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Ceratocystis polychroma sp. nov., a new species from *Syzygium aromaticum* in Sulawesi

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Abstract: Clove decline is the most serious disease affecting *Syzygium aromaticum* in North Sulawesi, Indonesia. The aetiology of this disease has never been established. Diseased *S. aromaticum* trees show symptoms of wilt and defoliation. Clove decline was found to affect 20–80 % of trees at 18 sites investigated during a recent survey of the disease. Dying trees are typically infested with the woodborer *Hexamitodora semivelutina*. Larval tunnels are associated with extensive discolouration of the xylem, which has a streaked appearance. Isolations from discoloured wood and larval galleries consistently yielded a *Ceratocystis* sp. morphologically similar to *C. fimbriata*. Comparisons of DNA sequence data for three gene regions showed that this *Ceratocystis* sp. resides in a clade distinct from *C. fimbriata* or any other *Ceratocystis* sp. It can also be distinguished from other *Ceratocystis* spp. based on colony morphology and a distinct ecology. We, therefore, describe it as a new taxon, *C. polychroma* sp. nov.

Taxonomic novelty: *Ceratocystis polychroma* M. van Wyk, M.J. Wingf. & E.C.Y. Liew sp. nov.
Key words: Coleoptera, Cerambycidae, die-back, DNA sequence comparisons, phylogeography, sap stain, woodborer.

INTRODUCTION

Cloves, produced from the unopened flower buds of the evergreen tree, *Syzygium aromaticum* L. Merr. & Perry (*Myrtaceae*) represent a commonly used spice and flavourant, worldwide (Nutman & Roberts 1971, Purseglove *et al*. 1981). The tree is indigenous to the Molucca Islands, but has been spread to many countries where it is now commercially cultivated. Most clove plantations are in developing countries, providing an important source of income to small-scale farmers. The trees flourish in tropical environments that are hot and humid with high annual rainfall (Nair 2000).

Syzygium aromaticum is affected by a number of pests and pathogens (Purseglove *et al*. 1981, Nair 2000). The best-known disease is Sumatra disease, which is caused by the bacterium *Pseudomonas syzygii* S.J. Roberts, Eden-Green, P. Jones & Ambler (Roberts *et al*. 1990). The woodborer, *Hexamitodera semivelutina* Hell. (Coleoptera: Cerambycidae) that infests living trees is the most serious insect pest of clove (Purseglove *et al*. 1981). This borer is particularly serious in Sulawesi, where it is commonly associated with extensive die-back of clove trees.

Although *H. semivelutina* is closely associated with clove die-back in Sulawesi, it has been hypothesised that this dramatic disease could be associated with other factors, including pathogens. A preliminary survey was conducted during September 2001 and December 2002, and isolations were made from symptomatic tissues (Fig. 1), especially those associated with woodborer damage. A *Ceratocystis* sp. was commonly found in the tunnels of *H. semivelutina* and consistently isolated from discoloured wood associated with the borer. This fungus was tentatively identified as *C. fimbriata* Ellis & Halst. (Liew *et al.* 2003) based only on morphological characteristics. The aim of this study was to identify the *Ceratocystis* sp. more comprehensively, based on morphology and DNA sequence comparisons.

MATERIALS AND METHODS

Fungal isolates

Diseased clove trees at 18 different locations in North Sulawesi were sampled during September 2001 and December 2002. These sites included Toliangoki, Lahendong, Leilum, Kiawa, Rumoong, Tumpaan, Munte, Kombi, Lalumpe, Tulap, Kakas, Tinoor, Kumelembuai, Motoling, Tambelang, Poopo, Koka, and Kembes.

Table 1. Isolates of *Ceratocystis* used in this study.

Species	Isolate no.[d]	Alternative no. [e, f]	GenBank accession no.	Date of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. albifundus	CMW 5329[a]	–	AF388947[g] –	1999	*Acacia mearnsii*	Uganda	–	J. Roux
	CMW 5943[a]	CBS 116323	–	2000	*A. mearnsii*	Uganda	–	J. Roux
C. fimbriata	CMW 2218[a,]	CBS 116351	AF395680[g] AY528974[i]	1991	*Platanus* sp.	France	–	C. Grosclaude
	CMW 2219[a]	–	AF395679[g] – AY528975[i]	1991	*Platanus* sp.	France	–	C. Grosclaude
C. pirilliformis	CMW 6569[a]	–	– – AY528982[i]	1991	*Eucalyptus nitens*	Australia	–	M.J. Wingfield
	CMW 6579[a]	–	– – AY528983[i]	1991	*E. nitens*	Australia	–	M.J. Wingfield
C. polychroma	CMW 11424[a,b,c]	PREM 57818 CBS 115778	AY528966[g] AY528970[h] AY528978[i]	2002	*Syzygium aromaticum*	Sulawesi, Indonesia	*Hexamitodera semivelutina*	E.C.Y. Liew & M.J.Wingfield
	CMW 11436[a]	PREM 57819 CBS 115777	AY528967[g] AY528971[h] AY528979[i]	2002	*S. aromaticum*	Sulawesi, Indonesia	*H. semivelutina*	E.C.Y. Liew & M.J. Wingfield
	CMW 11443[a, b,c]	PREM 57820 CBS 115776	–	2002	*S. aromaticum*	Sulawesi, Indonesia	*H. semivelutina*	E.C.Y. Liew & M.J. Wingfield
	CMW 11449[a,b,c]	PREM 57821 CBS 115775	AY528968[g] AY528972[h] AY528980[i]	2002	*S. aromaticum*	Sulawesi, Indonesia	*H. semivelutina*	E.C.Y. Liew & M.J. Wingfield
	CMW 11455[a]	PREM 57822 CBS 115774	AY528969[g] AY528973[h] AY528981[i]	2002	*S. aromaticum*	Sulawesi, Indonesia	*H. semivelutina*	E.C.Y. Liew & M.J. Wingfield
C. virescens	CMW 3276[a]	–	AY528984[g] AY528990[h] AY528991[i]	1963	*Quercus* sp.	U.S.A.	–	T. Hinds

[a]Isolates sequenced. [b]Isolates used for morphological descriptions.[c]Isolates included in the growth studies. [d]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. [e]CBS refers to the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. [f]PREM refers to the National Fungal Herbarium (PREM), Pretoria, South Africa. [g]GenBank accession numbers representing the ITS sequences. [h]GenBank accession numbers representing the β-tubulin sequences. [i]GenBank accession numbers representing the elongation factor 1-α sequences.

Trees were examined for signs of insect infestation and fungal infection. Larval tunnels were inspected on site, with the aid of a 10 × magnification hand lens, for fungal fruiting structures.

Adult and juvenile beetles, larvae and breeding galleries of *H. semivelutina*, as well as stem sections cut from infested trees were collected for further study. All samples were stored in plastic bags and transported to the laboratory. Dry bark samples were sprayed with distilled water and the bags sealed to create a moist environment, conducive to the sporulation of fungi. Reference specimens of *H. semivelutina* as well as larvae and dried specimens of the adults are maintained in the collection of the Department of Pests and Diseases, Faculty of Agriculture, University of Sam Ratulangi, Manado, North Sulawesi, Indonesia.

Ascomata typical of a *Ceratocystis* sp. commonly developed on wood samples and isolations were made directly from these structures. Isolations were also made from ascomata that formed on pieces of wood placed between carrots as described by Moller & DeVay (1968). Ascospore droplets at the apices of the ascomatal necks were transferred to 2 % malt extract agar (MEA) (20 % w/v) (Biolab, Midrand, South Africa). Ascospore masses were transferred from the primary isolation plates onto 2 % MEA supplemented with streptomycin sulphate (0.001 g/L (SIGMA, Steinheim, Germany) and thiamine (0.001 g/L) (SIGMA, Steinheim, Germany) to obtain pure cultures and to encourage sporulation. All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1) and representative isolates have been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. **Holotype** material of the new *Ceratocystis* sp. from Sulawesi, consisting of dried cultures on MEA has been lodged in the herbarium of the National Collection of Fungi, Pretoria (PREM), South Africa (Table 1).

PCR, sequencing and analysis

Mycelium from actively growing cultures on 2 % MEA plates, for each representative isolate chosen for DNA sequence comparisons (Table 1) was scraped into Eppendorf tubes and lyophilised for 2 d. The lyophilised mycelium was placed in liquid nitrogen and ground to a powder using a glass rod. DNA was extracted using the method described by Barnes *et al.* (2001).

Both ITS regions (ITS1 and ITS2) including the 5.8S gene of the ribosomal DNA (rDNA) operon of all selected isolates (Table 1) were amplified using primers ITS1 and ITS4 (White *et al.* 1990) at an annealing temperature of 55 ºC. Part of the β-tubulin gene was amplified using primers βt1a and βt1b (Glass & Donaldson 1995) at an annealing temperature of 56 ºC. The transcription elongation factor 1-α gene was amplified with primers EF1-728F and EF1-986R (Carbone & Kohn 1999) at an annealing temperature of 58 ºC.

Polymerase chain reaction (PCR) mixtures consisted of 200 nM of the forward and reverse primers, 200 µM of each dNTP, Expand High Fidelity PCR System enzyme mix (1.75 U) (Roche Diagnostics, Mannheim, Germany), 1 × Expand HF Buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme) and 2–10 ng DNA. Reaction volumes were adjusted to 25 µL with sterile water. The PCR programme was set at 96 ºC for 2 min, followed by 10 cycles at 94 ºC for 20 s, × ºC (× = the annealing temperature specified for each set of primers as noted above) for 40 s and 72 ºC for 45 s. A further 30 cycles were included with the annealing time altered to 40 s and a 5 s extension after each cycle. A final step of 10 min at 72 ºC completed the programme. Amplification of the respective fragments was confirmed by electrophoresis in a 2 % agarose (Roche diagnostics, Mannheim, Germany) gel containing ethidium bromide and visualised under UV light. After amplification, products for each gene were purified using Sephadex columns following the manufacturer's guidelines (1 g in 15 mL H_2O, SIGMA, Steinheim, Germany).

Purified PCR amplicons were sequenced in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit, following the manufacturer's protocols (Applied BioSystems, Foster City, California). Sequencing of the respective gene areas was done using the same primers as those used for the PCR reactions. Sequence products were cleaned using the same technique used for the PCR reactions. Sequence reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California) and sequence electropherograms were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California).

The sequences obtained for the *Ceratocystis* sp. from clove were compared with those of morphologically similar *Ceratocystis* spp. that were available in GenBank (Table 1). Sequences were aligned manually and analysed using PAUP v. 4.0b10 (Phylogenetic Analysis Using Parsimony) (Swofford 2002). Gaps were treated as "newstate" and trees were obtained via stepwise addition of 1000 replicates with the Mulpar option in effect. The heuristic search option based on parsimony with tree bisection reconnection was used to obtain the most parsimonious tree. Confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis virescens* (R.W. Davidson) C. Moreau was used as the out-group taxon.

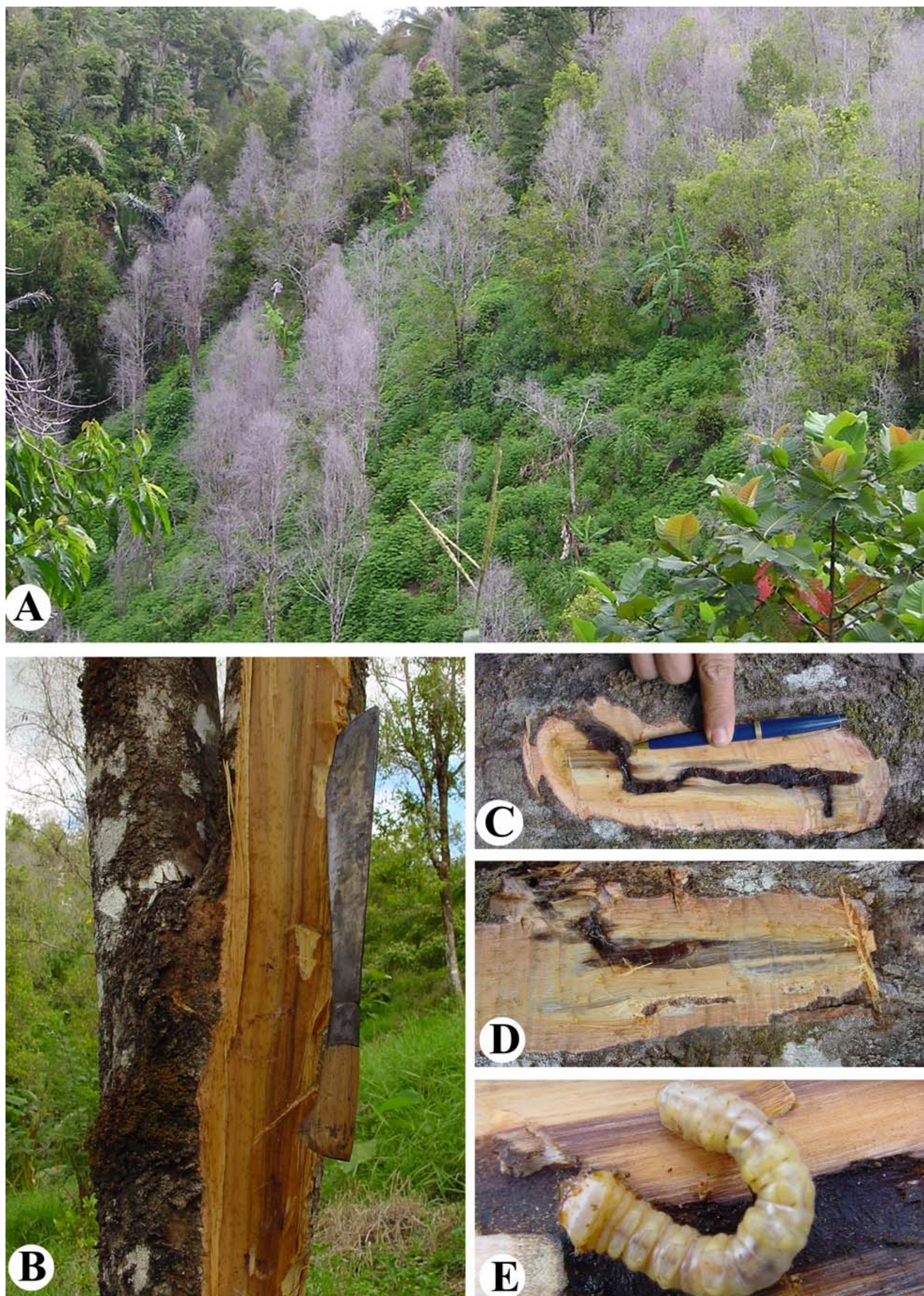

Fig. 1. Symptoms of disease caused by *C. polychroma* in Sulawesi. A. Diseased and dead *Syzygium aromaticum* trees. B–C. Internal symptoms showing colour streaked discolouration in the wood. D. Tunnels in the wood caused by *Hexamitodera semivelutina* larvae. E. *H. semivelutina* larva.

A partition homogeneity test (Swofford 2002) was used to determine whether the sequence data sets for the three different gene regions could be combined. The Markov Chain Monte Carlo (MCMC) method (Larget & Simon 1999), with a Bayesian framework was used to estimate the posterior probability of nodes in the phylogenetic tree. One hundred thousand random trees using the MCMC procedure were generated, sampling every 100th tree and printing every 10th tree. To avoid including trees that might have been sampled before convergence of the Markov chain, the chain was assessed for the number of trees that were formed before the stabilization, and these trees (5000) were discarded. For the analysis of the ITS gene sequence, gamma rate heterogeneity was set, and no codon specific sites were included. For the β-tubulin and elongation factor 1-α sequences, codon-specific sites were specified with a site-specific substitution rate and the site partition was treated as a by-codon.

Morphology and cultural characteristics

The growth rate of isolates CMW 11424, CMW 11443 and CMW 11449, representing the *Ceratocystis* sp. from clove, was determined on 2 % MEA in Petri dishes. Prior to the growth studies, the isolates were grown for two weeks at 20 ºC. Mycelial plugs were taken from actively growing cultures using a 5 mm cork borer and single plugs were transferred to the centres of 90 mm Petri dishes containing 2 % MEA. Five plates for each isolate were incubated at 4 ºC as well as at temperatures ranging from 10 ºC to 35 ºC, at five-degree intervals. Two measurements of colony diameter at right angles to each other were made every second day, for 16 d and averages were computed. The entire experiment was repeated once.

Morphological characteristics were described from 14-d-old cultures, on 2 % MEA supplemented with streptomycin sulphate (1 mg/L). For microscopic examination, fungal structures were mounted in lactophenol. Fifty measurements were made for each taxonomically relevant structure of isolate CMW 11424, and 10 measurements were made for each of the other isolates (CMW 11436, CMW 11443 & CMW 11449). Ranges, averages, and standard deviations of the corresponding measurements were calculated in Microsoft Excel. Microscopic observations were made using a Carl Zeiss microscope and the photographic images were captured with a Zeiss Axio Vision camera system. Colour descriptions were determined using the colour charts of Rayner (1970).

RESULTS

Fungal isolates

A *Ceratocystis* sp. was the only fungus consistently found associated with *H. semivelutina* larval tunnels or isolations made from the discoloration associated with them. In total, 120 isolates of the fungus were collected from 22 different trees at 18 sites. Some of the isolates were obtained directly from ascomata in the tunnels or associated wood that had been incubated. Others originated from baiting the discoloured wood with carrot slices.

PCR, sequencing and analysis

Amplification of the ITS regions and the 5.8S gene of the rDNA operon resulted in amplicons of ~500 bp in size. Amplification of the β-tubulin gene resulted in amplicons of ~500 bp while the amplification of the elongation factor 1-α resulted in amplicons of ~300 bp.

Partition homogeneity tests for the sequence data sets of all three genes resulted in a P-value of 0.05. The data of the three gene regions could thus be combined. The combined sequences of the ITS, β-tubulin and elongation factor 1-α gene resulted in a data set of 1383 characters. Of these characters, 804 were constant while 292 characters were parsimony-uninformative and 287 were parsimony-informative. Analysis of this data set resulted in three most parsimonious trees, of which one was selected for presentation (Fig. 4). This tree had a length of 825, a consistency index (CI) of 0.8994, a homoplasy index (HI) of 0.1006, a retention index (RI) of 0.8588 and a rescaled consistency index (RC) of 0.7724. The posterior probability of the branch nodes of the combined data sets, generated with the Bayesian inference programme, supported the bootstrap values.

The posterior probability for the branch nodes in the tree was 100 % for the *C. pirilliformis* Barnes & M.J. Wingf., *C. fimbriata* and *C. albifundus* M.J. Wingf., De Beer & M.J. Morris clades respectively. Isolates of the *Ceratocystis* sp. from clove resided in a discrete clade that grouped separately from all the other clades, with its own posterior probability of 100 % (Fig. 4).

Species of *Ceratocystis* included in the analyses resided in four well-resolved clades (Fig. 4). The *Ceratocystis* sp. from clove in Sulawesi did not group with any known *Ceratocystis* sp. analysed. It formed a single, well-supported sub-clade (bootstrap = 98 %). The other sub-clades represented isolates of *C. fimbriata*, *C. albifundus* and *C. pirilliformis* respectively (Fig. 4). This result was confirmed using the Bayesian analysis, with a branch node probability of 100 % (Fig. 4). DNA-based comparisons thus showed that the *Ceratocystis* sp. from Sulawesi represents a previously undescribed species of *Ceratocystis*.

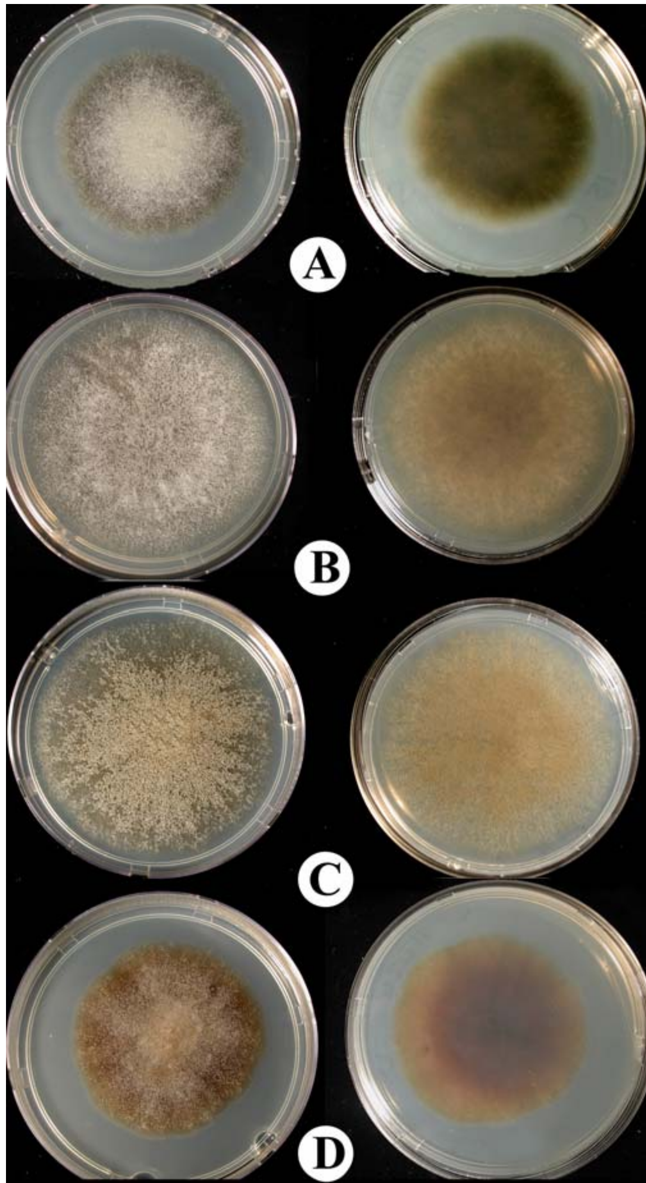

Fig. 2. Different colony colours of *C. polychroma* on 2 % MEA agar at different temperatures (Left surface, right reverse). A. 15 °C. B. 20 °C. C. 25 °C. D. 30 °C.

Anamorphe Thielaviopsis: conidiophora in mycelio singula, hyalina, basibus tumidis, apicem versus angustata, 53–81 (–103) µm longa, basi 4–6 µm lata, apicibus 3–4 µm lata. *Conidiogenesis* phialidica incremento parietis annulari; *conidia* duarum formarum: conidia primaria hyalina, aseptata, cylindrica, (13–)16–24(–26) × 3–5 µm; conidia secondaria hyalina, aseptata, doliiformia, 9–11 × 6–8 µm, in catenis portata. *Chlamydosporae* ellipsoideae, crassitunicatae, leves, brunneae, 11–14 × 8–14 µm, in agaro submersae, singulae vel catenatae.

Taxonomy

The *Ceratocystis* sp. isolated from the tunnels and infections associated with *H. semivelutina* infesting clove in Sulawesi is morphologically distinct from all other described species of *Ceratocystis*. Sequence data for three different gene regions support the morphological differences observed. The following description is, therefore, provided for the fungus from clove.

Ceratocystis polychroma M. van Wyk, M.J. Wingf. & E.C.Y. Liew, **sp. nov.** MycoBank MB500074. Figs 2, 3.

Anamorph: *Thielaviopsis* sp.

Etymology: Reflecting the different colours of cultures at different temperatures.

Coloniae ad 15 ºC olivaceo-virides, infra olivaceae, ad 20 ºC albae vel fulvo-brunneae, infra mellinae, ad 25 ºC supra infraque mellinae, et ad 30 ºC supra infraque avellaneae. *Mycelium* plerumque in medio immersum; mycelium aerium album parcum. *Crescit* optime ad 20–25 ºC, nullo incremento supra 35 ºC. *Hyphae* leves, ad septa non constrictae, 3–5 µm latae. *Bases ascomatum* atrobrunneae vel nigrae, globosae, hyphis ornatae, bases (208–)217–261 (–269) µm diam. *Colla ascomatum* basi atrobrunnea vel nigra, apicem versus pallidiora, (837–)849–1070(–1190) µm longa, basi (42–)44–54(–57) µm lata, ad apicem (15–)16–18(–20) µm lata. *Hyphae ostiolares* divergentes, hyalinae, (31–)33–43(–46) µm longae. *Asci* non visi. *Ascosporae* e latere visae galeiformes, aseptatae, hyalinae, vagina investitae, vagina inclusa 5–7 × 3–4 µm, vagina exclusa 4–5 × 3–4 µm. Ascosporae in massis mucilagineis fulvo-luteis ad apices collorum ascomatum cumulantes.

Colonies (Fig. 2A–D) olive-green (23m), reverse olive (21''m) at 15 ºC, at 20 ºC colonies white to buff-brown (17'''I), reverse honey (19''k), at 25 ºC colonies honey (19''k), reverse honey (19''k), and at 30 ºC colonies hazel (11'k), reverse hazel (11'k) in colour. *Mycelium* mostly submerged in medium, sparse white aerial mycelium present. *Optimal temperature* for growth 20–25 ºC, reaching 90 mm diam in 16 d. Cultures reaching an average of 10 and 45 mm in 16 d, at 10 and 30 ºC, respectively. No growth above 35 ºC. *Hyphae* smooth, not constricted at septa, 3–5 µm wide. *Ascomatal bases* dark brown to black, globose, ornamented with hyphae, bases (208–)217–261(–269) µm diam (Fig. 3). *Ascomatal necks* dark brown to black at the base, becoming light brown towards the apex, (837–)849–1071(–1187) µm long, (42–)44–54(–57) µm wide at the base, (15–)16–18(–20) µm wide at the apex. *Ostiolar hyphae* divergent, hyaline, (31–)33–43(–46) µm long. *Asci* not observed. *Ascospores* cucullate in side view, aseptate, hyaline, invested in a sheath, 5–7 × 3–4 µm with sheath, 4–5 × 3–4 µm without sheath. Ascospores accumulating in buff-yellow (19d) mucilaginous masses at the apices of ascomatal necks.

Thielaviopsis anamorph: conidiophores occurring singly on mycelia, hyaline, from swollen base tapering towards the apex, 53–81(–103) µm long, 4–6 µm wide at base, 3–4 µm wide at the apices. Phialidic *conidiogenesis* through ring wall building, *conidia* of two types: primary conidia hyaline, aseptate, cylindrical, (13–)16–24(–26) × 3–5 µm, secondary conidia

hyaline, aseptate, barrel-shaped, 9–11 × 6–8 µm, borne in chains. *Chlamydospores* oval, thick-walled, smooth, argus-brown (13m), 11–14 × 8–14 µm, embedded in agar, formed terminally, singly or in chains.

Specimens examined: **Indonesia**, Sulawesi, Toliangoki, isolated from larval tunnel of *Hexamitoderma semivelutina* (Coleoptera: Cerambycidae) on *Syzygium aromaticum*, Dec. 2002, E.C.Y. Liew, **holotype** Herb. PREM 57818, culture ex-type CMW 11424 = CBS 115778; Sulawesi, Kiawa, isolated from larval tunnels of *H. semivelutina* on *S. aromaticum*, Dec. 2002, **paratype** PREM 57820, culture ex-paratype CMW 11443 = CBS 115776; same collection data, **paratype** PREM 57817, culture ex-paratype CMW 11419; Rumoong, isolated from larval tunnel of *H. semivelutina* on *S. aromaticum*, Dec. 2002, **paratype** PREM 57821, culture ex-paratype CMW 11449 = CBS 115775.

Morphology and cultural characteristics

Ceratocystis fimbriata is taxonomically and morphologically the species most similar to *C. polychroma.* There were no visually obvious morphological differences between *C. polychroma* and *C. fimbriata*. However, the diameter of ascomatal bases of *C. fimbriata* is 121–255 µm (Upadhyay 1981) while those of *C. polychroma* are considerably larger (217–261 µm). The ascomatal necks of *C. fimbriata* are 950 µm long while those of the clove fungus range form from 849 to 1071 µm in length and are 18–35 µm wide, in contrast to those of *C. fimbriata* that are much wider (44–54 µm). The ostiolar hyphae of *C. fimbriata* are 18–75 µm long, while those of *C. polychroma* are consistently shorter (33–43 µm long). *Ceratocystis fimbriata* has ascospores that are 2–2.5 µm wide, while those of the clove fungus are 3–4 µm. The conidiophores of *C. fimbriata* are 35–130 µm, and those of *C. polychroma* are 53–81 µm long. The barrel-shaped conidia are narrower for *C. fimbriata,* 6–8 µm, than the *C. polychroma*, which are 5–15 µm in width (all according to Upadhyay 1981).

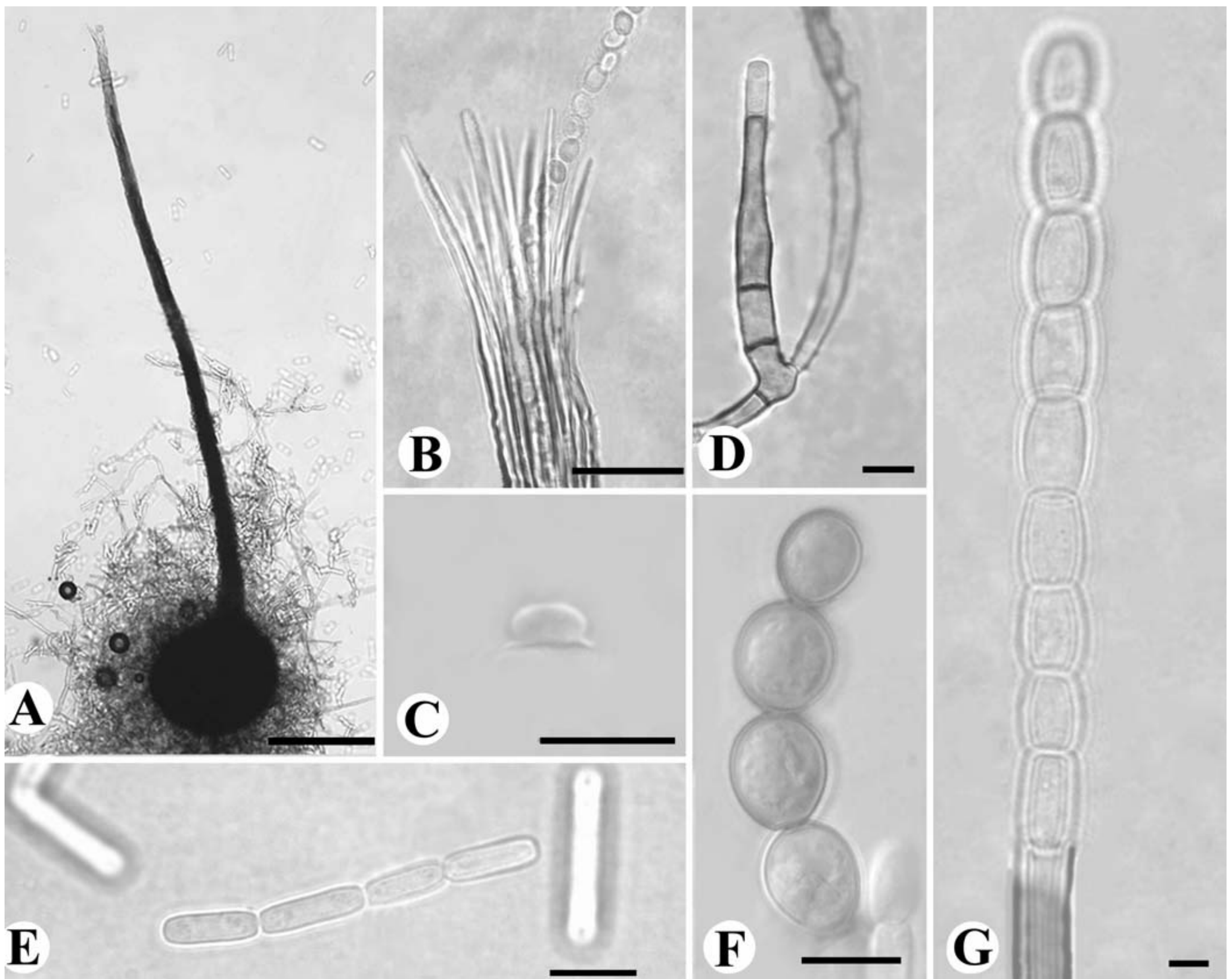

Fig. 3. Morphological characteristics of *C. polychroma* (CMW 11424). A. Globose ascomata. B. Divergent ostiolar hyphae. C. Hat-shaped ascospore in side view. D. Phialidic conidiogenous cell with emerging cylindrical conidium. E. Cylindrical conidia in a chain. F. Chain of chlamydospores. G. Barrel-shaped conidia in a chain. Scale bars: A = 200, B = 20, C, E, F = 10, D = 5 µm.

DISCUSSION

Ceratocystis polychroma represents a new taxon that is consistently found in the larval tunnels of *H. semivelutina* on dying clove trees in Sulawesi. This fungus can also easily be isolated from the red-brown streaked living wood that is found associated with the borer. Morphologically, *C. polychroma* most closely resembles *C. fimbriata*. This explains why Liew *et al*. (2003) tentatively identified the fungus as that species. Both species have characteristic globose to oval ascomatal bases covered with hyphae, and hat-shaped ascospores accumulating in slimy masses at the apices of the ascomatal necks. *Ceratocystis polychroma* is morphologically different from *C. albifundus* (Wingfield *et al*. 1996) and *C. pirilliformis* (Barnes *et al*. 2003), the two other *Ceratocystis* spp. in the *C. fimbriata* complex. Thus *C. albifundus* is easily recognised by its pale coloured ascomatal bases and *C. pirilliformis* is different from *C. polychroma* in having distinctly pear-shaped ascomatal bases.

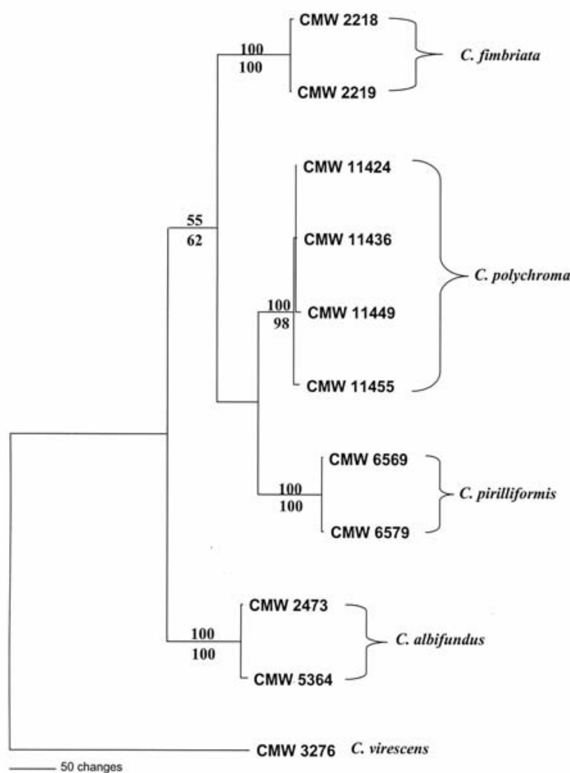

Fig. 4. Phylogenetic tree based on the combined sequence data from three gene regions; ITS, β-tubulin and elongation factor 1-α. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches while Bayesian values are indicated below the branches. *Ceratocystis virescens* is used as the out-group taxon.

Ceratocystis polychroma produces colonies that are white to green in colour, whereas isolates of *C. fimbriata* are typically olivaceous-green in culture. *Ceratocystis fimbriata* cultures tend to produce obvious aerial mycelium, which is different to those of *C. polychroma* that produce a sparse white mat of mycelium at the surface of cultures. The bases of the ascomatal necks are much wider in *C. polychroma* than in *C. fimbriata* and the barrel-shaped conidia are also much wider in the former than the latter species.

Comparisons of DNA sequence data for three gene regions, also confirmed that it resides in a discrete phylogenetic group, distant from all similar species.

Together with the new *C. polychroma,* there are seven *Ceratocystis* spp. with hat-shaped ascospores. Other species include *C. fimbriata* (Halsted 1890, Upadhyay 1981), *C. moniliformis* (Hedgc.) C. Moreau (Davidson 1935, Hunt 1956), *C. albifundus* (Morris *et al.* 1993, Wingfield *et al.* 1996), *C. moniliformopsis* Yuan & Mohammed (Yuan & Mohammed 2002), *C. pirilliformis* (Barnes *et al*. 2003) and *C. acericola* H.D. Griffin (Grylls & Seifert 1993). Of these fungi, only *C. fimbriata* (Upadhyay 1981), *C. pirilliformis* (Barnes *et al*. 2003) and *C. polychroma* produce chlamydospores. The ascomatal bases of *C. pirilliformis* (Barnes *et al*. 2003) are pear-shaped and thus unique. *Ceratocystis moniliformis* and *C. moniliformopsis* both have short conical spines covering their ascomatal bases, which are absent in *C. polychroma* and other species with hat-shaped ascospores (Davidson 1935, Yuan & Mohammed 2002). *Ceratocystis acericola* can be distinguished from all the above species by the absence of ostiolar hyphae (Upadhyay 1981).

Ceratocystis polychroma and *C. fimbriata* are clearly similar and they are also phylogenetically closely related. Sequence data for the ITS regions of the rDNA operon alone showed that *C. polychroma* is different from *C. fimbriata*. By adding sequence data for two other gene regions, we were able to gain substantial additional support for the view that *C. polychroma* represents a unique taxon, although it resides in the clade including *C. fimbriata*, *C. pirilliformis* and *C. albifundus*. This clade is strongly separated from the *C. coerulescens* clade (Witthuhn *et al.* 1998), which also includes species with hat-shaped ascospores.

Ceratocystis polychroma is closely associated with damage to clove trees caused by the cerambycid beetle, *H. semivelutina*. Association with an insect is not unusual for *Ceratocystis* spp., many of which require wounds for infection and are known to be vectored by insects (Upadhyay 1981, Kile 1993). Numerous species of *Ceratocystis,* such as *C. fimbriata*, produce fruity aromatics and are thus attractive to non-specific insects such as picnic beetles (Coleoptera: Nitidulidae) and flies (Diptera) that transport them to freshly made wounds on trees (Moller & DeVay 1968, Hinds 1972). This is very different from species such as *C. polonica* (Siemaszko) C. Moreau, *C. laricicola* Redfern & Minter and *C. rufipennis* M.J. Wingf., T.C. Harr. & H. Solheim that do not produce fruity aromas, but are specifically associated with the

bark beetles *I. typographus* L., *I. cembrae* Heer and *Dendroctonus rufipennis* Kirby, respectively (Redfern *et al*. 1987, Wingfield *et al*. 1997, Yamaoka *et al*. 1997). Although *C. polychroma* is closely associated with an insect, we do not believe that this borer acts as a vector for the fungus. This is because cerambycid beetles are ecologically poorly adapted to transmit such fungi (Wingfield 1987). Adult borers that emerge from dying clove trees and that might be carrying *C. polychroma* ascospores never again enter trees. Rather, they mate and female insects insert an ovipositor under the bark. This would not easily allow for the transmission of spores on their bodies, which do not come into close contact with the wood. One possibility is that they carry mites or other phoretic animals that might act as secondary vectors as suggested for vectors of the pine wood nematode *Bursaphelenchus xylophilus* Steiner & Buhrer (Wingfield 1987). Alternatively, other insects not specifically associated with *H. semivelutina* might enter the relatively long-lived galleries of this borer and thus act as vectors for the fungus. Further studies of insects associated with the galleries of *H. semivelutina* are planned to resolve this question.

Ceratocystis polychroma resides in a genus of very well known pathogens of woody plants (Kile 1993). Its association with dramatic die-back of cloves and the very characteristic discoloration of woody tissue associated with woodborer damage, suggests that it contributes to tree death. However, pathogenicity of the fungus remains to be demonstrated. This process is somewhat frustrated by the high value of single trees that belong to small-scale farmers. Nonetheless, pathogenicity tests are planned for the future and these will substantially enhance our understanding of the serious die-back disease of clove trees in Sulawesi.

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REFERENCES

Barnes I, Gaur A, Burgess T, Roux J, Wingfield BD, Wingfield MJ (2001). Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular Plant Pathology* **2**: 319–325.

Barnes I, Roux J, Wingfield MJ, Old KM, Dudzinski M (2003). *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865–871.

Carbone I, Kohn LM (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.

Davidson RW (1935). Fungi causing stain in logs and lumber in the Southern states, including five new species. *Journal of Agricultural Research* **50**: 789–807.

Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Grylls BT, Seifert KA (1993). A synoptic key to species of *Ophiostoma*, *Ceratocystis* and *Ceratocystiopsis*. In: *Ceratocystis and Ophiostoma*: *Taxonomy, Ecology, and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds.). APS Press, St. Paul, Minnesota: 261–268.

Halsted (1890). Some fungous disease of sweet potato. *New Jersey Agricultural College Experiment Station Bulletin* **97**: 14.

Hinds TE (1972). Insect transmission of *Ceratocystis* species associated with aspen cankers. *Phytopathology* **62**: 221–225.

Hunt J (1956). Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**: 1–58.

Kile GA (1993). Plant diseases caused by species of *Ceratocystis* sensu stricto and *Chalara*. In: *Ceratocystis and Ophiostoma*: *Taxonomy, Ecology, and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds.). APS Press, St. Paul, Minnesota: 173–183.

Larget B, Simon DL (1999). Markov Chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* **16**: 750–759.

Liew ECY, Wingfield MJ, Assa B, Paath J, Kandowangkossor D, Sembel DT, Summerell BA, Burgess LW (2003). *Ceratocystis fimbriata* associated with clove decline in North Sulawesi. In: '*8th International Congress of Plant Pathology, 2–7 February 2003*, Christchurch, New Zealand, ICPP 8' Abstract no. 19.40.

Moller W, De Vay J (1968). Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1508.

Morris MJ, Wingfield MJ, De Beer C (1993). Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* **42**: 814–817.

Nair KSS (ed.) (2000). *Diseases and pests of the Indonesian forests*. Centre for International Forestry Research. SMT Grafika Desa Putera, Indonesia.

Nutman FJ, Roberts FM (1971). The clove industry and the diseases of the clove tree. *Pest Articles News Summaries* **17**: 147–165.

Purseglove JW, Brown EG, Green CL, Robbins SRJ (eds.) (1981). Cloves. In: *Spices*. New York, London: 229–285.

Rayner RW (1970). *A Mycological Colour Chart*. Commonwealth Mycological Institute and British Mycological Society, Kew, Surrey.

Redfern DB, Stoakley JT, Steele H (1987). Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36**: 467–480.

Roberts SJ, Eden-Green SJ, Jones P, Ambler DJ (1990). *Pseudomonas syzygii*, sp. nov., the cause of Sumatra

disease of cloves. *Systematic and Applied Microbiology* **13**: 34–43.

Swofford DL (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods).* Version 4. Sunderland, Massachusetts: Sinauer Associates.

Upadhyay HP (1981). *A monograph of Ceratocystis and Ceratocystiopsis*. University of Georgia Press: Athens.

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

Wingfield MJ (1987). Fungi associated with the pine wood nematode, *Bursaphelechus xylophilus*, and cerambycid beetles in Wisconsin. *Mycologia* **79**: 325–328.

Wingfield MJ, De Beer C, Visser C, Wingfield BD (1996). A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematics and Applied Microbiology* **19**: 191–202.

Wingfield MJ, Harrington TC, Solheim H (1997). Two species in the *Ceratocystis coerulescens* complex from conifers in wester North America. *Canadian Journal of Botany* **75**: 827–834.

Witthuhn RC, Wingfield BD, Wingfield MJ, Wolfaardt M, Harrington TC (1998). Monophyly of the conifer species in the *Ceratocystis coerulescens* complex using DNA sequence data. *Mycologia* **90**: 96–100.

Yamaoka Y, Wingfield MJ, Takahashi I, Solheim H (1997). Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* f. *japonicus* in Japan. *Mycological Research* **101**: 1215–1227.

Yuan ZQ, Mohammed C (2002). *Ceratocystis moniliformopsis* sp. nov., an early coloniser of *Eucalyptus obliqua* logs in Tasmania, Australia. *Australian Systematic Botany* **15**: 125–133.