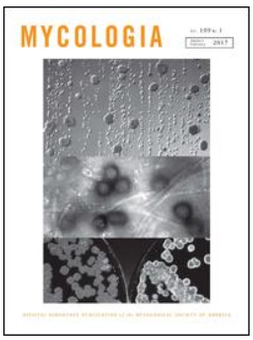

Mycologia

ISSN: 0027-5514 (Print) 1557-2536 (Online) Journal homepage: https://www.tandfonline.com/loi/umyc20

Serenomyces associated with palms in southeastern USA: isolation, culture storage and genetic variation

M.L. Elliott & E.A. Des Jardin

To cite this article: M.L. Elliott & E.A. Des Jardin (2014) *Serenomyces* associated with palms in southeastern USA: isolation, culture storage and genetic variation, Mycologia, 106:4, 698-707, DOI: 10.3852/13-353

To link to this article: https://doi.org/10.3852/13-353

Published online: 20 Jan 2017.

Mycologia, 106(4), 2014, pp. 698–707. DOI: 10.3852/13-353

Serenomyces associated with palms in southeastern USA: isolation, culture storage and genetic variation

M.L. Elliott[1]
E.A. Des Jardin
University of Florida – IFAS, Department of Plant Pathology, Fort Lauderdale Research and Education Center, 3205 College Avenue, Florida 33314

Abstract: *Serenomyces* is a genus belonging to the family Phaeochoraceae, which is known to occur only in association with the plant family Arecaceae (palms). It is presumed to be one of the causes of a leaf disease referred to as either rachis blight or petiole blight, depending on the palm species affected. The fungus is not readily observed, with few reports in the literature; it cannot be readily isolated from tissue, with only one known instance of it being cultured on artificial media and has no DNA sequences deposited in GenBank. Over an 8 y period, leaves symptomatic for rachis or petiole blight were obtained from Florida and South Carolina, USA. The fungus was induced to produce ascospores, and single-spore isolates were obtained in culture and, in some instances, induced to produce ascospores in culture. Based on ascospores size and ITS sequencing, *Serenomyces* from *Phoenix canariensis* and *P. dactylifera* form one group, *Serenomyces* from *Thrinax radiata* form a second group and *Serenomyces* from *Sabal palmetto* form a third. All three groups are most similar morphologically to *Serenomyces phoenicis*. Due to the observed instability of *Serenomyces* in culture, we have suggestions regarding the storage of this fungus.

Key words: Arecaceae, Phaeochoraceae, *Phoenix canariensis*, *P. dactylifera*, phylogeny, *Sabal palmetto*, *Thrinax radiata*

Introduction

Serenomyces Petr. was described first from a dead palm leaf (*Serenoa repens* = *Serenoa serrulata*) collected in Winter Park, Florida, (USA) in 1942 (Petrak 1952). It is one of four fungal genera belonging to the family Phaeochoraceae in the order Phyllachorales (Hyde et al. 1997, Hyde and Cannon 1999). The other genera include *Cocoicola*, *Phaeochora* and *Phaeochoropsis*. This relatively new Ascomycota family in class Sordariomycetes (Lumbsch and Huhndorf 2007) is known to occur only in association with the plant family Arecaceae (= Palmae) (Hyde et al. 1997, Hyde and Cannon 1999), a highly diverse group of arborescent monocotyledons primarily distributed across the tropics and subtropics (Dransfield et al. 2008). While members of this plant family contain economically important crops (e.g. *Cocos nucifera*, *Phoenix dactylifera*, *Elaeis guineensis*), palms also are highly prized and used as ornamental plants, especially in landscapes in the southern continental USA, tropical climates, Mediterranean climates and in botanical garden glasshouses (Riffles and Craft 2003).

Serenomyces is also a member of the ''tar spot'' group of fungi. This is an artificial grouping, with the biologically common feature that all produce fruiting bodies (stromata) with significant blackening of the surface layers due to melanin deposition (Barr et al. 1989, Barr et al. 1997, Hyde and Cannon 1999). While some tar spot fungi invade the palm leaf blade, others, including *Serenomyces*, invade only the petiole or rachis of the palm leaf (Hyde and Cannon 1999, Simone 2004). Hence the common palm disease names rachis blight or petiole blight.

Initial symptoms include chlorosis and necrosis of leaflets on one side of the rachis of a pinnate leaf palm or chlorosis and necrosis of some leaflet segments within the blade of a palmate or costapalmate leaf palm. The leaflet symptoms are accompanied by a reddish brown or brown stripe on one side of the petiole and rachis and internal discoloration of the same petiole and rachis (Simone 2004). Eventually the leaf dies. For unknown reasons, these symptoms are observed only on the oldest leaves in the palm canopy. While *Serenomyces* does not appear to cause a lethal disease of palms, this fungus can cause premature death of numerous older leaves, temporarily reducing the canopy. Complicating field diagnosis, the symptoms described above appear to be caused by a number of fungi (e.g. *Macrophoma*, *Phomopsis*, *Neodeightonia*) (Alfieri et al. 1994, Simone 2004). These are also the same symptoms of Fusarium wilt caused by formae speciales of *Fusarium oxysporum*, lethal pathogens of certain ornamental species of palms (Summerell et al. 2001, Elliott et al. 2010). Thus, confirmation of the pathogen associated with these general symptoms requires either isolation of the pathogen or examination of fruiting bodies and ascospores associated with the palm tissue. No anamorphic state has ever been observed for *Serenomyces* (Hyde and Cannon 1999).

Submitted 26 Oct 2013; accepted for publication 24 Jan 2014.
[1] Corresponding author. E-mail: melliott@ufl.edu

Because *Serenomyces* is not directly culturable from palm tissue, it could be easily missed if only culture-based isolation techniques are used in the diagnostic laboratory. Even if a moisture chamber is used to try to induce development of perithecia and ascospores, *Serenomyces* can be quickly overgrown by other fungi associated with the palm tissue and perhaps inhibited from developing fertile perithecia. This might explain why reports of *Serenomyces* in the literature are limited. To date, the fungus has been reported only on *Copernicia* × *burretiana*, *Latania loddigesii*, *Mauritia flexuosa*, *Phoenix dactylifera*, *P. canariensis*, *Sabal palmetto* and *Serenoa repens*, with *Phoenix* spp. the most commonly listed host (Dixon and Coile 1995, 1998, 1999; Hyde and Cannon 1999; Elliott and Des Jardin 2006; Farr and Rossman n.d.). Of the four known *Serenomyces* spp., *S. phoenicis* is the only one with more than one or two reported observations or specimens (Hyde and Cannon 1999). Even in the survey conducted by Fröhlich and Hyde (2000), which examined the microfungi associated with dead palm leaves collected from six palm genera in four tropical countries, *Serenomyces* was not observed. Thus, little is known about this presumed biotrophic fungal genus.

In 2005–2012 our laboratory received or collected palm leaf samples with the symptoms described above from 11 locations. Three of these samples have been described (Elliott and Des Jardin 2006). While initially unable to obtain single-spore cultures of *Serenomyces*, we ultimately were successful in obtaining such cultures and, in some cases, inducing ascospore production on artificial media. With the culturing of *Serenomyces* from these samples, we also began DNA amplification and sequencing of the internal transcribed spacer region (ITS) of the nuclear rDNA genes (White et al. 1990) to ensure the presumed single-spore isolates were derived from the *Serenomyces* ascospores present on the palm tissue.

Our goals with this study were: (i) to obtain single-spore isolates of *Serenomyces* derived from ascospores produced in palm tissue, (ii) to compare *Serenomyces* isolates from different palm hosts and geographic locations, using spore morphology and molecular techniques with ascospores produced in palm tissue and in culture media from single-spore isolates and (iii) to develop a protocol for storage of this genus, including single-spore isolates.

MATERIALS AND METHODS

Palm samples and fungal morphological characteristics.—Information regarding each sample or isolate is provided (TABLE I). Infected palm petiole or rachis pieces were placed in Riker mounts with the appropriate annotation and are available from the US National Fungus Collections (Beltsville, Maryland). If the surface of the petiole or rachis tissue was not already disrupted by the fungus, or ascospores were not already present, an approximately 10 cm section of petiole or rachis was washed under tap water, dried and wiped thoroughly with an alcohol-saturated tissue paper. It then was placed in a plastic container with a paper towel saturated with sterile deionized water. After sealing, the container was left at room temperature (~ 25 C) with room lighting. The palm tissue was monitored for fungal disruptions of the tissue surface and for ascospores and ascomatal necks associated with the asci. The time for this to occur ranged from a few days to as long as 3 wk. Spores (25 or 50 per sample) were examined under a microscope to measure length and width; necks also were measured for length.

When spores were observed on the palm tissue, they were collected and diluted in sterile deionized water and spread on 1.5% water agar (WA). These plates were incubated in the dark at 28 C and examined periodically for germination. Germinated spores were transferred to potato dextrose agar (PDA) (Difco). A preliminary study demonstrated that perithecia were produced only when the cultures were grown with light and that mycelial growth was about equal on PDA, V-8 agar (low sodium), V-8 agar supplemented with 20 g dextrose or an agar medium containing yeast extract (5 g), malt extract (5 g), peptone (5 g) and dextrose (20 g) (all from Difco). Thereafter, PDA plates with single-spore isolates were placed under lights (mix of 20 watt cool white fluorescent bulbs and 20 watt Verilux natural spectrum fluorescent bulbs) for 12 h each day at 26 C. It took 4–8 wk for viable ascomata to develop on PDA, if they developed at all. Ascospores produced (25 or 50 per isolate) were examined under a microscope to measure length and width; necks also were measured for length.

Storage methods and recovery from storage.—Two single-spore isolates were selected for each leaf sample, except for samples from *Copernicia* and *Latania*; spores collected from these two palms did not germinate. Mycelial growth from colonized PDA was stored using three methods. First, 4 mm agar plugs (approx.. 15) were stored in 1.5 mL sterile deionized water in a 2 mL cryogenic vial (Corning), which was sealed with Parafilm™ and stored in the dark at room temperature. Second, a single agar plug, obtained with a sterile glass Pasteur pipet (1 mm diam), was transferred to a one-fifth-strength PDA slant, sealed with Parafilm™ and, after some growth was evident, stored in the dark at 28 C. Third, three agar plugs, obtained with a sterile Pasteur pipet, were placed in 1.5 mL potato dextrose broth in a 2 mL cryogenic vial and incubated at 28 C for 2–4 wk before sealing with Parafilm™ and storing in the dark at room temperature.

In Oct 2012, each *Serenomyces* isolate except for PLM-657 and PLM-658, which had just been stored, was transferred from storage to determine which of these three methods was best for storage. The cultures had been stored 1–5 y. For each storage method, three pieces of fungal tissue were transferred to one-fifth PDA and incubated at 28 C in the dark for at least 4 wk. Recovery from storage was repeated in Feb 2013.

TABLE I. The palm host, sample location and year sample was obtained from the 11 leaf samples, along with length and width of ascospores from palm tissue and from single-spore isolates and GenBank accession numbers for ITS DNA sequences

Palm host	Location[a]	Date	U.S. National Fungus Collections No.	Palm tissue or isolates	Length ± SE[b] (μm)	Width ± SE[b] (μm)	GenBank accession No.[c]
Phoenix canariensis	Miami-Dade	2006	BPI 892703	Rachis	12.7 ± 0.1 fgh	7.4 ± 0.1 a	KF361443
				PLM-341S	12.7 ± 0.2 fgh	6.1 ± 0.1 efg	KF361445
				PLM-342S	13.0 ± 0.2 ef	6.6 ± 0.1 cd	KF361447
Phoenix canariensis	Orange	2011	BPI 892710	Rachis	12.4 ± 0.1 hi	6.0 ± 0.0 fg	KF361448
				PLM-594S	12.6 ± 0.1 gh	6.0 ± 0.0 fg	KF361450
				PLM-595S	12.9 ± 0.1 fg	6.1 ± 0.0 efg	KF361452
Phoenix canariensis	Palm Beach	2007	BPI 892708	Rachis	13.4 ± 0.2 de	6.3 ± 0.1 e	KF361463
				PLM-314S	12.0 ± 0.2 j	6.0 ± 0.1 fg	KF361465
				PLM-315S	12.6 ± 0.1 fgh	6.1 ± 0.1 efg	KF361467
Phoenix canariensis	Polk	2008	BPI 892707	Rachis	12.9 ± 0.2 fg	6.5 ± 0.1 cd	KF361453
				PLM-345S	13.5 ± 0.2 d	7.0 ± 0.1 b	KF361455
				PLM-347S	12.2 ± 0.2 ij	6.0 ± 0.0 g	KF361457
Phoenix dactylifera	Hillsborough	2012	BPI 892709	Rachis	13.5 ± 0.1 d	6.7 ± 0.1 c	KF361458
				PLM-657S	14.7 ± 0.2 b	7.4 ± 0.1 a	KF361460
				PLM-658S	14.0 ± 0.1 c	7.4 ± 0.1 a	KF361462
Copernicia × *burretiana*	Miami-Dade	2005	BPI 892702	Petiole	13.3 ± 0.2 de	6.3 ± 0.1 e	
Latania loddigesii	Pinellas	2005	BPI 892711	Petiole	13.4 ± 0.1 de	7.1 ± 0.1 b	KF361474
Thrinax radiata	Lee	2007	BPI 892705	Petiole	16.2 ± 0.1 a	6.5 ± 0.1 d	KF361468
				PLM-339S	16.6 ± 0.2 a	6.7 ± 0.1 cd	KF361470
				PLM-340S	16.3 ± 0.1 a	6.2 ± 0.1 ef	KF361472
Sabal palmetto	Manatee	2008	BPI 892706	Petiole	14.4 ± 0.2 bc	5.4 ± 0.1 i	KC898536
Sabal palmetto	Martin	2008	BPI 892704	Petiole	14.3 ± 0.1 bc	5.7 ± 0.1 h	KC898539
Sabal palmetto	South Carolina	2010	BPI 892712	Petiole	14.4 ± 0.1 bc	5.4 ± 0.1 i	KC898542
F Value					57.79	65.56	
P Value					<0.0001	<0.0001	

[a] Location is county within Florida (USA), unless otherwise stated.

[b] Data are the mean of 25 or 50 ascospores per sample or isolate. Means followed by the same letter within a column are not significantly different by the Waller-Duncan k-ratio method, k = 100, $P = 0.05$.

[c] These accessions represent sequences obtained using DNA from ascospores. More GenBank accessions were deposited, which represent ITS sequences obtained from mycelia cultures for all the isolates listed and for PLM-399M and PLM-400M (*S. palmetto*, Manatee County) and for PLM-403M and PLM-404M (*S. palmetto*, Martin County).

A fourth storage method was designed for archival storage of pure cultures (no palm tissue) producing perithecia with ascospores; it is a modification of the technique suggested by the U.S. National Fungus Collections (http://www.ars.usda.gov/Services/docs.htm?docid=9405). A single 7 mm agar plug was placed on a fresh PDA plate, sealed with Parafilm™ and grown at 26 C with 12 h light each day. After perithecia with ascospores were evident on the plate, small sections (25 × 30 mm) agar with perithecia were cut from the plate and glued to the bottom half of a chipboard slide mailer with Elmer's® Glue-All Multi-Purpose glue (non-toxic). The mailer section was placed in a rigid cardboard slide pillbox (tray interior 7.3 × 4.8 × 2.9 cm; Ted Pellas Inc.), left slightly open and placed in a desiccator. Once the agar had dehydrated, the box was closed and stored in a desiccator at room temperature.

Hyphal growth rate as affected by temperature.—An experiment was conducted with isolates PLM-314, PLM-340 and PLM-594 to determine effect of temperature on growth rate. Colonized mycelial agar plugs were placed in the center of fresh PDA plates, with four replicate plates per isolate per temperature. Plates were placed at 15, 20, 25, 30 and 35 C. Colony diameter was measured at 7, 14 and 21 d. The experiment was conducted twice.

Herbarium material for DNA amplification.—In 2007, these specimens were obtained from the U.S. National Fungal Collections (Beltsville, Maryland): *Serenomyces phoenicis* (BPI 579851, BPI 579852, BPI 579853, BPI 579854: collected in Lahore, Pakistan, 1970 on *P. dactylifera*), *S. mauritiae* (labeled *S. shearii*; BPI 579855: collected in 1942 in Florida, USA, on *Serenoa serrula*, renamed *S. repens*) and *S. virginiae* (BPI 749366: collected in 1995 in California, USA, on *P. dactylifera)*). All specimens were palm rachis with disruptions to the surface due to presumed *Serenomyces* infection. BPI 579852 was not used for DNA amplification because an annotation indicated that *Leposphaerella phoenicis* also was present on the specimen. BPI 579854 and BPI 579853 did have what appeared to be a dry mass of spores on the exterior surface of the rachis tissue, but none of the other specimens did. For DNA amplification (described below), spores were collected and placed in 300 µL sterile, ultrapure water in a 1.5 mL microfuge tube. If spores were not present, areas showing evidence of palm tissue disruption by the fungus due to development of fruiting bodies were collected and also placed in water. Samples were boiled 5 min (Elliott et al. 1993) to release DNA. This material was used as the DNA template for PCR.

DNA amplification and sequencing.—The methods used for DNA extraction and the polymerase chain reaction (PCR) assay varied slightly during 8 y of this study. Fresh ascospores from palm tissue or cultures were either collected in sterile, ultrapure water, boiled 5 min and the material used for PCR, or ascospores were collected in cell lysis solution for DNA extraction (Gentra PureGene cell kit, QIAGEN) and the DNA used for PCR. Single-spore-derived mycelial cultures were grown in 4 mL potato dextrose broth, without shaking, 6–10 d at 25 C and then DNA extracted with Gentra PureGene cell kit for PCR.

DNA was amplified with the ITS1 and ITS4 primers to target the ITS1-5.8S-ITS2 region (White et al. 1990), with an annealing temperature of 52 C for 35 cycles, to amplify the two noncoding regions (internal transcribed spacer regions 1 and 2) and 5.8S rDNA gene located between the 18S and 28S rDNA genes. Aliquots (5 µL) of PCR products were evaluated by electrophoresis. PCR products were purified on spin columns (Wizard PCR Preps DNA Purification System, Promega Corp.) and sequenced directly with primers ITS1 and ITS4. Sequencing was performed at the University of Florida's Core Sequencing Service Laboratory, Gainesville.

Sequences were edited and queried for similarity against nucleotide sequences in the GenBank database with the BLASTn tool. Sequences from all but the herbarium material were then aligned with Clustal W (Thompson et al. 1994), with sequences from *Phoenix* aligned first, then adding *Sabal* sequences, followed by *Thrinax* sequences and then *Latania* sequences. A phylogenetic tree was constructed with maximum parsimony (1000 bootstrap replicates) using MEGA 4 software (Baldauf 2003, Kolaczkowski and Thorton 2004, Tamura et al. 2007). Three fungal species representing each subclass within the Sordariomycetes were used to root the tree: *Glomerella cingulata* ATCC MYA-4136 (GenBank FJ746685), *Xylaria acuta* ATCC 56487 (GenBank AF163026) and *Neurospora crassa* ATCC MYA-4619 (GenBank GU327635). Sequences were deposited in GenBank.

Statistical analysis.—Data for ascospores length and width were analyzed by analysis of variance using PROC GLM and mean separations by the Waller-Duncan k-ratio method (SAS 9.1, SAS Inst., Cary, North Carolina).

RESULTS

Palm samples and fungal morphological characteristics.—There is no known anamorph of *Serenomyces*, and none was observed in this study. Information regarding *Serenomyces* from *Copernicia* × *burretiana*, *Latania loddigesii* and *Phoenix canariensis* (Miami-Dade sample in this study) has been reported (Elliott and Des Jardin 2006), but pertinent information is repeated herein for comparison purposes with material collected after that report.

Palm hosts, location and year the leaf samples were obtained is provided herein (TABLE I). No survey sampling was conducted; samples were provided by others for diagnostic purposes or obtained directly by the authors. Nine of the 11 palm leaves were living with symptoms of rachis or petiole blight. The two exceptions were the leaf from the *P. dactylifera*, which was necrotic when received, and the leaf from the *S. palmetto* from Martin County, which had discoloration of the entire petiole. Six of the 11 palm leaves had stromata present in the tissue. All but one sample were from Florida, and that one was from South Carolina. The *P. dactylifera* and *P. canariensis* samples were from the coastal southeast (Miami-Dade and

Palm Beach counties), central inland (Orange and Polk counties) and coastal west-central (Hillsborough County) areas of Florida. The *S. palmetto* samples were from opposite coasts of Florida (Martin [east coast] vs. Manatee [west coast] counties), plus coastal South Carolina. Remaining samples were from coastal southeast (Miami-Dade County), coastal west-central (Pinellas County) and coastal southwestern (Lee County) areas of Florida. Nine of the samples were from landscapes; the two *S. palmetto* samples from Florida were from natural areas. Thus, while only five palm species are represented, the geographical area represented is broad.

If stromata were already present at the time the sample was received, necks and ascospores were observed within 2–4 d after the palm tissue was placed in the moisture chamber. Otherwise, it was 2–3 wk before ascomata necks and ascopores were observed. Asci were never observed, which is consistent with previous observations that asci are evanescent in *Serenomyces* (Hyde and Cannon 1999). All ascospores looked similar, whether from palm samples or from cultures of single-spore isolates: gold to light brown, lenticular to ovoid single cells with obtuse ends. Ascomata necks were elongated, black and cylindrical. (See Elliott and Des Jardin 2006 for photographs.)

At the time of our initial report on *Serenomyces* in Florida (Elliott and Des Jardin 2006), no cultures of *Serenomyces* had been obtained from the Florida material. This was not too surprising in that only one study had been made regarding a *Serenomyces* culture (Barr et al. 1997). However, after numerous attempts with the *P. canariensis* sample from Miami-Dade County, germinated ascospores did produce pure cultures, which yielded perithecia with ascospores. No cultures were obtained from the *Copernicia* and *L. loddigesii* samples. Single-spore isolates were obtained from the remaining *Phoenix*, *S. palmetto* and *T. radiata* samples from Florida but not from the *S. palmetto* sample from South Carolina. All isolates produced perithecia and ascospores in culture, except for the isolates obtained from the *S. palmetto* samples. While the ascospores germinated and grew faster on PDA, they were easier to discern under the microscope for selection purposes on WA, and the WA reduced contamination from other fungi and bacteria.

Ascospore measurements from spores collected from palm tissues and spores produced in PDA culture are provided (TABLE I). The standard error for ascospore length and width demonstrated low variability among spores for each sample and each isolate. A comparison of mean length across all tissue samples and isolates indicated that the ascospores associated with *T. radiata* are significantly longer than those from all other palm hosts by almost 2 μm. The ascospores from *S. palmetto* also seemed to group separately from most other samples and isolates, relative to length, with the exception of the two isolates (but not the rachis tissue) from *P. dactylifera*. A comparison of mean width indicates that the ascospores from *S. palmetto* are significantly narrower than all other samples and isolates. Combined with the ascospore length, this would indicate that the ascospores produced in association with *S. palmetto* are different from all other palm hosts. Thus, the ascospores associated with *P. canariensis*, *P. dactylifera*, *Copernicia* × *burretiana* and *Latania loddigesii* form one morphological group, the ascospores from *T. radiata* form a second group and the ascospores from *S. palmetto* form a third group (FIG. 1).

All ascospores in the current study are < 8 μm diam. Based on the key in Hyde and Cannon (1999), the species observed in these samples is either *S. mauritiae* or *S. phoenicis*. The obtuse end of all ascospores would suggest *S. phoenicis* rather than *S. mauritiae*. Also, ascospore length would suggest *S. phoenicis* if the specimens not included in the type collection are used as references (Hyde and Cannon 1999). In the key, *S. phoenicis* is 8–12 μm long, but in the description provided for this species, notes indicate the specimens from Pakistan on *P. dactylifera* have longer ascospores (4–10 μm) and the original description by Rolland (1890, as stated in Hyde and Cannon 1999), presumed to be on a *Phoenix* sp., provides a length of 12–15 μm. However, this still means that the spores observed from *T. radiata*, both on the palm and in culture, are longer than reported.

While the ascospore size and shape generally matches *S. phoenicis*, the length of the ascomata neck does not. While neck length varied widely, both within a sample or culture and among samples, all were at least 700 μm long. The description in Hyde and Cannon (1999) indicates a neck length ''to 600 μm long'' for *S. phoenicis* but further states in the note that ''[i]n most collections seen, ascomatal necks were either completely absent or consisting of short papillate structures. As the necks are very fragile they may well have broken off at some stage …'' The neck length in our samples was most likely due to the fact we were examining freshly collected material (both from palm tissue and PDA culture) and Hyde and Cannon were examining herbarium specimens.

In culture the single-spore isolates appeared highly variable. All isolates had irregular colony morphology, with discrete but irregular colony edges, mostly appressed felt- or velvet-like mycelium but often with ridges within the colony. Most of the isolates exuded a dark brown pigment (presumed to be melanin) into the medium and were relatively slow growing. Colony

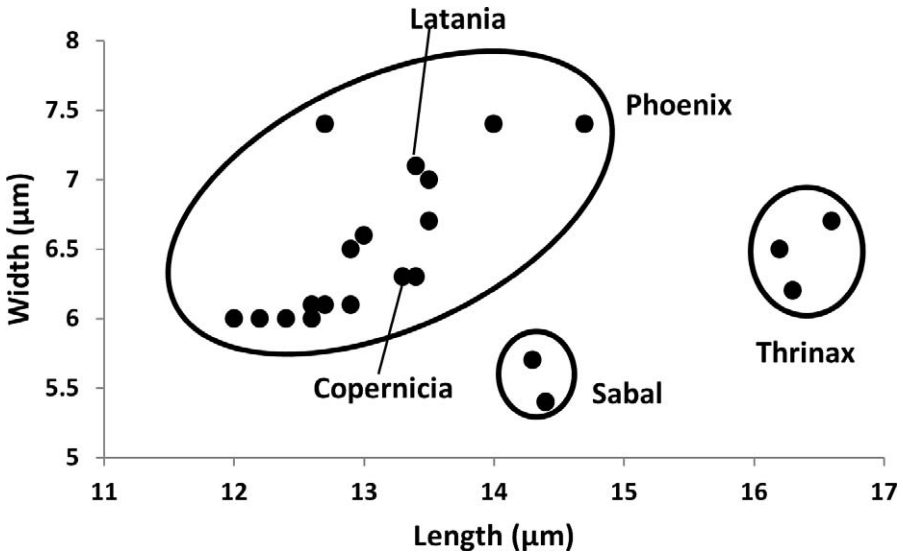

FIG. 1. Scatter plot illustrating grouping of ascospores (length × width in micrometers) obtained directly from palm material and from single-spore cultured isolates. Some dots represent two isolates with the same dimensions.

varied from white to gray to brown. Even after 4 wk, most isolates did not fill a 100 mm diam Petri plate. Also, most isolates stopped growing after only two or three serial transfers.

Recovery from storage.—In both Oct 2012 and Feb 2013, all *Serenomyces* isolates obtained before 2012, except PLM-399, were recovered from the colonized agar plugs that had been placed in sterile deionized water in cryogenic vials and stored at room temperature. PLM-399 was also the only isolate not recovered from mycelial growth in potato dextrose broth. Multiple attempts were made to recover PLM-399 from these storage techniques, but they were unsuccessful. Recovery from 1/5 PDA slants was inconsistent. Seven isolates were recovered in Oct 2012, and nine isolates were recovered in Feb 2013, with only five isolates recovered both times (PLM-340, 399, 403, 404, 594). Storage date did not appear to be a factor for the agar slants, in that isolates stored in 2007 and 2008 were recovered the same as isolates stored in 2011.

Archival deposition of fungal isolates.—Because the *Serenomyces* cultures did not appear to be stable, isolates that produced perithecia and ascospores in vitro have been deposited as archival material with US National Fungus Collections (BPI) using the rigid cardboard slide pillbox method. Duplicate archival material has been deposited with University of Florida Mycological Herbarium (FLAS) in Gainesville.

Hyphal growth rate.—Each time the experiment was conducted, growth from some colonized mycelial plugs did not occur at any temperature, others grew a little and stopped and others seemed to grow normally. One isolate grew consistently across all replicates the first time the experiment was performed but not the second time. Therefore, due to the lack of consistent growth within an isolate, only generalities can be provided. After 14 d, all three isolates examined had the greatest growth at 30 C. Growth was reduced slightly at 25 C, reduced even more at 20 C, and no growth was recorded at 15 C or 35 C. Even at 30 C, none of the isolates filled the plate. Colonies at 30 C were ~ 50 mm diam for PLM-314 and PLM-594 and ~ 20 mm diam for PLM-340.

Amplicon sequencing and phylogenetic analysis.—PCR amplicons were obtained from all the herbarium material evaluated except BPI 579854. Unfortunately the ITS sequences obtained from the remaining herbarium material matched (100% coverage, 99–100% maximum identity, E value = 0) ITS sequences in GenBank for *Candida* (BPI 579851, BPI 579855), *Rhodotorula* (BPI 579853) or *Cladosporium* (BPI 749366). Therefore these sequences were not included for phylogenetic analysis.

Amplicons were obtained from all freshly collected palm material and from single-spore isolates with the PCR assay using the ITS1 and ITS4 primers. The ITS sequence obtained from *Copernicia* closely matched (99% maximum identity) an ITS sequence from *Harzia cameroonensis* (CPC 22065) and was not included in the phylogenetic analysis. All other sequences generated from Florida and South Carolina palm material and derived isolates (either from

spores or hyphae) were deposited in GenBank as KC89536-KC898542 (*Sabal palmetto* material) and KF361443-KF361474 (all other material). None of these ITS region DNA sequences from *Serenomyces* matched nucleotide sequences in GenBank. The closest matches were to other Sordariomycetes, such as *Phaeoacremonium* (e.g. JQ418446: 62% coverage, 87% maximum identity, E value > 0; AF197979: 55% coverage, 89% maximum identity, E value > 0), which include species associated with woody dicot trees. There are no nucleotide sequences in GenBank of the other three genera in the Phaeochoraceae family.

A phylogenetic tree (FIG. 2) was developed using the maximum parsimony method with ITS region sequences from the ascospores obtained directly from palm tissue, ascospores obtained from cultures of single-spore isolates and, for single-spore isolates that did not produce perithecia in culture, mycelia obtained from liquid cultures of these isolates. The ITS sequences from *Glomerella cingulata* ATCC MYA-4136, *Xylaria acuta* ATCC 56487 and *Neurospora crassa* ATCC MYA-4619, representing each subclass within the Sordariomycetes, were used to root the tree. All sequences from *Phoenix* spp. isolates matched. The same is relatively true for the *S. palmetto* isolates and *T. radiata* isolates respectively. This indicates that the cultures obtained from single ascospores associated with the palm tissue do represent the original material. This analysis implies that the *Serenomyces* isolates in Florida and South Carolina fall into three clades, one that includes isolates from *Phoenix* spp., a second that includes isolates from *S. palmetto* and a third that includes isolates from *T. radiata* and *L. loddigesii*.

DISCUSSION

In continental USA, documented observations of *Serenomyces* associated with palm leaves are not common. One reason may be due to the fact that *Serenomyces* does not cause significant harm to palms. Unless disease symptoms are present and viewed to be harmful, nursery and landscape managers may never contact a laboratory regarding a diagnosis. All of the *Phoenix* samples in the current study were obtained because of concern that the palm was infected with *Fusarium oxysporum* f. sp. *canariensis*, a lethal pathogen of *P. canariensis*, which causes similar symptoms (Summerell et al. 2001). Except for the *P. dactylifera* sample, ascospores were not observed until the petiole or rachis was placed in a moisture chamber, even though stromata were observed. Of interest, the *P. dactylifera* sample was a completely necrotic leaf. Perhaps the fungus does not produce ascospores naturally until no additional living plant tissue remains to parasitize. In landscape situations, palm leaves often are removed while they still have green tissue. These leaves would either be composted, incinerated, removed to a landfill or chopped up for mulch—activities that are not conducive for initiating sporulation.

The five palm genera represented in this study all belong to the same palm subfamily Coryphoideae, but each genus belongs to a separate subtribe (Dransfield et al. 2008). Thus, all reported *Serenomyces* associations have been with genera of Coryphoideae, primarily *Phoenix* species, except for *Mauritia*, which belongs to the palm subfamily Calamoideae (Hyde and Cannon 1999, Elliott and Des Jardin 2006, Dransfield et al. 2008, Farr and Rossman n.d.).

Science recognizes four species of *Serenomyces* based on morphology (Müller and Dennis 1965, Barr et al. 1989, Barr et al. 1997, Hyde and Cannon 1999). Two species, *S. palmae* and *S. virginiae*, have relatively large ascospores (14–22 × 8–10 μm). The other two species, *S. mauritiae* (previously *S. sheari*) and *S. phoenicis*, have ascospores that are < 8 μm diam, which is similar to the *Serenomyces* ascospores observed in this study. *S. mauritiae* has ascospores, 11–18 μm × 4–6 μm, lenticular with somewhat acute ends and necks to 2500 μm long. Reports on the size of *S. phoenicis* ascospores vary, as noted, from 8–12.5 μm × 5–7 μm to 10–14 μm × 5–6.5 μm, ovoid, obtuse ends and necks to 600 μm long. Thus, except for the neck length, the ascospores associated with four of the five palm species most closely conform in size and shape to *S. phoenicis*. Given the fragility of the necks, this may not be a reliable characteristic for taxonomic purposes. Statistically there were three distinct groups of ascospores based on size. Ascospores from *T. radiata* were significantly longer than all other ascospores and do fall outside those reported for *S. phoenicis*, but the ascospore shape matches *S. phoenicis*. Ascopores from *S. palmetto* were significantly longer and thinner than all other ascospores, except those from *T. radiata*, but were within the range reported for *S. phoenicis*. The ascospores from the remaining three palm genera were diverse, but ascospores obtained directly from palm tissue was 12.4–13.5 μm long and 6.0–7.3 μm wide, which is also within the range reported for *S. phoenicis*.

Molecular characterization of the *Serenomyces* isolates in this study was obtained with the noncoding regions (internal transcribed spacer region) between the 18S and 5.8S (= ITS1) and 5.8S and28S (= ITS2) rDNA genes. The spacer regions are more variable than the actual rDNA genes and are considered to have evolved faster than the nuclear rDNA and often vary among species within a genus (White et al. 1990). Because there are no archival cultures of *Serenomyces*, the matching of ITS sequences from single-spore isolates cultured from the palm tissue (hyphae and

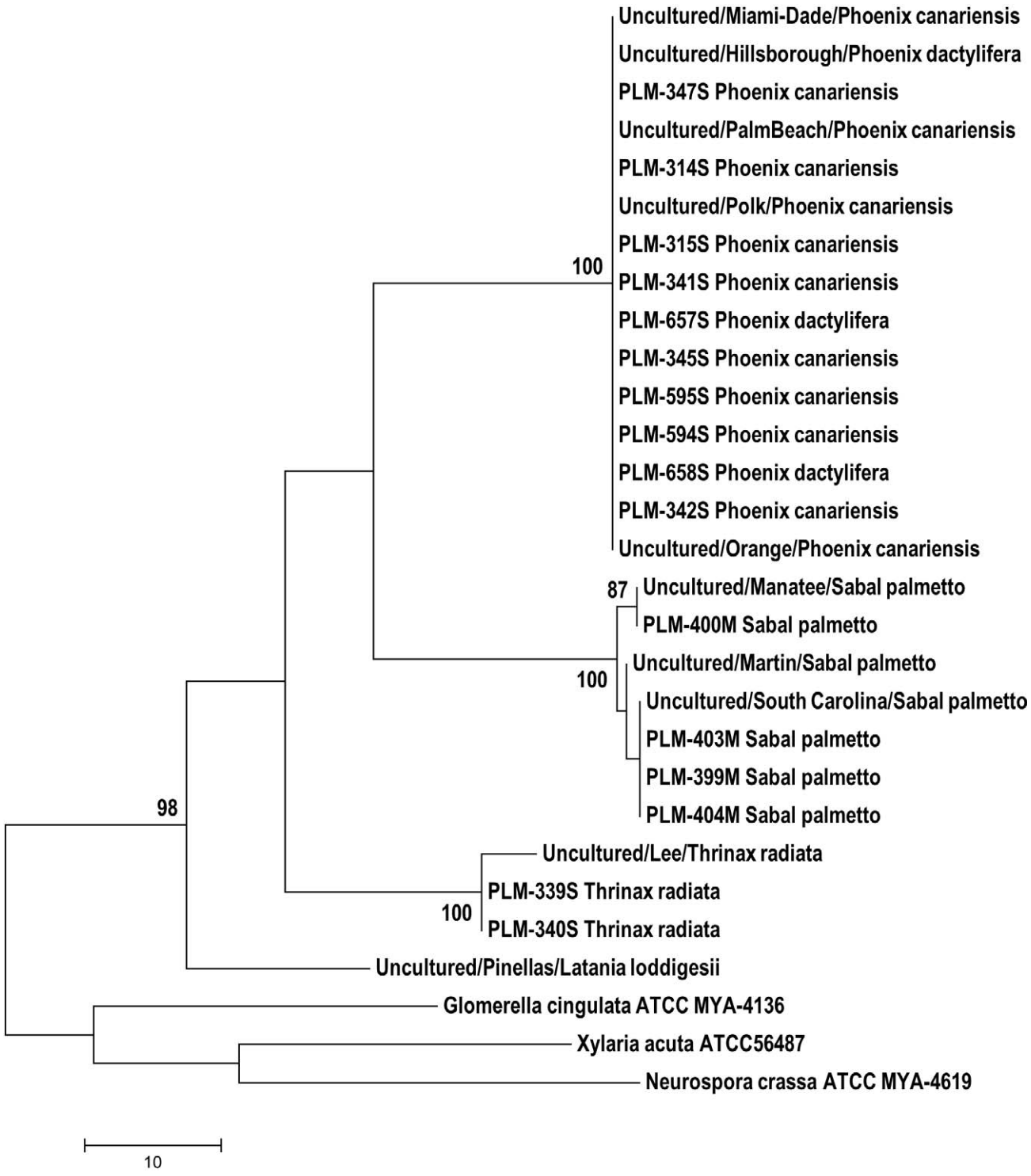

FIG. 2. Phylogenetic tree, constructed by the maximum parsimony method, using ITS DNA sequences from *Serenomyces* ascospores obtained directly from palm material (uncultured), ascospores obtained from cultures of single-spore isolates (PLM-xxxS) (TABLE I) and mycelia obtained from cultures of single-spore isolates (PLM-xxxM) that did not produce ascospores in culture. Palm host is provided, as is county or state for uncultured material. ITS sequences from *Glomerella cingulata* ATCC MYA-4136, *Xylaria acuta* ATCC 56487 and *Neurospora crassa* ATCC MYA-4619, representing each subclass of Sordariomycetes, were used to root the tree. Support values greater than 70% are shown. Length = 193, consistency index = 0.742, retention index = 0.906.

ascospores) and from the ascospores direct from the palm tissue provides assurance to other researchers regarding the archival fungal material deposited with the US National Fungal Collections and University of Florida Mycological Herbarium.

By culturing single-spore isolates, and in some cases inducing these isolates to produce viable ascospores on artificial media, followed by sequencing the ITS sequences from this fungal material, we are confident that the ITS sequences obtained for *Serenomyces* from infested *P. canariensis*, *P. dactylifera*, *T. radiata* and *S. palmetto* are correct. We are less certain about the ITS sequences obtained for *Serenomyces* from *L. loddigesii* in that no cultures were obtained. Still, it is evident that there are distinct differences in the *Serenomyces* obtained from each genera of palm. The molecular characterization of the ITS region and spore size might support separation of *Serenomyces* obtained from *Phoenix*, *Sabal* and *Thrinax* into three different species. Of note, *Phoenix* spp. are not native to USA, whereas *Sabal* and *Thrinax* are. It is possible that the *Serenomyces* sp. associated with the exotic palm genus is an Old World species, and the *Serenomyces* spp. associated with the native palm genera are New World species.

What is less clear is the morphological and molecular characterization of *Serenomyces* from *L. loddigesii* and *Copernicia*. The *Serenomyes* spores observed from the *Copernicia* and *L. loddigesii* leaves have morphology that is no different from the *Serenomyces* obtained from the *Phoenix* samples. For the *Serenomyces* from the *L. loddigesii* leaf, the ITS region is most similar to *Serenomyces* isolates from the *T. radiata* leaf. But, without more samples and isolates, especially from *Latania*, *Copernicia* and *Thrinax*, species identification within *Serenomyces* should remain as is for now. While *S. phoenicis* has been identified as a separate species since 1952 (Petrak 1952), the family *Phaeochoraceae* is new (Hyde et al. 1997) and there is still much to be learned about this group, especially at the molecular level.

Serenomyces from *Phoenix* spp., *Copernicia* × *burretiana*, *Latania loddigesii*, *Sabal palmetto*, *Serenoa repens*, *Mauritia flexuosa* have been reported (Dixon and Coile 1995, 1998, 1999; Hyde and Cannon 1999; Elliott and Des Jardin 2006; Farr and Rossman n.d.). However, this is the first report of a *Serenomyces* sp. from *S. palmetto* in South Carolina (USA) and on *Thrinax radiata* in the world. Considering that this small group represents palms from the Old World (*P. dactylifera*) and New World (*Mauritia*, *Sabal*, *Serenoa*, *Thrinax*) and palms from islands in the Indian Ocean (*Latania*), Atlantic Ocean (*P. canariensis*) and Caribbean Sea (*Copernicia*) (Riffles and Craft 2003), the genus *Serenomyces* is either universally present where palms grow (and just not observed to date) or has moved around the world as people have moved palm material. The former is just as likely as the latter because a fossil of the extinct palm *Uhlia allenbyensis* also included a *Serenomyces*-like fungus (*Paleoserenomyces allenbyensis*) beneath the epidermis (Currah et al. 1998). This fossil specimen is associated with the middle Eocene period and was found near Princeton, British Columbia, Canada.

With the sequencing of *Serenomyces* ITS region, data is now available for comparison with *Serenomyces* found associated with other palms species in USA and around the world. This also may allow for development of specific primers for *Serenomyces* and perhaps a reevaluation of the BPI herbarium material and material located in other collections (see Hyde and Cannon 1999). More important, it will help in the molecular evaluation of the systematics of the class Sordariomycetes and the order Phyllachorales (Wanderlei-Silva et al. 2003, Zhang et al. 2006).

To make it easier for others to study this genus we offer a few suggestions regarding storage. Based on our observations, *Serenomyces* is unstable in culture. While we could recover the fungus from storage, the growth was extremely slow and erratic for some isolates. Serial transfers of the fungus resulted in loss of viability, and this was shown again by the fact that recovery was poor when the fungus was stored on 1/5 PDA slants. The advantage of storing colonized mycelial plugs in water (no serial transfer) or mycelia grown in PDB is that this material can be used directly for future molecular characterization. This is especially true for the mycelia grown in PDB. Storing fungal plugs in water at room temperature is an older method (Boeswinkel 1976, Ellis 1979, Jones et al. 1991), but it is simple, cost effective and does not require electricity to run an ultralow-temperature freezer. As observed in Elliott (2005) in our laboratory with *Gaeumannomyces graminis*, using cryogenic vials aids long-term storage because they prevent evaporation of liquids compared to other types of vials.

The small, matchbox-like pillboxes are useful for storing dried sporulating cultures of *Serenomyces* and likely would be useful for similar fungi. The height prevents crushing of the fungal material and the material is protected within the box yet easily accessible. With careful packing, the boxes can be shipped easily. While we stored the boxes in a desiccator for further protection, they could be easily stored in herbarium cabinets also.

ACKNOWLEDGMENTS

The authors thank the U.S. National Fungus Collections group for providing valuable material.

LITERATURE CITED

Alfieri Jr SA, Langdon KR, Kimbrough JW, El-Gholl NE, Wehlburg C. 1994. Diseases and disorders of plants in Florida. Bulletin 14. Gainesville: Florida Department of Agriculture and Consumer Services, Division of Plant Industry.

Baldauf SL. 2003. Phylogeny for the faint of heart: a tutorial. Trends Genet 19:345–351, doi:10.1016/S0168-9525(03)00112-4

Barr ME, Ohr HD, Ferrin DM, Mundo-Ocampo M. 1997. A new species of *Serenomyces* from date palm in California. Mycotaxon 61:481–484.

———, ———, Murphy MK. 1989. The genus *Serenomyces* on palms. Mycologia 81:47–51, doi:10.2307/3759449

Boesewinkel HJ. 1976. Storage of fungal cultures in water. Trans Br Mycol Soc 66:183–185, doi:10.1016/S0007-1536(76)80119-2

Currah RS, Stockey RA, LePage BA. 1998. An Eocene tar spot on a fossil palm and its fungal hyperparasite. Mycologia 90:667–673, doi:10.2307/3761225

Dixon WN, Coile NC, eds. 1995. Plant pathology. Online. Triology 34. http://www.freshfromflorida.com/Divisions-Offices/Plant-Industry/Plant-Industry-Publications/Triology-FDACS-DPI/Triology-Vol.-34-No.-5

———, ———. 1998. Plant pathology. Online. Tri-ology. 37. http://www.freshfromflorida.com/Divisions-Offices/Plant-Industry/Plant-Industry-Publications/Tri-ology-FDACS-DPI/Triology-Vol.-37-No.-3

———, ———. 1999. Plant pathology. Online. Tri-ology 38. http://www.freshfromflorida.com/Divisions-Offices/Plant-Industry/Plant-Industry-Publications/Tri-ology-FDACS-DPI/Triology-Vol.-38-No.-2

Dransfield J, Uhl NW, Asmussen CB, Baker WJ, Harley MM, Lewis CE. 2008. Genera palmarum: the evolution and classification of palms. Richmond, Kew, Surry, UK. 732 p.

Elliott ML. 2005. Survival, growth and pathogenicity of *Gaeumannomyces graminis* var. *graminis* with different methods of long-term storage. Mycologia 97:901–907, doi:10.3852/mycologia.97.4.901

———, Des Jardin EA. 2006. First report of a *Serenomyces* sp. from *Copernicia* × *burretiana*, *Latania loddigessi* and *Phoenix canariensis* in Florida and the United States. Online. Plant Health Prog, doi:10.1094/PHP-2006-1213-02-BR

———, ———, Henson JM. 1993. Use of a polymerase chain reaction assay to aid in identification of *Gaeumannomyces graminis* var. *graminis* from different grass hosts. Phytopathology 83:414–418, doi:10.1094/Phyto-83-414

———, ———, O'Donnell K, Geiser DM, Harrison NA, Broschat TK. 2010. *Fusarium oxysporum f.* sp. *palmarum*, a novel forma specialis causing a lethal disease of *Syagrus romanzoffiana* and *Washingtonia robusta* in Florida. Plant Dis 94:31–38, doi:10.1094/PDIS-94-1-0031

Ellis JJ. 1979. Preserving fungus strains in sterile water. Mycologia 71:1072–1075, doi:10.2307/3759297

Farr DF, Rossman AY n.d. Fungal databases. Systematic Mycology and Microbiology Laboratory, ARS, USDA. Retrieved 6 Jul 2013 from http://nt.ars-grin.gov/fungaldatabases/

Fröhlich J, Hyde KD. 2000. Palm microfungi. Hong King: Fungal Diversity Press. 393 p.

Hyde KD, Cannon PF, Barr ME. 1997. *Phaeochoraceae*, a new ascomycete family from palms. Systema Ascomycetum 15:117–120.

———, ———. 1999. Fungi causing tar spots on palms. Mycological papers 175. Wallingford, UK: CABI Publishing. 114 p.

Jones RJ, Sizmur KJ, Wildman HG. 1991. A miniaturized system for storage of fungal cultures in water. Mycologist 5:184–186, doi:10.1016/S0269-915X(09)80482-5

Kolaczkowski B, Thornton JW. 2004. Performance of maximum parsimony and likelihood phylogenetics when evolution is heterogeneous. Nature 431:980–984, doi:10.1038/nature02917

Lumbsch HT, Huhndorf SM. 2007. Outline of ascomycota – 2007. Myconet 13:1–58.

Müller E, Dennis RWG. 1965. Fungi venezuelani: Plectascales, Sphaeriales, Loculoascomycetes VIII. Kew Bull 19:357–386, doi:10.2307/4108161

Petrak VF. 1952. Serenomyces n. gen., eine neue Gattung der Ceratostomaceen. Sydowia 6:296–298.

Riffles RL, Craft P. 2003. An encyclopedia of cultivated palms. Portland, Oregon: Timber Press Inc. 528 p.

Simone GW. 2004. Rachis blight. In: Elliott ML, Broschat TK, Uchida JY, Simone GY, eds. Compendium of ornamental palm diseases and disorders. St Paul, Minnesota: The American Phytopathological Society. p 33–35.

Summerell BA, Kistler HC, Gunn LV. 2001. Fusarium wilt of *Phoenix canariensis* by *Fusarium oxysporum* f. sp. *canariensis*. In: Summerell BA, Leslie JF, Backhouse D, Bryden DL, Burgess LW, eds. Fusarium: Paul E. Nelson memorial symposium. St Paul, Minnesota: The American Phytopathological Society. p 263–270.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA 4: molecular evolutionary genetics analysis. 4.0. Mol Biol Evol 24:1596–1599, doi:10.1093/molbev/msm092

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680, doi:10.1093/nar/22.22.4673

Wanderlei-Silva D, Neto ER, Hanlin R. 2003. Molecular systematic of the Phyllachorales (Ascomycota, Fungi) based on 18S ribosomal DNA sequences. Brazilian Arch Biol Tech 46:315–322.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego, California: Academic Press. p 315–322.

Zhang N, Castlebury LA, Miller AN, Huhndorf SM, Schoch CL, Seifert KA, Rossman AY, Rogers JD, Kohlmeyer J, Volkmann-Kohlmeyer B, Sung G-H. 2006. An overview of the systematic of the Sordariomycetes based on a four-gene phylogeny. Mycologia 98:1076–1087, doi:10.3852/mycologia.98.6.1076