CHAPTER 4

MORPHOLOGY AND PHYLOGENETIC STUDY OF LEAF SPOTTING FUNGAL SPECIES ASSOCIATED WITH TERRESTRIAL PALMS

4.1. Introduction

Leaf spots are a common disease affecting the foliage of ornamentals and shade trees. Economically, leaf spots reduce the yield and growth of plants by bringing a reduction in the available area necessary for photosynthesis process (Agrios, 2005). In most cases, leaf spots are not considered to be serious problem on the crops, but they may have economic effects on nurseries or floriculture industries such as palms industry. Palms are historically considered as valuable ornamental plants due to their exotic appearances.

Ornamentals plant is widely recognized as a plant cultivated for its beauty rather than for use. Ornamentals have been referred to as 'amenity crops', in the sense that they contribute to physical or material comfort or convenience or to a pleasant life (Baker and Linderman, 1979). Ornamental plantings used to control erosion, to provide shade and ameliorate environment around a home through control of temperature, wind and traffic noise, and to help purify the atmosphere of pollutants have become necessities of modern life. Some of them, e.g. areca palm (*Areca catechu*), coconut (*Cocos nucifera*), chamaedorea palm (*Chamaedorea angustisecta*), have a medicinal property.

Since 1979, the ornamentals industry has continued to be rocked by disease outbreaks (Daughtrey and Benson, 2005). In the greenhouse industry, bacterial blight

of geraniums caused by *Xanthomonas campestris*, *Impatiens necrotic spot* (INSV) and *Tomato spotted wilt* (TSWV) tospoviruses and the bacterial wilt caused by *Ralstonia solanacearum* (race 3, biovar 2) have had the most impact (Daughtrey and Benson, 2005). In the nursery industry, the effect of sudden oak death caused by *Phytophthora ramorum* has far outweighed that of any disease to date (Daughtrey and Benson, 2005). These, along with thousands of other host-pathogen interactions, make up the contagious disease challenges of plant health management for growers of ornamentals.

In the ornamentals palm industry, several necrosis symptoms caused by fungi such as leaf spot, blight, blotch and anthracnose are commonly found. Necrosis on palm leaflets such as leaf spot symptoms can be caused by species of *Astrosphaeriella* Syd. and P. Syd., *Bipolaris* Shoemaker, *Cercospora* Fresen., *Drechslera* S. Ito, *Exosporium* Link, *Guignardia* Viala and Ravaz, *Maculatipalma* J. Fröhl. and K. D. Hyde, *Mycosphaerella* Johanson and *Oxydothis* Penz. and Sacc. (Anderson *et al.*, 2000; Fröhlich, 1992; Fröhlich and Hyde, 1994, 1995a, b, c, 1998, 2000; Hyde and Fröhlich, 1995a; Hyde *et al.*, 1997). In this chapter, the morphology and phylogenetic elucidation of selected species of leaf spotting fungi on palms leaflet found during this study are presented.

4.2. Materials and Methods

Collection Sites and Morphological Examination

Specimens collected in this study were collected from several locations in Chiang Mai province. One or two 29 cm × 42 cm resealable plastic bags were used for each palm species. Specimen collection was carried out by observing leaf spot

symptoms on the leaves surface. Various types of symptoms such as frog eye spots, stripes, shot hole effect, discoloration or necrosis were collected and examined. Specimens were collected after observing the presence of fungal fruiting bodies on leaves using a 10Δ or 20× magnifying glasses. The specimens that showed the existence of fungal fruiting bodies were stored in the plastic bags. Collecting bags are sealed and labeled some following information: *name of host plants, collecting site, collector/s and collection date*. On returning to the laboratory, the material was examined immediately and the remains were incubated in a moist chamber for studying at a later date.

The specimens were observed using an Olympus BX50 photomicroscope system with differential interference contrast microscopy. Water is the medium used for all examinations, spore measurements, and most of the illustrations. Measurements are given as (minimum) mean ± standard deviation (maximum) (n=sample size). Specific reagents were used when necessary as follows: Melzer's reagent was used to investigate any reactions in the ascus. Ascomata sections of rehydrated fruiting structures were made with a Micron HM505E cryostat microtome or by hand. Lactophenol was added to the slides for permanent fixation. Dried herbarium specimens were deposited at CMU Herbarium (CMU), Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. Cultures were obtained in this study according to the modification of method of Choi *et al.* (1999) which is elucidated in chapter 2. An ex-type culture is maintained in the Molecular of Plant Pathology Culture Collection, Department of Plant Pathology, Chiang Mai University, Chiang Mai, Thailand.

Molecular Characterization

In this chapter, molecular characterization was carried out in order to analyze the phylogenetic relationship of selected isolates with other similar taxa. Total genomic DNA was extracted from mycelial cultures grown on malt extract agar (Difco) following a 2 × cetyltrimethylammoniumbromide (CTAB) protocol (Rogers and Bendich, 1994). DNA amplification of internal transcribed spacer (ITS) nrDNA region was performed by polymerase chain reaction (PCR) using ITS4 and ITS5 primers (White *et al.*, 1990) to generate about 587 nucleotides from the complete ITS region. The amplification conditions were performed in a 50 µl reaction volume as follows: 1 × PCR buffer, 0.2 mM each dNTP, 0.3 mM of each primer, 1.5 mM $MgCl_2$, 0.8 units Taq polymerase, and 10 ng DNA. PCR parameters for all the regions were performed as follows: initial denaturation at 94ºC for 3 min, 30 cycles of 94ºC for 1 min, 52ºC for 50 s, and 72ºC for 1 min, and final extension of 72ºC for 10 min.

The characterization of PCR products was performed via agarose gel electrophoresis on a TAE 1% agarose gel containing ethidium bromide (EtBr) as the staining agent. The PCR product was purified using Qiaquick purification kit (Qiagen) and DNA concentration of the PCR products was subjected to automatic sequencing (ABI PRISM Dye Terminator Cycle Sequencing and ABI PRISM Sequencer model 377, Perkin Elmer). The GenBank accession numbers of the sequences and taxa used to construct the phylogenetic trees in this chapter are shown in appendices 2 and 3.

Sequences were aligned in ClustalX version 2.0.3 (Larkin *et al.*, 2007) and BioEdit (Hall, 1999) using default parameters. The sequences alignments were checked and manual adjustment were made where necessary. Regions designated as

ambiguously aligned were excluded from the analyses. Gaps were treated as missing data. Phylogenetic analyses were performed in PAUP version 4.0b10 (Swofford, 2002).

Unweighted Maximum Parsimony (UMP) analysis was performed in this study. Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics (tree length [TL], consistency index [CI], retention index [RI], related consistency index [RC], homoplasy index [HI] and log likelihood [-ln L]) were calculated for trees generated under different optimality criteria. The Kishino-Hasegawa (KH) likelihood test (Kishino and Hasegawa, 1989) was carried out using PAUP to compare the best tree topology obtained by the nucleotide sequence data with a constrained tree. Clade stability was assessed in bootstrap analyses with 1000 replicates, each with 1000 replicates of random stepwise addition of taxa. Random sequence addition was used in the bootstrap analyses. Trees were figured in TreeView (Page, 1996). Other details are outlined in Cai *et al.* (2005).

4.3. Results and Discussion

4.3.1. ***Cercospora arecacearum*** Hidayat and Meeboon, *Mycol. Prog.* **8**: 116 (2009)

MycoBank No. MB 510616

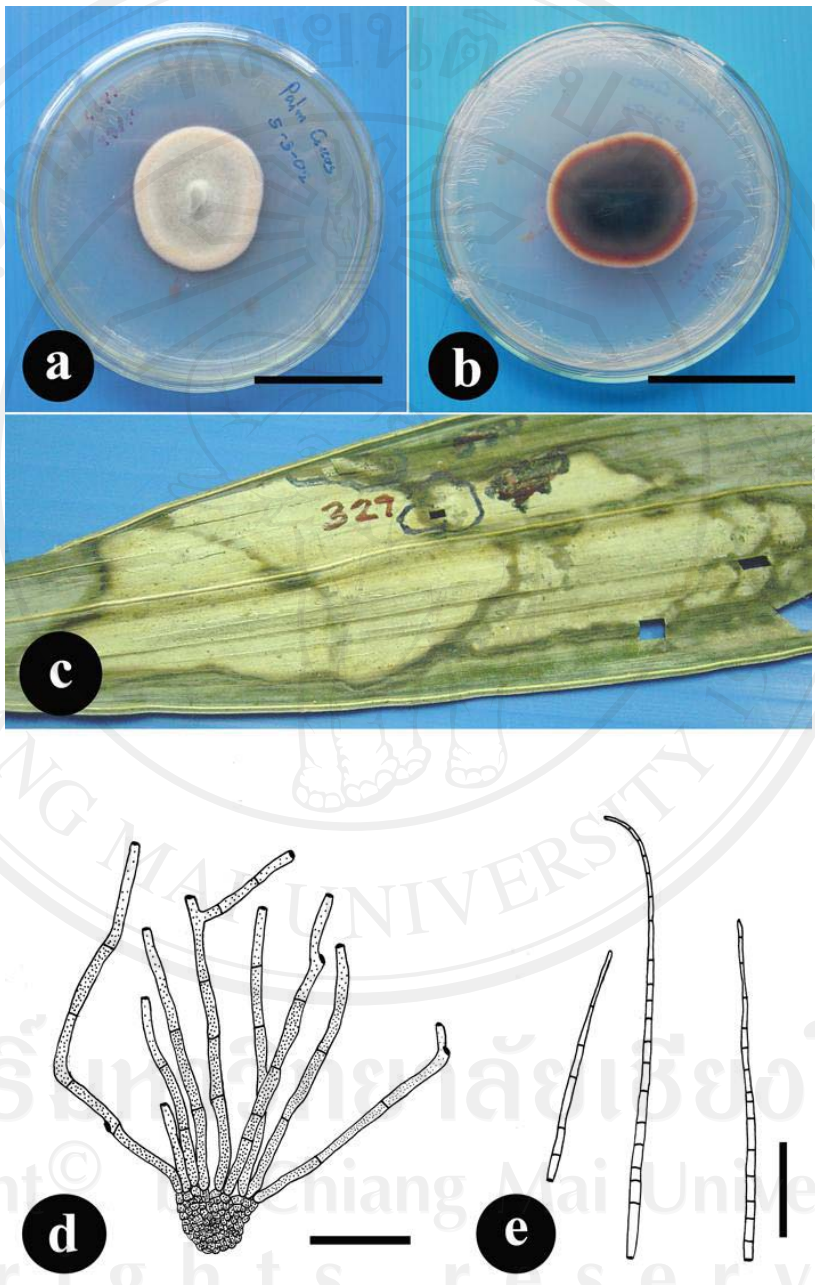
(Figs.4.1)

Differt a C. apii s. lat. (C. nucifera), C. palmae-amazonensis et C. raphiae stromatibus bene evolutis, (30) 64 ± 26 (100) µm diam., conidiophoris raro ramosis, multigeniculatis et a C. palmae-amazonensis et C. raphiae conidiis acicularibus.

Etymology: Derived from *Arecaceae*, the host family of this species.

Leaf spots amphigenous, irregular, 1-10 cm diam., brownish, dull grayish brown, finally pale grayish with a white center and dark margins, spots usually overlapping. ***Caespituli*** amphigenous, scattered, and dark yellowish. ***Stromata*** substomatal to intraepidermal, well-developed, subglobular, brown to blackish brown, (30) 64 ± 26 (100) µm diam. (n = 10). ***Conidiophores*** in rich fascicles, dense, arising from stromata, cylindrical, but narrowed towards the apex, straight, rarely branched, strongly geniculate, variable in length, (68.5) 165 ± 91 (310) × (4) 4.5 ± 0.5 (5) µm (n = 30), smooth, pale yellowish to brownish throughout, sometimes paler at the apex, 2-8-septate. ***Conidiogenous cells*** integrated, terminal, sympodially proliferating, (24.5) 37 ± 13 (67) × (4) 4 ± 0.4 (5) µm (n = 30). ***Conidiogenous loci*** conspicuous, thickened and darkened, (2.5) 3 ± 0.3 (3) µm diam. (n = 30). ***Conidia*** formed singly, acicular, straight, often curved at the apex, (140) 229 ± 56 (320) × (4) 5 ± 0.5 (5) µm

(n = 30), hyaline, 9-25-septate, thin-walled, smooth, tapered towards a subacute apex, base truncate, hilum thickened and darkened, (2.5) 3 ± 0.3 (3) µm diam (n = 30).



Figs. 4.1 *Cercospora arecacearum* (from holotype). a. Culture on the upper surface on PDA. b. Culture on the lower surface on PDA. c. Symptoms on the upper surface of the host. d. Conidiophores and stroma. e. Conidia (Scale bars: a = 3.5 cm, b = 4 cm, d = 75 µm, e = 60 µm).

Colonies on potato dextrose agar medium slowly growing, velvety, 3-4 cm after 30 days, tight to the agar, dark, covered by a grayish white aerial mycelium, reddish near the margin, with white margin, producing red pigmentation in the agar, no sporulation.

Teleomorph: *Mycospaherella* Johanson.

Material examined: THAILAND, Chiang Mai province, Mae Taeng district, Pa Pae village, Mushroom Research Centre, on leaf spots of *Areca catechu* L. (Arecaceae), 17 November 2006, Iman Hidayat (**Holotype**: CMU 27946).

Habitat: Leaf spots of *A. catechu*.

Distribution: Chiang Mai, Thailand.

Notes: According to Crous and Braun (2003), this species belongs to *Cercospora* s. str., which is characterized by having pigmented conidiophores, thickened and darkened conidiogenous loci, and hyaline scolecoid conidia. Furthermore, this fungus is distinct from the plurivorous *C. apii* s. lat. by having well-developed, large stromata and strongly geniculate, rarely branched conidiophores in rich fascicles (Crous and Braun, 2003).

About 12 species of *Cercospora* s. lat. are hitherto known on Arecaceae. Most of them have been excluded from *Cercospora* s. str. based on comprehensive re-examinations by several researchers (Anderson *et al.*, 2000; Chupp, 1954; Crous and Braun, 2003; Deighton, 1985; Goh and Hsieh, 1989; Hughes, 1952). Hughes (1952) excluded *C. palmivora* Sacc. (= *C. preisii* Bubák) from *Cercospora* and put it in *Stigmina* as *S. palmivora* (Sacc.) S. Hughes. Chupp (1954) excluded three additional species of *Cercospora* on Arecaceae by transferring *C. acrocomiae* J. A. Stev. to

Exosporium Link, *C. calamicola* Henn. to *Helminthosporium* Link, and by classifying *C. licualae* Syd. and P. Syd. (= *Cercospora virens* Sacc.) as helminthosporoid fungus. Deighton (1985) assigned *C. elaeidis* Steyaert to *Pseudospiropes* due to the type of lesions and pigmented conidia. Anderson *et al.* (2000) re-examined the type material of *C. palmicola* Speg. and renamed this fungus as *Drechslera palmicola* (Speg.) Anderson, Bianchinotti and U. Braun due to its tretic conidiogenesis, with wide and plainly thick-walled conidia. Goh and Hsieh (1989) introduced the new combination, *Pseudocercospora rhapisicola* (Tominaga) Goh and W. H. Hsieh, for *C. rhapisicola* Tominaga because of inconspicuous, unthickened conidial scars and hila.

Currently, only two species on *Arecaceae*, viz, *Cercospora palmae-amazonensis* Bat. and Cavalc. (Batista and Cavalcanti, 1964) and *C. raphiae* Deighton (1985), have been maintained in *Cercospora* s. str. (Crous and Braun, 2003). Another species of *Cercospora* s. str., *C. nucifera* R. K. Srivast., S. Narayan and A. K. Srivast. (Srivastava *et al.*, 1995), is now classified as *C. apii* s. lat. (Crous and Braun, 2003).

Cercospora arecacearum is distinct from *C. raphiae* by having amphigenous caespituli, rarely branched and strongly geniculate conidiophores as well as much narrower acicular conidia. Deighton (1985) characterized *C. raphiae* by having hypophyllous caespituli, unbranched, non-geniculate conidiophores and obclavate-cylindrical conidia with slightly thickened hila (table 4.1).

Table 4.1 Morphological comparison of *Cercospora arecacearum* with other *Cercospora* species from Arecaceae (Data from Batista and Cavalcanti, 1964; Deighton, 1985)

Morphological characteristics	***C. arecacearum***	***C. palmae-amazonensis***	***C. raphiae***
Leaf spots	Amphigenous	Amphigenous	Amphigenous
Colonies	Amphigenous	Amphigenous	Hypophyllous
Stromata	Well-developed, (30) 64 ± 26 (100) µm diam.	Small, 22-25 µm diam.	Well-developed, 25-40 µm diam.
Conidiophores	Densely fasciculate, strong geniculate, rarely branched, (68.5) 165 ± 91 (310) × (4) 4.5 ± 0.5 (5) µm	Densely fasciculate, simple to slightly geniculate, unbranched, 112-250 × 4-5 µm	Divergent fasciculate, simple to slightly geniculate, unbranched, up to 210 × 6.5 µm
Conidia	Solitary, acicular, hyaline, (140) 229 ± 56 (320) × (4) 5 ± 0.5 (5) µm	Solitary, obclavate, pigmented, 40-90 × 2.5-7.5 µm	Solitary, obclavate-cylindric, hyaline, 48-117 × 5.5-8 µm

Batista and Cavalcanti (1964) described *C. palmae-amazonensis* as having amphigenous caespituli, conidiophores in fascicles, oblong stromata (22-25 µm diam.), unbranched, simple, to slightly geniculate conidiophores (6-10 septate, 112-250 x 4-5 µm) and lightly brown obclavate conidia with truncate base (6-9 septate, 40-90 x 2.5-7.5 µm). The taxonomic affinity of this species remains uncertain due to the described pigmentation of the conidia. Crous and Braun (2003) examined authentic material of this species, but failed to find any fructification. *Cercospora arecacearum* is easily distinguishable from *C. palmae-amazonensis* by its large stromata, branched and strongly geniculate conidiophores with hyaline acicular conidia.

Molecular phylogenetic analysis of ITS DNA region of *C. arecacearum* was carried out in this report in order to confirm the morphological elucidation of the fungus with related taxa, particularly members of true cercosporoid fungi sensu Crous and Braun (2003). The ITS sequence of *C. arecacearum* was aligned with 34 sequences of species of *Cercospora*, *Pseudocercospora* Speg. and *Passalora* Fr. Sequences of *Cladosporium cladosporioides* (Fresen.) G. A. de Vries and *Davidiella tassiana* (De Not.) Crous and U. Braun were assigned as outgroup. The alignment data matrix consists of 35 taxa and 527 characters, of which 38 characters were excluded from the analysis due to ambiguity of the alignment. Of the remaining 489 included characters, 329 characters were constant, 45 characters were variable and parsimony-uninformative and 115 characters were parsimony-informative. Sum of minimum possible length was 207 and sum of maximum possible length was 729. The best parsimonious tree selected by KH test ($P < 0.05$) was generated in 264 steps (CI = 0.7, RI = 0.8, RC = 0.6, HI = 0.3).

Based on this analysis, three well-supported clades (*Cercospora* clade, *Pseudocercospora* clade and *Passalora* clade) were generated with bootstrap values greater than 75 % for each clade (fig. 4.2). These three clades, which are defined as true cercosporoid fungi by Crous and Braun (2003) formed a monophyletic clade with 100 % bootstrap support. The characteristics among these three clades were elucidated morphologically and phylogenetically by Stewart *et al.* (1999). The *Pseudocercospora* clade appeared as a sister group to the *Cercospora* clade with 80 % bootstrap support, and *Passalora* clade appeared as a sister group to the *Cercospora* and *Pseudocercospora* clades with 100 % bootstrap support. All of the *Cercospora* sequences showed a distinct and monophyletic clade with a well-supported bootstrap support value (95 %). In this clade, *C. arecacearum* together with non-*Cercospora apii* s. lat. taxa such as *C. acaciae-mangii* Crous, Pongpan. and M. J. Wingf., *C. agavicola* Ayala-Escobar, *C. asparagi* Sacc., *C. caricis* Oudem., *C. piaropi* Tharp, *C. polygonaceae* Ellis and Everh., *C. rodmanii* Conway, and *C. sorghi* Ellis and Everh. f. *maydis* Ellis and Everh., clustered alongside the *C. apii* s. lat. taxa with a bootstrap support value 62 %. On the other hand, only *C. zeae-maydis* Tehon and E. Y. Daniels was separated from this clade. Therefore, these data suggested that *Cercospora* species belonging to *C. apii* s. lat. do not generate a monophyletic clade based on the phylogenetic analysis of ITS sequence data. In conclusion, *C. arecacearum* is considered a new cercosporoid species, which is morphologically distinct from other similar taxa, and phylogenetically separated from related taxa.

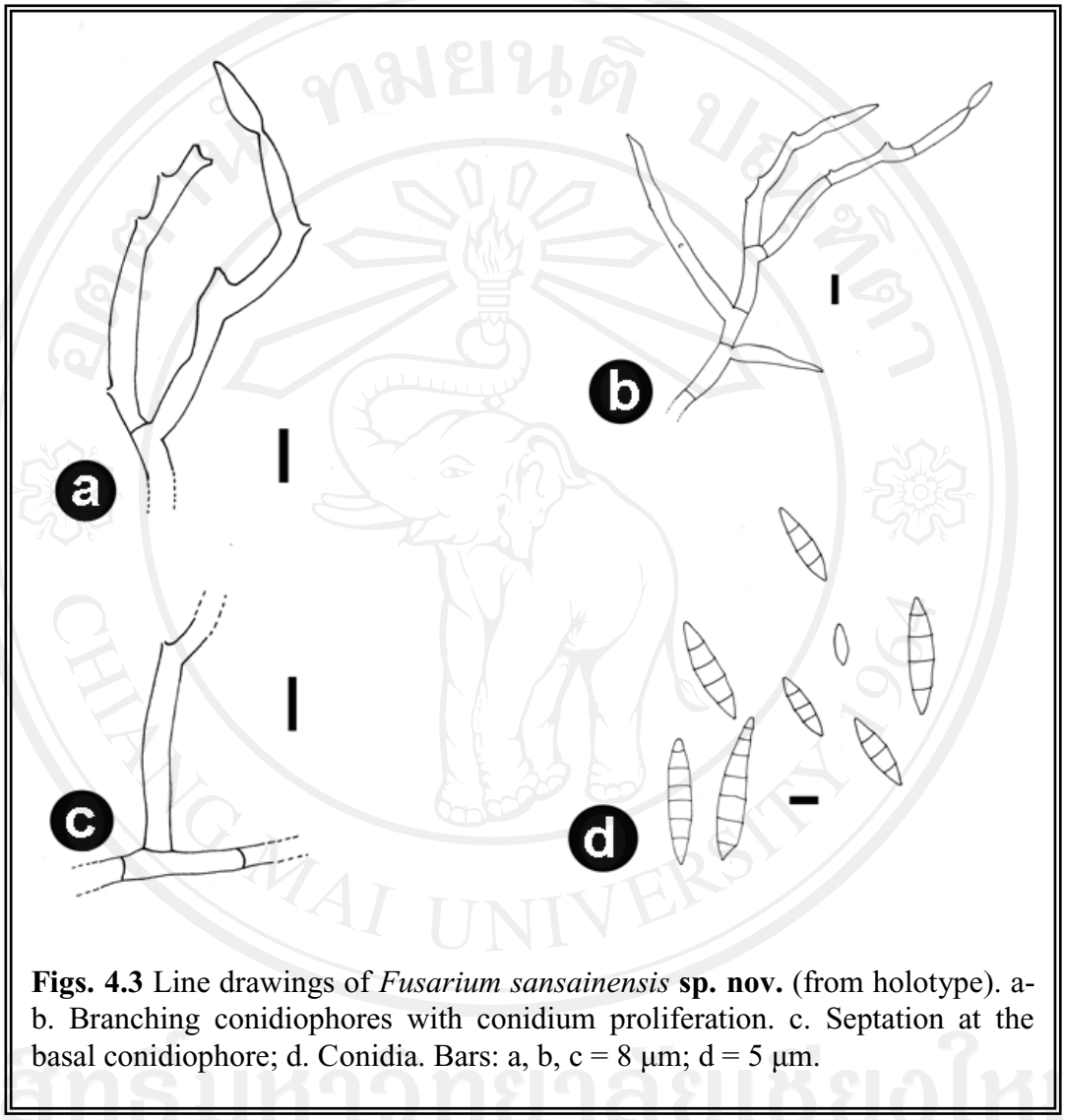
Fig. 4.2 Single parsimonious tree based on ITS nrDNA sequence data representing placement of *Cercospora arecacearum* within representatives of the family Mycosphaerellaceae. The tree is obtained from heuristic search with 1000 random taxon addition of the sequences alignment. Bootstrap values (>50%) from 1000 replicates of Unweighted Maximum Parsimony (UMP) analyses are shown above internodes.

4.3.2. ***Fusarium sansainensis*** Hidayat & To-anun, **sp. nov.**

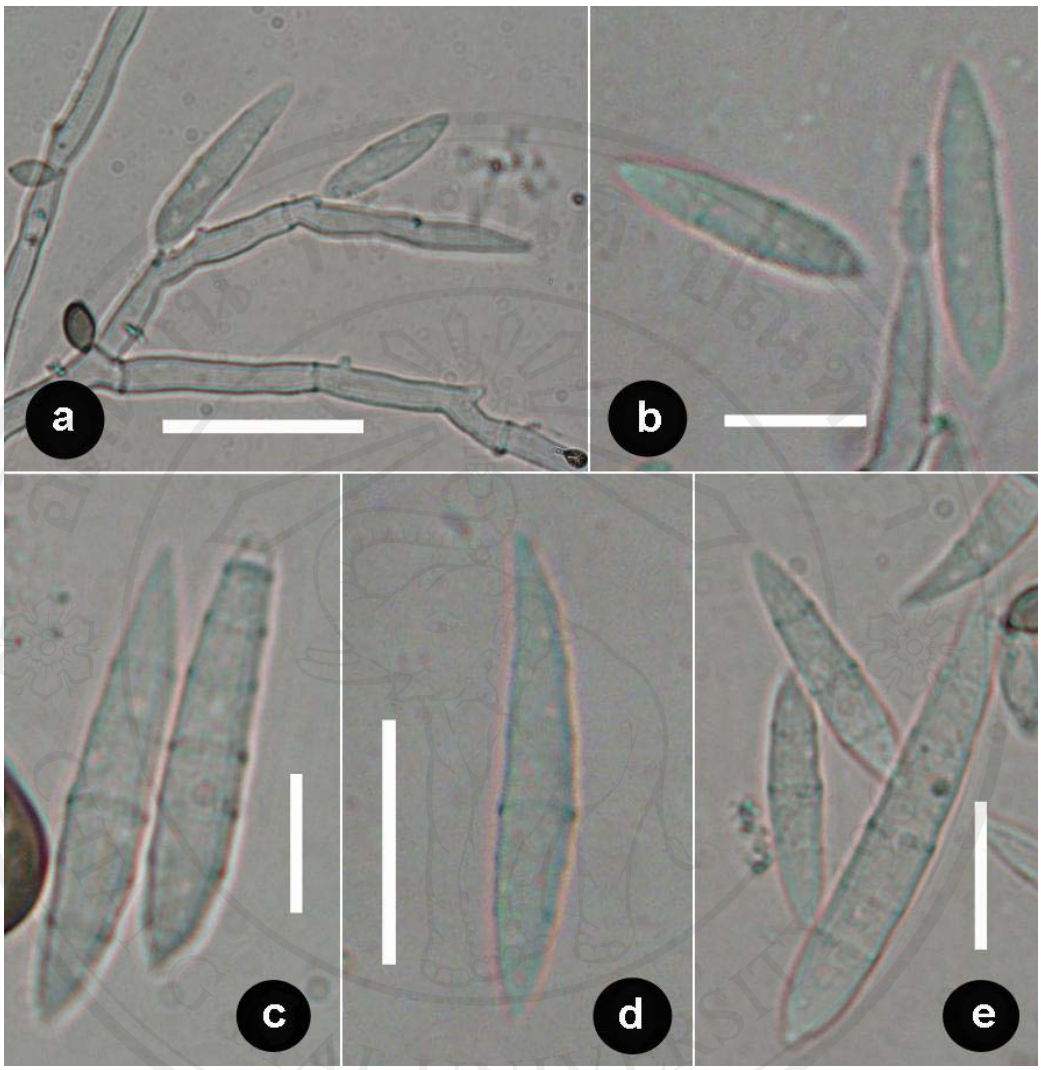
(Figs. 4.3; 4.4)

Coloniae albidae, effusae. Myceliae plerumque superficiale, hyalinae, septatis, ramosis, laevibus, hyalinis, (2) 3 ± 0.7 (4) µm latis, compositum. Conidiophora ex cellulis superficialis hypharum mycelialium inflatis lateraliter oriunda, mononematica, macronematica, hyalina, laevia, septata, recta, ramosa, (40) 182.7 ± 92.2 (320) × (3) 4.2 ± 0.7 (5) µm, apicale coangustare, cellulae conidiophorae (10) 20.1 ± 8.1 (30) × (3) 4.1 ± 0.7 (5) µm. Cellulae conidiogenae polyblasticae, discretae, laevia, sympodiales proliferatibus, denticulus cylindricis manifestis, (1) 2.2 ± 0.7 (3) × (0.5) 1.3 ± 0.5 (2) µm praeditae. Conidia hyalina, obclavatis vel obpyriformis, inequilaterale, recta, laevia, 3-6-septata (plerumque 3-septata), rarenter aseptata vel 7-septata, septa leviter compressa, apice abtusus, (12) 28.7 ± 12.1 (47) × (4) 5.9 ± 1.4 (8) µm, basi reliquiis cellulis conidiogenis praeditae.

Etymology: Refers to the place where the specimen was collected.



Figs. 4.3 Line drawings of *Fusarium sansainensis* **sp. nov.** (from holotype). a-b. Branching conidiophores with conidium proliferation. c. Septation at the basal conidiophore; d. Conidia. Bars: a, b, c = 8 µm; d = 5 µm.



Figs. 4.4 Micrographs of *Fusarium sansainensis* **sp. nov.** (from holotype). a. Branching conidiophores with distinct denticulates. b. Conidium proliferation. c-e. Conidia. Bars: a = 25 µm; b-c = 10 µm; d = 20 µm; e = 15 µm.

Colonies on the host surface pale, whitish, effuse. ***Mycelium*** generally superficial, composed of hyaline, septate, branched, smooth-walled hyphae, (2) 3 ± 0.7 (4) µm wide (n = 30). ***Conidiophores*** arising as lateral branches from swollen cells of the superficial mycelial hyphae, mononematous, macronematous, hyaline, smooth-walled, septate, erect, straight, branched, (40) 182.7 ± 92.2 (320) × (3) 4.2 ± 0.7 (5) µm (n = 30), narrowing to the apex, conidiophore cells (10) 20.1 ± 8.1 (30) ×

(3) 4.1 ± 0.7 (5) µm (n = 30). ***Conidiogenous cells*** polyblastic, discrete, smooth, sympodial proliferation, with conspicuous cylindrical denticles, (1) 2.2 ± 0.7 (3) × (0.5) 1.3 ± 0.5 (2) µm (n = 30). ***Conidia*** hyaline, obclavate to obpyriform, inequilateral, straight, smooth, thin-walled, 3-6-septate (mostly 3-septate), rarely aseptate and 7-septate, slightly constricted at the septum, obtuse at the apex, (12) 28.7 ± 12.1 (47) × (4) 5.9 ± 1.4 (8) µm (n = 30), with some remains of conidiogenous cells attached at the base.

Colonies on PDA fast growing, woolly, frequently growing in "blooms", pale to white, becoming yellowish to light brown centrally with the production of abundant chlamydoconidia. Reverse tan to brown.

Matreial examined: THAILAND, Chiang Mai Province, Mae Jo, Sansai, Palm Nursery, on leaflet spots of *Chamaedorea metallica* O. F. Cook and H. E. Moore (Arecaceae), 25 October 2006, Iman Hidayat and Jamjan Meeboon (**Holotype**: FIH 405).

Host: On leaf spot of *C. metallica*.

Distribution: Chiang Mai, Thailand.

Notes: The genus *Fusarium* Link (1809), now is approaching its third century as a genus that contains many pathogens. The members of this genus can incite directly diseases in plants, humans and domesticated animals (Desjardins, 2006). These fungi also may grow as apparently symptomless endophytes under many conditions. In plant pathology, for example, *Fusarium* plant diseases have had several major impacts, e.g. epidemics of *Fusarium oxysporum* f. sp. *ciceris* Matuo and K. Sato on chickpeas (*Cicer arietinum*) (Bateman *et* al., 1996) and *Fusarium* spp.

associated with *Gossypium* species which are threatening cotton production in Australia (Shivas, 1989).

Many plants have at least one *Fusarium*-associated disease (Leslie and Summerell, 2006). It is supported by data of the plant disease list maintained by the American Phytopathological Society (www.apsnet.org/online/common/search.asp) that insisted over 81 of the 101 economically important plants on the list had at least one associated *Fusarium* disease. However, as a genus, *Fusarium* lacks a large number of morphological characters that can be used to easily differentiate species. Thus, identifying the *Fusarium* strain present in diseased plants, usually to species level and sometimes further, has been and remains and important task in many pant diagnostic laboratories. Later, Leslie and Summerell (2006) revealed that the number of species of *Fusarium* exceeds 80 species. Although many morphological species concepts have changed, fortunately many have remained stable, even though tested by genetic and molecular criteria. The use of molecular approaches to differentiate species has been tried with a number of strains usually considered problematic and its providing new means of evaluating relatedness.

On plant family Arecaceae, according to the USDA fungal-host databases (http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm), approximately 28 species of the genus *Fusarium* have been recorded associated with various species of palms worldwide. Of them, seven species, namely, *F. chlamydosporum* Wollenw. & Reinking, *F. moniliforme* J. Sheld., *F. oxysporum* Schltdl., *F. pallidoroseum* (Cooke) Sacc., *F. roseum* Link, *F. semitectum* Berk. & Ravenel, *F. solani* (Mart.) Sacc. and *F. subglutinans* (Wollenw. & Reinking) P. E. Nelson, Toussoun & Marasas, are associated with palm genus *Chamaedorea*.

Based on morphological characteristics examination of *Fusarium sansainensis* with other closely related *Fusarium* species, *F. sansainensis* is much closer to *F. denticulatum* Nirenberg and O'Donnell due to denticulation of the conidiogenous cells and conidia (Nirenberg and O'Donnell, 1998; Leslie and Summerell, 2006). However, *F. sansainensis* is easily differentiated from *F. denticulatum* in having obclavate macroconidia and being recorded from palms (*F. denticulatum* is associated with *Ipomoea batatas*) (Nirenberg and O'Donnell, 1998). Leslie and Summerell (2006) insisted that the denticulation of conidiogenous cells and conidial shape as well as conidial septation are important characters of differentiating species within genus *Fusarium* which exceeds about 80 species. The morphological differences between *F. sansainensis* and *F. denticulatum* are supported by the molecular phylogenetic analysis study of ITS+5.8S rDNA regions (fig. 4.5). In this phylogenetic analysis, several other anamorphic and teleomorphic taxa of family Nectriaceae were included.

The molecular phylogenetic analysis of ITS DNA region of *F. sansainensis* with related taxa within family Nectriaceae was carried out in order to confirm the morphological elucidation, particularly members of *Fusarium* species. The ITS sequence of *F. sansainensis* was aligned with 54 sequences of species of *Fusarium*, *Calonectria* De Not., *Cylindrocladium* Morgan, *Glionectria* Crous & S. L. Schoch, *Leuconectria* Rossman, Samuels & Lowen, *Nectricladiella* Crous & S. L. Schoch, *Neocosmospora* E. F. Sm., *Neonectria* Wollenw., *Volutella* Fr., *Xenocalonectria* Crous & S. L. Schoch and *Xenocylindrocladium* Decock, Hennebert & Crous. Sequences of *Trichoderma longibrachiatum* Rifai and *Hypocrea orientalis* Samuels & Petrini were assigned as outgroup.

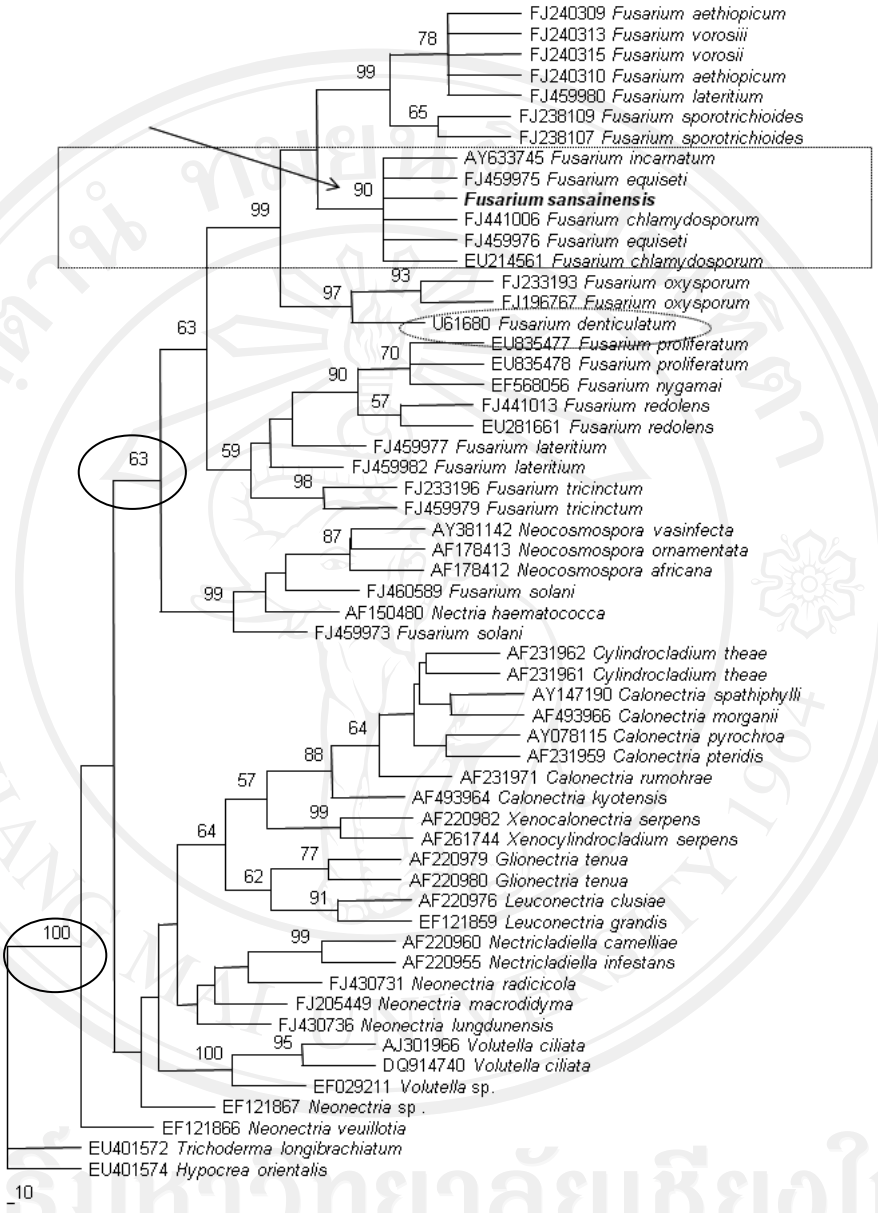


Fig. 4.5 The best parsimonious tree based on ITS nrDNA sequence data representing placement of *Fusarium sansainensis* within representatives of the family Nectriaceae. The tree is obtained from heuristic search with 1000 random taxon addition of the sequences alignment. Bootstrap values (>50%) from 1000 replicates of Unweighted Maximum Parsimony (UMP) analyses are shown above internodes.

The alignment data matrix consists of 57 taxa and 528 characters, of which 154 characters were excluded from the analysis due to ambiguity of the alignment. Of the remaining 374 included characters, 247 characters were constant, 12 characters were variable and parsimony-uninformative and 115 characters were parsimony-informative. Sum of minimum possible length was 199 and sum of maximum possible length was 1628. Of the 92 parsimony trees generated from the analysis, the best parsimonious tree selected by KH test ($P < 0.05$) was generated in 393 steps (CI = 0.5, RI = 0.8, RC = 0.4, HI = 0.5).

In general, the phylogenetic tree generated from the analysis showed the monophyletic of member in family Nectriaceae with 100 % Bootstrap support (fig.4.5). The member of genus *Fusarium* are nested together to form a monophyletic clade (63% Bootstrap support). In this large clade, the member of *Fusarium* showed anamorph-teleomorph connection with *Nectria* and *Neocosmospora* teleomorphs as previously also reported by Nirenberg and Samuels (2000). Recently, the genus *Neocosmospora* is linked with *Acremonium* anamorph, but lacking of phylogenetic analysis. Leslie and Summerell (2006) also reported *Fusarium* has a close relationship with *Gibberella*, *Nectria* and *Albonectria* teleomorphs.

A new species, *Fusarium sansainensis*, nested together with *F. chlamydosporum* Wollenw. & Reinking, *F. equiseti* (Corda) Sacc. and *F. incarnatum* (Desm.) Sacc., to form a small monophyletic clade with 90% bootstrap support. On the other hand, *F. denticulatum*, another denticulate species, is nested together with *F. oxysporum* Schltdl. (97% Bootstrap support). Therefore, this result supported the morphological distinction between *F. sansainensis* and *F. denticulatum* as described earlier.

The following is a key to the species of *Fusarium* within *Gibberella fujikuroi* (teleomorph) complex (Nirenberg and O'Donnell, 1998) with a modification and addition of *F. sansainensis*:

1a. 0- to 1-septate oval conidia without foot cell produced on the agar surface, never in the aerial mycelum …… ***F. bactridioides***

1b. 0- to 5-septate conidia without foot cell produced in the aerial mycelium … **2**

2a. Chlamydospores produced within 14 days in the dark … **3**

2b. Chlamydospores not produced within 14 days in the dark … **9**

3a. Chlamydospores mostly lateral or terminal, borne singly or in pairs, rarely in clusters, polyphialides absent … **section *Elegans***

3b. Chlamydospores mostly intercalary, typically borne in chains or clusters, polyphialides sometimes present … **4**

4a. Conidia forming chains and false heads in the aerial mycelium … **5**

4b. Conidia not forming chains in the aerial mycelium … **7**

5a. Some pyriform conidia are produced … **6**

5b. No pyriform conidia produced … ***F. nygamai***

6a. Clavate and pyriform conidia produced in long linear chains (> 30 conidia) on monophialides … ***F. napiforme***

6b. Obovoid and pyriform conidia produced, sometimes in short false chains (< 15 conidia) on mon- and polyphialides … ***F. pseudoanthophilum***

7a. Conidiophores often branched, each branch often ending with polyphialides ….. ***F. pseudoanthophilum***

7b. Conidiophores rarely branched, rarely forming polyphialides … **8**

8a. Sporodochial conidia mostly 3-septate with an uncinate apical and basal cell, never producing pyriform conidia … ***F. udum***

8b. Sporodochial conidia mostly 3-septate with an acute apical cell, never producing pyriform conidia in the aerial mycelium … ***F. acutatum***

8c. Sporodochial conidia mostly 5-septate with a slightly beaked apical cell, occasionally producing pyriform conidia in the aerial mycelium … ***F. dlaminii***

9a. Coiled sterile hyphae formed in and on the agar … **10**

9b. Coiled sterile hyphae not formed … **11**

10a. Conidia aggregated in false head, never in chains … ***F. circinatum***

10b. Conidia aggregated in false head and in short false chains (< 15 conidia) when cultivated under continuous black light … ***F. pseudocircinatum***

11a. Conidia adhering in chains and false head … **12**

11b. Conidia adhering only in false head, chains absent … **24**

12a. Conidia only borne on monophialides … **13**

12b. Conidia borne on mono- and polyphialides … **14**

13a. Conidia borne on conidiophores that often terminate verticillately with 3 phialides, cosmopolitan on numerous plant hosts, especially cereals …….. ***F. verticillioides***

13b. Conidia borne on conidiophores that usually terminate verticillately with 4 phialides, culture typically produce slimy dark violet plaques on PDA within 5 days, pathogenic on *Sorghum* spp. … ***F. thapsinum***

14a. Pyriform and clavate conidia produced in chains … **15**

14b. Pyriform conidia not produced … **16**

15a. Polyphialides frequent, sporodochia produced … ***F. proliferatum***

15b. Polyphialides rare, sporodochia not produced … ***F. nisikadoi***

16a. Globose conidia produced singly or in botryose clusters … ***F. globosum***

16b. Globose conidia not produced ... **17**

17a. Polyphialides rare, sympodially proliferating conidiophores common … **18**

17b. Polyphialides usually abundant, sympodially proliferating conidiophores rare …….. **19**

18a. Clavate conidia mostly 0-3-septate, sporodochial conidia up to 7-septate, on *Phyllostachis* and *Tritichum* … ***F. nisikadoi***

18b. Clavate conidia mostly 0-septate, rarely 1-septate, sporodochial conidia typically 3-5-septate, causing gibberellins induced bakanae disease on *Oryza sativa* … ***F. fujikuroi***

19a. Conidia adhering in short (< 15 conidia) to medium length chains (15-30 conidia) … **20**

19b. Conidia adhering in long chains (< 30 conidia) … **23**

20a. Conidial chains produced under any light condition … **21**

20b. Conidial chains produced either in the dark or under black light … **22**

21a. Conidial chains zigzaglike, of short (< 15 conidia) or medium length chains (15-30 conidia), causing fruits endosepsis in *Ficus carica* … ***F. lactis***

21b. Conidial chains linear, of short (< 15 conidia) or medium length chains (15-30 conidia), pathogenic to *Pennisetum typhoides* … ***F. pseudonygamai***

22a. Short (< 15 conidia) linear conidial chains formed abundantly only in the dark, pathogenic to *Sansevieria*, *Gasteria*, and *Dracaena* spp. … ***F. phyllophylum***

22b. Short (< 15 conidia) false conidial chains formed only under continuous black light … ***F. brevicatenulatum***

23a. Sporodochial conidia almost straight … ***F. proliferatum***

23b. Sporodochial conidia strongly recurved, some ring-shaped … ***F. annulatum***

24a. Conidiophores mainly erect, branched … **25**

24b. Conidiophores mainly prostrated, mostly branched … **28**

25a. Pyriform conidia produced … ***F. anthophilum***

25b. Pyriform conidia not produced … **26**

26a. Conidia oval to fusoid, mostly 0-3-septate, pathogenic to *Zea mays* …… ***F. subglutinans***

26b. Conidia obovoid or oval, mostly 0-septate … **27**

27a. 0-septate conidia mostly obovoid, pathogenic to *Ananas comosus* …… ***F. guttiforme***

27b. 0-septate conidia mostly ova, pathogenic to *Nerine*, *Vallota*, *Haemanthus* flowr bulbs … ***F. bulbicola***

28a. Conidiophores branched at two levels, pathogenic to *Ficus carica* …… ***F. ramigenum***

28b. Conidiophores branched at one levels … **29**

29a. Sporodochial conidia present, strongly recurved, some ring-shaped, pathogenic to *Succisa pratensis* … ***F. succisae***

29b. Sporodochial conidia absent, or when present almost straight … **30**

30a. Pyriform conidia produced, pathogenic to *Saccharum officinarum* ……. ***F. sacchari***

30b. Pyriform conidia not produced … **31**

31a. Conidiogenous cells denticulate … **32**

31b. Conidiogenous cells not denticulate … **33**

32a. Conidia falcate, but not obclavate, pathogenic to *Ipomoea batatas* …… ***F. denticulatum***

32b Conidia obclavate to pyriform, pathogenic to *Chamaedorea metallica* ……..… ***F. sansainensis*** **Hidayat & To-anun**

33a. Sporodochia not formed in wild-type cultures, pathogenic to *Saccharum officinarum,* causing pokkah boeng disease … ***F. sacchari***

33b. Sporodochia formed in wild-type cultures … **34**

34a. Sporodochial conidia mostly 3-5-septate, on *Musa sapientum* ……. ***F. concentricum***

34b. Sporodochial conidia mostly 3-septate, pathogenic to *Begonia* spp. ……. ***F. begonia***