soil depth, whereas R_u samples were collected at 15 to 20cm soil depth.

(NTPs), primers, polymerase, and other PCR mixture compounds to obtain highly purified DNA templates for sequencing. This procedure also allowed segments of DNA and RNA to be amplified. PCR and sequencing reactions were undertaking using the BigDye® Terminator v3.1 kit from Applied Biosystems. In total three PCRs primer pairs were utilised. ITS sequencing used TW81 and AB28 primer pairs; TEF1f and TEF1r primer pairs were used for partial TEF sequencing; and for the bacterium colony, 16S sequencing was conducted using 27f and 534r primer pairs. The amplicons generated in this study were 449 – 529 bp in length.

All identifications were based on comparisons with the European Molecular Biology Laboratory database via

Table 2. Endomycorrhizal fungi associated with *Mauritia flexuosa* in Trinidad.

Family/genus/species [Stage] and Location	Identification and description	Reference
(TRICHOCOMACEAE) ***Penicillium Lanata-Divaricata*** [Juvenile 1] ASESA	Using FASTA and BLAST together with CABI fungal database, the ITS sequence showed top matches at >98% identity to members of *Penicillium* section *Lanata-Divaricata* including 100% identity to sequence GU981580 from the type strain of *P. brefeldianum* (CBS 235.81) and 99.4% identity to sequence GU981568 from the type strain of *P. limosum*. Type strains from other members of this group gave slightly lower matches e.g. 98% identity to sequence GU981585 from the type strain of *P. janithellum* (CBS 340.48). From these results, *P. brefeldianum* was the most likely identity for the sample.	Type strains sequences have been published in Diao *et al.* (2018)
(HYPOCREACEAE) ***Trichoderma*** section ***Pachybasium*** clade ***Harzianum*** [Adult 2] ASESA	Internal transcribed spacer results gave >99% matches to many sequences to this section of the *Trichoderma* genus and predominantly to strains of *T. harzianum* DAOM 222136 [JN942884] and closely related species, including 99.6% to *Hypocrea lixii*. The ISTH TrichoKey online tool did not recognise this sequence. The partial TEF sequence gave >99% matches to several sequences assigned to members of the *T. harzianum* clade, including 99.0% match to *T. guizhouense* strain GJS 06-100 (FJ463289). It was not possible to determine a definitive species-level identification for this isolate.	*T. harzianum* strain DAOM 222136 [JN942884] has been cited by Schoch *et al.* (2012), whereas, *T. guizhouense* strain GJS 06-100 (FJ463289) has been cited by Błaszczyk *et al.* (2016).
(HYPOCREACEAE) ***Trichoderma*** [Young juvenile] Talparo	Top matches of >99% were seen to sequences assigned to members of this genus, including 99.4% to *T. gamsii* strain DAOM 231637 (EU280129). The ISTH online identification tool identified this as an 'unidentified Trichoderma species', and as such, it was not possible to determine a definitive species-level identification for this isolate.	*T. gamsii* strain DAOM 231637 (EU280129) has been cited by Hoyos-Carvajal *et al.* (2009).
(NECTRIACEAE) ***Acremonium*** and ***Pleiocarpon*** [Older seedling] St. Augustine	Internal transcribed spacer produced top matches of >95% to members of the family Nectriaceae including members of the genera *Acremonium* and *Pleiocarpon*. Several sequences of unspecified *Acremonium* strains featured in the top results with matches of >98% identity and unspecified Nectriaceae gave matches of 96.4% and 96.6% identity. However, the top match to a fully identified species was only at 95.8% identity to sequence HQ897806 from a reference culture collection strain of *A. macroclavatum* (CBS 123922). A similar match was obtained to 17 strains of *P. strelitziae* e.g. sequence KY304661 and sequence KY304661. However, there was no clear distinction between genera, therefore identification was given to family level.	*A. macroclavatum* strain (CBS 123922) was published by Gräfenhan *et al.* (2011). Seventeen published strains of *P. strelitziae* e.g. sequence KY304661 and sequence KY304661 were published by Aiello *et al.* (2017).
(PLEOSPORACEAE) ***Epicoccum nigrum*** [New seedling] Tunapuna	Internal transcribed spacer sequence obtained from this sample showed top matches at 100% identity to multiple sequences of *E. nigrum*. Best matches included the sequence AJ279448 from reference collection strain CBS 318.83.	*E. nigrum* sequence AJ279448 from reference collection strain CBS 318.83 was published by Shrestha *et al.* (2011).

ASESA, Aripo Savanna Environmentally Sensitive Area; FASTA, Fast-All; BLAST, Basic Local Alignment Search Tool; NCBI, National Centre for Biotechnology Information; ITS, Internal Transcribed Spacer; ISTH, International Subcommission on Trichoderma and Hypocrea.

Table 3. Other endophytes associated with *Mauritia flexuosa* in Trinidad.

FAMILY/genus [Stage] & Location	Identification and description	Reference
NECTRIACEAE ***Gliocephalotrichum*** [Seed] ASESA	Over 98% identity matches (via FASTA, BLAST and NCBI) to multiple sequences from members of the genus *Gliocephalotrichum* including *G. bacillisporum*, *G. longibrachium*, *G. cylindrosporum*, *G. mexicanum*, *G. nephelii*, *G. queenslandicum* and others. The ITS sequence (using BLAST and NCBI) showed top matches at 99% identity to the type strains of *G. longibrachium* (sequence NR 136977) *G. mexicanum* (sequence KF513289) and *G. queenslandicum*. The type strains of *G. cylindrosporum*, *G. simmonsii* and G. *bulbilium* gave slightly lower matches at 98% identity. As there was no clear distinction between species from the ITS sequence result, identification was given to genus level only.	The type strains sequences have been published in Lombard *et al.* (2014).
BURKHOLDERIACEAE ****Burkholderia*** [Young adult] ASESA	Over 99% identity matches (via FASTA, BLAST, NCBI and other database) were made to several species belonging the genus *Burkholderia*, and included the validated type strain sequences of *B. lata* [CP000150 – complete genome study], and the validated type strain of *B. multivorans* [Y18703] which both gave matches of 100%. Sequences derived from type material of *B. cepacia* [ATCC 25416] and *B. vietnamiensis* [LMG 10929] also gave matches of 100% and 99.8% respectively.	Type strains sequences have been published in (Vanlaere *et al.* 2009).

ASESA, Aripo Savanna Environmentally Sensitive Area; FASTA, Fast-All; BLAST, Basic Local Alignment Search Tool; NCBI, National Centre for Biotechnology Information; ISTH, International Subcommission on Trichoderma and Hypocrea.

*soil borne fungi associated with post-harvest spoilage

** rhizobacteria

FASTA (Fast-All) algorithm, European Bioinformatics Institute, National Centre for Biotechnology Information (NCBI), Centre for Agriculture and Bioscience International Fungal Reference Collection - International Mycological Institute (CABI - IMI), Basic Local Alignment Search Tool (BLAST) algorithms, other databases limited to sequences from type material, peer-reviewed published taxonomic descriptions, and matches to sequences published in peer-reviewed literature.

DISCUSSION

The results illustrate important symbiotic relationships between *M. flexuosa* and the following identified endophytes: *A. macroclavatum* (St. Augustine), *P. brefeldianum*, *T. guizhouense* and *T. harzianum* (Aripo Savannas), and *E. nigrum* (Tunapuna). *Trichoderma gamsii* and an unknown species (*Trichoderma* sp.) were also identified in a juvenile palm planted ex situ in Talparo. This suggests that further work should be done to establish the precise relationship between symbiotic microorganisms and *M. flexuosa* palms.

Although we treated samples with rose bengal and streptomycin to kill bacteria, there was still a high concentration of endomycorrhizal bacteria existing in the samples from the ASESA (Savanna 3). This may indicate that *Burkholderia* species exist within the ASESA at high densities in soil and root nodules; may be resistant to streptomycin (Rhodes and Schweizer 2016); or may be insensitive to sodium (Ahn *et al.* 2016). The genus *Burkholderia* includes plant growth rhizobacteria (*B. vietnamiensis*) and pathogens (*B. cepacia*, *B. multivorans* and *B. lata*) capable of both plant and animal diseases (Kirzinger *et al.* 2011).

Different endomycorrhizal fungi were found on fruit and seeds in the ASESA. New seedlings grown in Tunapuna (using seeds from the ASESA) did not share similar endophytes to the other samples. This suggests that endophyte variability within the different stage classes of *M. flexuosa* is high. *Epicoccum nigrum* (found in new seedlings in Tunapuna) is a ubiquitous endomycorrhizal fungus that is known to act as a control for pathogens in sugarcane (Shrestha *et al.* 2011), whereas *Pleiocarpon strelitziae* (found in St. Augustine) is a species recently known for causing basal stem rot (Aiello *et al.* 2017). Seedlings in St. Augustine and Tunapuna are likely to die, since they do not possess any plant-growth stimulating endophytes such as *Trichoderma gamsii* (found in young juvenile palms in Talparo). We speculate that palms with high mycorrhizal fungi and bacteria levels have a greater chance of surviving under stressful conditions. Endophytes, such as *T. guizhouense* and *T. harzianum,* are commercially

used to biologically control plant pathogens (Chaverri and Samuels 2002), whereas *B. vietnamiensis* is known to regulate nitrogen, promote growth, and enhance yields (Van Dommelen and Vanderleyden 2007).

Further studies are required to identify and investigate beneficial endophytes in *M. flexuosa* growing *ex situ*. The identification of host-specific endomycorrhizal associations will have the potential for allowing *M. flexuosa* to be planted closer to indigenous villages that depend on these palms for their livelihoods (Penn 1999, Horn *et al.* 2012.)

CONCLUSION

This study provides a preliminary checklist of endomycorrhizal fungi associated with *M. flexuosa* palms in Trinidad. It also records variability in endophyte species amongst study sites and within the different stage classes of the studied palm.

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