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Seed decaying Dothideomycetes in Thailand: *Zeloasperisporium pterocarpi* sp. nov., (Zeloasperisporiaceae, Zeloasperisporiales) on carpel of *Pterocarpus* sp. (Fabaceae) seed pod

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Abstract

In this study we introduce a new species, *Zeloasperisporium pterocarpi*, (Zeloasperisporiaceae) based on its morphology and LSU and SSU gene sequence data. The new species is similar to other known *Zeloasperisporium* spp. but differs from its close relative *Z. siamemse* in having a mycelium and granular spores. Multigene phylogenetic analysis also confirmed its novelty. We also provide ITS, *rpb2* and *tef1* sequence data for *Zeloasperisporium pterocarpi*, which are deposited in GenBank.

Keywords – 1 new species – granular – multigene phylogeny – mycelium – phylogenetic analysis

Introduction

Many fungal diversity studies have investigated various plant substrata, however, few have focused on fruits and seeds (Somrithipol et al. 2002), and therefore we have initiated a study of fungal diversity on fruits and seeds exposed on the natural forest floor. This is the third paper studying Dothideomycetes of decaying seeds and fruits (Jayasiri et al. 2017a, b). Dothideomycetes comprise 33 orders and 175 families with huge diversity on various hosts, ecosystems as well as in different parts of plants (Hyde et al. 2013, Wijayawardene et al. 2017, 2018).

Crous et al. (2015) introduced the new family Zeloasperisporiaceae, comprising the genera *Neomicrothyrium* and *Zeloasperisporium*. Both of these genera had not previously been linked taxonomically, but share few similar morphological characteristics. Crous et al. (2015) referred Zeloasperisporiaceae to the order Natipusillales. The order Zeloasperisporiales was introduced by Hongsanan et al. (2015) based on morphology, phylogeny and habitat differences with Natipusillales as a sister order. Based on their studies *Zeloasperisporium* is identical to *Neomicrothyrium*, therefore the genus *Neomicrothyrium* is synonymized under *Zeloasperisporium*. In this study we found another isolate of *Zeloasperisporium* from Thailand on a decaying seed pod of *Pterocarpus* sp.

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Material and Methods

Sample collection and specimen examination

The specimens were collected from at Chiang Rai, Thailand in 2016. Pods collected were brought to the laboratory and observed using a Motic SMZ 168 Series microscope. Hand sections of fruiting structures were mounted in water for microscopic studies and photomicrography. The fungus was examined with a Nikon ECLIPSE 80i compound microscope and photographed with a Canon 450D digital camera connected to the microscope. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for the figures were processed with Adobe Photoshop CS6 Extended version 10.0 software (Adobe Systems, USA). Isolations were made from single ascospores, following a modified method of Chomnunti et al. (2014). Cultures were incubated to study asexual structures.

Voucher specimens were deposited in the herbarium of Mae Fah Luang University (Herb. MFLU) and Kunming Institute of Botany Academia Sinica (HKAS). The living cultures were deposited in the culture collection of Mae Fah Luang University (MFLUCC), Thailand with duplicates in Kunming Institute of Botany Culture Collection (KMUCC). Faces of fungi and IF numbers were obtained as in Jayasiri et al. (2015) and Index Fungorum (2018).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the growing mycelium after 60 days on MEA at 18°C using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (Hangzhou, P.R. China). DNA amplifications were performed by Polymerase Chain Reaction (PCR). The partial large subunit nuclear rDNA (LSU) was amplified with primer pairs LROR and LR5 (Vilgalys & Hester 1990). The partial small subunit nuclear rDNA (SSU) was amplified with primer pairs NS1 and NS4 (White et al. 1990). The amplification procedure was carried in a 25 µl reaction volume containing 2 µl DNA, 12.5 µl PCR mix, 8.5 µl distilled water 1 µl of each primer. The PCR reactions for amplification of LSU, and SSU were performed under standard conditions (White et al. 1990). Purification and sequencing of PCR products were carried at Shanghai Sangon Biological Engineering Technology and Services Co. (China).

Sequence alignment and phylogenetic analysis

Sequences generated from the LSU and SSU gene regions were carefully verified before further analyses. Multiple sequence alignments were produced with MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html) and further improved manually where necessary. All introns and exons were aligned individually. Ambiguously aligned regions with many leading or trailing gaps were excluded in alignments prior to tree building.

The final phylogenetic tree used to infer the taxonomic placement of our new taxon was generated based on DNA sequence analyses of a concatenated dataset of LSU and SSU. A maximum likelihood analysis was performed at CIPRES using RAxML v.7.2.8 as part of the "RAxMLHPC2 on TG" tool (Stamatakis et al. 2008, Miller et al. 2010). The general time reversible model (GTR) using proportions of invariable sites were applied with a discrete gamma distribution and four rate classes. The best scoring tree was selected with a final likelihood value of -32505.313425. Maximum likelihood bootstrap support (MLBS) equal or greater than 70% are given near to each node (Fig. 1).

The model of evolution was performed using jModeltest 2.1.7 (Guindon & Gascuel 2003, Darriba et al. 2012). GTR + I + G model were the best-fit model of each locus for Bayesian analysis and maximum likelihood as determined by MrModeltest. Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. MCMC heated chain was set with a "temperature" value of 0.2. All sampled topologies beneath the asymptote (20%) were discarded as part of a burn-in procedure; the remaining trees were used for

calculating posterior probabilities in the majority rule consensus tree. Bayesian Posterior Probabilities (BP) equal or greater than 0.95 is given near to each node (Fig. 1). Phylogenetic trees were drawn using FigTree v. 1.4 (Rambaut & Drummond 2008). The sequences of novel species is deposited in GenBank (Appendix 1) and the final matrices used for phylogenetic analyses were saved in TreeBASE (ID 23196).

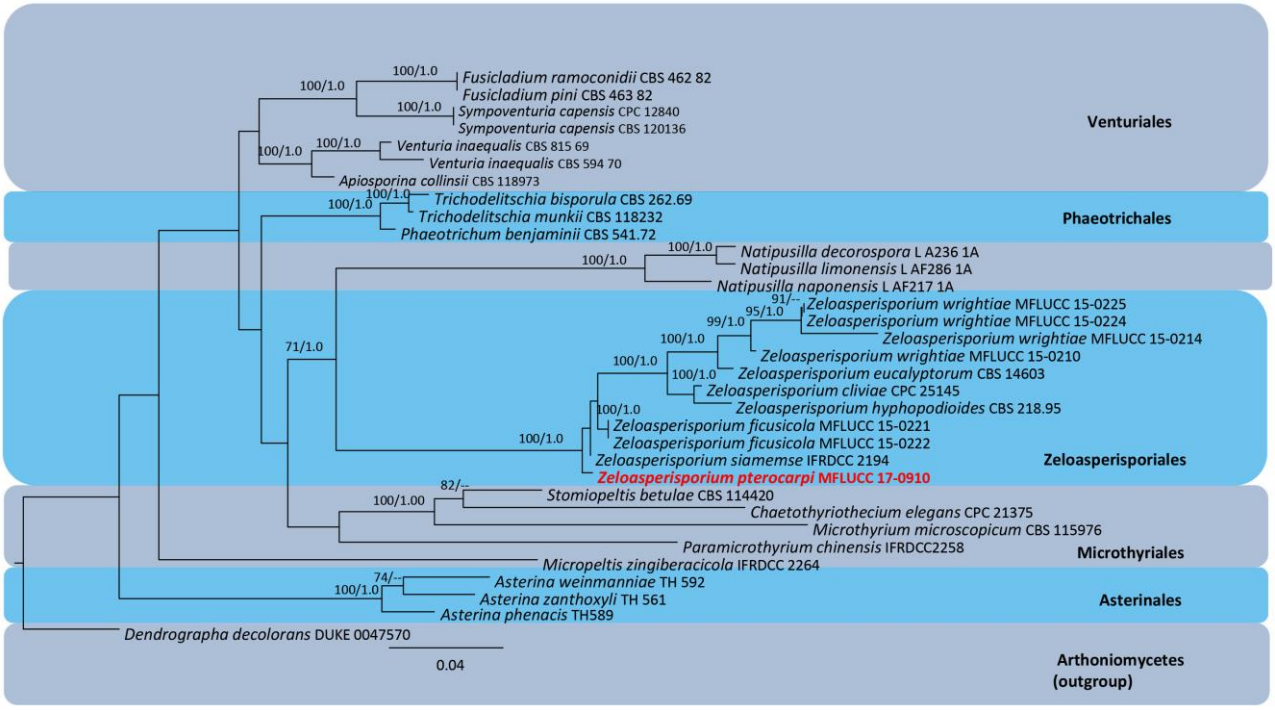

Fig. 1 – Simplified phylogram showing the best RAxML maximum likelihood tree obtained from the combined multigene (LSU and SSU) matrix of 32 taxa including the orders Asterinales, Microthyriales, Natipusillales, Phaeotrichales and Venturiales. MLBS above 70 % and Bayesian posterior probabilities above 0.95 are given at each branch. The tree is rooted with *Dendrographa decolorans* (DUKE 0047570). The type species of each genus are in bold and the new isolate is in bold and blue.

Results

Phylogenetic analyses

Multiple genes (LSU and SSU) were used for the phylogenetic analysis. The topologies of the obtained trees for each gene were compared manually, to verify that the overall tree topology of the individual datasets was congruent with the tree obtained from the combined alignment. The Bayesian analyses showed similar tree topologies and were congruent to those obtained in the ML analysis. The combined gene analysis of (LSU and SSU) sequence data representing the taxa including the orders Asterinales, Microthyriales, Natipusillales, Phaeotrichales and Venturiales is shown in Fig. 1, which included 32 strains, representing 25 species and consisted of 2065 characters. *Dendrographa decolorans* (DUKE 0047570) is the out group taxon. The Bayesian analysis resulted in 8000 trees after 1,000,000 generations. The first 2000 trees, representing the burn-in phase of the analyses were discarded, while the remaining tree was used for calculating posterior probabilities in the majority rule consensus tree and is shown in Fig. 1. A best scoring RAxML tree resulted with the value of likelihood: -32505.313425. Phylogenetic trees obtained from ML and Bayesian analysis yielded trees with similar overall topology at the species level and in agreement with previous studies based on maximum likelihood and Bayesian analysis (Hongsanan et al. 2015). The new strain of *Zeloasperisporium* species clusters with other species of this genus. It forms a sister clade to *Zeloasperisporium siamemse* IFRDCC 2194 with high

statistical support (MLBS 100%, BPP 1.0). Therefore, the new strain is introduced as a new species in the genus *Zeloasperisporium.*

Taxonomy

Zeloasperisporium pterocarpi Jayasiri, E.B.G. Jones & K.D. Hyde, sp. nov. Figs 2, 3

Index Fungorum number: IF555358; Facesoffungi number: FoF04885

Etymology – "*pterocarpi"* referring to the host genus which the taxon was found.

Holotype **–** MFLU 18–1506

Saprobic on the carpel of fallen pod of *Pterocarpus* sp. forming minute black spots. *Mycelium* present (Fig. 2d). Sexual morph: *Thyriothecia* 140–150 μm diam, superficial, coriaceous, solitary or scattered, appearing as circular, scattered, flattened, brown to dark brown spots, upper wall composed of ellipsoid angular cells, arranged in parallel radiating lines from the center to the outer rim covering the host, with a poorly developed basal layer and an irregular margin. Outer cell layers darker than central cell layers, ostioles lacking. *Hamathecium* of parallel asci in mucilage,

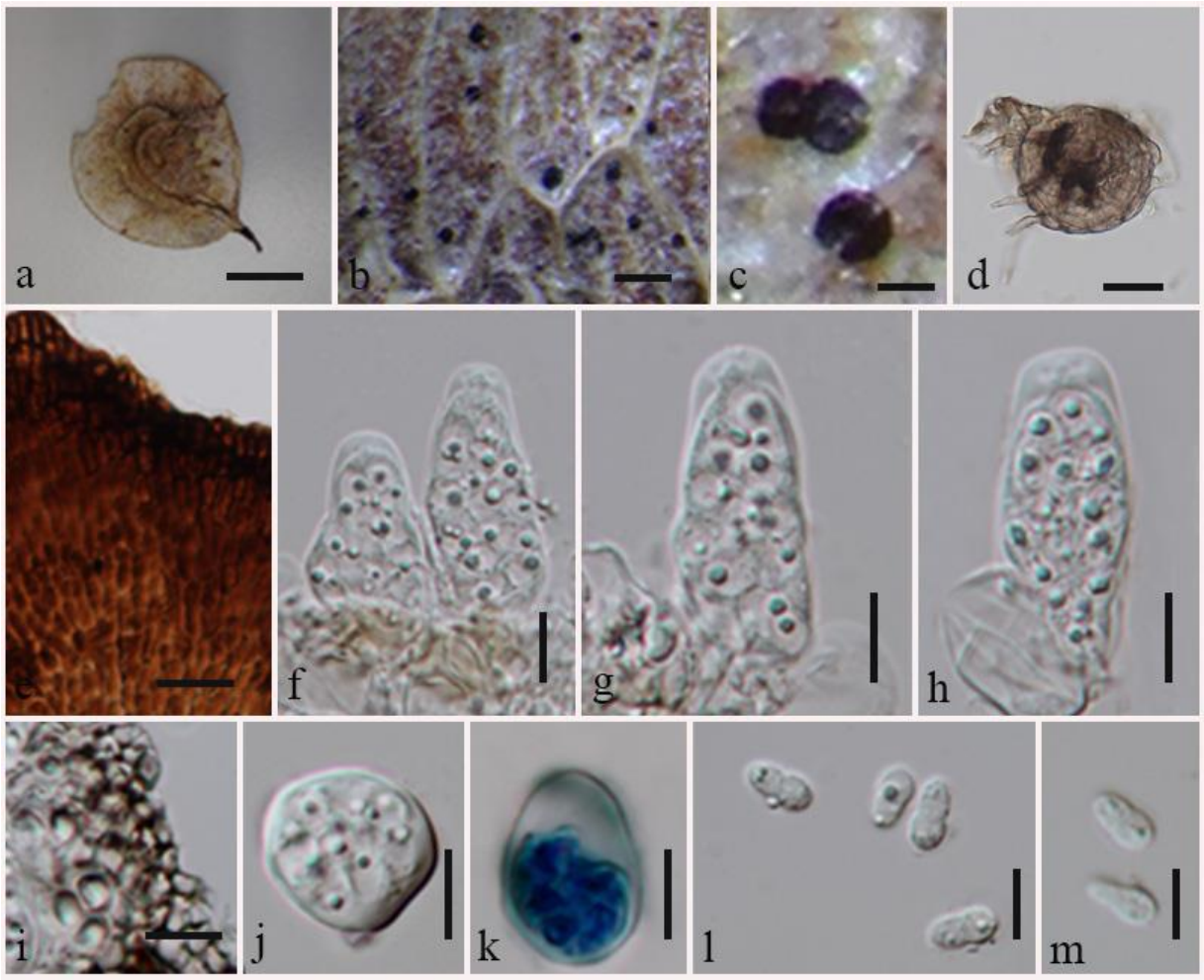

Fig. 2 – *Zeloasperisporium pterocarpi* (holotype 18**–**1506). a Host of *Pterocarpus* sp. pod. b, c, d Superficial thyriothecia on substrate. Squash mounts showing thyriothecial walls. f-h, j, k Asci. i Peridium. l, m Ascospores. Scale bars: b = 500 μm, c, d = 200 μm, e = 20 μm, f-i = 10 μm, h-k = 20 μm, j-m = 10 μm.

pseudoparaphyses not observed. *Asci* 16–36 × 15–9 μm ($\bar{x}$ = 23 × 12 μm, n = 20), 8-spored, bitunicate, subglobose to broadly obpyriform, apedicellate, straight, with small ocular chamber,

thick outer wall. *Ascospores* 7–10 × 3.1–4.3 µm ($\bar{x}$ = 9.1 × 3.6 µm, n = 20), irregularly arranged, overlapping in the ascus, hyaline, oval to obovoid with obtuse ends, 1-septate, two size cells, with granular appearance. Asexual morph: *Hyphae* 2–3 µm diam., branched, septate, constricted at the septa, hyaline to pale brown, smooth-walled, *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 12–18 × 2–4 µm ($\bar{x}$ = 15 × 3.5 µm; n = 10), micronematous, arising as lateral hyphal branches, cylindrical to subcylindrical, straight or slightly curved, unbranched, slightly tapering towards the apex, brown, slightly thick-walled. Conidial proliferation sympodial, with one to several conidiogenous loci, mostly crowded at the apex, protuberant conidial scars thickened-refractive. *Conidia* 14–16 × 3–5 µm ($\bar{x}$ = 15 × 4.5 µm; n = 10), fusiform to obclavate or cylindrical, straight to curved, 1-septate, two different size cells, slightly constricted at the septum, somewhat thickened and darkened refractive, hyaline to pale brown, smooth-walled, many small granules.

Culture characteristics – Ascospores germinating on MEA at 18°C in the dark, hyphae emerging from both cells of the ascospores, septate, strongly constricted at the septa forming monilioid cells in culture, hyaline to brown initially, becoming dark brown to black, bluish reverse iron gray later. Slow growth, colonies reaching 1 cm diam. after 14 days on MEA at 18°C, colony superficial to erumpent, surface verrucose, velvety, difficult to remove, asexual morph structures was produced in MEA after 21 days incubation (Fig. 3).

Material examined – THAILAND, Chiang Rai (20° 2' 51" N, 99° 53' 43" E), on carpel of *Pterocarpus* sp. (Fabaceae) seed pod, 19 December 2016, Subashini C. Jayasiri, C 206 (MFLU 18–1506, holotype); (HKAS102412, isotype), ex-type living culture, MFLUCC 17–0910

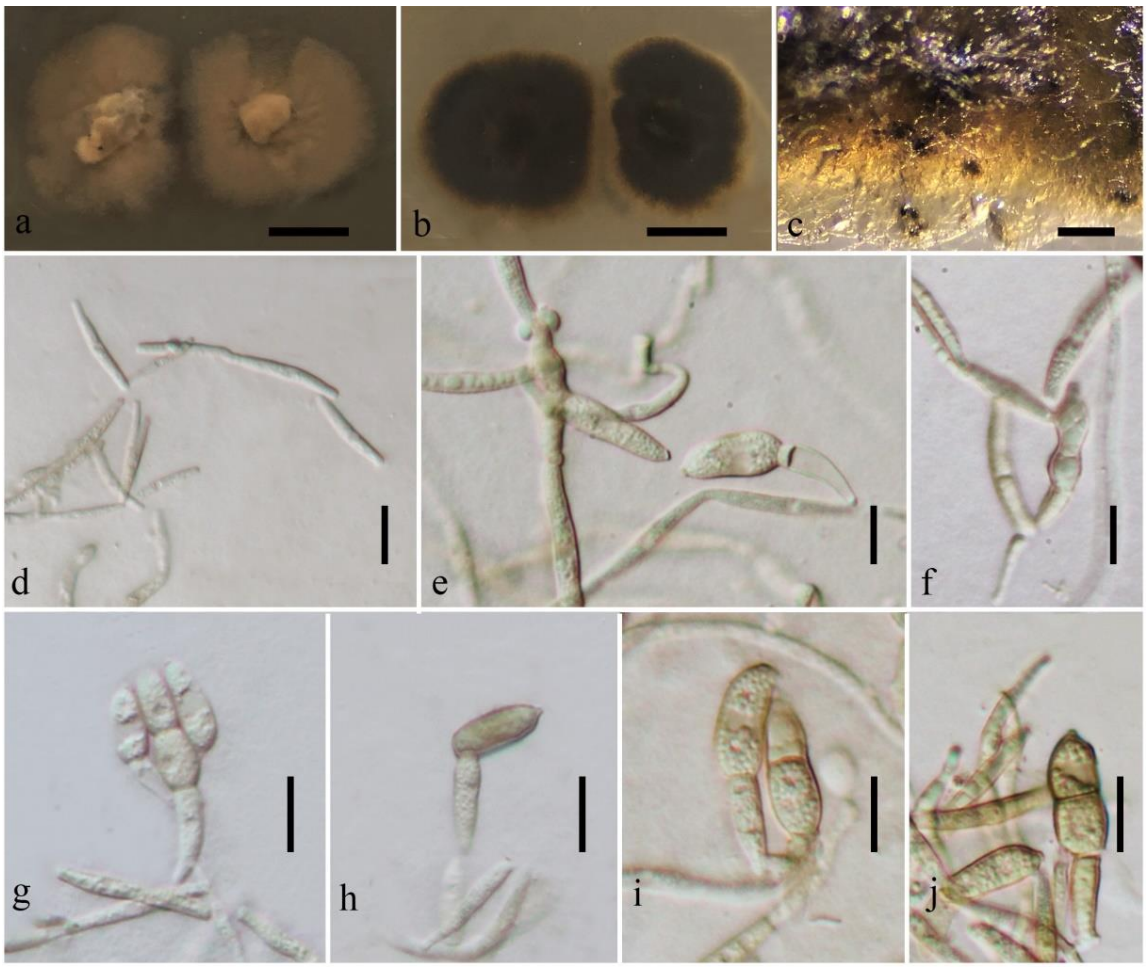

Fig. 3 – *Zeloasperisporium pterocarpi* (asexual morph from culture). a, b Colonies on MEA. d Conidia and conidiogenous cells in culture. d, e Micronematous conidiogenous cells. f-h Conidia on conidiogenous cell with sympodial proliferation. i, j Conidia with 1 septum. Scale bars: a, b = 1 cm, c = 500 µm, d-j = 10 µm.

Discussion

A new species was introduced in this study based on morphology and phylogeny of all known *Zeloasperisporium* spp. *Zeloasperisporium pterocarpi* fits with the generic concept of *Zeloasperisporium* in having superficial thyriothecia without ostiole, globose to ovoid or clavate, apedicellate asci and 1-septate, hyaline, asymmetric ascospores (Fig. 2). Our strain forms a sister clade to *Zeloasperisporium siamense* with high statistical support (MLBS 100, BPP 1.0, Fig 1). However, *Zeloasperisporium pterocarpi* shares different morphological features to *Z. siamense*. *Zeloasperisporium pterocarpi* differs from *Z. siamense* in having mycelial thyriothecia, cell wall with different strata and granular appearance ascospores. In addition, our strain was isolated from decaying seed pod of *Pterocarpus* sp. Based on Hongsanan et al. (2015), *Zeloasperisporium* species can be found on dead and living leaves in Thailand, and are frequently found on plants during the cold season (December to February). *Zeloasperisporium siamense* (Wu et al. 2011) has also been collected during this period. However, we found *Zeloasperisporium* species on decaying seed pod of *Pterocarpus* sp. (Fabaceae) in the same season and locality (Thailand). Thus, *Zeloasperisporium* species are distributed in different hosts as well as different parts of the plant and not only on leaves.

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Appendix 1 GenBank accession numbers used in this study. GenBank accessions marked in bold represent new sequences generated in the current study.

Species	**Strain**	**Accession numbers**	
		LSU	**SSU**
Apiosporina collinsii	CBS 118973	GU301798	GU296135
Asterina phenacis	TH589	GU586217	GU586211
A.weinmanniae	TH 592	GU586218	GU586212
A. zanthoxyli	TH 561	GU586219	GU586213
Chaetothyriothecium elegans	CPC 21375	KF268420	
Fusicladium pini	CBS 463.82	EU035436	
F. ramoconidii	CBS 462.82	EU035439	
Micropeltis zingiberacicola	IFRDCC 2264	JQ036227	JQ036222
Microthyrium microscopicum	CBS 115976	GU301846	GU296175
N. decorospora	L A236 1A	HM196369	HM196376
N. limonensis	L AF286 1A	HM196370	HM196377
N. naponensis	L AF217 1A	HM196371	HM196378
Paramicrothyrium chinensis	IFRDCC2258	KF636760	JQ036224
Phaeotrichum benjaminii	CBS 541.72	NG 057709	AY016348
Schimatomma decolorans	DUKE 0047570	NG 027622	
Stomiopeltis betulae	CBS 114420	GU214701	GU214701S
Sympoventuria capensis	CPC 12840	DQ885904	
S. capensis	CBS 120136	DQ885906	

Appendix 1 Continued.

Species	**Strain**	**Accession numbers**	
		LSU	**SSU**
Trichodelitschia bisporula	CBS 262.69	GU348996	GU296202
T. munkii	CBS 118232	DQ384096	DQ384070
Venturia inaequalis	CBS 815.69	GU301878	GU296204
V.inaequalis	CBS 594.70	GU301879	KF156093
Zeloasperisporium cliviae	CPC 25145	KR476781	KR476748
Z. eucalyptorum	CBS 14603	GQ303329	
Z. ficusicola	MFLUCC 15-0221	KT387733	KT387734
Z. ficusicola	MFLUCC 15-0222	KT387735	KT387736
Z. hyphopodioides	CBS 218.95	EU035442	
Z. pterocarpi	**MFLUCC 17-0910**	**MH763755**	**MH763756**
Z. siamemse	IFRDCC 2194	JQ036228	JQ036223
Z. wrightiae	MFLUCC 15-0210	KT387739	KT387743
Z.wrightiae	MFLUCC 15-0214	KT387741	KT387745
Z. wrightiae	MFLUCC 15-0224	KT387740	KT387744
Z. wrightiae	MFLUCC 15-0225	KT387737	KT387738
Z. cliviae	CPC 25145	NG058173	